THE EPIDEMIOLOGY, DIAGNOSIS AND PREVENTION OF INFECTIOUS DISEASES IN LIVESTOCK

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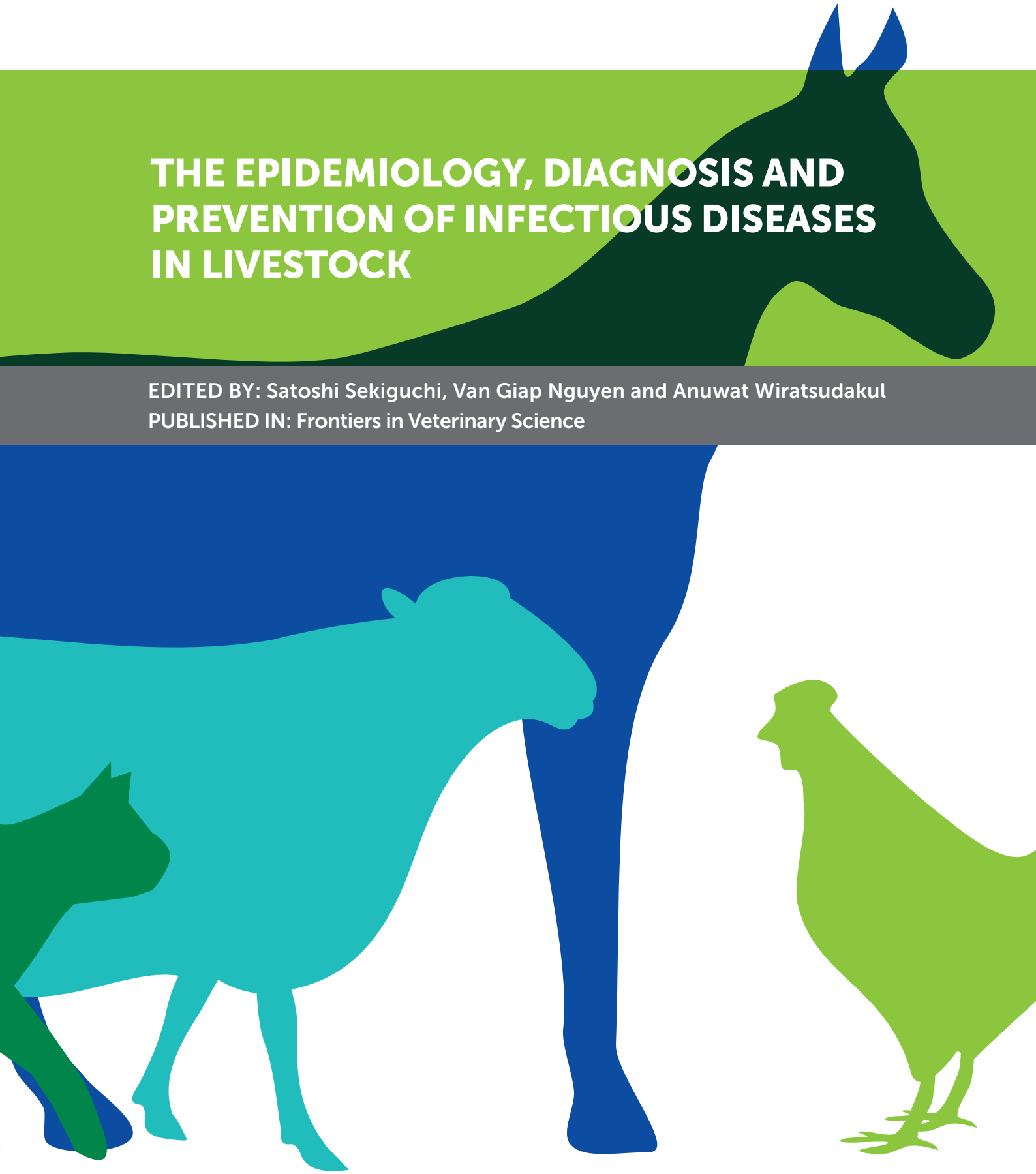
PUBLISHED IN: Frontiers in Veterinary Science



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frontiers

ISSN 1664-8714
ISBN 978-2-88974-518-0
DOI 10.3389/978-2-88974-518-0

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THE EPIDEMIOLOGY, DIAGNOSIS AND PREVENTION OF INFECTIOUS DISEASES IN LIVESTOCK

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Citation: Sekiguchi, S., Nguyen, V. G., Wiratsudakul, A., eds. (2022). The Epidemiology, Diagnosis and Prevention of Infectious Diseases in Livestock. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88974-518-0

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frontiers in Veterinary Science

EDITORIAL
published: 24 January 2022
doi: 10.3389/fvets.2021.840635

Editorial: The Epidemiology, Diagnosis and Prevention of Infectious Diseases in Livestock

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Keywords: epidemiology, diagnosis, infectious diseases, prevention, livestock

Editorial on the Research Topic

The Epidemiology, Diagnosis and Prevention of Infectious Diseases in Livestock

The infectious disease in livestock plays a vital role in the economy and food security of many developing countries. An approach in veterinary epidemiology to animal infectious diseases focuses on controlling and managing disease in the livestock population. Additionally, the risk of transmission and spread of emerging diseases is increasing because of intensive human traffic. In such circumstance, those outbreaks cause severe economic losses when the livestock industry become affected. Therefore, a major component of animal disease policies and disease management strategies are a viable prevention measure. However, the same prevention doesn't work everywhere due to strategies has changed over time. An emergency preparedness needs to do before outbreaks were reported.

This Research Topic yielded 45 articles of which 10 were brief research report, 30 original research articles, one clinical trial, one opinion, two systematic reviews, and one review, involving 321 authors from 17 countries. The two areas of research cover by this Research Topic are included: (i) infectious disease and control or eradication in livestock; (ii) development of diagnosis, monitoring, and surveillance program.

A first line of research includes the infectious disease and control or eradication in livestock. Intervention can vary depending on clinical fields and local circumstances. In this part, their works presents the appropriate framework needed for strengthening a surveillance program in each region. Serology has been performed for many years and the present of antibody refer as a marker to identify reservoir that may boost the outbreaks. In their work, Sun et al. report of *Chlamydia* seroprevalence in domestic, black-boned sheep and goats in southwest China. Involving same methods, Ullah et al. also reported anti-*Brucella* antibodies in sheep and goats at these livestock farms in Punjab, Pakistan. Furthermore, serological cross-reactivity was detected between alphaherpesvirus-2 and alphaherpesvirus−1 in a calf located in Central Italy by Petrini et al. However, this method is only required for early diagnosis and infection occurring in previously vaccinated animals may not be detectible. To overcome the limitation mentioned above, the molecular epidemiology of infectious diseases is also discussed in this Research Topic. For examples, isolation and characterization of a porcine transmissible gastroenteritis coronavirus in Northeast China by Yuan et al.; epidemiology of bovine tuberculosis in Ethiopia by Tulu et al. Similarly, an interesting work led by Hamada et al. described the new investigation on molecular prevalence of bovine leukemia virus in Egyptian cattle. In an article by Shi et al., they revealed bovine pestivirus isolates from cattle have extensive genetic variations in Central China.

There were still many studies highlighted the evaluation of the prevention of infectious disease in livestock. As the case in Vietnam, Mai et al. find out the possible risk factors for transmission pattern

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *21 December 2021*
Accepted: *30 December 2021*
Published: *24 January 2022*

Citation:
Sekiguchi S, Wiratsudakul A and Nguyen VG (2022) Editorial: The Epidemiology, Diagnosis and Prevention of Infectious Diseases in Livestock. Front. Vet. Sci. 8:840635. doi: 10.3389/fvets.2021.840635

of PEDV in an endemic region. They indicated that there have three of these 29 variables such as farrow-to-wean production type, distance from the farm to the slaughterhouse, and the presence of chickens on site. Recently, Makita et al. also examined the decision-making process for farm biosecurity among livestock farmers through elevated attitudes and self-efficacy. An integrated community-based intervention is also applied for control and prevention of epizootic lymphangitis in mules, Bahir Dar, Ethiopia, which was reported by Duguma et al.

This Research Topic is further investigating the role of wildlife in the epidemiology of infectious diseases. In addition, the identification of microorganisms by vectors has been useful for identifying reservoirs and verifying modes of transmission. An outcome of the papers by Chang et al. indicated pigeons play an important role in transmission pattern of Pigeon paramyxovirus type I. Works reporting the evidence of wild animals, as the case in Korea, wild leopard cat and Asian badger may spread feline parvovirus and its related viruses, which was reported by Kim et al. or in Germany, Q fever is presented by Winter et al. in ruminant. In particular, the work led by Shimizu et al. shown that infected wild boars are a major source of infection for the current classical swine fever occurred in Japan. A retrospective survey by Zhou et al. indicated that the blue fox's outbreak of abortions was derived from brucellosis which caused by *B. melitensis* strain. Moreover, a survey by Kivali et al. identified trypanosome species in cattle and their spatial distribution in western Kenya.

It was well-known that those novel pathogens are also responsible for emerging infectious diseases. A work led by Yu et al. shown that, as in goat, the impact of endogenous and exogenous factors affecting susceptibility to orf virus will likely reflect the host's specific in term of a particular strain such as NZ7-like orf viruses. An additional paper by Shimizu et al., presented the report of genetic variability of orf viruses in Japan, which can be spread to sheep or farmers and can be transmit to Japanese serows. Along this line, Su et al. showed that a coinfection porcine astrovirus with PEV and GARV in diarrheic piglets in China. Furthermore, Getah virus was reported by Ren et al. in southern China.

A second related aspects of research that drew the development of diagnosis, monitoring, and surveillance program. The application of new innovative diagnostic technologies for rapidly detect of pathogens is required for limit the economic impact of emerging infectious animal diseases. The desirable characteristics are fast, simple, cost-effective, highly sensitive, and specific. In these lines, a rapid detection of PCV2 and direct identification of PCV2 genotypes from clinical samples was developed by Wang, Song, Shin, Kim et al., examined the performance of two newly available methods, multiplex real-time PCR assay and PCR-reverse blot hybridization assay. These techniques can also yield results in 2–3 h for differentiate between the PCV2 genotypes and detect PCV2 from clinical specimens. Continuing with the search of novel diagnostic, Wang, Song, Shin, Choi et al. also highlighted that using the new clinical molecular assay for diagnosis in porcine cytomegalovirus (PCMV) can be used to screen with dramatic reduction in false positives and negatives. Besides, Arrieta-Villegas et al. examined the applicability of the P22 antigen complex as a complementary tool for TB diagnostics in goats with a cost-effective alternative. The review by Rodríguez-Hernández et al. also highlight application of Volatilome analysis to the diagnosis of mycobacteria infection in livestock, which was fulfills part of the mycobacterial diagnosis requirements.

The remaining works further highlighted the novel methods such as one-tube nested RT-PCR which required ∼1.5 h for completion for the characterizing pathogen responsible for porcine cytomegalovirus infection (Wang, Song, Shin, Choi et al.); or multiplex PCR is design to provide a rapid, specific and sensitive detection method for the identification of four pathogenic bacteria in minks (Li et al.) and thermal image scanning for the early detection of fever induced by highly pathogenic avian influenza virus infection in chickens and ducks (Noh et al.). Similarly, a procedure for partial or full genome sequencing of peste-des-petits-ruminants virus is described by Torsson et al. The use of a portable laboratory such as miniPCR and MinION to the field and to the production of a full genome with the results just 24 h of collection. On the other hand, Ruggeri et al. indicated that the newly scoring system could be used to exam pathological lesions in the respiratory tracts in porcine respiratory disease complex.

Taken together, all the published papers in this Research Topic reflects that a combined effort across borders is needed to control infectious diseases in livestock on such epidemiology, diagnosis, and prevention and coordinated action around the globe required to fill gaps in the literature regarding control and eradication programs for infectious diseases.

AUTHOR CONTRIBUTIONS

VN and SS wrote the first part of the infectious disease and control or eradication in livestock. AW wrote the second part of the development of the diagnosis, monitoring, and surveillance program. All authors contributed to conception, design of the project, manuscript revision, read, edited, added sections, provided critical comments of the manuscript, and agreed on the submitted version.

ACKNOWLEDGMENTS

The authors would like to thank the reviewers and scientists who contributed to this Research Topic.

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ORIGINAL RESEARCH
published: 03 April 2020
doi: 10.3389/fvets.2020.00152

TRIM62 From Chicken as a Negative Regulator of Reticuloendotheliosis Virus Replication

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Emerging evidence suggests that the tripartite motif containing 62 (TRIM62), a member of the TRIM family, plays an important role in antiviral processes. The objective of the study was to explore the role of TRIM62 in reticuloendotheliosis virus (REV) infection and its potential molecular mechanism. We first demonstrated that the REV infection affected the TRIM62 expression first upregulated and then downregulated in CEF cells. Next, we evaluated the effect of TRIM62 on viral replication. Overexpression of TRIM62 decreased REV replication. On the contrary, silencing of endogenously expressed TRIM62 increased viral replication. Then, to explore the necessity of domains in TRIM62's negative regulation on viral replication, we transfected CEF cells with TRIM62 domain deletion mutants. Deletion domain partially abolished TRIM62's antiviral activity. The effect of SPRY domain deletion was the highest and that of coiled-coil was the lowest. Further, we identified 18 proteins that coimmunoprecipitated and interacted with TRIM62 by immunocoprecipitation and mass spectrometry analysis. Strikingly, among which, both Ras-related protein Rab-5b (RAB5B) and Arp2/3 complex 34-kDa subunit (ARPC2) were involved in actin cytoskeletal pathway. Altogether, these results strongly suggest that chicken TRIM62 provides host defense against viral infection, and all domains are required for its action. RAB5B and ARPC2 may play important roles in its negative regulation processes.

Keywords: TRIM62, negative regulation, reticuloendotheliosis virus, domain deletion, RAB5B and ARPC2

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *20 January 2020*
Accepted: *03 March 2020*
Published: *03 April 2020*

Citation:
Li L, Niu D, Yang J, Bi J, Zhang L, Cheng Z and Wang G (2020) TRIM62 From Chicken as a Negative Regulator of Reticuloendotheliosis Virus Replication. Front. Vet. Sci. 7:152. doi: 10.3389/fvets.2020.00152

INTRODUCTION

TRIM62 (tripartite motif containing 62) is a member of the TRIM family proteins and is also known as DEAR1 (ductal epithelium-associated RING chromosome) (1). TRIM family proteins, also known as RBCC proteins, contain conserved RING finger, B-box, coiled-coil domains, and a variable C terminus (2). Despite their common domain structure, TRIM proteins play critical roles in distinct cellular processes such as intercellular signaling, innate immunity, transcription, autophagy, and carcinogenesis (3, 4). Members of the TRIM family of E3 ligases exhibit antiviral activities (5, 6). More than 20 TRIM proteins, which affected the entry or release of retrovirus such as human immunodeficiency virus 1 (HIV), murine leukemia virus (MIV), or avian leucosis virus (ALV), were screened (6). Expression of low amounts of TRIM62 enhanced HIV gene expression and release, and the E3 mutant of TRIM62 inhibited HIV release more potently than the wild-type

protein (6). However, TRIM62 from orange-spotted grouper (EcTRIM62) negatively regulated the innate antiviral immune response against fish RNA viruses (7).

Reticuloendotheliosis virus (REV) is an avian retrovirus that can induce immunosuppression, runting syndrome, lymphomas, and acute reticulum cell neoplasia (8, 9). The occurrence of reticuloendotheliosis (RE) has an immunosuppressive effect and REV as the contaminant within vaccines against Marek's Disease (MD) (10), fowlpox (11, 12), and Gallid herpesvirus 2 (GaHV-2) (13), which may lead to vaccination failures and co-incidence of RE with other secondary infectious agents. Since breeder and layer flocks are commonly vaccinated against MD, the possible congenital transmission of REV between chickens was also be taking into account. The occurrence of RE has major economic importance. So far, no effective vaccines have been developed against RE; thus, the only protection remains flock renewal with elimination of affected birds or application of experimental antiviral treatment. In a previous study, we have confirmed that TRIM62 possesses restriction of avian leukosis virus subgroup J (ALV-J) replication (14). ALV-J is another avian retrovirus. At present, no data are available regarding the role of TRIM62 from chicken in REV infection.

To explore the role of TRIM62 in REV infection, in the present study, we detected and analyzed the association of TRIM62 expression with viral replication. Then, we evaluated the effects of TRIM62 on viral replication by overexpression, silencing, and domain deletion of TRIM62 in CEF cells with REV infection. Further, with TRIM62 overexpression, we screened key proteins that interacted with TRIM62. Our study provided evidence that chicken TRIM62 negative regulated the REV replication.

TABLE 1 | Primers used for quantitative reverse transcription-PCR.

Gene target	Primer sequence	Fragment size (bp)
TRIM62	Forward: TACTGGGAGGTGGTGGTGTC	246
	Reverse: CGTCGGCGTTGTAGAAGATG	
REV (env)	Forward: TTGTTGAAGGCAAGCATCAG	330
	Reverse: GAGGATAGCATCTGCCCTTT	
RAB5B	Forward: CCCCAGCATCGTCATTG	101
	Reverse: GGCTGTTGTCATCTGCGTAA	
ARPC2	Forward: CGGAAAGGTGTTTATGC	223
	Reverse: CAGGTAGTCTCGGAATGTG	
GADPH	Forward: GAACATCATCCCAGCGTCCA	132
	Reverse: CGGCAGGTCAGGTCAACAAC	

MATERIALS AND METHODS

Cell Culture and Viral Infection

The CEF cells' cultural protocol was conducted as described in a previous study (14). CEF cells were incubated with a diluted stock

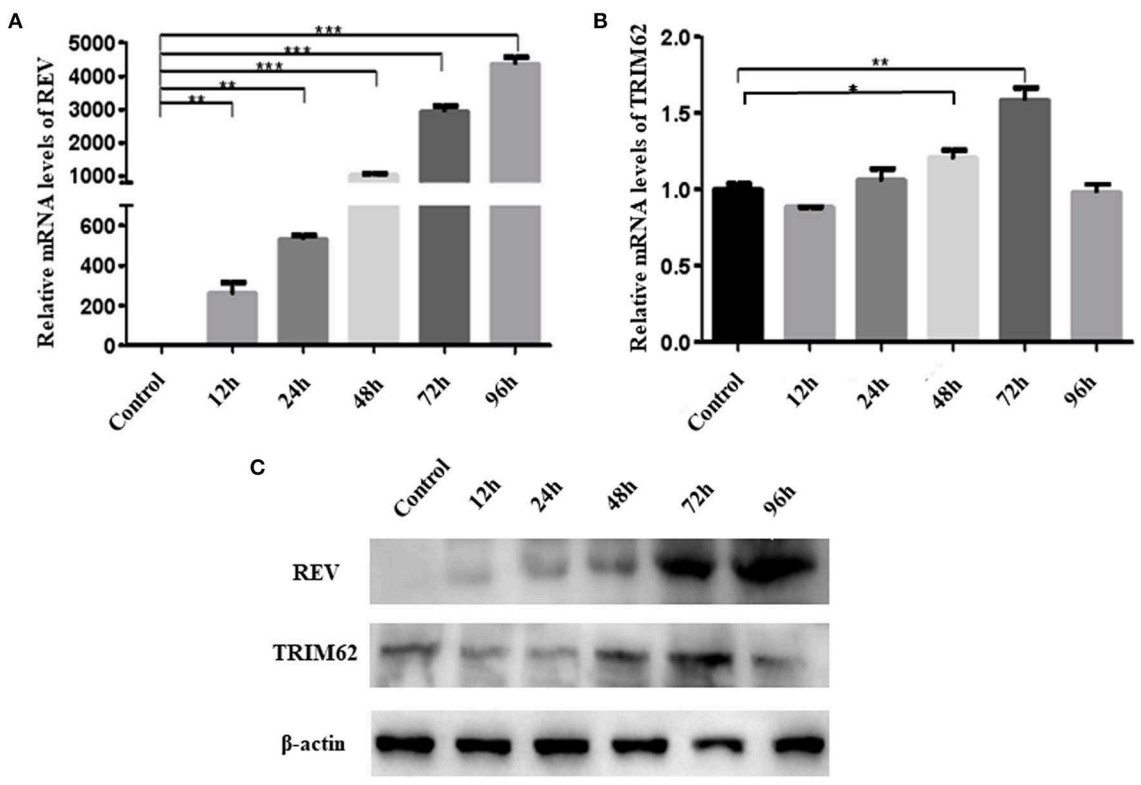

FIGURE 1 | Effect of REV infection on TRIM62 expression. CEF cells were infected with REV. The RNA expression levels of REV **(A)** and of TRIM62 **(B)** at 12, 24, 48, 72, and 96 h post-infection in CEF cells were assessed by qRT-PCR. **(C)** The protein expression levels of REV and TRIM62 were detected by WB. Uninfected CEF cells were used as the control. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

of SNV strain of REV (China strain: JX0927, maintained in our laboratory) at a multiplicity of infection (MOI) of 0.1. The mRNA expression of TRIM62 and REV in CEF cells were detected at different times (12, 24, 48, 72, and 96 h post-infection). The mRNA expression of TRIM62 in the supernatant was also detected in parallel at the same sampling times.

Plasmid of Chicken TRIM62 and Short Hairpin RNA (shRNA) Transfection

To identify the antiviral function of TRIM62, CEF cells were seeded on six-well-plates for 12 h and transfected with plasmids using a lentiviral vector for TRIM62 overexpression and silence. The transfected plasmids containing chicken TRIM62 (pTRIM62)/domain deletion mutants were for TRIM62 overexpression, and those containing short hairpin RNA targeting TRIM62 (shTRIM62) were for TRIM62 silence. The gene sequence of chicken TRIM62 was obtained from GenBank (XM_015297235.2) (14). These plasmids fused to a Flag tag. The transfected empty vector CEF cells were used as control. The pTRIM62/shTRIM62/pTRIM62-ΔR/B/C/S and empty vector were purchased from Jikai Gene Technology, Inc. (Shanghai, China). After stably expressing TRIM62/shTRIM62 for 12 h/24 h, the transfected CEF cells were incubated with REV. After 72 h, the TRIM62 and viral mRNA/protein levels were detected by qRT-PCR/WB.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA from CEF cells was isolated using the Tiangen RNeasy mini kit according to the manufacturer's instructions. RNA was reverse transcribed to cDNA using the TaqMan Gold Reverse Transcription kit (Applied Biosystems). qRT-PCR was performed according to a previously described protocol (15, 16). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a control for basal RNA levels. Primer sequences are listed in **Table 1**.

Western Blotting

The CEF cells were lysed in RIPA lysis buffer [25 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol] containing protease and phosphatase inhibitor cocktails (Novasygen, Beijing, China). The lysis was separated by SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, USA) as reported previously (17, 18). The target proteins were detected with specific primary antibodies against TRIM62 (19) and REV env (primary antibodies were prepared by our laboratory, anti-REV gp90) at a 1:200, 1:500, and 1:3,000

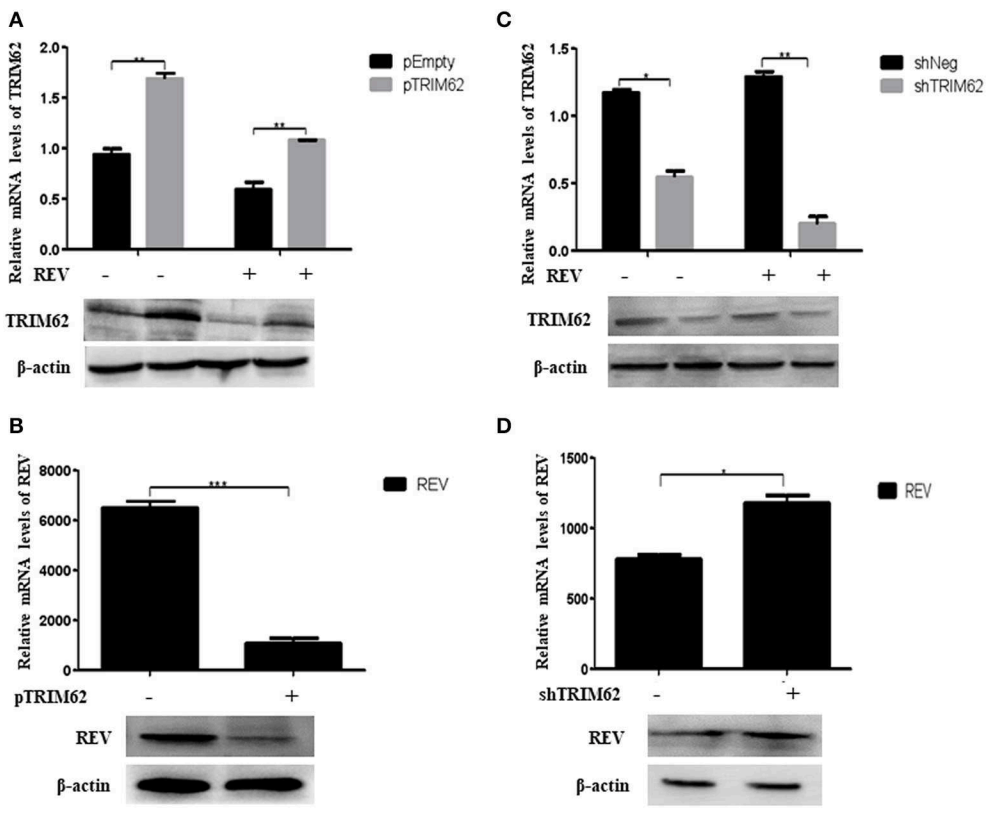

FIGURE 2 | Restriction of REV replication in CEF cells induced by TRIM62. **(A,B)** CEF cells were transfected with pTRIM62 or empty vector before infection with REV. The expression levels of TRIM62 **(A)** and REV **(B)** were assessed by qRT-PCR and Western blotting. **(C,D)** CEF cells were transfected with shTRIM62 or a negative-control shRNA before infection with REV. The expression levels of TRIM62 **(C)** and REV **(D)** were determined by qPCR and Western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

dilution, respectively. The secondary antibodies were horseradish peroxidase (HRP)-conjugated enhanced chemiluminescence (ECL) goat. The blots were visualized by the ECL-enhanced chemiluminescence kit (Roche, Basel, Switzerland).

Immunocoprecipitation

According to the instructions of Pierce Co-Immunoprecipitation (Co-IP) Kit (Thermo, Thermo Fisher Scientific, Massachusetts, USA), the 12-h pTRIM62 transfected CEF cells were subjected to REV infection for 72 h. The REV-infected CEF cells were then harvested and lysed. The anti-Flag label antibody was incubated with AminoLink coupling resin for 2 h at room temperature for antibody immunobilization. After centrifugation of cell lysates, supernatants were immunoprecipitated with coupling antibody. After another centrifugation of immunoprecipitated supernatants, the protein complex was washed three times with RIPA lysis buffer. Immunoprecipitates were incubated with elution buffer for 5 min at room temperature and centrifuged. The prey complex was collected and stored at −80°C. CEF cells were transfected with empty vector infected with REV as control.

Liquid Chromatography-Mass Spectrometry Analysis

Ten micrograms of the above prey complex was incubated in SDS-PAGE sample buffer (Solarbio, Beijing, China) for 5 min at 95°C. Proteins of cell lysates were separated by SDS-PAGE gels for 10 min and formed into a line. Each of the SDS-PAGE gel lanes was cut and subjected to trypsin digestion. Mass spectrometry (MS) analysis was performed according to a previously reported protocol (20) for detecting polypeptide

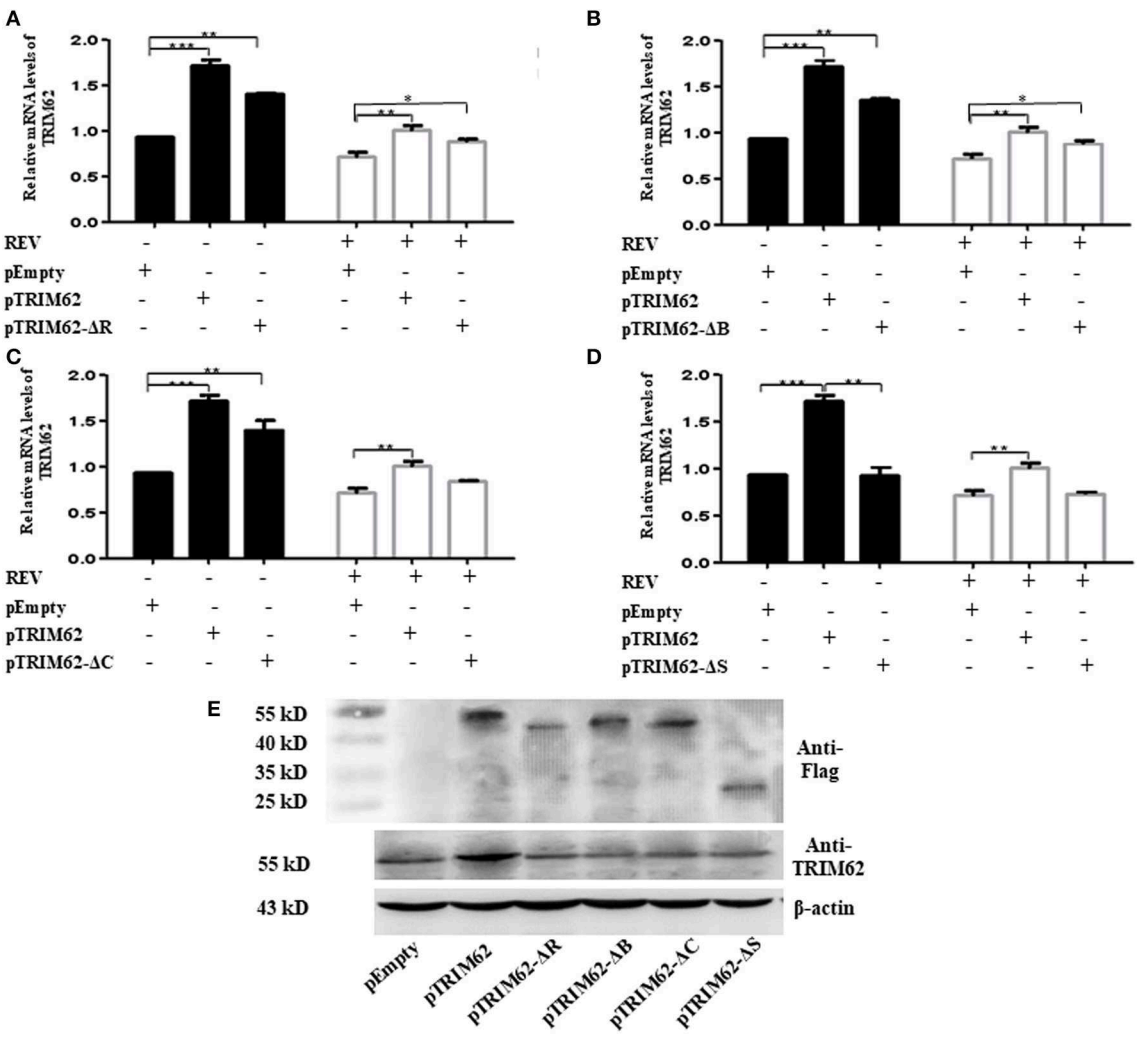

FIGURE 3 | Effect of domain deletion on TRIM62 expression. The relative mRNA expression of TRIM62 in CEF cells transfected with RING **(A)**, B-box **(B)**, coiled-coil **(C)**, or SPRY **(D)** domain deletion mutant with or without REV infection were measured by qRT-PCR using pEmpty and pTRIM62 as controls. **(E)** TRIM62 protein expression levels were detected with specific primary antibodies against Flag label and TRIM62 by Western blotting, respectively. pEmpty represent empty vector, pTRIM62 represent TRIM62 full length, pTRIM62-ΔR represent RING domain deletion, pTRIM62-ΔB represent B-box domain deletion, pTRIM62-ΔC represent coiled-coil domain deletion, and pTRIM62-ΔS represent SPRY domain deletion. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

sequence of proteins. Independent triplicate samples were analyzed. Polypeptide sequence was identified against using the Gallus (chicken) database and viral database (uniport-Gallusgalluschicken_REV_Combined.fasta) using Proteome Discoverer 1.4 (Thermo Fisher Scientific, Massachusetts, USA) software.

Statistical Analysis

Results are presented as the means ± standard deviations (SD) of at least three sample replicates. Statistical analysis was performed using SPSS 19.0 statistical software, and $p < 0.05$ was considered statistically significant.

RESULTS

REV Infection Affected TRIM62 Expression in CEF Cells

To assess association between the expression of TRIM62 and REV, we measured the TRIM62 expression and REV replication in REV-infected CEF cells at the transcriptional and translational level. Time course infection of REV in CEF cells showed that the REV mRNA levels were significantly increased ($p < 0.001$) from 48 to 96 h post-infection (**Figure 1A**), and the TRIM62 mRNA levels were also significantly upregulated at 48 h ($p < 0.1$) and 72 h ($p < 0.1$) as compared to that in uninfected CEF cells. However, compared with control, there were no changes of TRIM62 mRNA levels at another time point (**Figure 1B**). The REV mRNA expression was upregulated and reached the plateau at 96 h post-infection in CEF cells (17).

The protein levels of REV and TRIM62 were detected by WB, which were consistent with the dynamic changes of mRNA levels. Compared with control, the viral protein levels were significantly increased ($p < 0.001$) from 48 to 96 h post-infection, and TRIM62 protein levels were also significantly upregulated at 48 and 72 h ($p < 0.01$). However, there were no changes of TRIM62 protein levels at another time point observed (**Figure 1C**).

TRIM62 Restricted REV Replication in CEF Cells

We overexpressed or silenced TRIM62 to detect the role of TRIM62 in REV replication in infected CEF cells. Compared with non-transfected cells, despite REV infection, the TRIM62 mRNA level was significantly greater ($p < 0.01$) in pTRIM62-transfected CEF cells (**Figure 2A**) and lower ($p < 0.01$) in shTRIM62-transfected CEF cells (**Figure 2C**). The TRIM62 overexpression decreased the REV mRNA expression in transfected cells (**Figure 2B**), whereas silence of TRIM62 increased the mRNA expression of REV (**Figure 2D**). These results were confirmed in protein levels by Western blotting (**Figures 2A–D**). Our results strongly suggest the role of TRIM62 in restricting REV infection.

Domain Deletion-Induced TRIM62's Antiviral Activity Abolished Partially

To further investigate the effect of domain on negative regulation of TRIM62, we explored the effect of TRIM62 domain deletion mutants on TRIM62 expression and REV

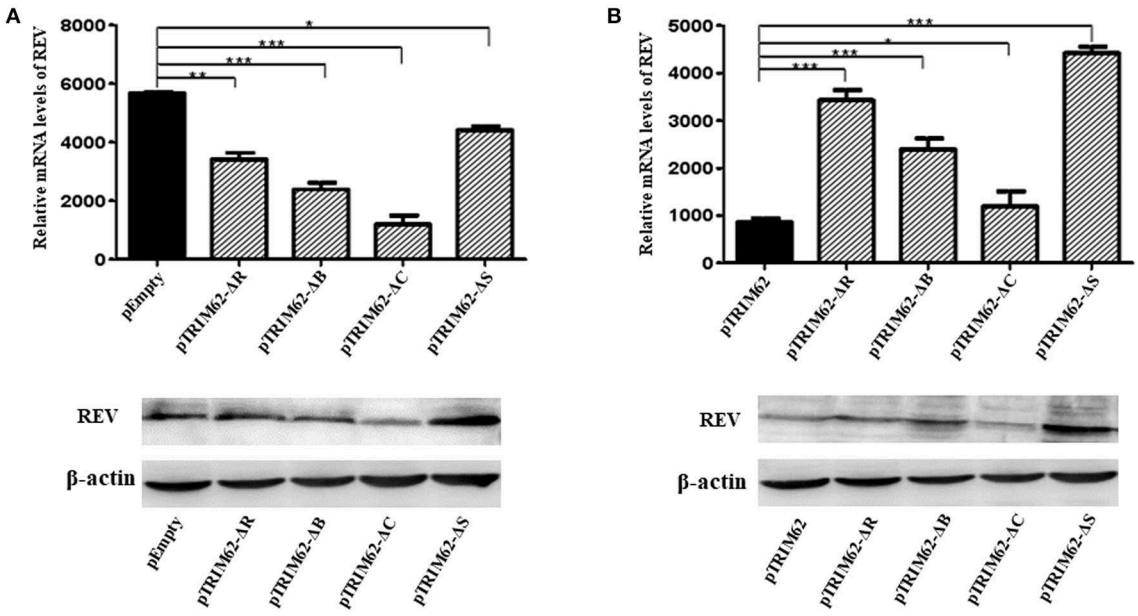

FIGURE 4 | Inhibition of REV RNA expression in CEF cells by TRIM62 RING, B-box, coiled-coil, and SPRY domain deletion mutants. The relative mRNA expression levels of REV in CEF cells transfected with RING, B-box, coiled-coil, or SPRY domain deletion mutants were measured by qRT-PCR and Western blotting. pEmpty **(A)** and pTRIM62 **(B)** were used as controls. pEmpty represent empty vector, pTRIM62 represent TRIM62 full-length, pTRIM62-ΔR represent RING domain deletion, pTRIM62-ΔB represent B-box domain deletion, pTRIM62-ΔC represent coiled-coil domain deletion, and pTRIM62-ΔS represent SPRY domain deletion. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

replication. The mutants were prepared as previously described (14). Regardless of REV infection, the expression of TRIM62 was significantly higher in cells transfected with deletion of RING (**Figure 3A**) and B-box (**Figure 3B**) than empty vector ($p > 0.1$). When transfected with coiled-coil domain deletion mutant, viral infection decreased the expression of TRIM62 in cells (**Figure 3C**). These results indicated that the effect of REV infection on TRIM62 may associate with the coiled-coil domain. Compared with empty vector, there was no difference obtained of TRIM62 expression in cells transfected with deletion of SPRY domain mutant (**Figure 3D**). The qRT-PCR primers located in the SPRY domain may explain this result that deletion of SPRY domain did not result in difference of TRIM62 mRNA expression. To further confirm the results, we measured the TRIM62 protein expression with specific primary antibodies against TRIM62 (**Figure 3E**). The expression of TRIM62 in mutant-transfected cells is lower than that in complete TRIM62-transfected cells.

As compared with pEmpty vector-transfected cells, the level of REV mRNA expression was significant lower ($p < 0.1$) in CEF cells transfected by any of the TRIM62 domain deletion mutants (**Figure 4A**). The effect of coiled-coil domain deletion was the highest (77.9 ± 2.3%), and that of SPRY domain deletion was the lowest (22.8 ± 2.5%). Compared with complete TRIM62, the mRNA expression of REV was significantly higher in cells transfected with domain deletion mutants (**Figure 4B**). The viral expression in SPRY domain deletion-transfected cells was the highest (435.5 ± 6.2%), and that in coiled-coil domain deletion-transfected cells was the lowest (53.2% ± 6.4%). These results suggested that the deletion of domains partially abolished the antiviral activity of TRIM62.

TABLE 2 | Identified proteins interacting with TRIM62.

No.	Accession	Gene	Protein	Coverage
1	A0A1L1RRL2	ACTB	Actin, cytoplasmic 1	30.12
2	A0A1L1RSN4	ACTC1	Actin, alpha cardiac muscle 1	25.61
3	F1NJ08	VIM	Vimentin	17.39
4	E1C2H4	LEMD2	LEM domain containing 2	4.79
5	A0A1L1RWD5	RPL15	Ribosomal protein L15	17.16
6	F1NSP8	HNRNPU	Heterogeneous nuclear ribonucleoprotein U	1.78
7	A0A1D5PW24	RPL19	Ribosomal protein L19	14.05
8	P01994	HBAA	Hemoglobin subunit alpha-A	10.56
9	A0A1L1RLB1	PKM	Pyruvate kinase PKM	5.81
10	**A0A1D5PKU6**	**RAB5**	**Ras-related protein Rab-5**	**5.14**
11	Q05744	CTSD	Cathepsin D	4.52
12	**A0A1L1RPQ9**	**Arp2/3**	**Arp2/3 complex 34 kD subunit**	**3.90**
13	A0A1L1RM78	PPIA	Peptidyl-prolyl cis-trans isomerase	12.00
14	A0A1D5NYW5	N/A	Peptidylprolyl isomerase	7.35
15	A0A1D5PZE3	APOA1	Apolipoprotein A-I	5.70
16	F1NK96	PDIA6	Protein disulfide isomerase family A member 6	6.04
17	A0A1D5PJM6	NUP214	Nucleoporin 214	0.59
18	F1NWX0	LOC425049	Tubulin alpha chain	6.46

The bold means the two proteins are involved in the skeleton pathway.

Identification of Cell Proteins That Interact With TRIM62

The REV infection affected the TRIM62 expression first upregulated and then downregulated in CEF cells. We hypothesized that other important interacting proteins besides the domains were involved in TRIM62's negative regulation of REV replication. To identify host cell proteins that interact with TRIM62, we used a tandem affinity purification approach coupled with mass spectrometry-based proteomics technology. These experiments identified 18 cell proteins that coimmunoprecipitated with transiently expressed Flag-tagged TRIM62 (**Table 2**). Decades of HIV research have testified to the integral role of the actin cytoskeleton in both establishing and spreading the infection (21). Of the TRIM62-interacting cell proteins identified, we focused on cytoskeletal proteins RAB5B and ARPC2. To verify the interaction between TRIM62 and RAB5B/ARPC2, CEF cells were transfected with a plasmid expression Flag-tagged RAB5B/APRC2 before REV infection, immunoprecipitated with anti-Flag antibody, and immunoblotted with anti-TRIM62 antibody. CEF cells were

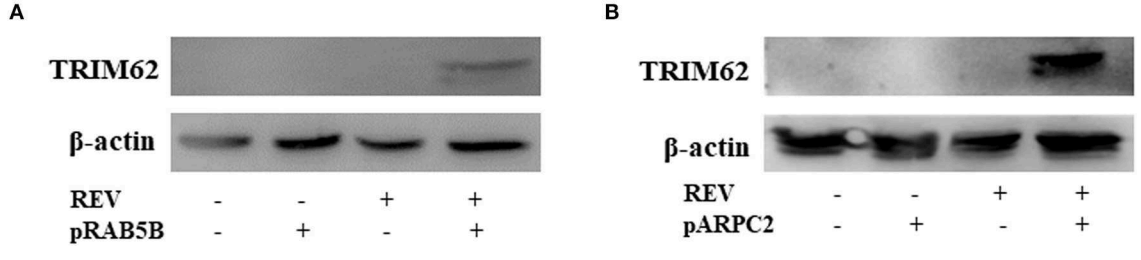

FIGURE 5 | Validation of the interaction of TRIM62 with ARB5B and ARPC2. CEF cells were transfected with a plasmid expression Flag-tagged RAB5B **(A)** and APRC2 **(B)** before REV infection/uninfection and immunoprecipitated with anti-Flag antibody. The TRIM62 in immunoprecipitates was detected by Western blotting with anti-TRIM62 antibody.

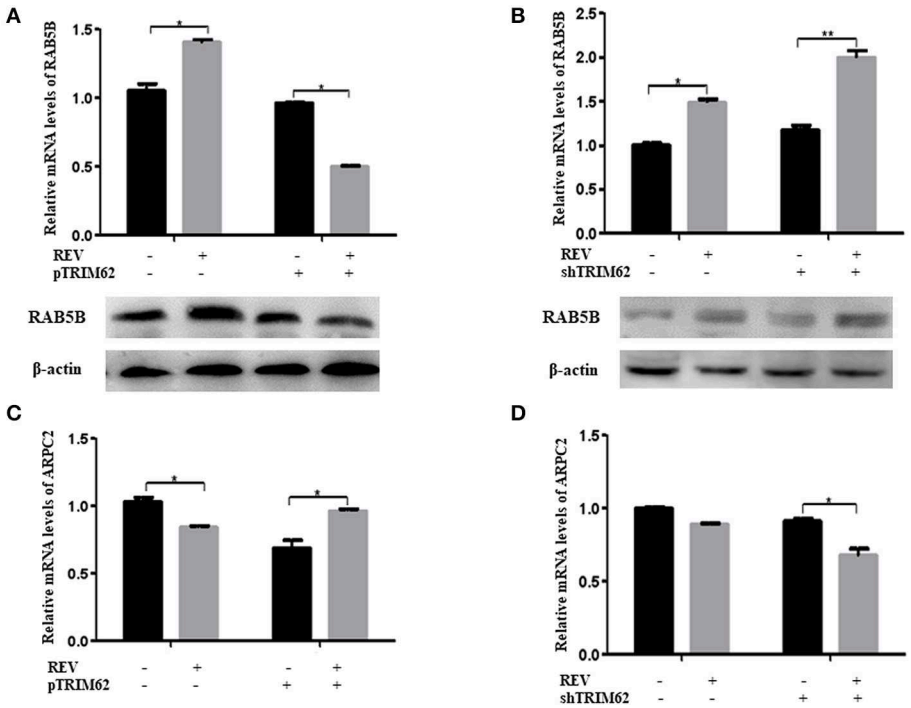

FIGURE 6 | Effect of TRIM62 on the expression of RAB5B and ARPC2. **(A,B)** The relative mRNA and protein expression of RAB5B and in CEF cells were transfected with pTRIM62 **(A)** or shTRIM62 **(B)**. **(C,D)** The relative mRNA expression of ARPC2 in CEF cells were transfected with pTRIM62 **(C)** or shTRIM62 **(D)**. $^{*}p < 0.05$, $^{**}p < 0.01$.

transfected with plasmid or empty vector uninfected with REV and transfected with empty vector infected with REV as control, respectively. Upon REV infection, anti-TRIM62 coprecipitated Flag-tag RAB5B (**Figure 5A**) and Flag-tag ARPC2 (**Figure 5B**).

Further, we found that TRIM62 affected the expression of RAB5B and ARPC2. As shown in **Figures 6A,B**, in CEF cells infected with REV, overexpression of TRIM62 exhibited a decrease of RAB5B expression, and silencing of TRIM62 enhanced the expression of RAB5B. On the contrary, in CEF cells infected with REV, the overexpression of TRIM62 resulted in an increase of ARPC2 expression, and silencing of TRM62 reduced the expression of ARPC2 (**Figures 6C,D**). These results indicated that RAB5B and ARPC2 play important roles in the negative regulation of TRIM62 on REV replication.

DISCUSSION

The host innate immune system senses antigen and elicits local antiviral defense to control infection. However, the REV can evade surveillance by the immune system after infection. TRIM62 is an innate immune regulator (6). In this study, we found that the TRIM62 expression levels were first up and then down with the increase of REV replication in CEF cells. These results suggest that as an innate immune factor, TRIM62 was activated and induced a short periodic significant upregulation at the fast replication period of REV ($p < 0.001$), and REV may circumvent TRIM62's restrictive effects by reducing its expression levels. However, we did not obtain valid data of TRIM62 expression in supernatant because the RNA concentration was too low. The reason that TRIM62 expression was first increased and then decreased needed further confirmation.

In the current study, TRIM62 has been identified as a protein that negatively regulates REV replication. Gene silencing of TRIM62 enhanced REV infection, which indicated that TRIM62 contributes to the endogenous restriction of REV in CEF cells. The further characterization of mechanisms by which REV reduces TRIM62 expression is warranted.

Owing to the presence of similar domains, the TRIM proteins were involved in similar antiviral biological processes. However, the domains of TRIM proteins played complicated roles in the inhibition of different viruses. Deletion of the PRY-SPRY domain of TRIM62 from human did not compromise its signal transduction properties. Rather, in addition to the RING domain, both B-box and coiled-coil domains were required for NF-κB and AP-1 induction by TRIM62 (5). Thus, the B-box and/or coiled-coil domains that confer oligomerization/dimerization properties (22) are indispensable for TRIM62-mediated signaling (5). The SPRY domain mediated the affinity for the viral capsid and the function of RING domain to TRIM5α restriction of retrovirus infection was determined by the target virus (23). The antiviral activity of fish TRIM36 required both RING and SPRY domains (24). The RING and C-terminal tail were essential for TRIM56's antiviral activity against flaviviruses (25). Interestingly, the inhibitory effect of fish TRIM62 on interferon immune and inflammation response to negatively regulate virus replication was also dependent on RING and SPRY domains (7). In our

study, this varied expression level of mutants (**Figure 3**) could be due to variation in transfection and expression efficiency of different plasmids in primary cells. Even though our results demonstrated that RING, B-box, coiled-coil, and SPRY domains contribute to the antiviral activity of chicken TRIM62, the effect of RING and SPRY was more significant than that of B-box and coiled-coil (**Figures 4A,B**). These results indicated that RING and SPRY domains are related to the antiviral activity of TRIM proteins.

Retroviruses are considered to use vesicular trafficking in infected cells (18, 26, 27). RAB5B is an isoform of RAB5, which is a member of the RAB family, a small GTPase family. RAB5B regulates fusion and motility of early endosomes, and is a marker of the early endosome compartment (28). RAB5B is a major regulator of hepatitis B virus (HBV) production (29). APRC2 is a component of Arp2/3 complex. The T cell-specific deletion of ARPC2 results in compromised peripheral T cell homeostasis (30). T cell survival and proliferation are mediated by complex homeostatic signals. Peripheral T cells are maintained at a constant cell number so that they can efficiently recognize foreign antigens and protect the host from pathogen invasion (31). Thus, of the TRIM62-interacting cell proteins identified, we focused on RAB5B and ARPC2. The interaction between RAB5B/ARPC2 and TRIM62 indicated that they played important roles in TRIM62 negative regulation on REV replication. A detailed understanding of host restriction may lead to antiviral therapies aimed at strengthening the innate immunity to retroviruses at the cellular and molecular level.

In addition, TRIM62 is a putative tumor suppressor and the TRIM62 levels represent an important prognostic marker in lung tumor (32) acute myeloid leukemia (AML) (33) and cervical cancer (34). ARPC2 promotes proliferation and metastasis (35, 36). REV is an oncogenic retrovirus. Further study is warranted to investigate the potential function of chicken TRIM62 and ARPC2 on REV infection inducing tumor formation.

CONCLUSION

We demonstrated that TRIM62 is a new suppressor that negatively regulates REV replication. The deletion of RING, B-box, coiled-coil, and SPRY domains partially affected TRIM62's antiviral activity, and the effects of RING and SPRY deletion were more significant than that of B-box and coiled-coil. The results suggested that all domains, RING, B-box, coiled-coil, and SPRY domains, are required for chicken TRIM62 to provide host defense against viral replication. Furthermore, both RAB5B and ARPC2 interacted with TRIM62, and maybe played important roles in TRIM62 negative regulation on REV replication. Our study provided a potential antiviral strategy targeting this novel regulator.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

GW designed the experiments. LL, DN, and JY performed the experiments. GW, LL, and DN prepared the manuscript. JB, LZ, and ZC analyzed the data. All authors have read and approved the final version of the manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 31772703) and the Natural Science Foundation of Shandong Provincial (No. ZR2017MC011).

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Conflict of Interest: JB was employed by the company China Animal Husbandry Industry Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 April 2020
doi: 10.3389/fvets.2020.00197

Genetic Diversity of Bovine Pestiviruses Detected in Backyard Cattle Farms Between 2014 and 2019 in Henan Province, China

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OPEN ACCESS

Edited by:
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University of Miyazaki, Japan

Reviewed by:
Norikazu Isoda,
Hokkaido University, Japan
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 03 January 2020
Accepted: 25 March 2020
Published: 17 April 2020

Citation:
Shi H, Li H, Zhang Y, Yang L, Hu Y, Wang Z, Duan L, Leng C, Yan B and Yao L (2020) Genetic Diversity of Bovine Pestiviruses Detected in Backyard Cattle Farms Between 2014 and 2019 in Henan Province, China. Front. Vet. Sci. 7:197. doi: 10.3389/fvets.2020.00197

Bovine pestiviruses include *Pestivirus A* (BVDV-1), *Pestivirus B* (BVDV-2), and *Pestivirus H*, which was originally called *HoBi-like pestivirus*. We conducted an epidemiological investigation for pestiviruses circulating in backyard cattle farms in central China. RT-PCR assays and sequences analysis were conducted on 54 nasal swabs, 26 serum samples, and three lung samples from cattle with respiratory infections and identified 29 pestivirus strains, including 24 *Pestivirus A* and five *Pestivirus H* strains. Phylogenetic analysis based on partial 5′-UTR and Npro sequences showed that the genotypes of 24 *Pestivirus A* strains included *Pestivirus A* 1b (six isolates), *Pestivirus A* 1m (six isolates), *Pestivirus A* 1q (two isolates), *Pestivirus A* 1u (one isolates), and *Pestivirus A* 1o (nine isolates, a putative new sub-genotype). In addition, a single *Pestivirus H* agenotype included all five *Pestivirus H* strains. This study revealed extensive genetic variations within bovine pestivirus isolates derived from cattle in backyard farms in Central China, and this epidemiological information improves our understanding of the epidemics of bovine Pestiviruses, as well as will be useful in designing and evaluating diagnostic methods and developing more effective vaccines.

Keywords: *Pestivirus A*, *Pestivirus H*, cattle, genotype, China

INTRODUCTION

Pestiviruses are single-stranded, positive-sense, enveloped RNA viruses with a genome of ∼12.3 kb, which belong to the family *Flaviviridae*, genus *Pestivirus*. According to the proposed revision to its taxonomy, the *Pestivirus* genus includes 11 species, namely *Pestivirus A* (*bovine viral diarrhea virus 1,* BVDV-1), *Pestivirus B* (*bovine viral diarrhea virus 2,* BVDV-2), *Pestivirus C* (*classical swine fever virus*, CSFV) and *Pestivirus D* (*border disease virus*, BDV), *Pestivirus E* (*pronghorn pestivirus*), *Pestivirus F* (*Bungowannah virus*), *Pestivirus G* (*giraffe pestivirus*), *Pestivirus H* (*Hobi-like pestivirus*), *Pestivirus I* (*Aydin-like pestivirus*), *Pestivirus J* (*rat pestivirus*), and *Pestivirus K* (*atypical porcine pestivirus*) (1). Among these species, *Pestivirus A*, *Pestivirus B*, and *Pestivirus*

H aroused great concern because these cause significant economic losses in the cattle industry worldwide (2–4). *Pestivirus A* and *Pestivirus B* are major viruses associated with a number of clinical manifestations that range from mild to severe in feedlot cattle, including respiratory disease, digestive disease, and/or reproductive system disturbances and suppression of the immune system (5–7). Natural infections in cattle involving *Pestivirus H* showed similar clinical signs as those of *Pestivirus A* or *Pestivirus B* infections (8–11).

To date, at least 23 genotypes of *Pestivirus A* (12–16) and six genotypes (17) of *Pestivirus B* have been classified based on sequence comparison analyses and the palindromic nucleotide substitutions (PNS) genotyping method (18, 19). *Pestivirus H*, first isolated from fetal calf serum (20), has spread to different continents, including North America, South America, Europe, and Asia (21–26). In China, nine genotypes of *Pestivirus A* (1a,1b, 1c,1d, 1m, 1o, 1p,1q, and 1u) (27–30), two genotypes (2a, 2b) of *Pestivirus B* (31–33), and *Pestivirus H* (24, 34) have been reported. Cattle production by backyard farming is a widespread cattle-keeping pattern in developing countries. In central China, which includes Henan Province, more than 3,720,000 cattle have been raised (35), and previous data showed that over 20% of cattle were kept in small farms (cattle number <10), including a large number of backyard farms in China (36), especially in the southern region of Henan Province, where free-range cattle farms are key economic sectors (35). However, the limited biosecurity measures in these farms usually lead to the introduction and spread of exotic or endemic disease (37–39). Furthermore, in backyard cattle farms in China, most of the animals graze in the wilderness, and thus come into contact with infected cattle. To our knowledge, information on the epidemiology of pestiviruses in cattle in backyard farms in China is limited. The aim of this study was to investigate the distribution of pestiviruses that are associated with respiratory disease from backyard farms in Henan Province, China.

MATERIALS AND METHODS

Samples

From November 2014 to April 2019, a total of 54 nasal swabs and 26 serum samples were collected from different cattle in 41 backyard farms in Henan Province in Central China; these animals had never been vaccinated against *Pestivirus A* and were diagnosed with respiratory infections by rural veterinarians and treated with antibiotics for days, resulting in slow recovery. In addition, three lungs of deceased calves were collected in 2015, 2016, and 2018. All samples were stored at −80°C until analysis.

Primer Selection

The nested RT-PCR primers for genotyping bovine pestiviruses, including *Pestivirus A*, *Pestivirus B*, and *Pestivirus H* (40) were used to detect the pestivirus genome in the samples. For phylogenetic analysis, the BVDV-positive samples were further subjected to 5′-UTR and Npro RT-PCR using primers 324/326 (41) and BD1/BD2 (42), respectively. Because the sequences of *Pestivirus H*-positive samples were most closely related to the HN1507 strain (43), the positive samples further subjected to RT-PCR covering a partial 5′-UTR fragment and the entire Npro region with the primers HN-F (sense; 5′-CCTTCAGTAGGACGAGCATAA-3′) and HN-R (antisense; 5′- AGACGGGCTATACCACAATAA-3′), corresponding to nt 109–1,107 of *Pestivirus H* strain HN1507 (GenBank accession number: KU563155).

RNA Extraction, Amplification, and Sequencing

The three lung samples were first homogenized, then RNA was extracted from the lung homogenates, nasal swabs, and serum samples using an EasyPure Viral DNA/RNA Kit (Transgen Biotech, China) according to the manufacturer's instructions. The RNA was resuspended in DEPC-treated water and kept until analysis. cDNA was synthesized from RNA using Easyscript Reverse Transcriptase kit (Transgen Biotech, China) using random 9-mers as reverse transcription primer.

nRT-PCR to detect the pestivirus genome was performed as described elsewhere (40). Then, the BVDV-positive samples were further subjected to 5′-UTR and Npro RT-PCR earlier described (41, 42). The *Pestivirus H*-positive samples were subjected to RT-PCR in a 50-μL reaction mixture similar to the *Pestivirus A* reaction mixture according using the following conditions: reverse transcription at 50°C for 60 min, then denaturation at 93°C for 3 min; followed by 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min; and a final extension at 72°C for 10 min. Then the amplified products were recovered from the agarose gel using a gel extraction kit (Omega Bio-Tek, China), and the purified amplicons were directly sequenced in both directions using an ABI automated A373 sequencer (ABI, USA). Lastly, all of the sequences were compared to the NCBI databases using a BLAST search.

Phylogenetic Analysis

The nucleotide regions of the 5′-UTR were compared and aligned using CLUSTAL W program. Molecular Evolutionary Genetics Analysis version 6 (MEGA6) (44) was used for phylogeny inference according to the neighbor-joining criterion and the Kimura 2-parameter model. The robustness of the hypothesis was tested with 1000 non-parametric bootstrap analyses.

Following strains were used for 5′-UTR Phylogenetic analysis: NADL [M31182] and Singer [L32875] are the references for the Pestivirus A 1a genotype, strains Osloss [M96687], and Draper [L32880] are the references for the Pestivirus A 1b genotype and strains Europa [AB000898], and F [AF298065] are the reference for the Pestivirus A 1.3 genotype. Strains 438/02 [AY159540], PT42-03 [AY944293], 23-15 [AF298059], so CP/75 [AB042661], Shitara-02-06 [LC089876], IS25CP/01 [AB359931], AQGN96B15 [AB300691], Bega [AF049221], Manasi [EU159702], KM [AF298068], G [AF298066], SD0803 [JN400273], isolate 6 [JX276543], 10-84 [AF298054], 3186V6 [AF298062], 11207/98 [AJ304390], 22146/81 [AJ304376], 2561 [JQ920287], 17P [AF244954], KS86-1ncp [AB042713], Deer [AB040132], TR70 [MG670547], TR75 [MG670549], ZM-95[AF526381], TJ0801 [GU120255], BJ1305 [KF925505], XZ-24 [KJ578918], TR-2007-Gu-175454-4695 [EU716150], TR16 [MG670548], TR72 [MG670546], J [AF298067], W [AF298073],

BJ0702 [GU120248], BJ0703 [GU120249], A [AF298064], L [AF298069], CH-01-08 [EU180024], 71-03 [KF205294], PG/13a/07 [not deposited], GXBH-EB34 [KJ578813], GXLZ-BB4 [KJ578814], 130/15-4215 [KY085998], 130/15-5364 [KY085999], Rebe [AF299317], SuwaCp [AF117699], SuwaNcp [AF117700], CH-05-b1 [EU180030], and S153 [KF006964] are references for the Pestivirus A 1.4 to Pestivirus A 1.23.

Following strains were used for Npro Phylogenetic analysis: NADL [M31182], Oregon C24V [AF091605] and SD-1 [M96751] are the references for the Pestivirus A 1a genotype, strain Osloss [M96687] is the reference for the Pestivirus A 1b genotype. Strains F [AF287284], 10JJSKR [KC757383], 23/15 [AF287279], 58-1 [KF023454], 2541 [JQ920342], so CP/75 [AB105590] are references for the Pestivirus A 1.3, 1.5 and 1.6 genotypes. Strains IS25CP/01 [AB359931], IS26NCP/01 [AB359932], Bega [AF049221], 519 [AF144464], Deer-NZ1 [U80903], G [AF287285], CH-SM09/20 [AY895007], SD0803 [JN400273], isolate 6 [KC207072], 3186V6 [AF287282] and 26-V639 [AF287282] are references for the Pestivirus A 1.7 to Pestivirus A 1.11 genotypes. Strains Deer-GB1 [U80902] and KS86-1ncp [AB078950] are references for the genotypes Pestivirus A 1.13. Strains TR70 [KF154779], TR73 [KF154777] and TR75 [KF154778], reported as genotype R (Yesilbag et al., 2014), are references for genotype 1.14. Strains BJ1305 [KF925522], TJ0801 [GU120262] and ZM-95 [AF526381] are references for the genotype Pestivirus A 1.15. Strains TR16 [EU163964], TR27 [EU163975], TR29 [EU163977] and TR72 [KF154776] are references for the genotype Pestivirus A 1.16. Strains J [AF287286], W [AF287290], BJ0701 [GU120259], BJ0702 [GU120260], BJ0703 [GU120261], A [AF287283], L [AF287287], CH-01-08 [EU180033], 71-03 [KF205326], M31182 [JQ799141], 441/09 [KY040435], CH-Bohni [AY894997] and CH-Suwa [AY894998] are references for the Pestivirus A 1.17 to Pestivirus A 1.22 genotypes.

RESULTS

Using first-round nRT-PCR to identify bovine pestivirus by amplification of a 1,013-bp fragment, 83 samples were screened and the pestivirus genome was detected in 17 out of the 54 nasal swab samples, 10 out of the 26 serum samples, and two out of three lung samples. The results of the second-round nRT-PCR showed that in 17 positive nasal swab samples, 14 were positive for *Pestivirus A* and the other three were positive for *Pestivirus H*; in the 10 positive serum samples, nine were positive for *Pestivirus A* and one was positive for *Pestivirus H*; in the two positive lung samples, one was positive for *Pestivirus A* and the other was positive for *Pestivirus H*; no *Pestivirus B*-positive samples were detected. The 29 pestivirus-positive samples are presented in **Figure 1**.

The sequences detected by 5′-UTR and Npro RT-PCR in 24 BVDV-positive samples were deposited in GenBank under accession numbers: MN442360–MN442383 and MN442389–MN442412. Sequence alignment of the 5′-UTR and Npro region of the 24 samples using CLUSTAL W indicated a sequence identity within the range 82.0–100% and 70.0–99.8%, respectively. BLAST analysis of the 5′-UTR and Npro sequences showed that all 24 BVDV-positive isolates belonged to *Pestivirus A*. The comparative analysis among Henan isolates and the reference strains of *Pestivirus A* (NADL, VEDEVAC) shared a 5′-UTR and Npro region sequence identity within the range 82.9–98.8%, 72.2–97.6%, respectively.

The sequences detected by 5′-UTR and Npro RT-PCR in the five *Pestivirus H*-positive samples were deposited in GenBank as accession numbers MN442384–MN442388 and MN442413–MN442417. Sequence alignment using CLUSTAL W program of the five samples revealed a 5′-UTR and Npro region respective sequence identity within the range 95.8–99.5% and 98.4–99.6%. BLAST analysis of the 5′-UTR and Npro sequences showed that all five *Pestivirus H*-positive isolates belonged to *Pestivirus H*. Comparative analysis of the 5′-UTR and Npro region of the Henan isolates and the reference strains of *Pestivirus H* (TH/04_KhonKaen, HN1507) revealed a sequence identity within the range of 87.5–99.5% and 90.3.2–99.4%, respectively. In particular, the nucleotide homologies between these isolates and the other strain (HN1507) (24, 43) isolated from goat in the same area were 97.9±1.6% and 98.6 ± 0.4% in the above two regions.

All 24 isolates from the BVDV-positive samples were classified as *Pestivirus A*, and on the basis of phylogenetic analysis of 5′-UTR and Npro genes (**Figure 2**) further classified into five genotypes: *Pestivirus A* 1b (six isolates), *Pestivirus A* 1m (six isolates), *Pestivirus A* 1q (two isolates), *Pestivirus A* 1u (one isolate), and the other nine *Pestivirus A* isolates cluster in the same genotype with Chinese strain XH-2 which was assigned as *Pestivirus A* 1o, but phylogenetic analysis showed this cluster of isolates were under different cluster from other *Pestivirus A* 1o strains, further analyzed by the PNS software which available at www.pns-software.com (45), these cluster should be members of a new sub-genotype (1.7.2) within the genotype 1o (1.7). In addition, five isolates from *Pestivirus H*-positive samples were classified as *Pestivirus H*, and all further were classified into genotype "*Pestivirus H* a" based on the results of phylogenetic analysis of the 5′-UTR and Npro genes (46) (**Figure 2**).

DISCUSSION

China is one of the countries that have the largest domesticated ruminant population in the world, including a large number of backyard farms (35). In 2017, commercial *Pestivirus A or Pestivirus B* vaccines were released to the market. However, vaccination is not mandatory, and awareness of the importance of immunization to prevent Pestivirus infections among backyard farm keepers is rare, despite the occurrence of pestivirus epidemics in large-scale farms in China in recent years (27–29, 47). It is thus essential to investigate the genetic diversity of pestiviruses in backyard farms. Furthermore, the new genotype pestivirus might result in the immune failure of pestivirus vaccine (48). For these above reasons, the genetic diversity of 29 pestivirus-positive samples derived from infected calves in backyard farms from central China was investigated by phylogenetic analysis of the 5′-UTR and Npro partial genomic regions.

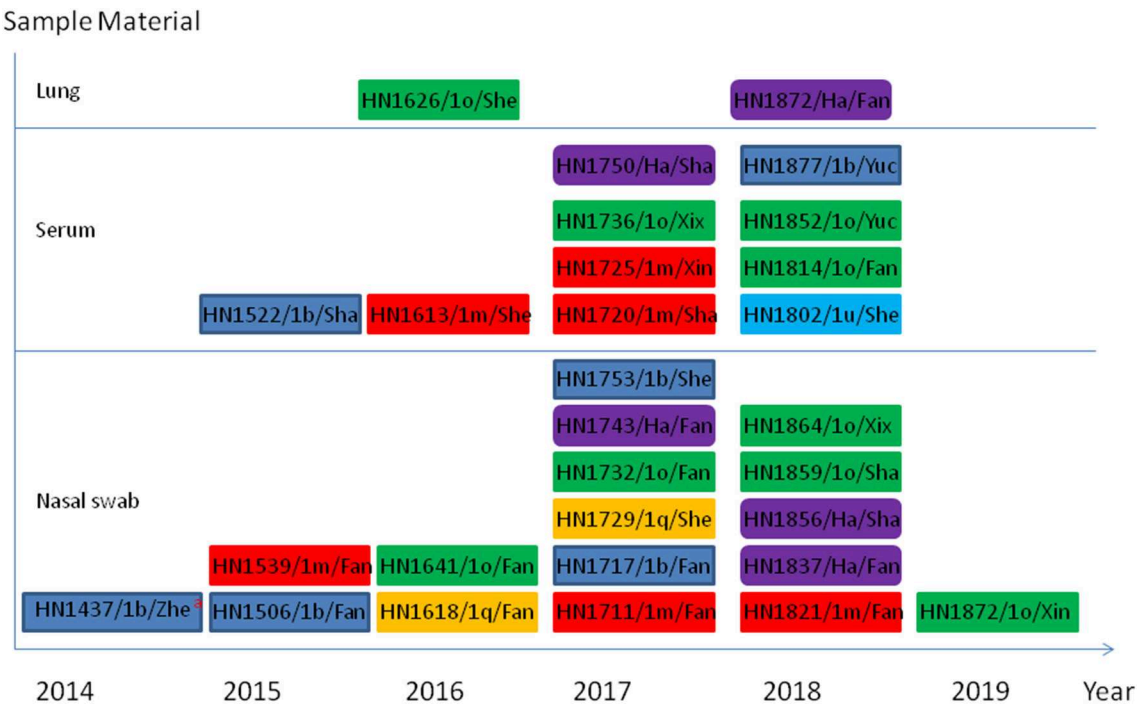

FIGURE 1 | Schematic representation of 29 bovine *Pestivirus* positive in this work. [a]indicate the information of sample including sample number/genotype/county. The county names: Zhe, Zhenping; Fan, Fangcheng; Sha, Shangcai; She, Sheqi; Xin, Xinye; Xix, Xixia; Yuc, Yucheng. The blue rectangle represents *Peativirus A* 1b genotype; the red rectangle represents *Peativirus A* 1m genotype; the yellow rectangle represents *Peativirus A* 1q genotype; the green rectangle represents *Peativirus A* 1o genotype; the light blue rectangle represents *Peativirus A* 1u genotype; the purple rounded rectangle represents *Peativirus H* a genotype.

The results of phylogenetic analysis showed that *Pestivirus A*−1o, *Pestivirus A*−1b, and *Pestivirus A*−1m were the predominant genotypes in our samples, followed by *Pestivirus A*−1q and *Pestivirus A*−1u. Furthermore, among these three predominant genotypes, *Pestivirus A*−1b and *Pestivirus A*−1m are frequently reported in China (27–30, 49, 50) the *Pestivirus A* 1a found in Henan province (51) was not detected in this work.

Six isolates of the *Pestivirus A* 1b genotype were detected in Zhenping, Fangcheng, Shangcai, Sheqi, and Yucheng in this study (**Figures 1**, **2**). This widespread distribution of *Pestivirus A* is not surprising, as the first genotype of *Pestivirus A* was isolated in China in 1983. Subsequently, *Pestivirus A*−1b was later detected in most of the provinces, including Henan Province and the neighboring provinces numerous reports (27–29, 47, 49, 52–55). Meanwhile, a recent study has shown that 31.6% (2193:6939) of the corresponding *Pestivirus A* isolates around the world were *Pestivirus A*−1b (16). These reports indicate the strong spreading ability of *Pestivirus A*−1b among large-scale farms, and the high detection rate (6/24) in our study also suggests that *Pestivirus A*−1b is a predominant genotype among backyard farms.

Six isolates of the *Pestivirus A* 1m genotype were detected in Fangcheng, Sheqi, Shangcai, and Xinye in this study (**Figures 1**, **2**). The first *Pestivirus A*−1m strain ZM-95 in China was isolated from pigs in 1995 (56). Subsequent reports revealed that *Pestivirus A*−1m is emerging in most of provinces and is considered to be a predominant *Pestivirus A* genotype in herds (27, 30, 50, 54, 57), and also detected in Henan province and neighbor province (29). In addition, sequences of *Pestivirus A*−1m strains in different regions showed high-nucleotide homology, indicating that these strains share the same origin (27, 49). Recently, other surveys on goats uncovered that the *Pestivirus A*−1m could infect goats naturally and cause diarrhea (58). In this study, *Pestivirus A* 1m strains were detected in different backyard farms that shared grassland with goats, this feeding method provided more chances for interspecies transmission of BVDV-1m, further accelerating the evolution of these viruses and more widely spreading disease.

The other nine *Pestivirus A* isolates shared the highest sequence identities (97–98%) in strains such as XH-6, XH-5, XH-1, XH-2, and BJ09 that were isolated from other provinces in China which was assigned as *Pestivirus A* 1o. Furthermore, the Npro sequences of these strains were not found in GenBank, and thus, confirmation could not be done based on the Npro phylogenetic tree analysis. The Npro sequences of the 21 isolates were also analyzed by BLAST, and the highest identities (86–91%) were observed in strain IS26/01ncp from Japan, and BJ0703, BJ0702, BJ0701, and JS12/02 that were isolated from other provinces including the Jiangsu province near to the Henan province in China and were classified as BVDV-1o or BVDV-1p (27, 58, 59). Then the PNS method was used to analyze the existing strain such as XH-2, then the genotype *Pestivirus A* 1.7 (1o) was verified, but the cluster in phylogenetic tree this cluster

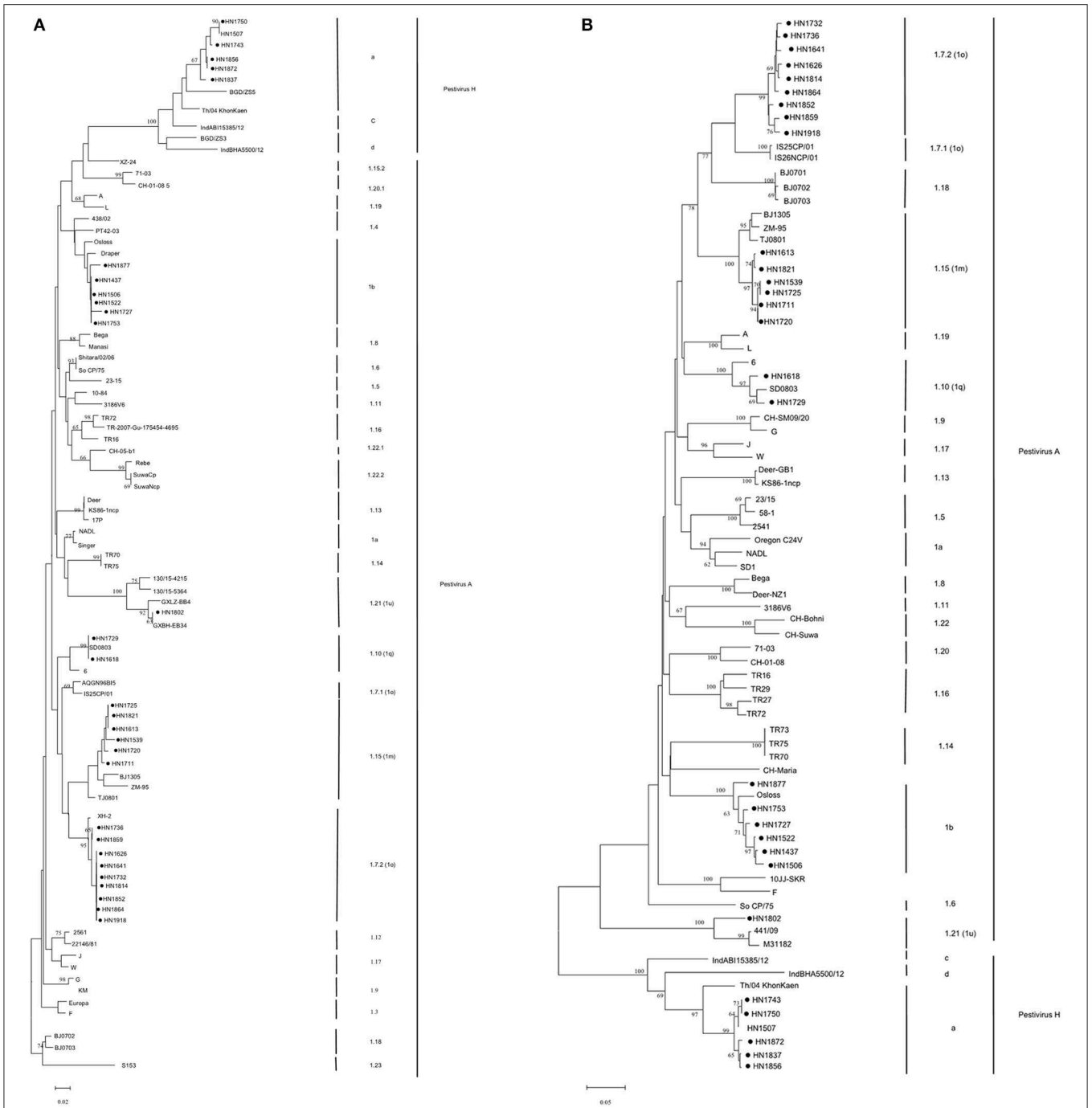

FIGURE 2 | Phylogenetic analysis of bovine *Pestivirus* from cattle in backyard farms in Central China, and reference strains using the 5′-UTR **(A)** and Npro **(B)** sequences. Bootstrap analyses that were supported by >60% of 1,000 replicates are indicated in nodes. Henan pestivirus isolates in this work are highlighted with a symbol (•).

was in different cluster from other *Pestivirus A* 1.7 (1o) strains, this result indicated these strains form a new sub-genotype (1.7.2). (**Figures 2A,B**). *Pestivirus A* 1o was first isolated from a calf that developed a mucosal disease and from PI calves in Japan (60), and has been detected in camels, goats, and pigs in China (30, 50, 58). In this study the new sub-genotype *Pestivirus A* 1o were detected in different backyard farms in a few of counties, and the *Pestivirus A* 1o could infect goats and sheep, it is in need that necessary measures should be taken to avoid this new sub-genotype *Pestivirus A* 1o spread in other hosts.

Five isolates of the *Pestivirus H* "a" genotype were detected in Fangcheng and Shangcai in this study (**Figures 1**, **2**). Before

this research, Pestivirus H has been previously detected in goats and sheep in Fangcheng (24), and phylogenetic analysis showed that these five isolates were closely related to the HN1507 strain isolated from goat (43). These results indicated that these isolates shared the same origin. Furthermore, considering that these strains were all isolated from animals raised in backyard farms where livestock commonly grazed in the mix, this specific feeding method provides a convenient route for interspecies transmission of *Pestivirus H*. To date, in China, *Pestivirus H* has been reported in contaminated cells, commercial FCS, goats, and sheep (24, 34, 61). This study showed that *Pestivirus H* could be detected in cattle immediately after being detected in goats and sheep in Central China (24).

Bovine pestivirus isolates in backyard farms exhibited a high level of genetic diversity, as indicated in the novel epidemic genotypes of *Pestivirus A* and *Pestivirus H* first emerging in cattle in China. These results indicate that backyard cattle farms could be a special reservoir for the evolution of bovine Pestivirus and provide an important complement to understand the epidemics of bovine Pestivirus. Furthermore, this study will be useful in designing and evaluating diagnostic methods and in developing more effective vaccines.

CONCLUSION

Several genotypes of *Pestivirus A* and *Pestivirus H* infections were identified in cattle with respiratory diseases and kept in backyard farms by RT-PCR, sequencing, and phylogenetic analysis. This is the first report on the molecular evidences on natural infections of *Pestivirus H* in cattle in China.

DATA AVAILABILITY STATEMENT

Datasets are in a publicly accessible repository: The datasets generated for this study can be found in GenBank: https://www.ncbi.nlm.nih.gov/genbank/. The Genbank accession numbers are mentioned in the Results of the article.

ETHICS STATEMENT

The processes of nasal swabs and blood from cattle were approved by their hosts, and all lungs were from animals found dead in the study. The study was approved by the Animal Welfare and Ethics Committee of Nanyang Normal University (No 14027).

AUTHOR CONTRIBUTIONS

HS participated in the design of the study, and drafted the main parts of the manuscript. HL, YZ, and LYan participated in the sample collection and PCR detection. YH, ZW, LD, and CL participated in the data analyzing. BY and LYao participated in revised the manuscript and supervised the project.

FUNDING

The National Natural Science Foundation of China (Grant no. 31902263 and 31870917), the program for Innovative Research Team of Science and Technology in University of Henan Province (No. 20IRTSTHN024), the Henan Provincial Scientific and technological research project (Grant no. 182102110084) and Nanyang Normal University (CN) (Grant no. 15081) supported this study.

ACKNOWLEDGMENTS

We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 24 April 2020
doi: 10.3389/fvets.2020.00188

Genetic Variability of 3′-Proximal Region of Genomes of Orf Viruses Isolated From Sheep and Wild Japanese Serows (*Capricornis crispus*) in Japan

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *20 January 2020*
Accepted: *23 March 2020*
Published: *24 April 2020*

Citation:
Shimizu K, Takaiwa A, Takeshima S, Okada A and Inoshima Y (2020) Genetic Variability of 3′-Proximal Region of Genomes of Orf Viruses Isolated From Sheep and Wild Japanese Serows (Capricornis crispus) in Japan. Front. Vet. Sci. 7:188. doi: 10.3389/fvets.2020.00188

Orf virus is a prototype species of the genus *Parapoxvirus*, subfamily *Chordopoxvirinae*, family *Poxviridae*. Japanese orf viruses, infecting sheep and wild Japanese serows (*Capricornis crispus*), have been considered to be genetically closely related based on the sequence identities of the open reading frames (ORFs) 11, 20, and 132 in their genomes. However, since the genome size of orf viruses is about 140 kbp long, genetic variation among Japanese orf viruses remains unclear. In this study, we analyzed the sequences of ORFs 117, 119, 125, and 127 located in the 3′-proximal region of the viral genome using two strains from sheep and three strains from Japanese serows isolated from 1970 to 2007, and compared them with the corresponding sequences of reference orf viruses from other countries. Sequence analysis revealed that ORFs 125 and 127, which encode the inhibitor of apoptosis and viral interleukin (IL)-10, respectively, were highly conserved among the five Japanese orf viruses. However, high genetic variability with deletions or duplications was observed in ORFs 117 and 119, which encode granulocyte macrophage colony-stimulating factor and IL-2 inhibition factor (GIF), and inducer of cell apoptosis, respectively, in one strain from sheep and two strains from Japanese serows. Our results suggest that genetic variability exists in Japanese orf viruses even in the same host species. This is the first report of genetic variability of orf viruses in Japan.

Keywords: genetic variability, Japanese serows, nucleotide sequence, orf virus, sheep

INTRODUCTION

Orf virus is a prototype species of the genus *Parapoxvirus*, subfamily *Chordopoxvirinae*, family *Poxviridae* (1). Orf virus has a linear double-stranded DNA genome (134–139 kbp) with high GC content (∼63–64%) and encodes 132 putative gene products (2). Orf virus is the causative agent of orf disease, also known as contagious pustular dermatitis, contagious ecthyma, or scabby mouth mainly in sheep and goats, and can be transmitted to humans (3). In Japan, the first reports of orf virus infections in sheep and wild Japanese serows (*Capricornis crispus*) were published in

1952 (4) and 1979 (5), respectively. Previously, we have reported nucleotide sequence homology in three open reading frames (ORFs) 11, 20, and 132 among 13 orf viruses isolated or polymerase chain reaction (PCR)-detected from sheep and wild Japanese serows (6). These ORFs encode viral envelope (7), virus interferon resistance (8), and viral vascular endothelial growth factor (VEGF) (9), respectively. The amino acid sequences derived from ORFs 11 and 20 were identical among the 13 orf viruses, and only one amino acid substitution was found in ORF 132 in an orf virus isolated from sheep (6). Therefore, the three viral genes of Japanese orf viruses are highly conserved. However, since only a part of the whole genome (∼140 kbp) has been sequenced so far, the degree of genetic variation in other regions remains unclear.

To explore genetic differences between Japanese orf viruses, we conducted next-generation sequencing (NGS) of some strains of these orf viruses. However, whole genome sequences were not obtained, due to the large number of unmapped reads in the 3′-proximal region of viral genome (**Figure 1**). We hypothesized that the 3′-proximal region of a viral genome has genetic variation. Thus, in the present study, we characterized four ORFs 117, 119, 125, and 127, which are located in the 3′-proximal region of the viral genome. ORFs 117, 119, 125, and 127 encode granulocyte macrophage colony-stimulating factor and interleukin 2 (IL-2) inhibition factor (GIF) (11), inducer of cell apoptosis (12), inhibitor of apoptosis (13, 14), and viral IL-10 (15), respectively.

MATERIALS AND METHODS

Viruses

For epidemiologic and genetic characteristics of Japanese orf virus, five Japanese strains isolated from 1970 to 2007 were used. Two strains of Iwate (16) and HIS (17) were isolated from sheep and three strains of S-1 (18), R-1 (19), and GE (6) were isolated from wild Japanese serows (**Table 1**). Viruses were propagated in fetal lamb lung cells (kindly provided by Dr. H. Sentsui, Nihon University, Japan) at 37°C in Dulbecco's modified Eagle's medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (PAA Laboratories, Pasching, Austria).

Whole Genome Re-sequencing and Assembly

Total DNA extracted from virus-infected cells using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) were used for constructing the libraries using Nextera XT DNA sample Prep Kit (Illumina, San Diego, CA, USA), and sequenced using an Illumina MiSeq (Illumina). Obtained short read sequences collected in FASTQ files were aligned to the orf virus strain NZ2 (DQ184476) as the reference genome using Burrows–Wheeler transformation (BWA) ver 0.7.12-r103 software (25) and constructed binary version of the sequence alignment/map (bam) file using SAM tools ver. 0.1.19-96b5f2294a software (26).

Abbreviations: GIF, granulocyte-macrophage colony-stimulating factor and IL-2 inhibition factor; IL-2, interleukin 2; ORF, open reading frame; PCR, polymerase chain reaction; VEGF, vascular endothelial growth factor.

Analysis of ORF

DNA was extracted from virus-infected cells using a DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's instructions. Four different PCRs were carried out with GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA) using Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR conditions and analyzed ORFs are provided in **Supplementary Table S1** and **Figure 1**, respectively. PCR products were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Duren, Germany), and the nucleotide sequences were determined by direct sequencing using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA). Sequence analysis was carried out using the software Genetyx-Win version 13 (Genetyx, Tokyo, Japan), and phylogenetic analysis was performed using the MEGA7 program (27). Phylogenetic trees were constructed using maximum likelihood methods, and the reliability of the branches was evaluated by 1,000 replicates. Sequence and phylogenetic analyses were compared with the reference orf viruses (**Table 1**).

RESULTS

NGS was performed using total DNA extracted from the concentrated virus. However, whole genome sequences were not obtained, possibly due to the large number of deletion in the 3′-proximal region of viral genome (**Figure 1**).

Specific PCR products of 799 bp for ORF 117 and 537–652 bp for ORF 119 were obtained from the four strains (Iwate, HIS, S-1, and GE) and from all five Japanese orf viruses, respectively. High genomic variability was seen in ORFs 117 and 119 in Japanese orf viruses. In ORF 117, 96.6–100% nucleotide identity was observed among four strains. Surprisingly, R-1 strain from Japanese serow completely lacked ORF 117 (**Figure 2A**). Partial deletion in ORF 117 was also observed in the amino acid sequences in reference orf virus strain NA1/11 isolated from sheep in China. In ORF 119, deletions were observed in the first half of the amino acid sequences in S-1 and R-1 strains as well as the reference Chinese NA1/11 strain (**Figure 2B**). Two and 12 amino acid deletion was observed in HIS and three reference strains from sheep and goat (NZ2, IA82, and YX), respectively. In the Iwate strain, 10 amino acids were found to be duplicated.

Specific PCR products of 522 and 561 bp were obtained for ORFs 125 and 127 from all of five Japanese orf viruses. Amino acid sequences derived from these ORFs from four Japanese orf viruses (Iwate, S-1, R-1, and GE) were found to be 100% identical. The sequence from HIS strain revealed only two and seven amino acid substitutions in ORFs 125 and 127, respectively (**Supplementary Tables S2, S3**). The sequences of ORFs 125 and 127 were highly conserved among Japanese orf viruses. In the phylogenetic analysis, there were mainly two branches, and all Japanese orf viruses were classified into the same group (**Figure 3**). Our results indicate that Japanese orf viruses are closer to the IA82 and NZ2 strains isolated in the United States and New Zealand, respectively, than other reference strains. Sequences obtained in this study were

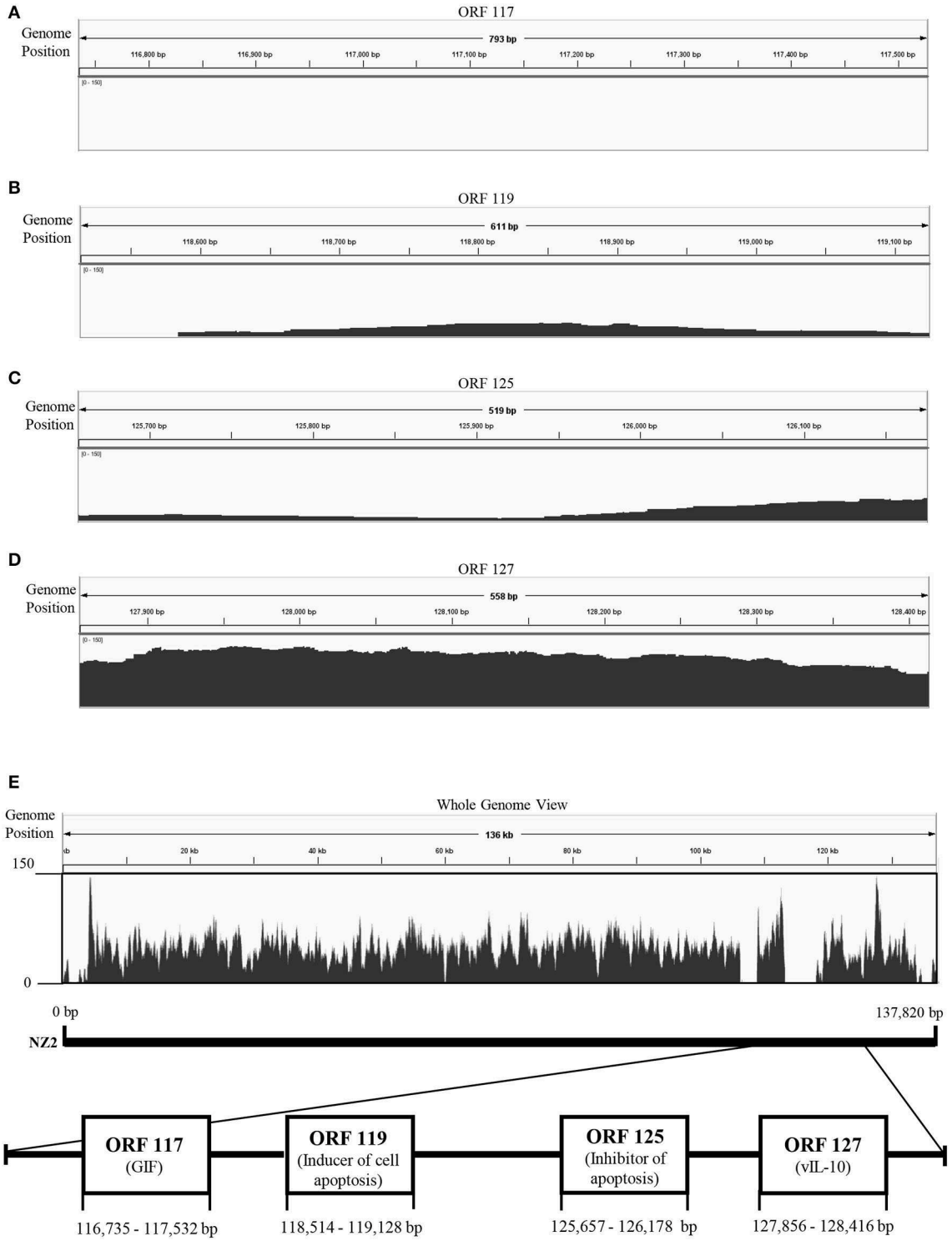

FIGURE 1 | Visualization of next-generation sequencing (NGS) coverage of Japanese orf virus R-1 strain and the details of the open reading frame (ORF) analyzed in this study. NZ2 strain (GenBank accession no. DQ184476) was used as the reference strain. Binary version of the sequence alignment/map (bam) file was loaded onto the Integrated Genome Viewer (IGV) (10). The vertical axis shows the number of reads mapping to each location of the genome. Zoomed view of ORF 117 **(A)**, ORF 119 **(B)**, ORF 125 **(C)**, and ORF 127 **(D)**. Whole genome view **(E)**.

TABLE 1 | Japanese and reference orf viruses used in this study.

Strain	Host	Year of isolation	Country	Deposited and reference accession no.[a]				References
				ORF 111–119	ORF 125	ORF 127	Complete genome	
Iwate	Sheep	1970	Japan	LC487906	LC476578	LC476583		(16, 20, 21)
HIS	Sheep	2004	Japan	LC476574	LC476579	LC476584		(17, 21)
S-1	Japanese serow	1985	Japan	LC476575	LC476580	LC476585		(18, 20, 21)
R-1	Japanese serow	1999	Japan	LC476576	LC476581	LC476586		(19, 20)
GE	Japanese serow	2007	Japan	LC476577	LC476582	LC476587		(6)
NZ2	Sheep	1982	New Zealand				DQ184476	(2)
IA82	Sheep	1982	USA				AY386263	(22)
SA00	Goat	2000	USA				AY386264	(22)
NA1/11	Sheep	2011	China				KF234407	(23)
GO	Goat	2012	China				KP010354	(24)
YX	Goat	2012	China				KP010353	(24)

[a] *ORF, open reading frame.*

submitted to DDBJ/EMBL/GenBank, and the accession numbers are given in **Table 1**.

DISCUSSION

In this study, we carried out ORF sequence analysis for five Japanese orf viruses, and our results revealed that the sequences of ORFs 125 and 127 were highly conserved. However, high genomic variability was seen in ORFs 117 and 119. Observed genetic variability was found to be the 3′-proximal region of Japanese orf viruses. To the best of our knowledge, this is the first report on the genetic variability of Japanese orf viruses.

In the phylogenetic analysis of ORF 125, Japanese orf viruses isolated from Japanese serows were classified into a group isolated from sheep. It has been reported that analyses of the phylogenetic tree of 47 ORFs including ORF 125 were found to assist in easily distinguishing between goat- and sheep-originated orf viruses (24). These results indicate a possibility that sheep orf virus may have infected Japanese serows. Furthermore, analyses of the phylogenetic tree of ORFs 125 and 127 clearly showed that the Japanese orf viruses were closer to IA82 and NZ2 strains than to other reference strains. In Japan, sheep are frequently imported from the United States and New Zealand for improved growth and to encourage breeding (28). Therefore, it is possible that these orf viruses came along with the imported animals and were introduced into breeding sheep and wild Japanese serows in Japan.

Our results showed genetic variability in ORFs 117 and 119 in the Japanese orf viruses, suggesting that there is heterogeneity even in viruses infected with the same host species. In addition, deletions in ORF 119 were observed in Japanese (HIS, S-1, and R-1) and reference (NZ2, IA82, NA1/11, and YX) strains. Based on the previous comparative analysis, it is presumed that genes in the central region of the orf virus genome are more conserved, whereas those in the terminal region show remarkably high variability (29). Notably, this variability is accompanied by a high frequency of gene recombination and nucleotide deletions (23). The genetic analysis of ORFs 117 and 119 may help to characterize or differentiate strains that are otherwise shown to be identical by the envelope coding genes (30). A previous study demonstrated that viruses with high deletion in ORFs 114–120 showed low virulence in animal inoculation experiments and that genomic deletions attenuate virulence (24). At present, the relationship between the deletion of ORF 117 and virulence in the R-1 strain is unknown. Therefore, there is a need to analyze the correlation between genetic variability and virulence in more detail.

In this study, it was revealed that there were differences in conservation and variability among ORFs. Viral IL-10 encoded by ORF 127 shares remarkable similarity to mammalian IL-10. Mammalian IL-10 is highly conserved across all mammalian species (15). IL-10 is a multifunctional cytokine that has suppressive effects on inflammation, antiviral responses. Orf virus produces viral IL-10 by itself and avoids host's inflammatory and immune response by it (31). This suggests that viral IL-10 encoded by ORF 127 might require high conservation in orf virus. On the other hand, GIF encoded by ORF 117 does not resemble any known mammalian granulocyte-macrophage colony–stimulating factor (GM-CSF)- or IL-2-binding proteins, and indeed, there are no reports of any other protein capable of binding both GM-CSF and IL-2. In addition, human GM-CSF does not respond in sheep cells due to its inability to bind to ovine receptor (32). Therefore, GIF was thought to have evolved a unique binding specificity in sheep, the natural host of the orf virus (33). This suggests that GIF encoded by ORF 117 is gene whose necessity changes depending on the host species. It is thought that differences in necessity of gene may affect conservation and variability of the gene encoded by ORFs.

Japanese serows are wild animals and a natural monument that is endemic in Japan (34). Japanese serows are often witnessed in mountain villages and can come into contact with livestock sheep. There have been reports that a single strain of orf virus caused outbreak of proliferative dermatitis in various ruminant species at a zoo (35). Orf virus from Japanese serows can be

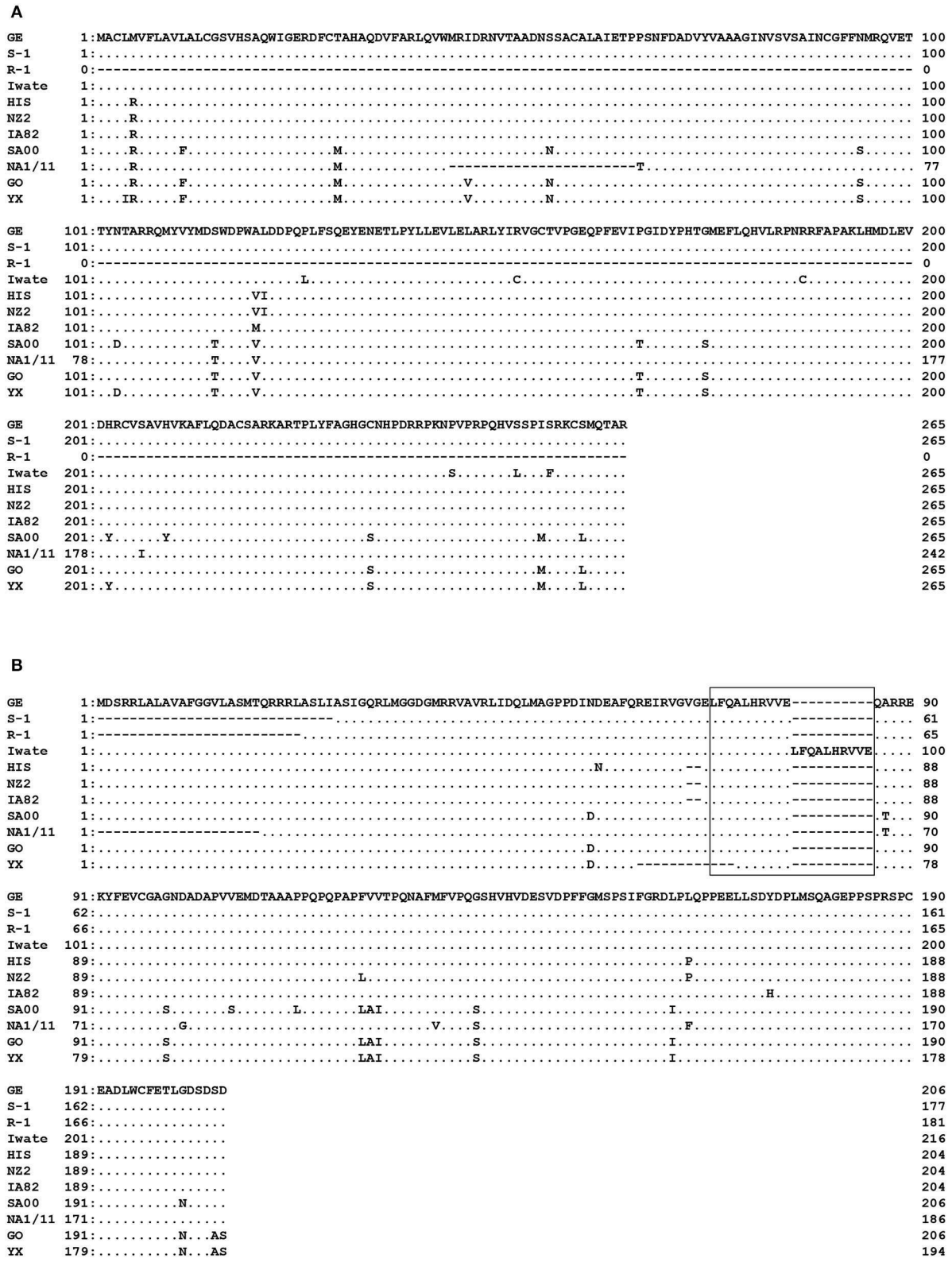

FIGURE 2 | Alignment of the amino acid sequences derived from ORF 117 **(A)** and ORF 119 **(B)**. Amino acids identical to GE strain at the given positions are represented by dots. R-1 strain completely lacked ORF 117. In ORF 119, deletions were observed in the first half of the nucleotide sequences in S-1 and R-1 strains. In the box, duplicate in Iwate strain is shown.

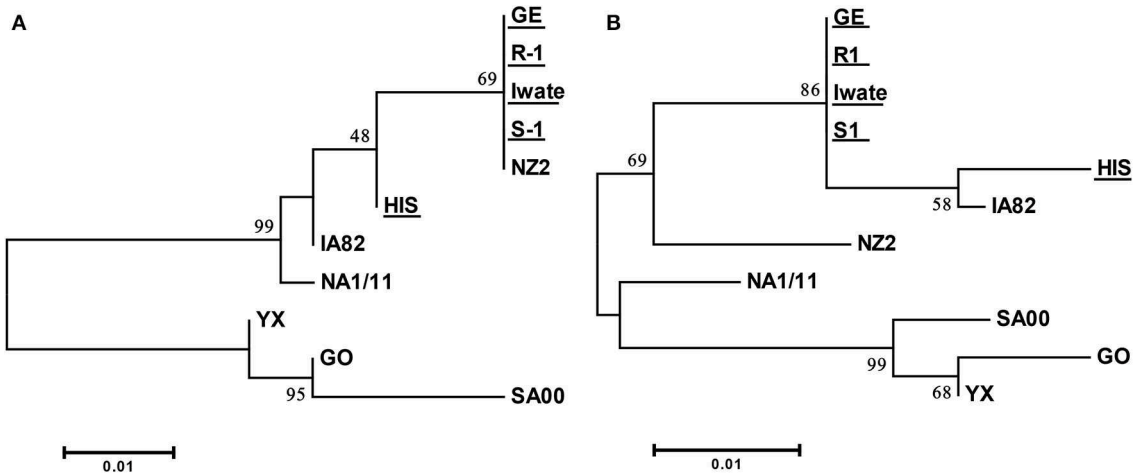

FIGURE 3 | Phylogenetic tree based on deduced amino acid sequences derived from ORF 125 **(A)** and ORF 127 **(B)**. Strains used in this study are underlined. The percentage bootstrap values calculated from 1,000 replicates are indicated above the internal nodes.

spread to sheep or farmers, or orf virus from sheep can be spread to Japanese serows. It is important to know the characteristics of Japanese orf viruses in order to reduce the spread risk.

We tried NGS analysis, but it was unsuccessful. NGS results indicated the 3′-proximal region of the genome of Japanese orf viruses has genetic variation. Our results obtained by Sanger sequencing for variable region of Japanese orf viruses may be useful for understanding the characteristics of these viruses. However, we analyzed the limited region of the viral genomes, and sequencing other regions using improved methods for NGS might be required to better understand the characteristics of Japanese orf viruses.

DATA AVAILABILITY STATEMENT

Sequence data obtained in this study is available in the DDBJ/EMBL/GenBank (accession nos. LC476574–LC476587 and LC487906).

AUTHOR CONTRIBUTIONS

KS and YI analyzed all data and were major contributors in writing the manuscript. KS, AT, ST, and AO performed the nucleotide/amino acid sequencing and phylogenetic analysis. All authors read and approved the final manuscript.

FUNDING

This study was partly supported by the Grant for Joint Research Program of the Research Center for Zoonosis Control, Hokkaido University, from the Ministry of Education, Culture, Sports, Science and Technology, Japan. This study was also supported, in part, by the Research Grant from The Okawa Foundation and by the Grant for Environmental Research Projects from The Sumitomo Foundation.

ACKNOWLEDGMENTS

We would like to acknowledge Dr. Hiroshi Sentsui (Nihon University, Japan) for providing fetal lamb lung cells.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00188/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 30 April 2020
doi: 10.3389/fvets.2020.00200

Comparison of Multiplex Real-Time PCR and PCR-Reverse Blot Hybridization Assays for the Direct and Rapid Detection of Porcine Circovirus Type 2 Genotypes

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Porcine circovirus type 2 (PCV2), the causative agent of porcine circovirus-associated diseases (PCVAD), poses a serious economic threat for the swine industry. Currently, PCV2 is classified into five major genotypes: PCV2a, PCV2b, PCV2c, PCV2d, and PCV2e. The aim of this study is to evaluate the performance of two commercially available methods, multiplex real-time PCR assay and PCR-reverse blot hybridization assay (REBA), for the rapid detection of PCV2 and direct identification of PCV2 genotypes from clinical samples as well as to compare the results with that of sequence analysis. Molecular diagnostic methods were used to evaluate a total of 180 samples, including tissues and blood samples from pigs that were suspected of PCVAD infection. The results of this study showed that the detection rate for positive PCV2 was 48.3% ($n = 87$) in both multiplex real-time PCR and PCR-REBA methods. Using sequence analysis, which is the gold standard, and multiplex real time PCR assay, the sensitivity, specificity, positive predictive value, and negative predictive value of PCV2 genotyping were found to be 97.1% ($n = 67$, 95% CI 0.894–0.998, $p < 0.001$), 100% ($n = 93$, 95% CI 0.966–1.000, $p < 0.001$), 100% (95% CI 0.953–1.000, $p < 0.001$), 97.9% (95% CI 0.921–0.998, $p < 0.001$), respectively. The results of PCR-REBA were found to be consistent with those of sequence analysis for all the samples and showed good agreement ($\kappa = 1$). The most prevalent genotypes detected in this study were PCV2d ($n = 53$, 60.9%), followed by PCV2a ($n = 17$, 19.5%), PCV2b ($n = 14$, 16.1%), and PCV2a/b co-infection ($n = 3$, 3.5%). Both the methods required ∼3 h for completion. Therefore, we conclude that two molecular methods are rapid and reliable for the characterization of the causative pathogen with PCV2 genotypes.

Keywords: porcine circovirus type 2, multiplex real-time PCR, PCR-REBA, ORF2, diagnosis

OPEN ACCESS

Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

Reviewed by:
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Francisco Rivera-Benítez, National Institute of Forestry, Agriculture and Livestock Research (INIFAP), Mexico

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *28 January 2020*
Accepted: *25 March 2020*
Published: *30 April 2020*

Citation:
Wang H, Song JK, Shin S and Kim H (2020) Comparison of Multiplex Real-Time PCR and PCR-Reverse Blot Hybridization Assays for the Direct and Rapid Detection of Porcine Circovirus Type 2 Genotypes. Front. Vet. Sci. 7:200. doi: 10.3389/fvets.2020.00200

INTRODUCTION

Different from the non-pathogenic porcine circovirus (PCV) type 1 strain (1), PCV type 2 is considered to be an important emerging pathogen that causes porcine circovirus associated diseases (PCVAD) including postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), enteritis,

reproductive failure (2, 3), and one of the most economically important swine diseases worldwide (4). Whenever there are outbreaks of respiratory clinical signs, wasting, and granulomatous inflammation of lymphoid tissues in pigs, PMWS is clinically suspected (5). PCV2-associated systemic infection is clinically characterized by wasting, dyspnea, and lymphadenopathy, and in some cases, might be associated with diarrhea, pallor, and jaundice (6). PCV2, belonging to the genus *Circovirus* of the family *Circoviridae*, is a small non-enveloped virus with a circular single-stranded DNA genome (7). The PCV2 genome is ∼1.7 kb nucleotide long and encodes for two open reading frames (ORFs) (8, 9). In the viral genome, ORF1 codes for the replicase (Rep) protein and ORF2 for the capsid (Cap) protein. Rep is a non-structural protein and is responsible for the viral replication, while the structural Cap protein controls the immunogenicity of the virus (10–13). With emerging viral strains, PCV2 has undergone much genetic variation in recent years and has been divided into five genotypes, namely PCV2a-e strains, which are classified based on the diversity level of the ORF2 nucleotide sequences (14, 15). Continuous mutations in the PCV2 genome have made the identification of PCV more difficult, especially by traditional molecular detection methods (16, 17). It has also been demonstrated experimentally that subclinical PCV2 infection might be associated with decreased vaccine efficacy (18). Therefore, PCV2 subclinical infection is not only the most common form of infection in pigs but is also resistant to the effect of vaccines. Hence, rapid and early identification of PCV2 subclinical infection is very important for the effective prophylaxis against PCVAD (19).

Until now, commercial diagnostic tests based on ELISA (9, 12) and PCR (19) have only been developed to confirm the presence or absence of PCV2. Although it has the advantage of being able to detect PCV2 in a short time, it is required expensive antibodies for diagnostic purposes, and the PCV2 genotypes cannot be distinguished simultaneously, so most PCV2 genotypes have been identified separately using PCR-based Restriction Fragment Length Polymorphism (RFLP) (20) or sequence analysis (13, 17, 20–22). In this study, a novel diagnostic assay based on multiplex real-time PCR (Opti PCV2-genotyping; Optipharm, Osong, Republic of Korea) was developed for the rapid and accurate identification of PCV2 as well as to discriminate between the PCV2 a/e, b, and d genotypes. The PCR-based reverse blot hybridization assay (PCR-REBA, REBA PCV2-genotyping; Optipharm) was to detect PCV2 and distinguish between PCV2a, PCV2b, PCV2c, PCV2d, and PCV2e genotypes. In this study, we evaluated the clinical applicability of multiplex real-time PCR and PCR-REBA methods and compared their efficiency to that of the sequence analysis method used for detecting PCV2 and differentiating the different PCV2a-e genotypes directly from the serum and tissue samples of pigs.

METHODS

Preparation of DNA Samples

To evaluate the diagnostic performance of the multiplex real-time PCR and PCR-REBA methods, a total of 180 samples suspected to be infected with PCVAD including 109 tissues and 71 bloods were provided by the Optipharm Animal Disease Diagnostic Center, which was commissioned from January to December, 2019. DNA was extracted from 200 μL of serum or 20 mg of organ tissue homogenate using a commercial automated system (Miracle-AutoXT Automated Nucleic Acid Extraction System, intronbio, Seongnam, Republic of Korea) according to the manufacturer's recommendations. To avoid cross contamination, all the samples were processed individually and stored at −20°C. The content and purity of the extracted DNA were assayed by measuring absorbance at 260 and 280 nm using an Infinite 200 NanoQuant (Tecan, Switzerland) spectrophotometer.

Multiplex Real-Time PCR Assay

Detection of PCV2 and identification of genotypes in clinical samples was performed with Opti PCV2-genotyping (Optipharm), a quantitative multiplex real-time PCR-based assay, using the CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA) for thermocycling and fluorescence detection. Both the detection and genotype identification of PCV2 can be performed in a single tube using this assay [PCV2 (Cy5), PCV2a/e (FAM), PCV2b/d (CAL Flour Red 610), and PCV2d (HEX)] by incorporating specific TaqMan probes labeled with different fluorophores. Real-time PCR amplification was performed in a total reaction volume of 20 μL containing 10 μL of 2× Thunderbird probe qPCR mix (Toyobo, Osaka, Japan), 5 μL of a mixture of primer and TaqMan probe that were labeled with different fluorophores, and 5 μL template DNA. The real-time PCR kits consisted of an internal control (IC) DNA, which was used to indicate successful nucleic acid extraction, the quality of the sample and to check for the presence of PCR inhibitors in the reaction. The IC DNA is designed to have minimal sequence similarity with the target gene and also facilitates detection of false negatives. Therefore, it does not directly compete with the amplification of the species-specific target in multiplex real-time PCR. Positive (Plasmid DNA with mixed PCV2a, b, d genotypes) and negative controls consisting of molecular grade (DNAse/RNAse-free) water (Ultra pure water; Welgene, Gyeongsan, Republic of Korea) without template DNA were included in each assay and the assay was performed under the following conditions: 95°C for 3 min followed by 40 cycles of 95°C for 20 s and 55°C for 40 s. Each sample was tested in duplicate by running the PCR cycles twice. The viral load was quantified by determining the cycle threshold (C_T), and the number of PCR cycles required for the fluorescence to exceed a value significantly higher than the background fluorescence. Positive result was indicated when the C_T value was <38.

PCR-Reverse Blot Hybridization Assay (PCR-REBA)

Oligonucleotide primers corresponding to both strands of the ORF2 region of PCV2 (**Figure 1**) were designed by Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primers were made as probes corresponding to the complementary strand and were used exclusively thereafter. To validate the efficiency of the selected probes, target DNA samples amplified from the PCV2

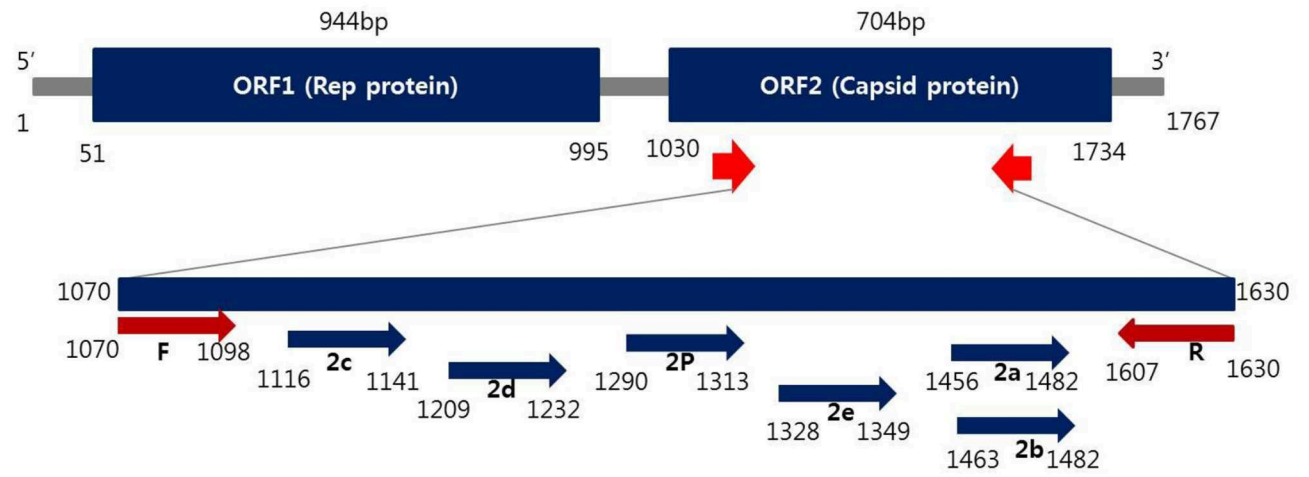

FIGURE 1 | Schematic representation of the ORF2 gene to distinguish PCV2 genotyping from positions of primer and probes used in this study.

strains were applied to the REBA membrane strips and spotted with the selected probes. Two types of DNA samples (PCV2c and PCV2e) were synthesized (Bioneer, Daejeon, Republic of Korea) and amplified with custom PCR primers (PCV2c, F-5′-TAAGTGGGGGGTCTTTAAGA-3′ and R-5′-TCCTCCGCC GCCGCCCCTGG-3′; PCV2e, F-5′-TAAGTGGGGGGTCTT TAA-3′ and R-5′-CTTGGCCATATCCTCCGCC-3′), resulting in amplicons of 630 and 640 bp, respectively. The resultant products were mutagenized after subcloning into the pBHA vector. Two plasmids were extracted from the transformants, and the mutated sequences were confirmed by sequence analysis (CosmoGenetech, Daejeon, Republic of Korea). PCR was performed using a 20 μL reaction mixture (GeNet Bio, Daejeon, Republic of Korea) containing 2× master mix (10 μL), 2 μL of primer mixture, 5 μL sample DNA, and 3 μL Ultra pure water (Welgene) to make up the final volume. The reactions were run on a Verity thermocycler (Applied Biosystems, CA, USA) under the following conditions: one cycle at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, annealing 60°C for 30 s, initial extension at 72°C for 30 s, and a final extension of 72°C for 10 min to complete the synthesis of all strands. The amplified target was visualized as a single band corresponding to a length of 620 bp using the ChemiDoc system (Vilber Lourmat, Eberhardzell, Germany).

For REBA PCV2 genotyping, the hybridization and washing processes were performed as follows; each sample was tested in duplicate and all PCR-REBA runs were performed twice. In brief, biotinylated PCR products were denatured at 25°C for 5 min in denaturation solution and then the denatured single-stranded PCR products suspended in hybridization solution were incubated with REBA PCV2-genotyping membrane strips at 55°C with shaking at 90 rpm in a blotting tray for 30 min. The strips were then washed twice with gentle shaking in 1 ml of washing solution for 10 min at 55°C, incubated at 25°C with 1:2,000 diluted streptavidin-alkaline phosphatase (AP) conjugate (Roche Diagnostics, Mannheim, Germany) in conjugate diluent solution (CDS) for 30 min, and finally washed twice with 1 ml CDS at room temperature for 1 min. The colorimetric hybridization signals were visualized by adding a 1:50 dilution of nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl (NBT/BCIP) AP-mediated staining solution (Roche Diagnostics), and then incubated until a color change was detected. Finally, the band pattern was read and interpreted visually.

Sequence Analysis

To confirm the results of the two molecular diagnostic methods, the PCR amplicons of all the clinical samples were sequenced using an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) and the ABI Prism BigDye Terminator (Applied Biosystems) system (CosmoGenetech, Republic of Korea). The primer set used to amplify the target ORF2 gene was 5′-TCTGAATTGTACATACATRGTTAYACGG-3′ (1070F) and 5′- TACCGYTGGAGAAGGAAAAATGG-3′ (1630R), which resulted in a 560-bp PCR product. The obtained sequences were compared with sequences in the National Center for Biotechnology Information (NCBI) GenBank database for species identification.

RESULTS

Analytical Sensitivity and Specificity of the Multiplex Real-Time PCR and PCR-REBA Methods

Analytical sensitivity of the two molecular methods for the detection of PCV2 was determined by using 10-fold diluted (1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg) samples for obtaining the standard curve for the DNA extracted from PCV2 strains. The detection limit of the multiplex real-time PCR assay for PCV2-P (PCV2 species-specific probe), PCV2a/e-P (PCV2a/e genotype-specific probe), PCV2b/d-P (PCV2b/d genotype-specific probe),

PCV2c (PCV2c genotype-specific probe), PCV2d-P (PCV2d genotype-specific probe), and PCV2e-P (PCV2e genotype-specific probe) ranged from 100 to 10 fg DNA per reaction. The C_T values for PCV2-P, PCV2a/e, PCV2b/d, PCV2c, PCV2d, and PCV2e for each DNA concentration ranged from 17 to 36.8, 16.5 to 34.9, 16.7 to 35.1, 16.1 to 35, 16.8 to 35.3, and 15.5 to 33.1, respectively (**Supplementary Figures 1A–D**). The PCR-REBA detection limit for PCV2a, PCV2b, PCV2c, PCV2d, and PCV2e was ∼100 fg to 10 fg DNA per reaction (**Supplementary Figures 1E–J**). In addition, the detection limit for mixed co-infection PCV2 subtypes in multiplex real-time PCR was 1 pg DNA per reaction, and the C_T value was found to be 17.97–32.86 (Data not shown). The PCR-REBA detection limit for mixed co-infection PCV2 subtypes detected ∼100 fg DNA per reaction (**Supplementary Figure 1K**).

To determine the specificity of the two molecular assays, primers and probes for detecting PCV2 positive genotypes were used for testing 55 DNA samples, respectively, extracted from specific pathogen-free swine serum samples and used as negative controls. The multiplex real-time PCR and PCR-REBA assay for detecting PCV2 positive genotypes yielded negative results with all strains except PCV2 strains (including mixed co-infection PCV2 subtypes), hence, the cross reactivity was not detected (**Supplementary Table 1**).

Detection of PCV2 DNA Using Multiplex Real-Time PCR and PCR-REBA Methods in Clinical Samples

To evaluate the performance of the multiplex real-time PCR and PCR-REBA assay, a total of 180 clinical samples including tissue ($n = 109$, 60.6%) and whole blood ($n = 71$, 39.4%) were analyzed. Of 180 clinical samples, 87 (48.3%) samples were positive for PCV2, and 93 (51.7%) samples were negative as detected by both multiplex real-time PCR and PCR-REBA (**Table 1**).

Multiplex Real-Time PCR and PCR-REBA Methods for the Detection of PCV2 Genotyping in Clinical Samples

Of the 87 PCV2 positive samples, 53 (60.9%), 17 (19.5%), 12 (13.8%), and 3 (3.5%) samples showed positive fluorescence signals for PCV2d, PCV2a, PCV2b, and PCV2a/b co-infections, respectively, as evaluated using multiplex real-time PCR assay (**Figure 2A**) while no PCV2 genotypes were detected in 2 cases (2.3%). All the clinical samples showed positive IC signals and the C_T values of the 87 positive and 93 negative samples ranged from 23.62 to 32.7 (mean 24.89, SD ± 1) and 22.47 to 33.4 (mean 25.36, SD ± 0.47), respectively. The C_T values of the PCV2d, PCV2a, PCV2b, and PCV2a/b co-infections samples ranged from 15.75 to 35.08 (mean 23.43, SD ± 5.6), 20.97 to 33.85 (mean 26.5, SD ± 4.07), 22.89 to 34.37 (mean 28.81, SD ± 5.78), and 21.2 to 30.73 (SD ± 2.4), respectively. PCR-REBA, which is another method for performing the molecular identification of PCV2 genotypes, was performed with the same clinical samples (**Figure 2B**). Of the 87 positive samples, the following PCV2 genotypes were identified using PCR-REBA: PCV2d was the most prevalent at 60.9% ($n = 53$), followed by PCV2a ($n = 17$, 19.5%), PCV2b ($n = 14$, 16.1%), and PCV2a/b co-infections ($n = 3$, 3.5%), respectively (**Table 2**).

Comparison of the Results Between the Two Molecular Assays and Sequence Analysis for Identification of the PCV2 Genotypes in the Clinical Samples

To confirm the results obtained from the multiplex real-time PCR and PCR-REBA assay, sequence analysis was performed using the same clinical samples (**Figure 2C**). Only 69 (79.3%) of the 87 PCV2 positive samples could be identified for PCV2 genotyping by sequence analysis. Therefore, 18 samples including 11 PCV2d, 2 PCV2a, 4 PCV2b, and 1 PCV2a/b co-infected samples identified by multiplex real-time PCR and PCR-REBA were excluded from the comparative analysis. The results of the multiplex real-time PCR and sequence analysis methods were consistent except for two cases. In these two cases, while PCV2 genotypes were not detected using multiplex real-time PCR, the samples were identified as PCV2b-positive by sequence analysis. The results of the PCR-REBA for PCV2 genotyping of all 69 samples were consistent with the sequencing results (**Table 2**). The phylogenetic tree was constructed using Phylogeny.fr software (23) after alignment of the 69 sequenced results. Analysis of the phylogenetic tree indicated that the sequences could be divided into three genotypes (PCV2d, PCV2a, and PCV2b), which accounted for 60.9, 24.6, and 14.5%, respectively (**Figure 3**).

DISCUSSION

Although the severity of the economic losses caused by PCV2 infection has been mitigated by vaccination, PCVAD is still detected quite often and is an important porcine pathogen. It is important to distinguish between the PCV2 genotypes for the laboratory diagnosis of PCV2 as different PCV2 genotypes have been found in samples from pigs affected with other PCVAD (24). The currently used PCR-based methods for diagnosis, including nucleotide sequencing, PCR, nested PCR, and PCR-RFLP (25–28), are time consuming for general veterinary clinical applications; they can only be used in specialized diagnostic institutes as these methods require specialized equipment and reagents, and need to be inspected by professionals to differentiate the subtype virus. Therefore, there is an increasing requirement for diagnostic techniques that can be complemented with traditional methods in clinical diagnostic laboratories.

In the present study, two molecular assays were developed for detecting PCV2 genotypes. The purpose of the present study was to evaluate the clinical efficacy of the multiplex RT-PCR assay (Opti PCV2-genotyping) and PCR-REBA (REBA PCV2-genotyping) for rapid and accurate detection as well as identification of PCV2 genotypes based on the ORF2 regions. We also compared the results of these two molecular assays with those obtained from conventional methods such

TABLE 1 | Detection of porcine circovirus 2 DNA in 180 clinical samples suspected of PCVAD infection using the multiplex real-time PCR and PCR-REBA assay.

Sample	Total no. (%) of samples	Multiplex real-time PCR		PCR-REBA	
		Positive (%)	Negative (%)	Positive (%)	Negative (%)
Tissue	109 (60.6)	54 (49.5)	55 (50.5)	54 (49.5)	55 (50.5)
Blood	71 (39.4)	33 (46.5)	38 (53.5)	33 (46.5)	38 (53.5)
Total	180 (100)	87 (48.3)	93 (51.7)	87 (48.3)	93 (51.7)

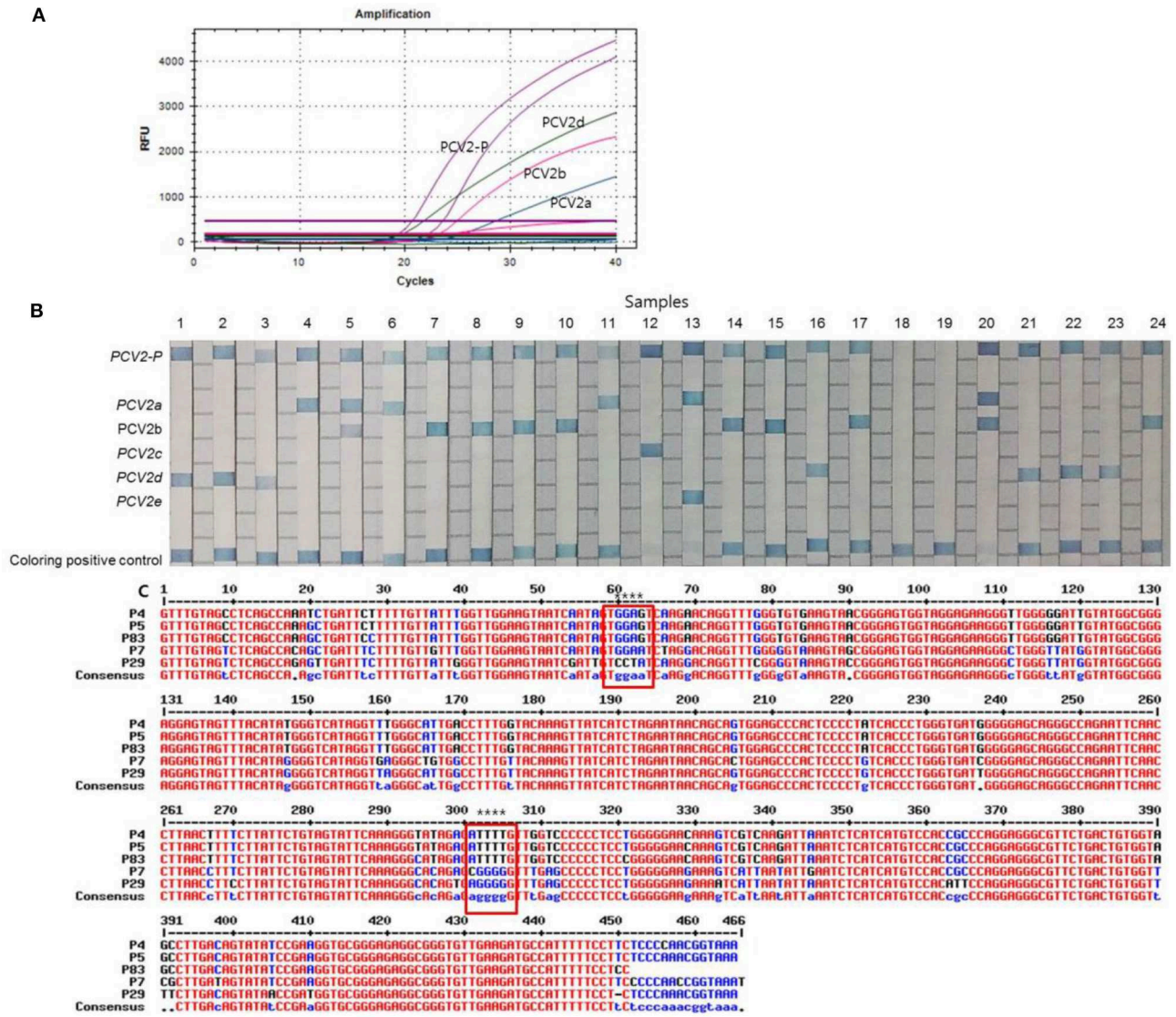

FIGURE 2 | Typical results of the multiplex real-time PCR, PCR-REBA, and sequence analysis with clinical samples. **(A)** Overall results for PCV2-positive, PCV2a, PCV2b, and PCV2d. Fluorescent dyes of specific TaqMan probes for multiplex real-time PCR were used PCV2 (Cy5), PCV2a/e (FAM), PCV2b/d (CAL Flour Red 610), and PCV2d (HEX), respectively. **(B)** Results of PCR-REBA; Lanes 1–3, 16, 21–23: PCV2d; lanes 4, 6, and 11: PCV2a; lanes 5 and 20: PCV2a and PCV2b co-infection; lane 7–10, 14–15, 17, and 24: PCV2b; lane 12: PCV2c; lane 13: PCV2e; lane 18 and 19: negative. PCV2c and PCV2e were used to synthesize the DNA as a control. **(C)** Sequence alignment results of a fragment of the genomic sequence of the clinical samples; P4, PCV2a; P7, PCV2b, P29, PCV2d, P5 and P83 showed that the two samples detected as PCV2a/b co-infection positive by the multiplex real-time PCR and PCR-REBA methods were shown as only PCV2a positive by sequence analysis; The red boxes indicate the position where three genotypes (PCV2a, 2b, and 2d) can be identified.

TABLE 2 | Comparison of multiplex real-time PCR, PCR-REBA, and sequence analysis results for the detection of PCV2 genotypes in 180 clinical samples suspected of PCVAD.

PCV2 genotyping	Molecular methods						Sensitivity (%)	95% CI	Specificity (%)	95% CI
	PCR-REBA		Multiplex real-time PCR		Sequence analysis					
	Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	N/A (%)	Consistent results			
PCV2 positive (*n* = 87)	**87 (48.3)**	**0 (0)**	**85 (97.7)**	**2 (2.3)**	**69 (79.3)***	**18 (20.7)**	**69 (100)**	100	0.954–1.000	100	0.979–1.000
Single infection (*n* = 84)	**84 (96.6)**	**0 (0)**	**82 (96.5)**	**2 (100)**	**67 (97.1)**	**17 (94.4)**	**67 (100)**	100	0.953–1.000	100	0.972–1.000
PCV2d	53 (63.1)	0 (0)	53 (63.1)	0 (0)	42 (79.2)	11 (20.8)	42 (100)	100	0.927–1.000	100	0.884–1.000
PCV2a	17 (20.2)	0 (0)	17 (20.2)	0 (0)	15 (88.2)	2 (11.8)	15 (100)	100	0.784–1.000	100	0.940–1.000
PCV2b	14 (16.7)	0 (0)	12 (14.3)	2 (2.4)	10 (71.4)	4 (28.6)	10 (100)	100	0.787–1.000	100	0.954–1.000
Multiple infection (*n* = 3)	**3 (3.4)**	**0 (0)**	**3 (3.5)**	**0 (0)**	**2 (2.9)**	**1 (5.6)**	**2 (100)**	100	0.425–1.000	100	0.961–1.000
PCV2a and PCV2b	3 (100)	0 (0)	3 (100)	0 (0)	2 (66.7)	1 (33.3)	2 (100)	100	0.369–1.000	100	0.953–1.000
PCV2 negative (*n* = 93)	**0 (0)**	**93 (100)**	**0 (0)**	**93 (100)**	–	–	–	–	–	–	–
Total (*n* = 180)	87 (48.3)	93 (51.7)	85 (47.2)	95 (52.8)	69 (79.3)	18 (20.7)	–	–	–	–	–

*N/A, not applicable; *A total of 18 samples including 11 PCV2d, 2 PCV2a, 4 PCV2b, and 1 PCV2a/b co-infection identified by multiplex real-time PCR and PCR-REBA were excluded because they were not sequenced; 95% CI, 95% confidence interval.*

as sequence analysis. The characteristics of the multiplex real-time PCR and PCR-REBA used in this study were similar as they were both rapid (turnaround time of 2–3 h), sensitive, specific, and comparatively easy to perform without requiring any specialized laboratory equipment, other than a PCR machine and water bath.

The Opti PCV2-genotyping assay is designed to simultaneously detect PCV2 and to distinguish PCV2a/e, PCV2b, and PCV2d genotypes. Multiplex real-time PCR, a recognized technique, is faster and more effective for the rapid detection of bacterial or viral infection compared to conventional PCR and other detection methods. Multiplex real-time PCR assay is a rapid method with a turnaround time of ∼1.5–2 h, which includes 30 min for DNA preparation and 1.5 h for target DNA amplification. The combination of excellent sensitivity and specificity as well as ease of handling enable rapid and simultaneous detection of multiple species, and minimizes the possibility of contamination by eliminating the need for additional post-PCR processing of the samples, which has made this technology appealing for clinical microbiology laboratory applications (29). PCR-REBA is a highly sensitive and specific probe-based method in which multiple oligonucleotide probes are immobilized on nitrocellulose strips, hybridized with biotin-labeled PCR products, and can be used to derive rapid results within 4 h (30). In addition to the time required for target DNA amplification (1.5 h), PCR-REBA is a 3-step process with a hybridization step (30 min), a washing step (20 min), and a chromogenic detection and data interpretation step (40 min). It also requires a fully automated system for the washing, hybridization, and interpretation steps. The PCR-REBA molecular diagnostic assay can be used to isolate all types of PCV2 genotypes as well as detect PCV2 directly from serum or tissue samples. In addition, PCR-REBA has the advantage of the flexibility to add more specific-probes to the membrane strip for increasing the range of PCV2 genotypes detected.

In this study, the concordance rate of the multiplex real-time PCR assay and sequence analysis was 98.8% (95% confidence interval [CI] 0.953–0.999, $p < 0.001$). Using sequence analysis as the gold standard, the sensitivity, specificity, and positive and negative predictive values of the PCV2 genotyping results by multiplex real-time PCR assay were 97.1% ($n = 67$, 95% CI 0.894–0.998, $p < 0.001$), 100% ($n = 93$, 95% CI 0.966–1.000, $p < 0.001$), 100% (95% CI 0.953–1.000, $p < 0.001$), 97.9% (95% CI 0.921–0.998, $p < 0.001$), respectively. The results of PCR-REBA were found to be consistent with those of sequence analysis and showed good agreement ($\kappa = 1$).

Studies have shown that the most common PCV2 genotypes detected worldwide are PCV2b (53.1%) and PCV2a (34.4%) in Taiwan (24), PCV2b (87.5%) and PCV2a (12.5%) in Mexico (31), and PCV2d (45.3%) and PCV2b (41.1%) in China (13). In this study, the most prevalent genotypes detected were PCV2d ($n = 53$, 60.9%), followed by PCV2a ($n = 17$, 19.5%), PCV2b ($n = 14$, 16.1 %), and PCV2a/b co-infection ($n = 3$, 3.5%). Co-infection of PCV2a and PCV2b in clinical samples has been suggested to be the primary cause of other PCVAD while dual heterologous infection of

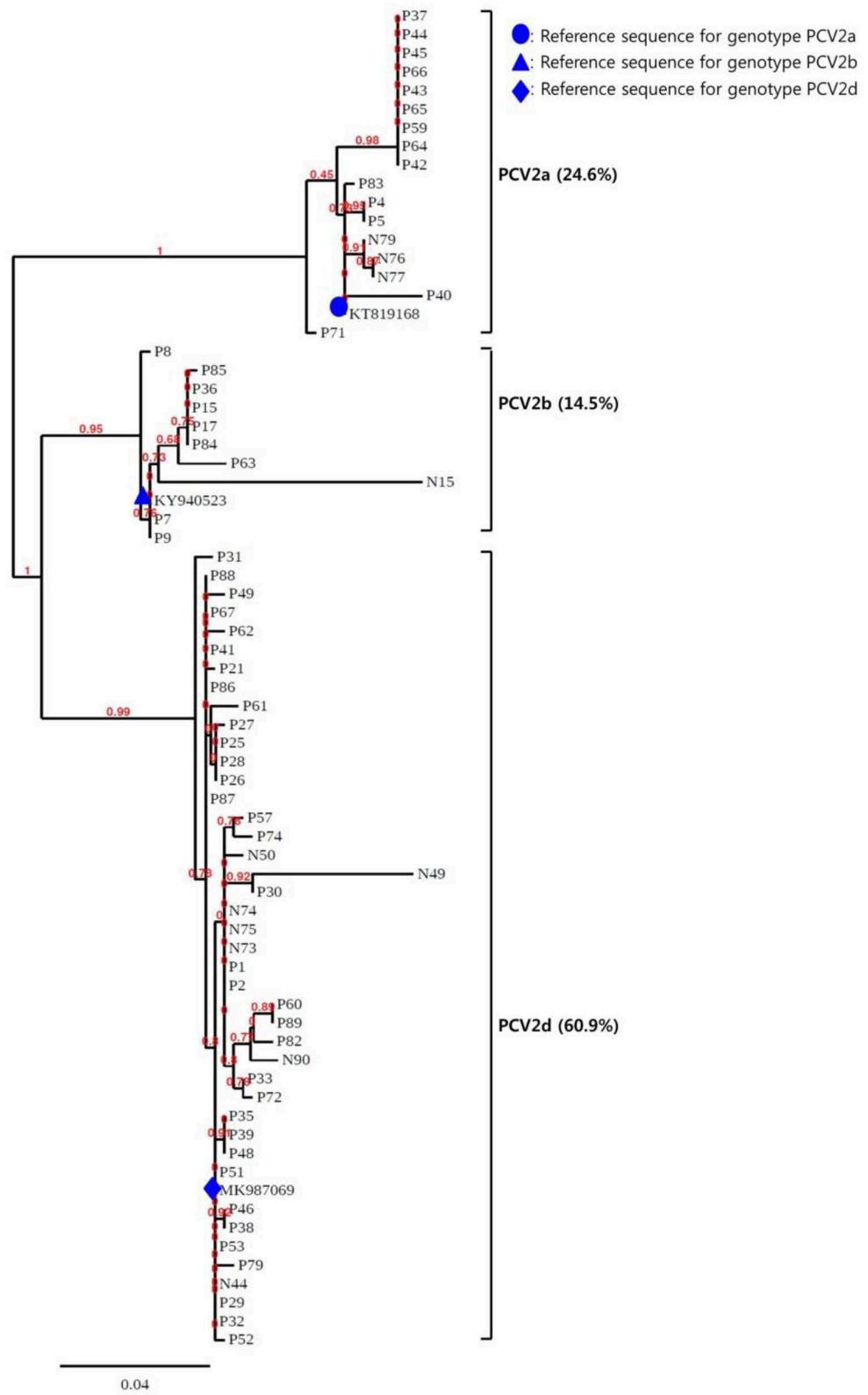

FIGURE 3 | Phylogenetic analysis of the 69 PCV2 isolates. The phylogenetic tree was constructed using Phylogeny.fr software after alignment of the 69 sequenced results. The PCV2genomes were mainly assigned to three genotypes (PCV2a, PCV2b, and PCV2d).

PCV2a and PCV2b in gnotobiotic pigs has been shown to induce severe clinical symptoms (24, 32). Therefore, rapid identification of co-infection of PCV2a and PCV2b is crucial. Generally, when identified samples from dually infected pigs were sequenced, only the predominant PCV2 genotype was detected. Our results also showed that only PCV2a could be identified by sequence analysis method in the three samples in which PCV2a/b co-infection was detected using the two molecular diagnostic methods.

There are potential limitations in this study. Firstly, the multiplex real-time PCR assay cannot distinguish between PCV2a type and PCV2e type, and does not include PCV2c type that has not yet been detected in Korea. Therefore, there should be an additional tube to include all of these genotypes. Secondly, although PCR-REBA detect all other genotypes in addition to PCV2, additional steps are required after PCR. Thirdly, the PCV2c and PCV2e genotypes were not detected in this study and further investigations of the samples are required.

CONCLUSIONS

The two recently developed molecular assays are accurate, rapid, and convenient tools for identifying PCV2. These assays can also discriminate between the PCV2 genotypes and directly detect PCV2 from clinical samples in only 2–3 h. Therefore, these two molecular assays can provide essential information that can help expedite therapeutic decisions for early and appropriate vaccinations during the acute phase of PCV2 infection. We believe that these assays can reduce the labor and time for PCV2 diagnosis in industrial animal area.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

All samples used in this study were animal diagnostic samples submitted by the clients and there was no animal handling involved.

AUTHOR CONTRIBUTIONS

HW performed evaluation of the experiments, analyzed the data, and drafted the manuscript. JS and SS provide clinical samples and clinical information. HK revised the manuscript. All authors have read and approved the final manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00200/full#supplementary-material

Supplementary Figure 1 | Detection limits of the multiplex real-time PCR and PCR-REBA methods evaluated using 10-fold serial diluted samples. Serially diluted PCV2-P, PCV2a/e, PCV2b, and PCV2d DNA samples ranging from 1 ng to 1 fg per reaction were used to determine the detection limit of the multiplex real-time PCR and PCR-REBA methods. In the multiplex real-time PCR assay, the amplification curve of the specific probe **(A)** for detecting PCV2 ($R^2 = 0.997$), PCV2a/e probe **(B)**, PCV2b probe **(C)**, and PCV2d probe **(D)**, for detecting PCV2 genotypes ($R^2 = 0.999$) are shown. The overall detection limit of this assay for the PCV2 genotypes ranged from ∼100 to 10 fg DNA per reaction. C_T was plotted against the input of the quantity of PCV2, 2a/e, 2b, 2c, 2d, and 2e DNA (repeated 40 times). The intensity of fluorescence is shown on the Y-axis (R^2 = reporter signal/passive reference signal). RFU, relative fluorescence unit and R^2, fluorescence units. Serially diluted PCV2-P **(E)**, PCV2a **(F)**, PCV2b **(G)**, PCV2c **(H)**, PCV2d **(I)**, PCV2e **(J)**, and 5 mixed co-infection PCV2 subtypes **(K)** with DNA amounts from 1 ng (lane 1), 100 pg (lane 2), 10 pg (lane 3), 1 pg (lane 4), 100 fg (lane 5), 10 fg (lane 6), and 1 fg (lane 7) were used to determine the detection limit of the PCR-REBA **(E–J)**. N, negative control. PCV2c and PCV2e used synthesized DNA as a control.

Supplementary Table 1 | Analytical specificity of the multiplex real-time PCR and PCR-REBA assay for detecting PCV2 and PCV2 genotypes with 25 strains and 30 normal serum samples, respectively, obtained from pigs. ATCC, American type culture collection, PCV2, Porcine circovirus type2.

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Conflict of Interest: All authors were employed by Optipharm, Inc during the preparation and execution of the study.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 03 July 2020
doi: 10.3389/fvets.2020.00374

Evaluation of P22 Antigenic Complex for the Immuno-Diagnosis of Tuberculosis in BCG Vaccinated and Unvaccinated Goats

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Current eradication strategies of tuberculosis (TB) in goats mainly rely on the single intradermal tuberculin test (SIT) and single intradermal cervical comparative tuberculin tests (SICCTs). TB vaccination has been proposed as a cost-effective option in high-prevalence herds or countries where economic compensation for the slaughter of positive animals is not affordable. However, TB vaccination compromises the efficiency of tuberculin-based diagnostic tests. In this study, the performance of a new diagnostic platform, based on the P22 antigenic complex, was assessed for skin test (ST), interferon-gamma release assay (IGRA), and serology under different TB scenarios. The sensitivity (Se) of diagnostic tests was assessed in TB-infected goats from the same farm (herd A, $N = 77$). The specificity (Sp) was assessed in two TB-negative farms (both vaccinated against paratuberculosis): one TB unvaccinated (herd B, $N = 77$) and another vaccinated with bacille Calmette-Guérin (BCG) (herd C, $N = 68$). The single (s) P22-IGRA showed the highest Se among IGRA tests (91%), and the comparative (c) P22-ST showed the highest Sp (100% in herd B and 98% in herd C). Combined interpretation of techniques enabled the best diagnostic performances. Combining the SICCT + sP22-IGRA improved Se (97%) compared to SICCT + tuberculin-based IGRA (95%), with a reduction of Sp (95 and 100%, respectively). Besides, combination of P22-ELISA with cP22-ST or SICCT elicited a similar performance in the non-vaccination context (Se: 94 and 95%; Sp: 95 and 95%, respectively), but Sp was significantly higher for the combination with cP22-ST compared to SICCT in the TB vaccination context (95 and 79%, respectively). The combination of serological tests based on P22 and MPB83 showed higher complementarity and improved 13 percentage points the Se of P22-ELISA alone. These findings suggest that either cell-mediated or antibody-based diagnostic techniques, using the P22 antigen complex, can contribute to improve the immunodiagnostics of TB in goats under different TB control strategies.

Keywords: tuberculosis, diagnosis, goats, bacille Calmette-Guérin (BCG), skin test, interferon-gamma release assay (IGRA), serology, P22

OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
Christian Menge, Friedrich Loeffler Institute, Germany
Faten Abdelaal Okda, St. Jude Children's Research Hospital, United States

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *06 March 2020*
Accepted: *28 May 2020*
Published: *03 July 2020*

Citation:
Arrieta-Villegas C, Infantes-Lorenzo JA, Bezos J, Grasa M, Vidal E, Mercader I, Singh M, Domingo M, de Juan L and Pérez de Val B (2020) Evaluation of P22 Antigenic Complex for the Immuno-Diagnosis of Tuberculosis in BCG Vaccinated and Unvaccinated Goats. Front. Vet. Sci. 7:374. doi: 10.3389/fvets.2020.00374

INTRODUCTION

Tuberculosis (TB) in goats is a chronic infectious disease, mainly caused by *Mycobacterium bovis* and *Mycobacterium caprae*, members of the *Mycobacterium tuberculosis* complex (MTBC). This disease entails important economic costs for livestock industries (1) and could be a source of TB for cattle (2), other domestic animals (3, 4), wildlife (5), and humans (6).

Spain has the second-highest goat census of the EU, with 2.7 million goat heads (data extracted from FAOSTAT on 17/02/2020). Besides, the high TB burden in goats could explain a number of new bovine TB breakdowns, hampering the goal of TB eradication in cattle (7). Therefore, some regions with a high concentration of caprine herds carry out TB eradication campaigns in caprine flocks (8); however, goat herds are still not subjected to a national eradication program, except for those epidemiologically linked with cattle (9).

The cornerstone of an efficient caprine TB eradication program is the diagnosis. The Spanish bovine TB eradication program effectiveness is highly dependent on the routine tuberculin skin testing (10). Current bovine TB testing is based on the single intradermal tuberculin test (SIT) and single intradermal cervical comparative tuberculin tests (SICCTs), and the interferon-gamma release assay (IGRA). However, in goats under certain epidemiological contexts, those diagnostic tests have some drawbacks in terms of sensitivity (Se) and specificity (Sp) (8, 11).

Another concern for TB diagnostics is the vaccination against *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which has been largely implemented in small ruminants, to prevent the development of clinical disease (12). Nevertheless, even though MAP vaccines are authorized (e.g., Gudair® vaccine), it has been demonstrated that paratuberculosis (PTB) vaccination interferes with STs and IGRA used for TB diagnosis (13, 14). In addition, the efficacy of *M. bovis* bacille Calmette-Guérin (BCG) vaccine has also been assessed in goats during the last decade in different vaccination trials (15–19). Even though these trials showed that BCG conferred certain protection to experimentally and naturally infected goats, it was evidenced that vaccination interfered with current TB diagnostic tests (16, 20).

To overcome diagnostic interferences due to BCG vaccination, defined antigens to differentiate infected from vaccinated animals (DIVA) have been developed (14, 21); nevertheless, those antigens have shown lower Se compared to tests based on standard tuberculins (22). Recently, a new multi-protein complex called P22, obtained from purified protein derivative of *M. bovis* (PPD-B) by affinity chromatography, has been developed (23), yielding high Se and variable Sp, depending on the animal species and epidemiological contexts (24). To date, this antigen has been tested to detect humoral response against MTBC in different species (25–30); however, there is a lack of information regarding its performance for cell-mediated immunity (CMI)-based diagnostics.

The aim of this study was to evaluate the performance of different cell-mediated and humoral immunodiagnostic tests, based on the P22 antigenic complex, for the diagnosis of TB in goats under different epidemiological and control scenarios.

MATERIALS AND METHODS

Herds and Experimental Design

A total of 222 goats from three herds were included in the study (**Table 1**): 77 infected goats (infection was confirmed postmortem by gross lesions, histopathology or mycobacterial culture, or both) from a TB-positive herd of murciana-granadina goats (herd A); 77 goats belonging to an officially TB-free herd of alpine goats (herd B) that were vaccinated against PTB with Gudair (CZ Vaccines, Porriño, Spain), around 2 years before sampling; and 68 goats from another TB-free herd (herd C) of Blanca de Rasquera autochthonous breed, that were vaccinated against PTB (Gudair®) and against TB with *M. bovis* BCG Danish 1,331 strain (ATCC, Ref. 35733) as described previously (15). In herd C, 50% of goats were vaccinated with BCG and Gudair® 9–10 months before sampling, and the remaining 50% were vaccinated more than 1 year before. STs, IGRAs, and immunoglobulin G (IgG) enzyme-linked immunosorbent assays (ELISAs) were carried out in the 77 infected goats, as well as in 138, 142, and 142 noninfected goats, respectively (**Table 1**).

Two TB control scenarios were hypothesized in order to study the performance of each diagnostic test: the conventional (TB unvaccinated) scenario, using data from herds A and B, and the BCG-vaccinated (TB-VAC) scenario, using data from herds A and C. Se was calculated using data from herd A, and Sp was calculated using data from herds B and C depending on TB control scenario (**Table 2**).

Antigens

M. tuberculosis var. *bovis* (PPD-B) and *M. avium* (PPD-A) tuberculins (2,500 IU/ml) were obtained from CZ Vaccines

TABLE 1 | Herd and treatment distribution of tested animals.

				No. of animals tested		
Herd	**TB status**	**BCG[1]**	**Gudair®[2]**	**ST**	**IGRA**	**ELISA**
A	Positive	No	No	77	77	77
B	Free	No	Yes	77	74	74
C	Free	Yes	Yes	61	68	68

[1]*BCG, bacilli Calmette-Guérin Mycobacterium bovis vaccine.* [2]*Gudair vaccine, vaccine against paratuberculosis (Mycobacteium avium subspecies paratuberculosis).*
TB, tuberculosis; ST, skin test; IGRA, interferon-gamma release assay.

TABLE 2 | TB control scenarios distribution of tested animals.

				No. of animals tested		
Control scenario	**Herds**	**BCG[1]**	**Gudair®[2]**	**ST**	**IGRA**	**ELISA**
Conventional[a]	A+B	No	Yes	154	151	151
TB-VAC[b]	A+C	Yes	Yes	138	145	145

[a]*Conventional scenario: composed by TB unvaccinated goats.* [b]*TB-VAC Scenario: TB negative animals from herd C were vaccinated with BCG and TB-positive animals from herd A were not vaccinated.* [1]*BCG, bacilli Calmette-Guérin Mycobacterium bovis vaccine.* [2]*Gudair vaccine, vaccine against paratuberculosis (Mycobacteium avium subspecies paratuberculosis).*
TB, tuberculosis; ST, skin test; IGRA, interferon-gamma release assay.

and used at concentrations recommended by the Spanish Ministry (9). The protein complex P22 was produced by immunopurification of PPD-B (CZ Vaccines) as described previously (23) and prepared at a concentration of 500 μg/ml (unpublished data). The DIVA reagent based on a cocktail of recombinant ESAT-6 and CFP-10 proteins (500 μg/ml) (31) and the recombinant MPB83 (MPT83) protein (500 μg/ml) (32) were purchased from Lionex (Braunschweig, Germany).

Skin Tests

SIT was performed by intradermal inoculation of 0.1 ml of PPD-B in the left-hand side of the neck by using a Dermojet® syringe (Akra Dermojet, Pau, France). In the same way, SICCT was performed by intradermal inoculation of 0.1 ml of PPD-B and PPD-A, both in the left-hand side of the neck, at the proximal and distal parts of the neck, respectively. Besides, 0.1 ml of P22 (at 500 μg/ml) was inoculated in the right-hand side of the neck. The increase in skinfold thickness (SFT) was measured just before the inoculation and after 72 h. Severe interpretations of SIT and SICCT were performed, as previously described in the manual of the Spanish bovine TB eradication program (9). Briefly, positive criterion for SIT: SFT PPD-B $>$ 2 mm (severe); and for SICCT: positive to SIT and SFT PPD-B - SFT PPD-A $>$ 1 mm (severe) or presence of clinical signs in the PPD-B inoculation site. P22 single and comparative STs (sP22-ST and cP22-ST) were interpreted using the same criteria as SIT and SICCT, respectively, i.e., considering SFT P22 and SFT P22 - SFT PPD-A measures, respectively.

Whole-Blood Interferon-Gamma Release Assays

Blood samples were collected from the jugular vein prior to ST performance using heparinized tubes and were processed as described previously (16). Shortly, blood samples were stimulated with PPD-B, PPD-A, and P22 at a final concentration of 20 μg/ml, and with DIVA reagent (ESAT-6/CFP-10) at 20 μg/ml, while PBS was added as an unstimulated control. Samples were incubated at 37 ± 1°C with 0.5% CO_2 overnight. Finally, plasma supernatant was collected and analyzed by ELISA (BOVIGAM®, Thermo Fisher Scientific, Waltham, MA, USA) and read at 450 nm using a spectrophotometer (Biotek Power Wave XS). The interpretation of tuberculin-based IGRA (STAND-IGRA) results was performed according to the cutoff point recommended by the manufacturer, i.e., the criterion for positivity: PPD-B OD – PBS OD $\geq$ 0.05 and PPD-B OD $>$ PPD-A OD. Similarly, cP22-IGRA was considered positive when P22 OD – PBS OD $\geq$ 0.05 and P22 OD $>$ PPD-A OD, whereas sP22-IGRA and DIVA-IGRA were considered positive when P22 OD – PBS OD $\geq$ 0.05 and DIVA OD – PBS OD $\geq$ 0.05, respectively.

Antibody Detection Tests

Plasma samples were analyzed for antibody detection by using two in-house indirect ELISA, one for detecting MPB83 antigen, performed and interpreted as described previously (33), and another one for detecting P22, performed as described previously (24, 25). P22-ELISA was interpreted as follows: ELISA percentage (E%) = [mean sample OD/(2 × mean negative control OD)] × 100. A sample E% $<$100% was classified as negative, and a sample E% $\geq$100% was classified as positive.

Post-mortem Examination

Seventy-seven goats from the positive herd (herd A) were euthanized after ST reading by intravenous injection of a sodium pentobarbital overdose. A complete necropsy procedure was conducted for TB lesion examination. Lesions were collected and immediately fixed in 10% buffered formalin for histopathological confirmation by hematoxylin/eosin staining. Mediastinal and tracheobronchial lymph nodes (LNs) were removed and stored at −20°C for bacterial culture.

Bacteriology

Whole pulmonary LNs of each animal were thawed, pooled, homogenized, and decontaminated as previously described (34) and plated on Middlebrook 7H11 medium (BD diagnostics, Sparks, MD, USA). Then, cultured plates were incubated at 37°C for 28 days. Finally, plates were read, and colonies were confirmed as MTBC by multiplex PCR (35).

Data Analysis

The Sp was calculated in TB-free farms (herds B and C) using the formula Sp = True negatives/(True negatives + False positives). The Se was calculated in the TB-infected farm by the formula Se = True positive/(True positive + False negative). Clooper-Pearson 95% confidence intervals were calculated for Sp and Se. Differences in diagnostic results, between tests, were evaluated by the McNemar test. Moreover, agreement between tests was calculated by Cohen's Kappa coefficient (k) and interpreted as follows: $<$0.00 poor, 0.00–0.20 slight, 0.21–0.4 fair, 0.41–0.60 moderate, 0.61–0.80 substantial, and 0.81–1.00 almost perfect. The diagnostic performance of each test was calculated using the diagnostic odds ratio (DOR) (36). All statistical tests and 95% confidence intervals were calculated using the Epitools calculator (Sargento, ESG, 2018, Epitools Epidemiological Calculators, Ausvet, Pty., Ltd., Australia; available in www.epitools.ausvet.com.au).

RESULTS

The results of Se of herd A and Sp of herds B and C are summarized in **Table 3**. The TB-positive status of all animals from herd A was confirmed by positive mycobacterial culture and/or positive lesions in histopathological analysis.

Skin Tests

The Se of the cP22-ST was the lowest among tests, but the Sp in herd B was the highest, being identical to the Sp of the SICCT, and a 6 percentage point (p.p.) and 8 p.p. more specific than the SIT and the sP22-ST, respectively. Regarding the herd C, the cP22-ST and the sP22-ST displayed similar Sp, being significantly more specific than the SIT (31 p.p. of increase, $p < 0.001$, and 30 p.p. of increase, $p = 0.005$, for cP22-ST and sP22-ST, respectively) and the SICCT (18 p.p. of increase, $p = 0.0026$, and 17 p.p. of increase, $p = 0.0094$, for cP22-ST and sP22-ST, respectively).

TABLE 3 | Sensitivity (Se) and specificity (Sp) of diagnostic tests.

Diagnostic test	TB positive (farm A)		Unvaccinated (farm B)		BCG vaccinated (farm C)	
	N[9]	Se (95% CI)[10]	N	Sp (95% CI[11]	N	Sp (95% CI)[11]
sP22-ST[1]	77	87% (77–94)	77	92% (84–97)	61	97% (89–100)
cP22-ST[2]	77	74% (63–83)	77	100% (95–100)	61	98% (91–100)
SIT[3]	77	94% (85–98)	77	94% (85–98)	61	67% (54–79)
SICCT[4]	77	91% (82–96)	77	100% (95–100)	61	80% (68–89)
sP22-IGRA[5]	77	91% (82–96)	74	95% (87–99)	68	84% (73–92)
cP22-IGRA[6]	77	86% (76–93)	74	96% (89–99)	68	85% (75–93)
STAND-IGRA[7]	77	77% (66–86)	74	100% (95–100)	68	96% (88–99)
DIVA-IGRA[8]	77	71% (60–81)	74	100% (95–100)	68	100% (95–100)
P22-ELISA	77	74% (63–83)	74	93% (85–98)	68	96% (88–99)
MPB83-ELISA	77	75% (64–84)	74	92% (83–97)	68	94% (86–98)

[1]*sP22-ST, single P22 skin test;* [2]*cP22-ST, comparative P22 skin test;* [3]*SIT, single intradermal tuberculin test;* [4]*SICCT, single intradermal cervical comparative tuberculin test;* [5]*sP22-IGRA, single P22 IGRA test;* [6]*cP22-IGRA, comparative P22 IGRA test;* [7]*STAND-IGRA, standard tuberculin IGRA test;* [8]*DIVA-IGRA, differentiating Infected from Vaccinated animals (ESAT-6/CFP-10 peptide cocktail) IGRA test.* [9]*Number of animals tested.* [10]*Clopper–Pearson 95% confidence interval for Se.* [11]*Clopper–Pearson 95% confidence interval for Sp.*
TB, tuberculosis; BCG, bacille Calmette-Guérin; IGRA, interferon-gamma release assay.

Interferon-Gamma Release Assays

The sP22-IGRA showed the highest Se among tests, being a 5, 14, and 20 p.p. more sensitive than the cP22-IGRA, the STAND-IGRA, and the DIVA-IGRA, respectively. Indeed, the sP22-IGRA detected 12 positive goats more than the STAND-IGRA, without significant agreement between tests ($k = 0.4$, $p = 0.098$) and diagnostic results significantly different (**Supplementary Data**, $p = 0.005$). The sP22-IGRA and the cP22-IGRA showed similar specificities in both herds B and C, being a 4–5 p.p. less specific than the STAND-IGRA and the DIVA-IGRA. In herd C, both cP22-IGRA and sP22-IGRA were a 10–9 p.p. and a 16–15 p.p. less specific than the STAND-IGRA and the DIVA-IGRA, respectively.

Serological Tests

In terms of Sp and Se, diagnostic results of P22-ELISA were similar to diagnostic results of MPB83-ELISA. In herd A, the MPB83-ELISA detected 10 TB positive animals more than the P22-ELISA, and the P22-ELISA detected nine TB positive animals more than the MPB83-ELISA, and the agreement between tests was considered fair although statistically significant ($k = 0.35$, $p = 0.001$). In herd B, diagnostic results of Sp showed a moderate but significant agreement between ELISA tests ($k = 0.51$, $p < 0.001$), but in herd C, no agreement was observed ($k = -0.05$, $p = 0.33$).

TABLE 4 | Sensitivity (Se) and specificity (Sp) combined results of P22-based diagnostic tests.

Diagnostic tests	TB positive (farm A)		Unvaccinated (farm B)		BCG vaccinated (farm C)	
	N[8]	Se (95% CI)[9]	N	Sp (95% CI)[10]	N	Sp (95% CI)[10]
SIT[1] + sP22-IGRA[2]	77	97% (91–100)	73	89% (80–95)	61	59% (46–71)
SIT + cP22-IGRA[3]	77	97% (91–100)	73	90% (81–96)	61	61% (47–73)
SIT + P22-ELISA	77	96% (89–99)	73	89% (80–95)	61	66% (52–77)
SICCT[4] + sP22-IGRA	77	97% (91–100)	73	95% (87–98)	61	67% (54–79)
SICCT + cP22-IGRA	77	97% (91–100)	73	96% (88–99)	61	67% (54–79)
SICCT + P22-ELISA	77	95% (87–99)	73	95% (87–98)	61	79% (66–88)
sP22-ST[5] + sP22-IGRA	77	95% (87–99)	73	88% (78–94)	61	82% (70–91)
sP22-ST + P22-ELISA	77	94% (85–98)	73	88% (78–94)	61	93% (84–98)
cP22-ST[6] + sP22-IGRA	77	95% (87–99)	73	95% (87–98)	61	84% (72–92)
cP22-ST + P22-ELISA	77	94% (85–98)	73	95% (87–98)	61	95% (86–99)
sP22-IGRA + STAND-IGRA[7]	77	92% (84–97)	74	95% (87–99)	68	84% (73–92)
P22-ELISA + sP22-IGRA	77	95% (87–99)	74	89% (80–95)	68	79% (68–88)
P22 ELISA + cP22-IGRA	77	95% (87–99)	74	91% (81–96)	68	81% (70–89)
P22-ELISA + MPB83-ELISA	77	87% (77–94)	74	92% (83–97)	68	90% (80–96)
P22-ELISA + STAND-IGRA	77	90% (81–95)	74	93% (85–98)	68	91% (82–97)
SIT + STAND-IGRA	77	95% (87–99)	73	93% (85–98)	61	67% (54–79)
SICCT + STAND-IGRA	77	95% (87–99)	73	100% (95–100)	61	80% (68–89)

[1]*SIT, single intradermal tuberculin test;* [2]*sP22-IGRA, single P22 IGRA test;* [3]*cP22-IGRA, comparative P22 IGRA test;* [4]*SICCT, single intradermal cervical comparative intradermal tuberculin test;* [5]*sP22-ST, single P22 skin test;* [6]*cP22-ST, comparative P22 skin test;* [7]*STAND-IGRA, standard tuberculin IGRA test.* [8]*Number of animals tested;* [9]*Clopper–Pearson 95% confidence interval for Se.* [10]*Clopper–Pearson 95% confidence interval for Sp.*
TB, tuberculosis; BCG, bacille Calmette-Guérin; IGRA, interferon-gamma release assay.

Complementarity of Diagnostic Tests

Combined interpretation of P22-based tests was evaluated. Results of Sp and Se of complementarity of diagnostic tests are shown in **Table 4**. In general, complementarity between tests yielded an overall rise of Se with a variable reduction in the Sp.

The combination of cP22-ST + P22 ELISA improved the Se in 20 p.p. and displayed a similar Sp in both herds B and C, being the combined interpretation with the best results in all situations. The combination of SICCT + P22 ELISA showed similar results of Se and Sp in herd B. In herd C, the latter combination detected

10 false-positives more than the cP22-ST + P22-ELISA, reducing its Sp in 16 p.p., and with diagnostic results significantly different between tests ($p = 0.004$). The combination of cP22-ST + cP22-IGRA improved the Se and Sp in herd B at a similar level than the combined interpretations above described, but in herd C, the Sp was reduced in 11 p.p. respect to the cP22-ST + P22-ELISA test.

The combination of current diagnostic tests, e.g., SIT and SICCT, with other diagnostic tests increased the Se but not the Sp, except for the SICCT + STAND-IGRA. The latter combination improved the Se in 4 and 18 p.p. compared to the SICCT and the STAND-IGRA alone, respectively, and maintained the Sp in herd B but not in herd C (reduction of 16 p.p. compared to the STAND-IGRA alone). In herd A, the combined results of MPB83-ELISA + P22-ELISA improved the Se in 12 and 13 p.p. with respect to the MPB83-ELISA and the P22-ELISA alone, respectively, and maintained the Sp in herd B, and in herd C showed a mild reduction of Sp (4 and 6 p.p. of reduction with respect to the MPB83-ELISA and the P22-ELISA alone, respectively). Other combinations of tests did not improve the Se and the Sp, as did the aforementioned combined interpretations.

Performance of Diagnostic Tests

The results of DOR to assess the diagnostic performance for each test are represented in **Figure 1**. In general, a reduced DOR in TB-VAC scenario was observed compared to the conventional one (0.47, 95% CI: 0.28–0.654, of mean reduction in log DOR). In the conventional context, SICCT + STAND-IGRA (3.38, 95% CI: 2.35–4.41), SICCT alone (3.16, 95% CI: 2.36–3.97), SICCT + cP22-IGRA (2.94, 95% CI: 1.12–4.76), and SICCT + sP22-IGRA (2.81, 95% CI: 1.08–4.54) showed the best performances (**Figure 1A**). In TB-VAC context, the best performances were observed in DIVA IGRA (2.53, 95% CI: 1.98–3.8), cP22-ST + P22 ELISA (2.44, 95% CI: 0.97–3.92), and sP22-ST + P22 ELISA (2.31, 95% CI: 0.95–3.67) (**Figure 1B**).

DISCUSSION

Efficient and accurate diagnosis is of paramount importance for the success of eradication programs based on test and slaughter strategy. Here, the performance of new P22 antigenic complex-based cell-mediated and humoral tests for the diagnosis of TB in goats was assessed under different epidemiological and TB control scenarios.

Recently, the P22 antigenic complex has been evaluated for the detection of IgG in ELISA tests in different species: cattle goat, sheep, pigs, and wild boar (24–27), red deer (28), badgers (29), and alpacas and llamas (30). In the present study, the performance of the P22 antigenic complex for diagnostic tests based on CMI, namely, STs and IGRA, was evaluated for the first time in goats. Indeed, the use of P22 for IGRA tests has only been reported in red deer experimentally infected with *M. bovis* (37).

The combined interpretation of tests leads to a substantial improvement of Se at the expense of a variable loss of Sp. As expected, in the conventional context, the SICCT alone or combined with the STAND-IGRA (8, 11, 38) showed the best performances by DOR analysis. The performances of tuberculin-based tests were followed by the combinations of SICCT with the P22-IGRAs, which increased Se at the cost of a certain loss in Sp. Moreover, the combination of cP22-ST + P22-ELISA clearly increased the Se with the benefit of a minimal decrease of Sp, showing similar results than the combination of SICCT + P22-ELISA. These findings are in concordance with previous studies of P22-ELISA and tuberculin-based skin testing. In cattle, the combination of SIT + P22-ELISA showed an improvement of Se of 30 and 6 p.p. compared to the SIT and the P22-ELISA alone, respectively (25). In another study conducted in goats (39), the same combination improved the Se of the SIT and the P22-ELISA in 19 and 9.5 p.p., respectively. Also, in the same study in goats, the combination of SICCT + P22-ELISA improved the Se of the SICCT in a 24 p.p. These results confirmed the benefits of the strategic use of serological and CMI-based diagnostic tests in parallel to maximize the Se in infected settings.

In the TB-VAC context, the combination of P22-ELISA with the two P22-based STs showed similar performances than the DIVA-IGRA. However, the latter showed considerably lower Se than the combinations of P22-ELISA with P22-based STs (reduction in 23–24 p.p.). Previous studies reported the excellent Sp (16) and the lack of Se (40) of DIVA-IGRA, although the DOR analysis tended to overestimate the Sp in this study. The Se of vaccine-associated diagnostic tests is an essential requirement for the development of an integral vaccination strategy (41), and the combination of cP22-ST + P22-ELISA showed an efficient and innovative diagnostic approach in the TB-VAC context, showing the highest combined Se and Sp values (94 and 95%, respectively).

Concerning the use of the ST in solitary, the P22-based STs showed lower Se compared to both the SIT and the SICCT tests, although previous studies in dairy goat flocks, with larger samples and different epidemiological situations, have shown lower Se for SIT (65%, 95% CI: 63.3–68.2) (8) and SICCT (44.5%, 95% CI: 35–55) (42). However, the Se of the cP22-ST (74%, 95% CI: 63–83) was similar to Se observed in two previous studies using DIVA STs (based on the peptide cocktails ESAT-6, CFP-10, and Rv3616c) developed for the diagnosis of TB in cattle: 76%, 95% CI: 59–93 (43) and 75%, 95% CI: 47.7–97.7 (44). In the latter, the addition of the Rv3020c peptide improved the Se to reach 87.5% (95% CI: 61.7–98.5), being similar to the Se of sP22-ST (87%, 95% CI: 74–94) obtained in the present study. On the other hand, in BCG-vaccinated animals, the Sp of SIT and SICCT decreased dramatically (27 and 20 p.p. of reduction, respectively), whereas the Sp of sP22-ST and cP22-ST remained high (97 and 98%, respectively). These findings again highlight the suitability of P22-based STs as TB vaccine-associated diagnostic candidates, although improvements to increase the Se should be necessary.

Moreover, herd PTB status and MAP vaccination may also affect the interpretation of the results. MAP infection was not reported in farms B and C, and no recent clinical history of PTB was observed by the veterinarians. Despite this, vaccination against MAP is a common practice in small ruminants in Spain (12), and diagnostic interferences due to MAP vaccination

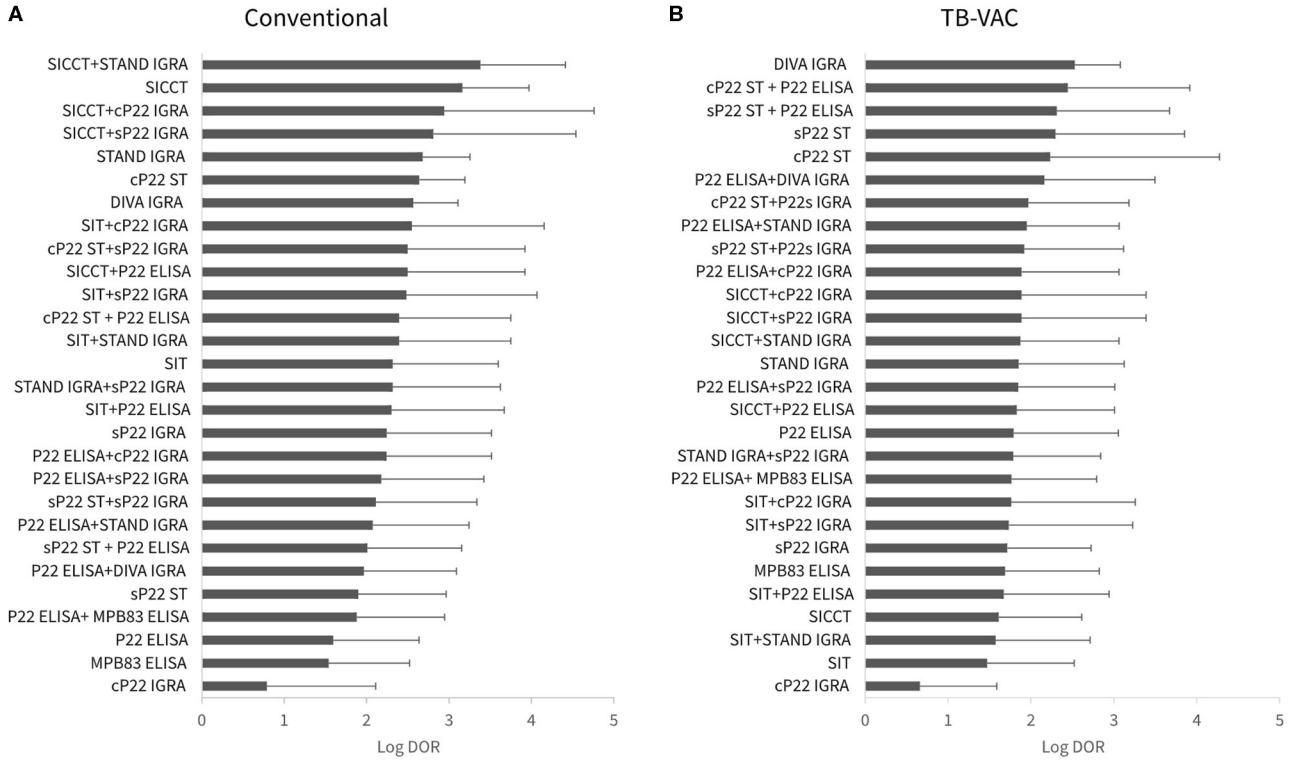

FIGURE 1 | Diagnostic test performance measured by diagnostic odds ratio (DOR). **(A)** Conventional (unvaccinated scenario). **(B)** Tuberculosis (TB)-VAC scenario, animals were vaccinated with *Mycobacterium bovis* bacille Calmette-Guérin (BCG). sP22-ST, single P22 skin test; cP22-ST, comparative P22 intradermal skin test; SIT, single intradermal tuberculin test; SICCT, single intradermal cervical comparative tuberculin test; sP22-IGRA, single P22 interferon-gamma release assay (IGRA) test; cP22-IGRA, comparative P22 IGRA test; STAND-IGRA, standard tuberculin IGRA test; DIVA-IGRA, differentiating infected from vaccinated animals (ESAT-6/CFP-10 peptide cocktail) IGRA test.

on TB diagnosis cannot be ruled out in these two MAP-vaccinated herds. In this sense, strong reactions to PPD-A were observed at skin testing (**Supplementary Data**), but the results of comparative tests (cP22-ST and SICCT) showed higher Sp compared to their respective single STs (i.e., sP22-ST and SIT). These findings indicate that some degree of cross-reactivity due to MAP vaccination was still maintained. Similarly, interferences of MAP vaccination on TB diagnosis, mainly in CMI-based diagnostic tests, were previously observed in MAP-vaccinated goats (14, 45).

Surprisingly, the P22-based IGRAs, particularly the sP22-IGRA, showed higher Se compared to STAND-IGRA and even higher compared to DIVA-IGRA. However, the Se of sP22-IGRA was similar to that previously observed by the STAND-IGRA (92%, 95% CI: 84–96) in other studies conducted in goats (26). The results of Se of the cP22-IGRA in the present study were also similar to those previously observed in experimentally *M. bovis*-infected red deer (37). However, a slight loss of Sp in the P22-IGRAs was detected compared to STAND-IGRA. Even so, the Sp was within ranges (95–100%) described for STAND-IGRA in previous studies (11, 38, 45). This mild reduction in Sp could be explained by the high concentration of P22 used for stimulation of whole blood (20 μg/ml) and by the fact that P22 complex contains 21 proteins also present in *M. avium* (23), which can cause cross-reactivity with MAP vaccination and/or infection. Indeed, the interference of MAP vaccination on STAND-IGRA has been previously observed in adult MAP-vaccinated goats (13, 14, 45). The Sp of P22-IGRAs considerably decreased in BCG-vaccinated herds compared to that previously described for the STAND-IGRA (16). Overall, the results of sP22-IGRA suggest that this test could be a potentially valuable tool for TB eradication in endemic areas, although further studies to determine the optimal concentration of P22 are required to improve its Sp with a minimal loss of Se.

Serological diagnostics is a cost-effective alternative for TB diagnostics. However, the Se of antibody-based diagnostic tests was generally lower compared to tests based on CMI (46, 47). In the present study, the Se of P22-ELISA was slightly lower than that in previous studies in goats and cattle (25, 39). This loss of Sp might be explained by the fact that animals from herd A were not vaccinated against MAP nor subjected to frequent intradermal testing, factors that could enhance humoral responses against MTBC antigens (48). Interestingly, the Se was significantly enhanced when using P22 and MPB83 ELISAs in parallel. Thus, even though MPB83 is

a major component of the P22 complex, specific IgGs of some infected animals were only detectable using the MPB83 purified recombinant protein alone, while others were only detected using the P22 complex, which contains additional serodominant epitopes (23).

Finally, Sp of the P22-ELISA reached considerably higher Sp in MAP-vaccinated (and BCG-unvaccinated, i.e., herd B) goats (93%) compared to that previously found in Spanish (78%) and Norwegian MAP-vaccinated goats (58%) (24). In the latter study, besides MAP vaccination, MAP coinfection and/or contact with environmental mycobacteria was not discarded as a source of diagnostic interference. Interestingly, in the present study, the Sp was also high in BCG- and MAP-vaccinated goats (96%), suggesting that BCG vaccination does not induce antibody responses that cause interference on the diagnosis by the P22-ELISA. The absence of antibody responses was consistent with the fact that the BCG Danish strain used for vaccination expresses low levels of MPB83 and MPB70 (49), which are the most abundant proteins of the P22 antigenic complex (23). Moreover, tuberculin skin testing after 42 days of MAP or BCG vaccination caused a boosting effect on humoral responses against tuberculin antigens, resulting in false-positive cattle for an MPB83-based ELISA (50). Here, minimal or no boosting effects of MAP/BCG vaccination due to skin testing were observed on the P22-ELISA. Indeed, goats from herd B were sampled around 2 years after vaccination against MAP, and ST was performed once or twice after MAP vaccination. Also, 34/68 goats from herd C were vaccinated with BCG and Gudair® at 9–10 months before the sampling, whereas the rest of the animals were vaccinated more than 1 year before, and no ST was performed since. Based on the results herein, the P22-ELISA seemed to be a useful ancillary diagnostic tool, either in BCG or MAP vaccination context, although it should be confirmed in further studies with larger sized herds.

In conclusion, this study reinforces the applicability of the P22 antigen complex as a complementary instrument for TB diagnostics in goats under different control scenarios. The P22 serological diagnostic is a cost-effective alternative, and combined interpretation with STs, either with PPD-B or P22, showed promising results. Moreover, the use of P22 antigenic complex in CMI-based diagnostic tests showed encouraging results, being suitable for further research on the improvement of TB diagnostics.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the **Supplementary Material**.

ETHICS STATEMENT

All animals included in this study belonged to commercial farms and were not experimental animals. All sampling and handling procedures were carried out by authorized veterinarians according to standard farm methods and in conformity with Spanish legislation (Royal Decree 2611/1996 and amendments) and European Union laws for the protection of animals used for scientific purposes (2010/63/EU). Test and slaughter of positive animals, as well as *post-mortem* sampling to confirm the disease, were conducted according to the regulations defined by the Catalan Government (Resolution AAM/1314/2014). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

BP and JB contributed to conceptualization. CA-V, BP, and MG contributed to data curation. CA-V and BP performed the formal analysis. BP and LJ acquired funding. CA-V, BP, MG, EV, MD, IM, JI-L, and JB contributed to the investigation. CA-V, BP, JI-L, EV, and MD contributed to the methodology. BP and LJ contributed to project administration. JI-L and MS acquired resources. CA-V and BP wrote the original draft. JB, LJ, JI-L, EV, and MD contributed to writing, reviewing, and editing.

FUNDING

This work was funded by a grant from *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria* (INIA), reference number RTA2015-00043-C02-00 (FEDER co-funded). IRTA is supported by CERCA Program/Generalitat de Catalunya. Claudia Arrieta-Villegas is recipient of a PhD grant from INIA (FPI-INIA 2016: CPD2016-0109) funded by the Spanish Ministry of Economy and Competitiveness.

ACKNOWLEDGMENTS

We are grateful to Maite Martín, Zoraida Cervera, Carlos Lopez-Figueroa, and the staff of Field Studies Unit of IRTA-CReSA for their technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00374/full#supplementary-material

Supplementary Data | Raw data diagnostic tests.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 10 July 2020
doi: 10.3389/fvets.2020.00352

Monitoring *Coxiella burnetii* Infection in Naturally Infected Dairy Sheep Flocks Throughout Four Lambing Seasons and Investigation of Viable Bacteria

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 29 March 2020
Accepted: 20 May 2020
Published: 10 July 2020

Citation:
Álvarez-Alonso R, Zendoia II, Barandika JF, Jado I, Hurtado A, López CM and García-Pérez AL (2020) Monitoring Coxiella burnetii Infection in Naturally Infected Dairy Sheep Flocks Throughout Four Lambing Seasons and Investigation of Viable Bacteria. Front. Vet. Sci. 7:352. doi: 10.3389/fvets.2020.00352

Progression of *Coxiella burnetii* infection in four naturally infected sheep flocks, and in their farm environment, was monitored throughout four lambing seasons. Flocks with an active infection were selected based on the presence of *C. burnetii* DNA in bulk-tank milk (BTM) and a high seroprevalence in yearlings during the previous milking period (Spring 2015). During four consecutive lambing seasons (2015/16–2018/19), samples were collected within 1 week after each lambing period from animals (vaginal swabs, milk and feces from ewes, and yearlings) and the environment (dust indoor sheep premises). BTM samples and aerosols (outdoors and indoors) were monthly collected between lambing and the end of milking. Real-time PCR analyses showed different trends in *C. burnetii* shedding in the flocks, with a general progressive decrease in bacterial shedding throughout the years, interrupted in three flocks by peaks of reinfection associated with specific management practices. A significant relationship was found between *C. burnetii* fecal shedding and the bacterial burden detected in dust, whereas shedding by vaginal route affected the detection of *C. burnetii* in indoor aerosols. Three genotypes were identified: SNP8 (three flocks, 52.9% of the samples), SNP1 (two flocks, 44.8% samples), and SNP5 (one flock, two environmental samples). *Coxiella burnetii* viability in dust measured by culture in Vero cells was demonstrated in two of the flocks, even during the fourth lambing season. The results showed that infection can remain active for over 5 years if effective control and biosafety measures are not correctly implemented.

Keywords: ***Coxiella burnetii*, Q fever, sheep, genotypes, viability, lambing, environment**

INTRODUCTION

Q fever is a widespread zoonosis caused by the intracellular bacterium *Coxiella burnetii*. Goats and sheep are considered the main reservoir of *C. burnetii*, and both have a significant role as source of human infection (1, 2). Q fever causes abortions in small ruminants (3), and once *C. burnetii* enters into a flock, infection spreads rapidly. Infected animals shed *C. burnetii* through birth products, vaginal fluids, feces, milk, and urine for several weeks after abortion or normal parturition

(4–10), but the bacterial load shed by aborted animals is higher than that shed by those that deliver normally (9). Abortion rates due to *C. burnetii* are especially high in goats (up to 70–90%) (4, 11) but lower in sheep (usually below 6%) (12). These low rates can be considered normal by the farmer, and consequently, samples of aborted animals are not submitted for diagnostic testing. Therefore, Q fever is not diagnosed, control measures are not implemented, and the infection can be maintained in a sheep flock throughout consecutive lambings. In a recent study carried out in dairy sheep flocks, *C. burnetii* shedding through milk was still observed in several flocks 10 years after first detection (13). This suggests that flock management practices together with lack of control measures implemented can cause periodical reactivation of *C. burnetii* infection (14–17).

Coxiella burnetii shedding by infected animals, together with their movements in indoor animal premises, promotes the formation of contaminated aerosols. Bacterial load in aerosols is the highest at the peak of abortions (4) and also correlates with the number of shedders in the flock (18). The progression of natural infection by *C. burnetii* in sheep flocks during several breeding seasons has not been fully investigated; thus, the length of time that the infection remains active in the flock is unknown. It is known that in the breeding seasons that follow an outbreak of abortion by Q fever, abortions decrease in sheep, and *C. burnetii* shedding naturally declines (19). Vaccination with phase I vaccine helped to limit bacterial shedding in ewes and yearlings from infected flocks in the two first years, resulting in a complete clearance of the infection after 4 years of vaccine implementation (20). However, *C. burnetii* DNA was still detected in dust samples in the fourth season after vaccination (20). Presence of *Coxiella* DNA in dust collected in farm premises has been reported in several studies (4, 9, 20, 21), but the time *Coxiella* remains viable has been scarcely investigated (4, 21). Kersh et al. (21) still found viable *Coxiella* in a goat farm in the kidding season that followed an abortion outbreak. In fact, the small-cell variant (SCV) is a spore-like form of *C. burnetii*, which can survive during long periods of time in the environment (22). Results pointed out that loads of viable *C. burnetii* are at the highest level during lambing/abortion period and progressively decrease thereafter until no viable bacteria are detected 2 months after the last parturition (4).

The genotype of *C. burnetii* could affect the course of infection (23, 24). In Spain, there is not much information about genotypes of *C. burnetii* involved in Q fever cases, neither in humans nor in animals. Recent studies carried out in Northern Spain identified goats rather than sheep as the main source of Q fever for humans, with pneumonia as the main symptom (4, 25–27), but interestingly, sheep and goats share the same *C. burnetii* genotypes in this area (4, 13, 27). Therefore, further studies are needed to better understand the epidemiological features of *C. burnetii* infection in sheep. This work was aimed at studying the progress of *Coxiella* infection throughout four lambing seasons in four dairy sheep flocks in semiextensive management systems in which no vaccination program was implemented. Genotyping of the strains involved and the investigation of *C. burnetii* viability in consecutive breeding seasons would help to better understand Q fever infection in sheep.

MATERIALS AND METHODS

Flocks Selection and Sampling Approach

Latxa is the dairy breed of sheep in the Basque Country. Lambing takes place once per year, between November and January for ewes and between March and April for yearlings. Animals are housed indoors in winter and at night and in rainy weather during the milking season, which ends in June–July. After that period, flocks are moved to communal mountain pastures where sheep share grazing areas with other sheep flocks, cattle, and horses, and wildlife, mainly wild boar, roe deer, badger, and foxes.

Four sheep flocks that tested real-time PCR positive to *C. burnetii* on bulk-tank milk (BTM) samples collected in March–April 2015 were selected. These flocks also showed a high seroprevalence against *C. burnetii* measured by ELISA in yearlings (**Table 1**). Both results suggested that *C. burnetii* infection was active in these flocks. Farmers were interested in collaborating and studying the evolution of the infection throughout four lambing seasons: 2015/2016, 2016/2017, 2017/2018, and 2018/2019. Flocks had never been vaccinated against this pathogen. It should be noted that, at the beginning of this study, there was a stock rupture in the production of the phase I inactivated vaccine, so vaccination could not be considered. A questionnaire was conducted to collect data on census, farm characteristics, management system, abortion history, and hygiene and biosecurity measures implemented in each farm. According to farmers' perception, significant abortions were not reported in the years prior to the study. However, one of them reported that a family member had suffered from pneumonia in the 2014/2015 production season, but since hospitalization was not needed, the etiological agent was not identified. For the correct management of the placentas, farmers were offered a freezer and biohazardous waste disposal containers, which, once filled, would be removed for incineration of infectious material. The goal was to quickly remove potentially infective material from the farm environment, thereby reducing possible environmental contamination. Three of the farmers performed this procedure, while the fourth (flock 2) had a type of slatted floor that resulted in placentas falling directly into the slurry pit. **Table 1** summarizes information about the selected flocks. Two of the flocks (flocks 2 and 3) used to buy animals from other flocks. Flock 4 had old animal premises and moved to a new farm in the lambing season 2018/2019.

Flocks were visited at lambing during four consecutive lambing seasons. During the first three seasons, samples were collected from ewes and yearlings and purchased animals (if any). During the last lambing season, only yearlings were sampled. Vaginal exudates (collected with swabs without medium), milk, and feces were taken from a maximum of 40 ewes and/or 40 yearlings within 1 week after parturition to evaluate *C. burnetii* shedding (**Table 1**). During these visits, environmental samples consisting of duplicates of 8–10 dust samples were taken from different surfaces of the animal premises to detect the presence of *C. burnetii* DNA and for further viability studies (one duplicate kept at −80°C). In addition, monthly visits were made until the end of the milking period, and a BTM sample was collected to monitor *C. burnetii* shedding at the flock level, as well as aerosols

TABLE 1 | General information of the sheep flocks included in the study.

	Flock 1	Flock 2	Flock 3	Flock 4
CENSUS				
Ewes	416	250	543	411
Yearlings	80	50	87	83
Communal pastures	Ewes	Ewes and yearlings	Ewes	Ewes and yearlings
Cattle in the farm	Yes	No	No	No
Goats in the farm	Yes	No	No	No
Purchase of animals	No	Yes	Yes	No
Abortions >3%	No	No	No	No
CHARACTERISTICS OF THE SHEEP PREMISES				
Year of construction	1995	2009	2007	1975*
Ventilation	Regular	Bad	Regular	Bad
Slatted floor	Yes	Yes	No	No
Straw bedding	Yearlings	No	Yes	Yes
Water source	Well	Well	Tap water	Tap water
Frequency of manure removal	Daily	1/year	2/year	2/year
BIOSECURITY MEASURES				
Management of placentas	Cremation	Manure	Cremation	Cremation
Exclusive cloth	No	No	No	No
Access of visits	Yes	Yes	Yes	Yes
Other measures	No	No	No	No
Q FEVER STATUS (2014/15)				
Seroprevalence (%)				
Ewes	26.7	40.0	13.3	26.7
Yearlings	66.7	46.7	46.7	53.3
BTM ELISA	Positive	Positive	Positive	Positive
BTM PCR	Positive	Positive	Positive	Positive
RANGE OF ANIMALS ANALYZED PER LAMBING SEASON				
Ewes†	30–40 (110)	30–61§ (131)	30–68§ (138)	30–40 (110)
Yearlings	28–40 (132)	7–28 (62)	7–28 (71)	30–40 (140)

**New sheep premises for the period 2018/19.*
†Ewes were not sampled in the 2018–2019 lambing season.
§Purchased ewes included.

collected indoor and outdoor sheep premises. The air sampler "MD8" Sartorius (Goettingen, Germany) was used, performing an aspiration of 100 L/min air for 10 min. The air passed through a gelatin filter adapted to the equipment, which was analyzed in the laboratory by real-time PCR in order to detect the presence of *C. burnetii* DNA in the aerosols generated at the farm.

Molecular Analyses

DNA Extraction and Real-Time PCR

DNA was extracted using the QIAmp DNA Blood Mini Kit (Qiagen Hilden, Germany) with some modifications. Briefly, milk or feces were mixed with 180 µl of ATL buffer (Qiagen, Hilden, Germany) and digested with 20 µl of proteinase K (8 mg/ml) for 30 min at 70°C before DNA extraction. Vaginal or dust swabs were treated with 300 µl of TE buffer (10 mM Tris base, 1 mM EDTA, pH 8) before being mixed with ATL and proteinase K for 1 h at 56°C. The initial treatment of the gelatin filters from the air sampler was done as previously described (18). Negative extraction controls were included every 10 samples to rule out DNA contamination. The presence of *C. burnetii* DNA was investigated by a real-time PCR procedure targeting the transposon-like repetitive region *IS1111* of *C. burnetii* genome (28). A commercial internal amplification control (IAC) (TaqMan® Exogenous Internal Positive Control, Thermo Fisher Scientific) was included in the assay to monitor for PCR inhibitors.

Genotyping

A selection of animal and environmental samples positive by real-time PCR with Ct < 31 were genotyped using a previously described single-nucleotide polymorphism (SNP) genotyping assay that detects 10 discriminatory SNPs by real-time PCR (29). SNPs were identified and selected by Huijsmans et al. (29) on the basis of both the consensus sequence generated from 100,000 bp of the five known whole genome sequences of *C. burnetii* (RSA493, RSA331, CbuG_Q212, Cbuk_Q154, and 5J108 111 Dugway) and an *in silico* investigation of their discriminatory power using BLAST (29). SNPs 769, 2287, 4439, 4557, 4844,

5423, and 6025 (positions indicated in the reference sequence RSA493, GenBank accession no. AE016828.2) are located within single-copy genes. SNPs 7087, 7726, and 7974 are located within the multicopy insertion sequence IS*1111* (positions within the first IS*1111* encountered, as indicated in the strain RSA493 reference sequence, GenBank accession no. AE016828.2), which is distributed throughout the *C. burnetii* genome. Ten real-time PCR reactions were performed per sample, each including two primers and two MGB® TaqMan probes to detect point mutations. Each 20 μl PCR mixture contained 625 nM of each primer, 125 nM of each probe, 1× Taq Mix ABsolute (Thermo Fisher Scientific), and 5 μl of template DNA. PCR reactions were run on a BioRad platform (CFX96™ RTi-PCR Detection System) using the following program: 15 min at 95°C, and 45 cycles of 3 s at 95°C, and 30 s at 60°C.

A selection of samples were also genotyped by multispacer sequence typing (MST) of eight spacers (Cox2, Cox5, Cox18, Cox22, Cox37, Cox51, Cox56, and Cox61) as previously described (30), with small modifications. Briefly, two four-plex PCR reactions were carried out followed by individual amplifications for each spacer region. Each amplicon was then purified and sequenced, and genotypes were identified by comparison with the database at https://ifr48.timone.univ-mrs.fr/mst/coxiella_burnetii/.

Serological Analyses: Enzyme-Linked Immunosorbent Assay

In order to evaluate seroprevalence against *C. burnetii*, individual milk samples were centrifuged, and milk sera were tested for Q fever antibodies using an ELISA test (LSIVET Ruminant Serum/Milk Q Fever kit; Thermo Fisher Scientific). An index (S/P) of the tested milk serum optical density to optical density of the positive control ratio was calculated according to the manufacturer's instructions. Individual milk samples with S/P indices $\leq$0.4 were considered negative, while samples with S/P $>$0.4 were considered positive.

Viability Studies

Ethics

Experimental studies were carried out in BSL3 facilities and consisted of experimental inoculations in 6-week-old BALB/c male mice combined with cell culture. Permission was obtained from the Ethical & Animal Welfare Committee (Bizkaiko Foru Aldundia, document 3/2017 v02, Reg. 32243 25 June 2018).

Isolation

Environmental viability of *C. burnetii* was assessed on dust samples collected after yearlings lambing under the assumption that dust deposited at that time would had been originated from aerosols generated during both ewes and yearlings lambing. Viability studies of C. *burnetii* in environmental samples were carried out by passage through mice and culture in Vero cells [African green monkey epithelial cells VERO C1008 (Vero 76, clone E6, Vero E6 ATCC® CRL-1586™)]. Dust samples were homogenized and prepared as detailed elsewhere (4). The quantification of *C. burnetii* genome equivalents (GE) in each homogenate was carried out by quantitative real-time PCR (qPCR) using 5 μl of DNA (in triplicates) and specific primers and a probe targeting the *IS1111* gene as described elsewhere (28). In each qPCR run, a standard curve was generated using 10-fold serial dilutions of a known concentration of Nine Mile (RSA439) phase II strain of *C. burnetii* DNA. After quantification, aliquots of 200–500 μl were prepared from each dust homogenate, containing approximately 10^2-10^3 *C. burnetii* GE. These aliquots were inoculated intraperitoneally in four mice each; a dust homogenate with viable *C. burnetii* (4) collected from a goat farm in 2017 and stored at −80°C was used as a positive control. As determined in previous studies (4, 31), mice were euthanized on days +14 and +21 p.i., and spleens were removed. The level of splenomegaly was determined from the ratio of the spleen weight to body weight. Half of the spleen from each mouse was processed for DNA extraction and real-time PCR amplification as fully detailed elsewhere (4). Positive samples were subjected to qPCR to quantify the number of *C. burnetii* GE detected in spleen in order to compare it with the number of GE inoculated; when the number of *C. burnetii* GE recovered from the spleen was equal or higher than the GE inoculated, *C. burnetii* was considered to have multiplied *in vivo*.

For qPCR-positive samples, the second half of the spleen was homogenized with 700 μl Dulbecco's modified Eagle's medium (DMEM) medium and 2% fetal bovine serum (FBS) in a TissueLyser. A hundred microliters of each homogenate were placed on shell vials (SV) containing Vero cells, as fully detailed elsewhere (4). Briefly, after harvesting *C. burnetii* from SV on day 6 p.i., three passages of 1,000 μl of harvested cells were transferred at weekly intervals into T25 culture flasks containing a Vero layer. At day 6 p.i. and before each passage, 200 μl was collected for DNA extraction and qPCR, following procedures described above. Cultures that maintained *C. burnetii* growth during the second or third passage were considered to be positive. Uninfected control cells were kept close to infected cells to rule out possible cross-contaminations.

Statistical Analyses

The possible influence of the different factors studied, i.e., flock (categorical; flock 1, flock 2, flock 3, flock 4), period of lambing (categorical; ewes/yearlings), and lambing season (categorical; 2015–2016, 2016–2017, 2017–2018, 2018–2019) over positive or negative *C. burnetii* animals shedding through vaginal fluids, feces, or milk was analyzed using a logistic regression. The final model was selected as the one with the lowest Akaike's information criterion (AIC) value from all of the models performed. Odds ratio values were computed by raising "e" to the power of the logistic coefficient over the reference category.

Cohen kappa statistics were used for assessing agreement between shedding by different excretion routes and ELISA results. The symmetry of disagreement between them was evaluated with McNemar's chi square test.

The risk of environmental (dust, indoor/outdoor aerosol) contamination by *C. burnetii* was evaluated with a data mining classification tree using "rpart" package (32); dust, indoor aerosols, and outdoor aerosols were continuous dependent variables (expressed in Ct values in real-time PCR). Classification and regression tree (CRT) identifies variables that divide

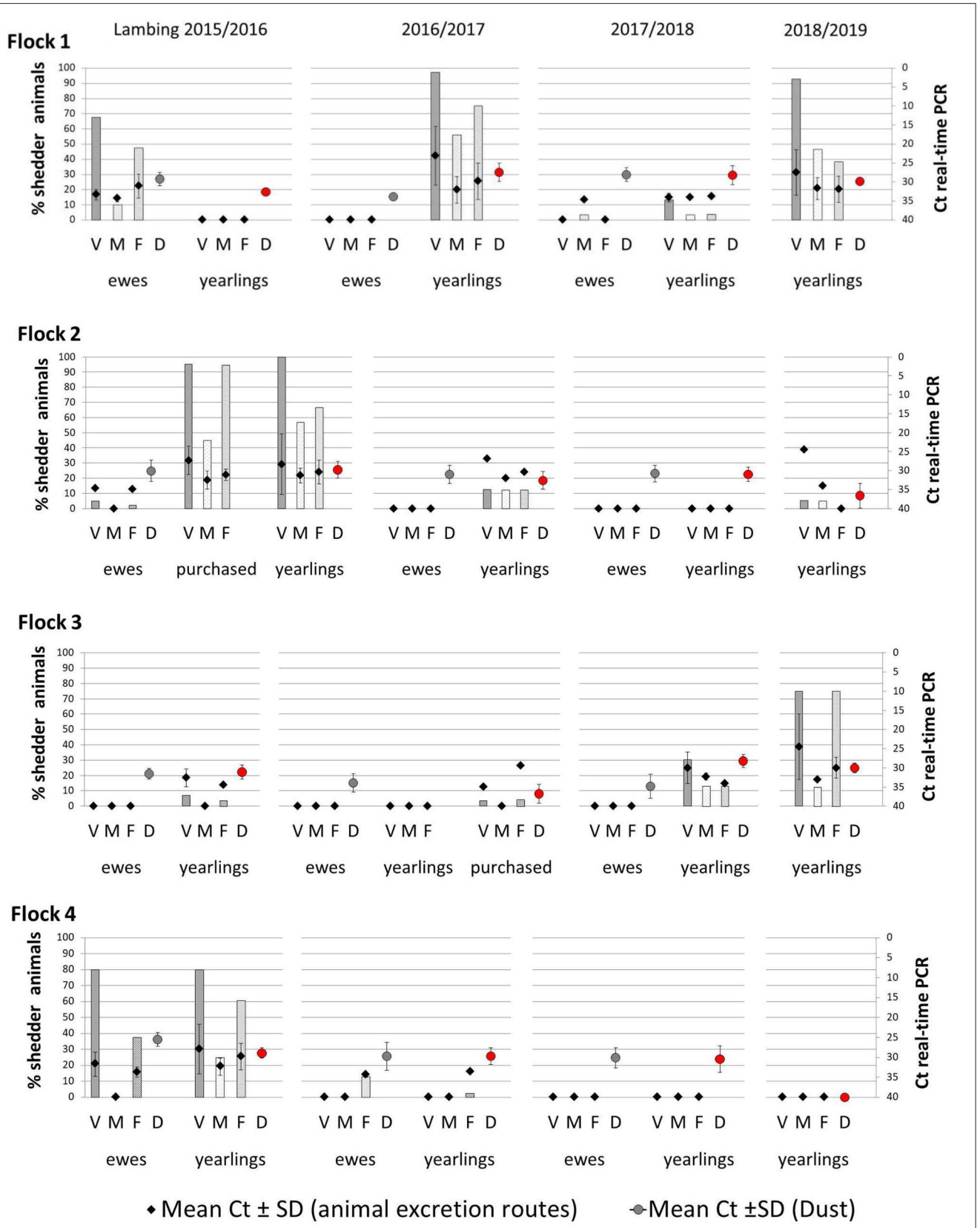

FIGURE 1 | Percentage of *Coxiella burnetii* shedders (ewes, yearlings, and, if applicable, purchased animals) throughout the four lambing seasons in the four studied flocks, through vaginal fluids (V), milk (M), and feces (F) with mean Ct values ± SD, represented with diamonds. The circles represent the mean of the Ct values ± SD obtained in real-time PCR from dust samples taken during ewes lambings (gray circles) and yearlings lambings (red circles). Ewes were not sampled in the fourth lambing season and are not represented.

environmental (dust/aerosols) results into homogeneous subgroups with distinct patterns of *C. burnetii* contamination. The CRT model provides a way to identify main factors. CRT evaluates all the values of the potential factor using, as a criterion, the significance of a statistical ANOVA test and split maximizing the between-groups sum of squares, selecting the best predictor variable to form the branch in a classification tree; successively splitting in data set makes increasingly homogeneous nodes in relation to the dependent variable. This process continues until the classification tree is fully grown. Figures for CRT were performed by "rpart.plot" package (33).

The degree of splenomegaly in experimental mice was evaluated with a Welch two sample *t* test to correct homoscedasticity. Linear regression was performed with the purpose of analyzing any relationship between *C. burnetii* GE load present in the inoculum injected to mice and the GE of *C. burnetii* recovered from the spleen of experimental animals. Log-transformed data were used in both analyses. All statistical analyses were performed using the statistical software R version 3.6.2 (34).

RESULTS

Coxiella burnetii Shedding and Serological Response in Ewes and Yearlings

Real-time PCR results showed different trends in *Coxiella* shedding throughout the 4-year study in the studied flocks (**Figure 1**). Overall, the percentage of *C. burnetii* shedders was significantly higher in the first lambing season (2015/2016) (**Table 2**). In the first lambing season, flock 1 showed a high-moderate percentage of ewes shedding *C. burnetii* by different routes (vaginal fluids > feces > milk), but no shedders were detected in the group of yearlings. The following lambing seasons (2016/2017 and 2017/2018) ewes barely shed *C. burnetii*, whereas yearlings showed a reactivation of the infection in the second and fourth lambing seasons. The situation in flock 2 was affected by the reported purchase of a group of 30 pregnant ewes in December 2015. These animals became infected when they entered into the contaminated animal premises and shed high *C. burnetii* loads at lambing. This caused the reactivation of infection in the flock, which mainly affected the yearlings while the proportion of ewe shedders of that season was low (**Figure 1**). Afterwards, in the following seasons, flock 2 showed a decrease in the percentage of animal shedders. Patterns of infection in flock 3 were the opposite, with a low proportion or absence of *C. burnetii* shedders during the first two lambing seasons and reactivation of infection in yearlings in the last two seasons. Flock 3 introduced a new group of pregnant ewes ($N = 62$) on the 20th March 2017, 1 week before the lambing of yearlings. Later on, purchased animals showed to be infected at lambing (7% animal shedders, 2/28, in samplings carried out between 27th March and 19th April). Flock 4 showed a high percentage of shedders during the first lambing season, in both ewes and yearlings, but a significant decrease occurred in the following lambings and no shedders were detected in the last two seasons (**Figure 1**). Overall, flock 4 had a significantly higher number of animal shedders than flocks 2 and 3, but lower than flock 1 (**Table 2**).

Shedders were more frequently found among yearlings than among ewes, but to a lesser extent than among newly introduced animals (purchased group) (**Table 2**). Regarding *C. burnetii* shedding loads expressed as Ct values in real-time PCR, the highest excretion levels (lowest Ct) were detected in yearlings, especially through vaginal fluids (**Figure 1**).

Comparison of seroprevalence in ewes throughout the lambing seasons showed marked differences between flocks (**Figure 2**). Thus, in flock 1, seroprevalence in ewes ranged between 58 and 80%; in flock 2, between 30 and 60%; in flock 3, 35–40%; and in flock 4, 13–63%. Thirty-two percent of the recently purchased ewes (9/28) in flock 3 had antibodies against *C. burnetii*, suggesting that ewes were already infected when introduced into the flock.

In yearlings, seroprevalence increased or decreased according to the trends of *C. burnetii* infection in each flock, showing in general lower seroprevalences than ewes. Flocks 2 and 4 showed a progressive decrease in seroprevalence during the study period (**Figure 2**). Independently of the shedding route (vaginal, feces, or milk), a high percentage of *C. burnetii* shedders was

TABLE 2 | Logistic regression model for the prevalence of shedders.

	Estimate	*Z* value	Pr (> |*t*|)	OR	95% CI
Intercept	0.4718	2.233	0.0255	1.60	1.06–2.43
Flock 4 (Ref.)					
Flock 1	0.6048	2.816	0.0050	1.83	1.20–2.80
Flock 2	−1.1090	−3.775	0.0001	0.33	0.18–0.58
Flock 3	−1.7177	−5.553	0.0001	0.18	0.10–0.32
Yearlings (Ref.)					
Ewes	−0.7806	−3.762	0.0001	0.46	0.30–0.68
Purchased	1.2847	4.353	0.0001	6.20	2.74–14.26
Lambing.season 2015–2016 (Ref.)					
Lambing.season 2016–2017	−1.7405	−7.708	0.0001	0.18	0.11–0.27
Lambing.season 2017–2018	−2.4730	−8.327	0.0001	0.08	0.05–0.15
Lambing.season 2018–2019	−0.6371	−2.166	0.0303	0.52	0.30–0.93

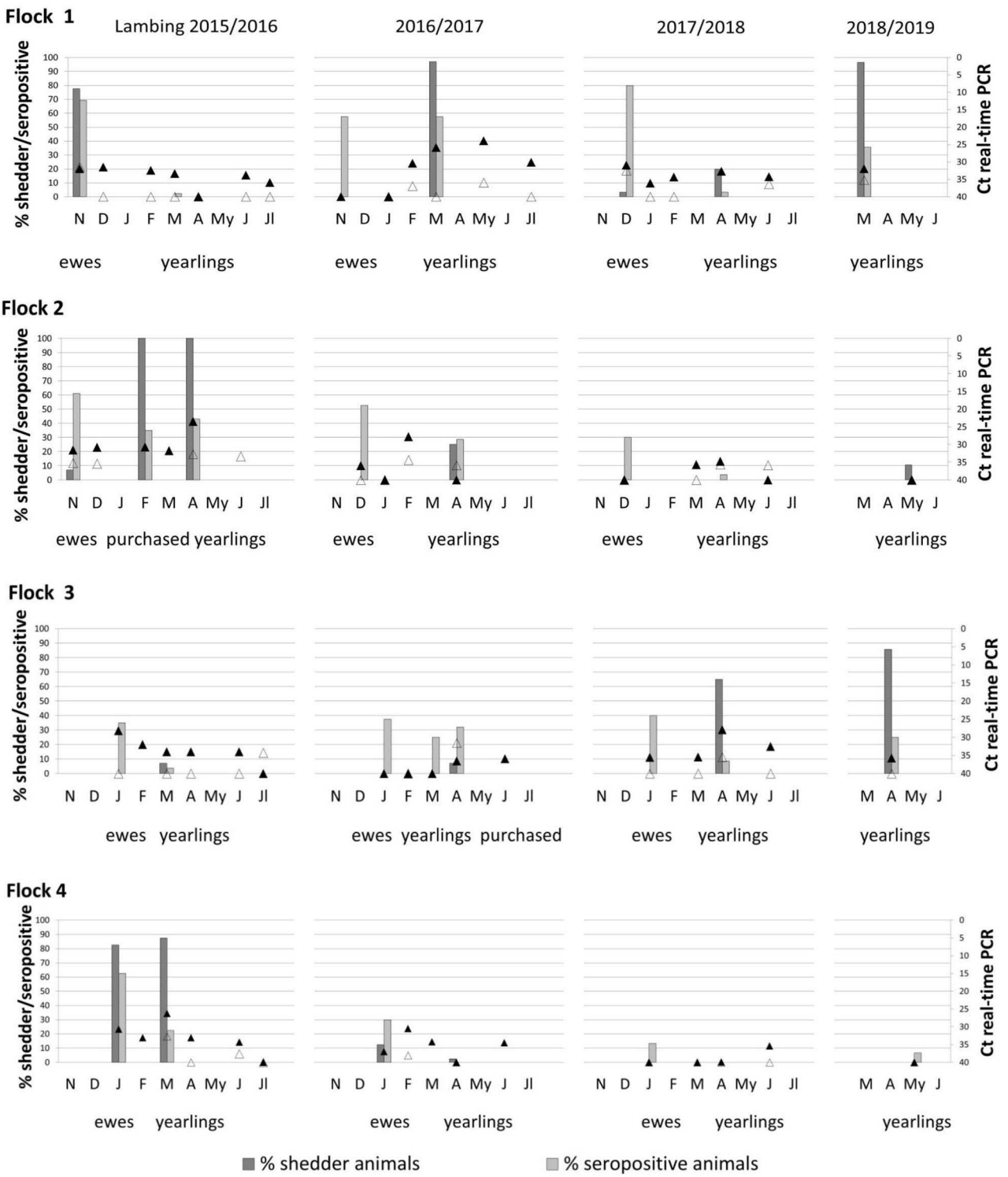

FIGURE 2 | Percentage of shedder animals by at least one excretion route (vaginal fluids, feces, or milk) and seroprevalence observed corresponding to ewes and yearlings of the four studied flocks during four lambing season (bars). *Coxiella burnetii* DNA present in aerosols taken outdoor (empty triangles) and indoor (black triangles) animal premises from lambing to the end of milking season are also represented. Ewes were not sampled in the fourth lambing season and are not represented.

not always positively correlated with seroprevalence (**Figure 2**). Cohen kappa (kappa = 0.142) and McNemar's test (McNemar's chi-squared = 10.75, $df = 1$, $P = 0.001$) statistics showed a poor relationship between *C. burnetii* shedding and ELISA results. Results of *C. burnetii* shedding as well as detection of antibodies in ewes and yearlings per flock and lambing season are compiled in **Table S1**.

The evolution of *C. burnetii* DNA presence in BTM samples during the four milking seasons is shown in **Figure 3**. In the first lambing season, bacterial shedding was very low in the four herds, always with Ct >30. In all milking seasons, bacterial load in BTM was higher after yearlings were included in milking (from February onwards). The highest bacterial load (Ct = 30) was found in flock 1 in the second lambing season. The reactivation of *C. burnetii* infection observed in yearlings from flocks 1 and 3 (see above) was also reflected in the bacterial load detected in BTM samples (**Figure 3** and **Table S1**).

Coxiella burnetii in Environmental Samples

Dust samples taken from different surfaces on animal premises during ewe and yearling lambings were *C. burnetii* DNA-positive throughout the 4 years of the study (ranges of Ct of 27–35), the only exception being flock 4, which became negative in the fourth lambing season. This flock had moved to new animal premises before lambing season 2018/2019 started, and *C. burnetii* DNA was not detected in dust thereafter. In the other three flocks, sporadic increases in dust bacterial load with respect to previous years were associated to increases in the number of shedders (**Figure 1**). The CRT algorithm stratified variables that played an important role in the amount of *C. burnetii* in dust (**Figure 4A**) and identified two determining factors for higher amounts of *C. burnetii* in dust (Ct = 29, 32% samplings), i.e., a percentage of fecal shedders higher than 1.2% (node 1), followed by the presence in the flock of more than 10% of shedders by vaginal route (node 3). When the presence of fecal shedders was low (node 2), the presence of *C. burnetii* in dust was determined by the lambing of ewes rather than yearlings (**Figure 4A**).

Results for the aerosols taken monthly indoor and outdoor of the farm from lambing until the end of the milking period are shown in **Figure 2**. Flocks 2 and 4 showed a progressive decrease in indoor environmental contamination during the course of the study, and in the fourth season, aerosols taken at yearling lambing were negative. On the contrary, flocks 1 and 3 showed sporadic increases in bacterial loads in aerosols due to reinfections in the flocks, and in the last lambing season, positive aerosols were still detected in the farm. The presence of *C. burnetii* in indoor aerosols was determined by the percentage of vaginal shedders (node 1) (**Figure 4B**), and when the percentage of shedders was below 10% (node 2), lambing season was identified as a determining factor (node 3); in the first lambing season, mean Ct value in indoor aerosols was lower than in the following seasons. From the second lambing period onwards, Ct values were determined by *C. burnetii* load shed through feces (node 5). Contamination of outdoor aerosols was less frequent, and when positive, the bacterial load was always lower (Ct > 32) than in aerosols taken indoors (**Figure 2**). Based on CRT algorithm results, rates of seroprevalence higher than 61% (node 1) determined the presence of positive aerosols outdoors (**Figure 4C**), and when seroprevalence in the flock was lower, a higher excretion of *C. burnetii* through feces (Ct < 40) (node 3) was the determining factor. Raw data of real-time PCR results obtained from dust and indoors/outdoors aerosols can be found in **Table S1**.

Genotypes of *C. burnetii*

A selection of samples (vaginal fluids, feces, milk, dust, and aerosols), with low Ct values in real-time PCR, was analyzed

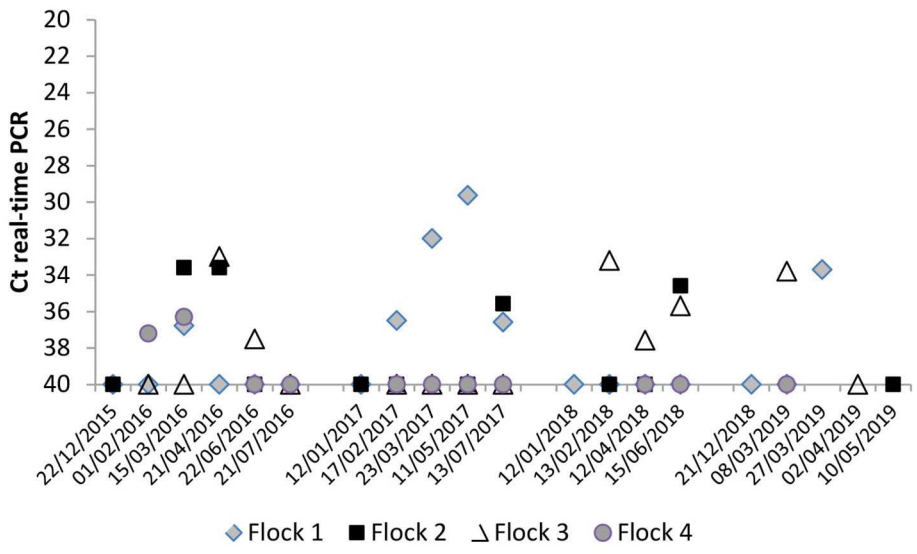

FIGURE 3 | Evolution of *C. burnetii* shedding through milk measured by bulk-tank milk (BTM) real-time PCR analyses throughout the four milking periods.

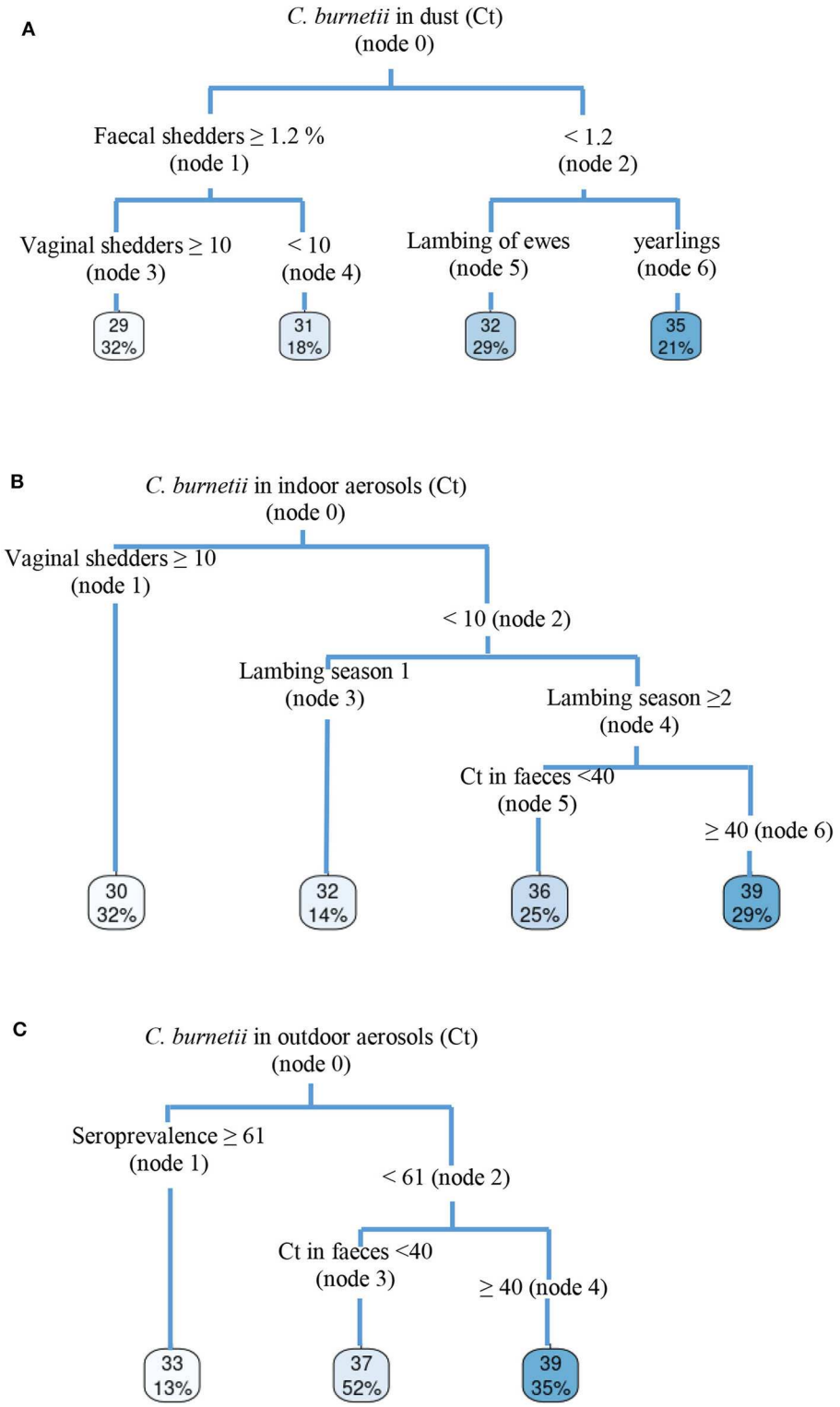

FIGURE 4 | Classification and regression tree (CRT) showing determining risk-factors for *C. burnetii* detection in **(A)** dust, **(B)** indoor aerosols, and **(C)** outdoor aerosols.

by SNP genotyping. The number of genotyped samples in each flock depended on the infection status shown in the course of the study. Thus, a total of 87 DNA samples could be genotyped (flock 1, 27 samples; flock 2, 25 samples; flock 3, 18 samples; flock 4, 17 samples), mainly from animals (61%) and dust (28%), and to a lesser extent, aerosols (11%) (**Table S2**). In flocks 1 and 4, only one genotype was identified (SNP8 in flock 1 and SNP1 in flock 4). In flock 2, three genotypes were identified (SNP1, SNP5, and SNP8), but SNP1 was the predominant genotype. In flock 3, two genotypes were identified (SNP1 and SNP8), but SNP8 was the predominant in both animal and environmental samples (**Table 3**). Overall, SNP8 was the most widespread genotype, found in three flocks and accounting for 52.9% of the samples; SNP1 was found in two flocks and 44.8% of the samples. For a selection of DNA samples ($N = 4$), the MST genotype was also determined. Two SNP1 samples from flocks 2 and 4 belonged to the MST13 genotype, while two SNP8 samples from flocks 1 and 2 corresponded with MST18. Unfortunately, no complete MST results were obtained from the only sample (dust) with SNP5 genotype.

Viability of *C. burnetii* in Dust

The duplicates of dust samples collected at each yearling lambing were pooled and processed to study *C. burnetii* viability using cell line culture. These accounted for 15 dust homogenates corresponding to the four lambings in flocks 1, 2, and 3, and three lambings in flock 4 (**Table 4**). Ct values of dust homogenates in real-time PCR ranged between 28.7 and 35.5 (**Table 4**). Prior to cell culture assays, each homogenate was inoculated into four 6-week-old male BALB/c mice. *C. burnetii* DNA was detected in the spleen of 20 of the 60 inoculated mice at +14 and +21 days p.i.; yet, in 3 of them, recovered GEs were below inoculated GEs. The presence of *C. burnetii* DNA was also confirmed in the four mice inoculated with the positive control (one mouse with lower GE than inoculated).

Comparisons among flocks suggested that viable *C. burnetii* were more widespread in flock 1, since 13 of the 16 mice spleens were positive after inoculation with dust collected in the four lambing seasons. In flock 2, only one mouse inoculated with a dust sample taken in the second lambing season was real-time PCR positive. In flock 3, six mice inoculated with dust collected in the first and fourth lambing seasons were positive in real-time PCR. Finally, mice inoculated with dust homogenates from flock 4 were all negative.

A significant correlation was observed between the *C. burnetii* GE inoculated and the *C. burnetii* GE recovered from spleen (adjusted $R^2 = 0.4398$, $P = 0.0042$). To assess splenomegaly, the ratio spleen of weight/live weight of mice was compared between positive mice (*C. burnetii* DNA detected in spleen by real-time PCR) and negative mice. Significant differences were observed ($t = -3.6449$; $df = 29.512$; $p = 0.0010$), and positive mice showed a ratio 1.53 times greater than negative mice.

Cultures in Vero cells of homogenates of spleens from the 20 real-time PCR-positive mice, as described above, resulted in *C. burnetii* growth in 7 mice (**Table 4**). Growth for at least two passages was only observed in those cases when shell vials were inoculated with at least 9.45×10^5 GE. *C. burnetii* isolates were successfully cultured from dust collected in flock 1 in the second and fourth lambing season, whereas in flock 3, viable *C. burnetii* was only recovered in dust collected in the fourth lambing seasons.

SNP genotypes were analyzed in positive spleens, and SNP8 was identified in mice inoculated with dust samples from flocks

TABLE 3 | Single-nucleotide polymorphism (SNP) genotyping results from selected DNA samples obtained in each flock from animals and the environment (dust and aerosols) during the four lambing seasons.

Lambing season	Origin of samples	Flock 1	Flock 2			Flock 3		Flock 4
		SNP8	SNP1	SNP5	SNP8	SNP1	SNP8	SNP1
2015–2016	Ewes/yearlings*	5	13	–	1	1	–	12
	Dust	–	3	–	–	–	3	3
	Aerosols	–	1	1	–	–	1	1
2016–2017	Ewes/yearlings	6	1	–	–	–	1	–
	Dust	2	1	1	–	–	1	1
	Aerosols	3	1	–	–	–	–	–
2017–2018	Ewes/yearlings	–	–	–	–	–	2	–
	Dust	2	–	–	1	–	2	–
	Aerosols	1	–	–	–	–	1	–
2018–2019	Ewes/yearlings	6	1	–	–	–	4	–
	Dust	2	–	–	–	–	2	–
	Aerosols	–	–	–	–	–	–	–

**Vaginal fluids/milk/feces.*

TABLE 4 | Investigation of viable *C. burnetii* in dust collected at yearling lambings in the studied flocks throughout the study using Balb/c mice and culture in Vero cell lines.

			BALB/c mice inoculation					Culture in cell lines (Vero E6) (GE/ml)					
Flock	**Lambing period**	**Ct real-time PCR (mean)**	**No. of GE[a] injected**	**No. of mice sacrificed at day +14 p.i.[b]**	**No. of mice sacrificed at day +21p.i.**	**No. of positive mice (day p.i.)**	**No. of GE in spleen**	**Inoculated**	**Day 6 p.i.**	**1st passage**	**2nd passage**	**3rd passage**	**Viable *C. burnetii***
1	2015–2016	32.9	3.8×10^2	2	2	2 (+14)	1.7×10^3	1.00×10^2	0.0	0.0	0.0	0.0	No
							1.0×10^3	1.00×10^2	0.0	0.0	0.0	0.0	No
						1 (+21)	1.3×10^4	1.00×10^2	0.0	0.0	0.0	0.0	No
	2016–2017	28.8	5.8×10^3	2	2	2 (+14)	3.4×10^4	1.54×10^2	3.44×10^2	0.0	0.0	0.0	No
							1.2×10^5	1.42×10^3	3.71×10^3	5.80×10^2	0.0	0.0	No
						2 (+21)	1.1×10^3	1.00×10^2	2.01×10^3	0.0	0.0	0.0	No
							9.8×10^6	9.50×10^5	8.16×10^5	1.43×10^6	2.88×10^5	2.26×10^5	Yes
	2017–2018	29.6	3.4×10^3	2	2	2 (+14)	2.5×10^2	1.00×10^2	0.0	0.0	0.0	0.0	No
							4.1×10^2	1.00×10^2	0.0	0.0	0.0	0.0	No
	2018–2019	28.7	6.2×10^3	2	2	2 (+14)	7.0×10^5	1.20×10^4	1.11×10^4	9.10×10^3	0.0	0.0	No
							4.5×10^7	9.45×10^5	4.23×10^5	1.94×10^5	2.22×10^5	0.0	Yes
						2 (+21)	2.7×10^6	3.82×10^6	2.18×10^6	4.70×10^5	5.24×10^5	1.00×10^5	Yes
							5.5×10^6	9.49×10^5	2.76×10^6	3.65×10^5	3.65×10^5	0.0	Yes
2	2015–2016	29.8	2.9×10^3	2	2	0	0						
	2016–2017	31.9	7.5×10^2	2	2	1 (+21)	8.8×10^3	2.52×10^3	2.10×10^3	0.0	0.0	0.0	No
	2017–2018	34.2	1.7×10^2	2	2	0	0						
	2018–2019	34.7	1.2×10^2	2	2	0	0						
3	2015–2016	30.5	2.0×10^3	2	2	1 (+14)	8.5×10^4	1.00×10^2	0.0	0.0	0.0	0.0	No
						2 (+21)	7.1×10^5	4.51×10^3	9.86×10^3	1.89×10^3	0.0	0.0	No
							1.2×10^4	5.19×10^3	1.42×10^4	1.85×10^4	0.0	0.0	No
	2016–2017	35.5	9.4×10^1	2	2	0	0						
	2017–2018	32.9	3.9×10^2	2	2	0	0						
	2018–2019	29.4	3.9×10^3	2	2	1 (+14)	7.1×10^7	4.45×10^6	1.11×10^6	4.63×10^5	4.46×10^5	1.00×10^5	Yes
						2 (+21)	1.5×10^7	3.79×10^6	1.25×10^6	1.99×10^6	3.30×10^6	1.32×10^6	Yes
							5.4×10^7	9.03×10^6	1.95×10^7	1.19×10^7	6.92×10^6	6.03×10^6	Yes
4	2015–2016	31.9	7.4×10^2	2	2	0	0						
	2016–2017	30.8	1.6×10^3	2	2	0	0						
	2017–2018	33.2	3.2×10^2	2	2	0	0						
Posit. Control (goats)	2017	20.9	10.7×10^5	2	2	2 (+14)	3.0×10^7	1.82×10^4	4.88×10^4	5.33×10^4	8.53×10^4	0.0	Yes
							9.1×10^6	2.88×10^6	6.90×10^5	8.37×10^5	2.76×10^5	6.91×10^5	Yes
						2 (+21)	4.8×10^6	2.30×10^4	2.05×10^4	2.86×10^4	0.0	0.0	No
							5.0×10^4	3.00×10^2	0.0	0.0	0.0	0.0	No

[a] *GE, genome equivalents of C. burnetii determined by quantitative real-time PCR targeting IS1111.*

[b] *p.i., postinoculation.*

1 and 3, whereas SNP1 was identified in the only mouse positive from flock 2. Interestingly, SNP5 detected in two environmental samples in flock 2 was not recovered from mice.

DISCUSSION

This study shows the patterns of *C. burnetii* infection in four naturally infected dairy sheep flocks throughout four lambing seasons. The presence of animal shedders in the previous milking season and high seroprevalence in yearlings suggested at the beginning of the study that infection was active in all flocks (1, 16). It is known that shedding of *C. burnetii* by vaginal fluids, feces, and milk can persist in the breeding season that follows infection onset in small ruminant farms, even when vaccination has been implemented (20). In this study, the low percentage or even the absence of shedders among ewes (flocks 2 and 3) and yearlings (flock 1) during the first lambing season supported the suspicion that *C. burnetii* infection had been present in these flocks before the 2014/2015 season. Besides, infection was still active in some flocks in the fourth lambing season (flocks 1 and 3), leading one to think that *C. burnetii* infection can remain in a flock for more than 5 years, probably due to periodical reinfections. In fact, a previous study hypothesized that infection could be maintained for 10 years (13). The outcome of infection showed the most desirable progression in flock 4, which started with the highest percentage of animal shedders and the highest bacterial shedding in ewes and yearlings during the first lambing season, followed by a significant decrease in the following years until the infection disappeared in the fourth lambing season. Movement of the flock to new uncontaminated farm premises before lambing season 2018/2019 undoubtedly helped to keep the flock free of infection.

The fact that the bacterial load shed throughout the 4 years of the study was lower in ewes than in yearlings also supports the idea that infection had established in the flocks some time before the study started. Had the infection onset occurred before, ewes would have had time to develop immunity, whereas yearlings would have been more susceptible to the infection (9).

This study also highlighted the risk of introducing naive animals into an infected flock, which, as shown in flock 2, can cause a reactivation of the infection. Purchasing infected animals also poses a risk when introduced into a negative flock (35, 36). This was probably the situation in flock 3, where purchased pregnant ewes could have been the source of a new infection. After lambings started, 1 week after purchase, the presence of shedders was detected and moderate seroprevalence was recorded, suggesting that those animals could have been infected previously and be the source of the infection observed in yearlings during the third and fourth lambing seasons.

The four flocks were managed under a semiextensive system where animals are housed during lambing and milking and, afterwards, graze on communal mountain pastures in contact with livestock and wildlife. In a region like the Basque Country, where Q fever is endemic and vaccination is not frequently implemented, this system poses a risk for infection and reinfection (14, 15, 17). Nevertheless, the infection peak observed in yearlings in flock 1 during the second and fourth lambing seasons is difficult to explain since yearlings did not share grazing pastures with other flocks. Roe deer frequented yearling grazing fields; however, their role as infection source was ruled out after testing *Coxiella* negative by PCR analysis of their feces (data not shown). However, 5.1% of the roe deer analyzed in the same region harbored *C. burnetii* DNA (37). Therefore, the role of roe deer and other wildlife species as source of *C. burnetii* infection as reported elsewhere (38) cannot be ignored. Besides, this was the only farm that also holds beef cattle and goats as potential source for yearling infection that unfortunately could not be tested. However, cattle and goats in the farm were managed under a year-round extensive system and did not share pastures with sheep, thus minimizing opportunities for sheep cross-infection. In any case, inappropriate implementation of biosafety measures is a risk factor for flock infections that cannot be ruled out (1, 16, 36).

The interpretation of the humoral immune response against *C. burnetii* is complex and has little value at individual level because a percentage of infected animals (25–50%) do not seroconvert (6, 39–41). In this study, a commercial ELISA kit was used to measure *C. burnetii* antibodies in milk samples, since a good correlation between the level of antibodies detected in individual milk with those present in blood serum has been reported (42, 43). The marked differences in seroprevalence observed in ewes and yearlings throughout the four lambing seasons among flocks were probably associated to the exposure of animals to different loads of viable bacteria. However, as seen in this study and others (16), seroprevalence is not correlated with bacterial shedding.

The abovementioned factors, such as animal purchase or grazing in communal pastures, can favor not only *C. burnetii* infection but also the probability of infection with more than one genotype. The presence of several *Coxiella* genotypes in a farm has been previously described (9, 44–46). Here, only one genotype was detected in flocks 1 and 4, whereas in flocks 2 and 3, three and two different genotypes were detected, respectively. Interestingly, flocks with more than one genotype were those that had purchased animals. Regardless of the flock, in all cases, the more frequently found genotypes were SNP1/MST13 and SNP8/MST18. Both genotypes had been found in the region in sheep flocks (13) or in Q fever outbreaks of caprine origin associated with pneumonia in humans (4, 27). In Europe, SNP1 and SNP8 genotypes have also been associated to Q fever outbreaks (29, 45), and SNP5, only detected here in two environmental samples (one dust, one aerosol) from flock 2, has been found in goats in Belgium and France (29, 45). Regarding any possible association between genotype and infection pattern, it is noteworthy that SNP1/MST13 was the predominant genotype in flocks where infection progressed toward a gradual decrease (flocks 2 and 4), whereas genotype SNP8/MST18 predominated in the two flocks where infection reactivated in yearlings (flocks 1 and 3). However, the effect of purchase of animals on that reactivation hampers any further conclusions. Similarly, since abortion rates were very low, no associations can be inferred between pathogenicity and genotype.

Inhalation of aerosols contaminated with *C. burnetii* is the main infection route in humans (23). Wind can easily spread *Coxiella* resistance forms when climatic conditions are favorable, and therefore, environmental contamination of the surroundings of infected farms is a hotspot of concern that has been addressed by many studies (4, 9, 21, 47, 48). In this study, *Coxiella* loads were higher in aerosols taken indoors than in those taken outdoors, and levels progressively decreased during the weeks that followed the lambing seasons. These results are in agreement with those found in similar studies (4, 18, 21, 47, 48). Interestingly, detection of *C. burnetii* in indoor aerosols was dependent on the proportion of animal shedders through vaginal fluids, as reported elsewhere (18). *C. burnetii* excretion by vaginal fluids is normally higher compared to feces or milk (9), especially when infection is recent in the flock. This study also pointed out that a high seroprevalence in the flock could be an indication of a recent infection by Q fever with a higher risk of detecting contaminated aerosols by *C. burnetii* outdoors.

Coxiella accumulates in the dust of infected farms, and its DNA remains in dust for long periods (20, 21). Here, levels of *C. burnetii* in dust seemed to depend mainly on the number of fecal shedders, and to a lesser extent of shedders by vaginal exudates. Nevertheless, the bacterial loads detected in dust samples were low in all four sheep flocks (ranges of Ct, 27–35) compared to the loads found in other goat and sheep flocks (4, 21, 49), but similar to the Ct values obtained in some farms from the Netherlands during the large Q fever outbreak (47, 48). The fact that, in the study herein, farmers rapidly discharged the placentas contributed to reduce environmental contamination at the sheep premises. Even though the inoculation procedure in mice was carried out at the same time and using the same conditions for all samples, inoculation of dust homogenates collected during the first lambing season of yearlings in flocks 2 and 4 did not produce positive results. Inoculated GE loads (2.9×10^3 in flock 2 and 7.43×10^2 in flock 4) were similar to those used with samples collected from the other two flocks that multiplied in mice tissues. Interestingly, the genotype identified in these samples was SNP1, which might suggests that multiplication of low concentrations of this particular genotype is not enough to grow in mice tissues.

Despite these low contamination loads in dust, isolation of *C. burnetii* was obtained from dust collected in the second and fourth lambing seasons in flock 1 and the fourth lambing season in flock 3. These results suggest that environmental contamination on the premises and surrounding areas of farms where *C. burnetii* infection has been present for several years and the number of shedders is low is markedly lower than at farms suffering a Q fever outbreak (4). However, despite the low loads of C. *burnetii* in dust, infection risk was still present since viable *C. burnetii* were detected during the fourth lambing season in two of the studied sheep flocks.

In conclusion, this study provided valuable epidemiological data on *C. burnetii* infection in sheep and opened new questions that require further investigation. The results obtained demonstrated that if *C. burnetii* infection is not controlled using a combination of vaccination and implementation of adequate biosafety and managing procedures, an active infection and continuous shedding of viable bacteria can persist in sheep flocks for over 5 years.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethical & Animal Welfare Committee (Bizkaiko Foru Aldundia, document 3/2017 v02, Reg. 32243 25 June 2018).

AUTHOR CONTRIBUTIONS

JB and AG-P: conceptualization. JB, IJ, and AG-P: methodology. CL: statistical analysis. RÁ-A, IJ, IZ, JB, and AG-P: investigation. AG-P and IJ: resources. RÁ-A and JB: data curation. RÁ-A: writing—original draft preparation. AH and AG-P: writing—review and editing. JB, AH, and AG-P: supervision. AG-P: project administration and funding acquisition. All authors contributed to manuscript revision and have read and approved the final manuscript.

FUNDING

This work was funded by INIA—Spanish National Institute for Agricultural and Food Research and Technology (RTA2017-00055-C02-00), the European Regional Development Funds (ERDF), and the Basque Government. RÁ-A is beneficiary of a Ph.D. contract funded by INIA (FPI-2015-014). The funders had no role in the study design, data collection and interpretation, or the decision to submit the work for publication.

ACKNOWLEDGMENTS

We thank the collaboration of the farmers.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00352/full#supplementary-material

Table S1 | Compilation of results of *Coxiella burnetii* shedding and detection of antibodies in ewes and yearlings per flock and lambing season; real-time PCR results obtained from BTM samples and from dust and indoors/outdoors aerosols.

Table S2 | Results of SNP genotyping obtained from animal and environmental samples.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 July 2020
doi: 10.3389/fvets.2020.00363

First Report of *Chlamydia* Seroprevalence and Risk Factors in Domestic Black-Boned Sheep and Goats in China

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The Gram-negative bacteria of the genus *Chlamydia* cause a wide range of diseases in humans and animals. The seroprevalence of *Chlamydia* in domestic black-boned sheep and goats in China is unknown. In this survey, a total of 481 serum samples were collected randomly from domestic black-boned sheep and goats from three counties in Yunnan province, southwest China, from July to August 2017. The sera were examined by an indirect hemagglutination assay (IHA). Antibodies to *Chlamydia* were detected in 100/481 [20.79%, 95% confidence interval (CI), 17.16–24.42] serum samples (IHA titer ≥1:64). The *Chlamydia* seroprevalence ranged from 12.21% (95% CI, 7.81–16.61) to 30.89% (95% CI, 22.72–39.06) across different regions in Yunnan province, and the differences were statistically significant ($P < 0.01$). The seroprevalence in male domestic black-boned sheep and goats (28.64%; 95% CI, 22.36–34.92) was significantly higher than that in the females (15.25%; 95% CI, 11.05–19.45) ($P < 0.01$). However, there was no statistically significant difference in *Chlamydia* seroprevalence in domestic black-boned sheep and goats between ages and species ($P > 0.05$). To our knowledge, this is the first report of *Chlamydia* seroprevalence in domestic black-boned sheep and goats in Yunnan Province, southwest China. These data provide baseline information for future implementation of measures to control *Chlamydia* infection in these animals.

Keywords: *Chlamydia*, domestic black-boned sheep and goats, indirect hemagglutination assay, seroprevalence, China

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Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

Reviewed by:
Si-Yang Huang, Yangzhou University, China
Si-Yuan Qin, Chinese Center for Disease Control and Prevention, China

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *05 January 2020*
Accepted: *26 May 2020*
Published: *17 July 2020*

Citation:
Sun L-X, Liang Q-L, Hu X-H, Li Z, Yang J-F, Zou F-C and Zhu X-Q (2020) First Report of Chlamydia Seroprevalence and Risk Factors in Domestic Black-Boned Sheep and Goats in China. Front. Vet. Sci. 7:363. doi: 10.3389/fvets.2020.00363

INTRODUCTION

Chlamydia, an obligate intracellular Gram-negative pathogen, is responsible for a broad spectrum of diseases in animals and humans (1, 2). *Chlamydia* grows and reproduces in the respiratory, urogenital, and gastrointestinal tracts (2). Two species of the genus *Chlamydia*, namely *Chlamydia abortus* and *Chlamydia pecorum*, can cause serious infections in sheep and goats (1). *Chlamydia* is a leading cause of abortion in sheep and goats, which caused significant economic losses to livestock industry (3–6). Additionally, as a zoonotic pathogen, humans can be infected via exposure to *Chlamydia* infected animals (7).

Chlamydia infection is prevalent in sheep and goats all over the world, especially in sheep-rearing areas, such as in Northern Europe and North America (8, 9). In China, *Chlamydia* infection in sheep has been reported in many provinces, such as Qinghai, Shandong, and Hubei (10). However, data about *Chlamydia* infection in domestic black-boned sheep and goats have been limited. Domestic black-boned sheep and goats have dark tissue compared to ordinary sheep and goats, which has been attributed to the presence of excessive melanin in domestic black-boned sheep and goats (11).

Domestic black-boned sheep and goats are indigenous animals to the Lanping County of Yunnan Province, China (11–13). Because of their unique characteristics of these breeds, black-boned sheep and goats have a strong adaptability, and they have been introduced into other provinces of China, such as Shandong, Henan, and Hebei (14). Therefore, in this study, we examined the seroprevalence and risk factors of *Chlamydia* infection in domestic black-boned sheep and goats in Yunnan Province, southwest China. Our results provide baseline data for future control strategies of *Chlamydia* infection in domestic black-boned sheep and goats in China.

MATERIALS AND METHODS

Ethical Statement

This study was approved by the Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences (approval no.: LVRIAEC-2017-06). Domestic black-boned sheep and goats, from which the blood samples were collected, were handled humanely in accordance with the requirements of the Animal Ethics Procedures and Guidelines of the People's Republic of China.

The Study Sites

The survey was conducted in Shilin County, Lanping County, and Yongsheng County in Yunnan Province, southwest China (**Figure 1**). Yunnan Province is the major producing region of domestic black-boned sheep and goats in China. In the present study, the sampling sites are all large-scale farms, which implement a free-range breeding mode for 5–8 h in daytime. The annual temperature difference in Yunnan Province is small, but the daily temperature difference is large.

Serum Samples

Between July and August 2017, a total of 481 blood samples were collected randomly from domestic black-boned sheep and goats from four intensive farms (n = 6,100), two of which were from Lanping county (n = 213), followed by Yongsheng county (n = 145) and Shilin county (n = 123), Yunnan province, southwest China. A standardized questionnaire was used to collect information about the region, gender, age, and species of each animal. Blood samples were transported to the laboratory, kept at room temperature for 2 h, and centrifuged at 3,000 g for 10 min, and the supernatants, which represent the serum samples, were collected and stored at −20°C until further analysis.

Serological Examination

A commercially available indirect hemagglutination assay (IHA) kit (Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences) was used to determine the level of *Chlamydia* antibodies in the serum of domestic black-boned sheep and goats. As a mature technology for detecting *Chlamydia* antibodies, the sensitivity and specificity of the IHA kit used in this study have been verified by the Ministry of Agriculture of China (NY/T 562-2002), which were 100% and 95%, respectively (15). The serological analysis was carried out according to the manufacturer's recommendations as previously described (16–19). Briefly, serum samples were added to 96-well V-bottomed polystyrene plates, which were diluted fourfold serially from 1:4 to 1:1,024. The detection antigen was added to each well, and the plate was then shaken slightly for 2 min followed by incubation at 37°C for 2 h. The samples were considered positive for *Chlamydia* antibodies when the agglutinated erythrocytes were formed in wells at dilutions of 1:64 or higher. Samples that had agglutination results between 1:4 and 1:64 were considered "suspect" and were retested.

Statistical Analysis

Differences in the seroprevalence of *Chlamydia* among domestic black-boned sheep and goats of different regions, genders, ages, and species were analyzed by a χ^2 test using the SPSS software (release 23.0 standard version; SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant. Odds ratios (ORs) with 95% confidence intervals (CIs) were also determined.

RESULTS

In the present study, 100 of the examined 481 serum samples of domestic black-boned sheep and goats (20.79%; 95% CI, 17.16–24.42) were seropositive for *Chlamydia* by IHA test at the cutoff titer of 1:64. The 100 positive samples included 26 samples (of 213) from Lanping Country (12.21%; 95% CI, 7.81–16.61), 36 (of 145) from Yongsheng Country (24.83%; 95% CI, 17.80–31.86), and 38 (of 123) samples from Shilin Country (30.89%; 95% CI, 22.72–39.06). The differences in *Chlamydia* seroprevalence between these regions were statistically significant ($\chi^2 = 18.59$, $df = 2$, $P < 0.01$; **Table 1**). As shown in **Table 1**, the investigation revealed that the seroprevalence in female and male animals was 15.25% (43/282; 95% CI, 11.05–19.45) and 28.64% (57/199; 95% CI, 22.36–34.92), respectively. The difference in *Chlamydia* seroprevalence was statistically significant between genders ($\chi^2 = 12.71$, $df = 1$, $P < 0.01$) of domestic black-boned sheep and goats. Seropositive black-boned sheep and goats were found in all four age groups and varied from 16.41% (21/128; 95% CI, 10.00–22.83) to 25.40% (48/189; 95% CI, 19.19–31.61). In terms of species, the seroprevalence was 22.76% (71/312; 95% CI, 18.11–27.41) in black-boned sheep and 17.16% (29/169; 95% CI, 11.48–22.84) in black-boned goats. There was no statistically significant difference in *Chlamydia* seroprevalence observed between age groups ($\chi^2 = 4.63$, $df = 3$, $P > 0.05$) and species ($\chi^2 = 2.09$, $df = 1$, $P > 0.05$) in domestic black-boned sheep and goats (**Table 1**).

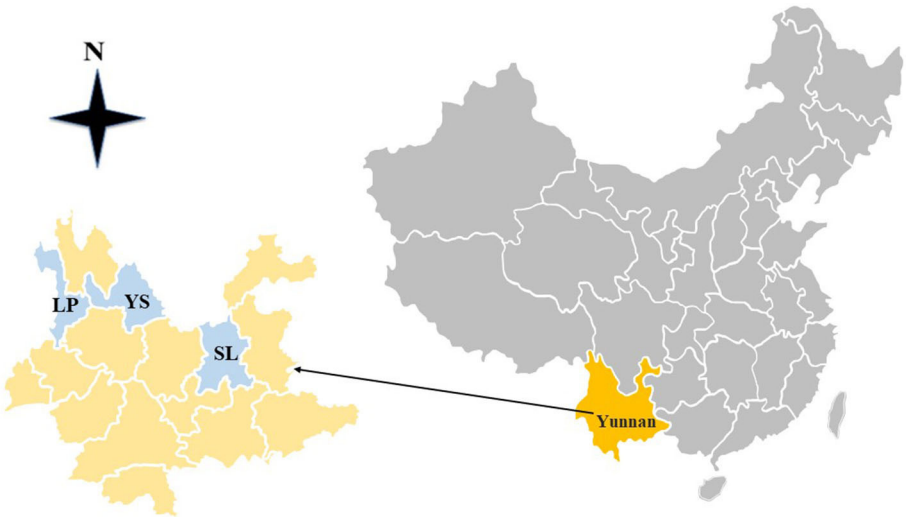

FIGURE 1 | The map of China showing the geographical regions in Yunnan province, where domestic black-boned sheep and goats were sampled. LP, Lanping County; SL, Shilin County; YS, Yongsheng County.

The antibody titers were diverse in domestic black-boned sheep and goats of different regions, genders, ages, and species, with the most frequent titers being 1:64 (87.00%), followed by 1:256 (10.00%) and 1:1,024 (3.00%; **Table 1**).

DISCUSSION

In this study, the seroprevalence of *Chlamydia* in domestic black-boned sheep and goats in Yunnan province was 20.79%, which was higher than the 8.45% reported in goats in Hunan Province, China (20), but was lower than that reported in sheep in Xinjiang Province (40.13%) in China (10). *Chlamydia* seroprevalence has been reported in sheep and goats worldwide. For example, 10.60% seroprevalence has been reported in sheep in India (21), and 33% seroprevalence has been reported in Spain (22). The different seroprevalences in different counties in our study is probably attributed to the differences in sanitation, husbandry practices, and animal welfare. In addition, other reasons for the variations of prevalence may include different ecological and geographical factors including temperature, rainfall, altitude, or level of vegetation. Furthermore, differences in the serological methods and cutoff titers used may be other factors that influence the seroprevalence of *Chlamydia* in different regions.

The overall *Chlamydia* seroprevalence in domestic black-boned sheep and goats in Shilin County was 30.89%, which was higher than the seroprevalence in Yongsheng County (24.83%) and in Lanping County (12.21%). There was significant difference in *Chlamydia* seroprevalence in domestic black-boned sheep and goats of different regions ($P < 0.01$). This result is consistent with a previous study that reported an 18.65% *Chlamydia* seroprevalence in Tibetan sheep in Gansu province (15). *Chlamydia* is significantly resistant under dry, cold (5–10°C), and dark conditions (23). Yunnan Province has a generally mild climate as diverse as its terrain. Shilin Country has an average annual temperature of 15°C and a mean annual rainfall of 1,010 mm. The warm temperature and appropriate precipitation in Shilin Country are favorable for the survival of *Chlamydia*. Therefore, the differences in *Chlamydia* seroprevalence in domestic black-boned sheep and goats across different regions are probably attributed to the variable climatic conditions in Yunnan Province.

Statistically, the *Chlamydia* seroprevalence in male (28.64%) domestic black-boned sheep and goats was significantly higher than in the females (15.25%). Statistical analysis showed a significant difference between genders ($P < 0.01$). Gender-related differences in *Chlamydia* seroprevalence were related to variations in immune response or antibody persistence between males and females (24). The result was different from a previous study, which reported no effect of the gender on the prevalence of *Chlamydia* infection in sheep (17).

The seroprevalence of *Chlamydia* varied across the different age groups of domestic black-boned sheep and goats. The highest seroprevalence was 25.40% in black-boned sheep and goats of the $0 <$ years ≤ 1 age group, and the lowest prevalence was 16.41% in the $2 <$ years ≤ 3 age group. But the differences were not statistically significant among different age groups ($P > 0.05$), which disagree with the study of Qin et al. (15), which reported positive association of *Chlamydia* seroprevalence with the ages of Tibetan sheep in Gansu Province. The higher seroprevalence in domestic black-boned sheep and goats of the $0 <$ years ≤ 1 age group may be due to the low levels of antibodies, which makes them more susceptible to infection. The different prevalence in different age groups indicates the possibility of horizontal transmission in investigated black-boned sheep and goat herds (25).

The seroprevalence of *Chlamydia* in domestic black-boned sheep (22.76%) was slightly higher than that in domestic black-boned goats (17.16%), which may be

TABLE 1 | Seroprevalence and risk factors for *Chlamydia* in domestic black-boned sheep and goats in Yunnan Province, southwest China, determined by indirect hemagglutination (IHA) test.

Variables	Categories	Antibody titers			No. tested	No. positive	Prevalence (%) (95% CI)	OR (95% CI)	*P*-value
		1:64	1:256	1:1.024					
Region	Lanping	22	1	3	213	26	12.21 (7.81–16.61)	Reference	<0.01
	Yongsheng	33	3	0	145	36	24.83 (17.80–31.86)	2.38 (1.36–4.15)	
	Shilin	32	6	0	123	38	30.89 (22.72–39.06)	3.22 (1.84–5.63)	
Gender	Female	40	1	2	282	43	15.25 (11.05–19.45)	Reference	<0.01
	Male	47	9	1	199	57	28.64 (22.36–34.92)	2.23 (1.43–3.49)	
Age (years)[a]	<0 to ≤1	42	6	0	189	48	25.40 (19.19–31.61)	1.73 (0.98–3.07)	0.2013
	1< to ≤2	17	1	0	103	18	17.48 (10.15–24.81)	1.08 (0.54–2.15)	
	2< to ≤3	18	1	2	128	21	16.41 (10.00–22.83)	Reference	
	>3	10	2	1	61	13	21.31 (11.03–31.59)	1.38 (0.64–2.98)	
Species	BBG[b]	28	0	1	169	29	17.16 (11.48–22.84)	Reference	0.1487
	BBS[c]	59	10	2	312	71	22.76 (18.11–27.41)	1.42 (0.88–2.30)	
	Total	87	10	3	481	100	20.79 (17.16–24.42)		

[a] *Year.*
[b] *Domestic black-boned goat.*
[c] *Domestic black-boned sheep.*
OR, odds ratio; CI, confidence interval.

related to the different susceptibility of goats and sheep to *Chlamydia*. Statistical analysis suggested that species may not be a crucial factor for *Chlamydia* infection in black-boned sheep and goats. The difference in *Chlamydia* seroprevalence in domestic black-boned sheep and goats may be caused by the sample bias, where more domestic black-boned sheep samples were examined than black-boned goats.

There are some limitations to the present investigation. The serum samples of black-boned sheep and goats examined in this study were collected from July to August 2017, a relatively short sampling time; thus, the reported *Chlamydia* seroprevalence may not fully reflect the true situation of long-term infection of *Chlamydia* in domestic black-boned sheep and goats. Given that domestic black-boned sheep and goats have been introduced into other provinces of China (14), further research should investigate *Chlamydia* seroprevalence in domestic black-boned sheep and goats in these provinces, which will provide global baseline data for the prevention of *Chlamydia* infection in black-boned sheep and goats in China.

CONCLUSION

The present study revealed that *Chlamydia* seroprevalence (20.79%) is relatively high in domestic black-boned sheep and goats in Yunnan Province, southwest China. This study also demonstrated that region and gender are the main risk factors for *Chlamydia* seroprevalence between domestic black-boned sheep and goats. To our knowledge, the present survey is the first to document the seroprevalence of *Chlamydia* infection in domestic black-boned sheep and goats in China, which provided baseline data for future prevention and control of *Chlamydia* in domestic black-boned sheep and goats.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Ethics and Administration Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

AUTHOR CONTRIBUTIONS

X-QZ and F-CZ conceived and designed the experiments. L-XS performed the experiments, analyzed the data, and wrote the paper. ZL, J-FY, and F-CZ participated in the collection of serum samples. Q-LL and X-HH participated in the implementation of the study. X-QZ and F-CZ critically revised the manuscript. All authors have read and approved the final version of the manuscript. All authors contributed to the preparation of the manuscript.

FUNDING

This project support was provided, in part, by the Agricultural Science and Technology Innovation Program (ASTIP) (Grant No. CAAS-ASTIP-2016-LVRI-03) and the Promotion Project of ESI Disciplines of Yunnan Agricultural University (Grant No. 2019YNAUESIMS03).

ACKNOWLEDGMENTS

The authors would like to thank Associate Professor Hany M. Elsheikha from the Faculty of Medicine and Health Sciences, University of Nottingham, UK, for improving the English of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 29 July 2020
doi: 10.3389/fvets.2020.00433

Evaluating the Risk Factors for *Porcine Epidemic Diarrhea Virus* Infection in an Endemic Area of Vietnam

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Porcine epidemic diarrhea virus (PEDV) causes enteritis, vomiting, watery diarrhea, and high mortality in suckling pigs, threatening the swine industry. Porcine epidemic diarrhea (PED) re-emerged globally in 2013 in many important swine-producing countries in Asia and the Americas. Several studies have identified the risk factors for the spread of PEDV in acute outbreaks. However, limited information is available on the risk factors for the transmission of PEDV in endemic regions. We hypothesized that poor biosecurity, location, and some social or cultural practices are the main risk factors for PEDV transmission in the Vietnamese pig population. The aim of this study was to evaluate the potential risk factors for the transmission of PEDV in an endemic area in Vietnam. In this case–control study, questionnaires containing 51 questions were completed for 92 PEDV-positive and 95 PEDV-negative farms. A logistic regression analysis was performed to assess the risk factors associated with PEDV infection. Province and the total number of pigs were included as random effects to determine their influence on the risk of PEDV infection. Twenty-nine variables of interest that have been associated with PEDV status were analyzed in a univariate analysis ($P < 0.20$), with backward stepwise selection. Only three of these 29 variables in four models remained significant PEDV risk factors in the final model: farrow-to-wean production type, distance from the farm to the slaughterhouse (< 1,000 m), and the presence of chickens on site ($P < 0.05$). This is the first study to identify the main risk factors for PEDV infection in an endemic area. Our findings suggest that hygiene measures should be strictly implemented on farms for the effective control and prevention of PEDV infection.

Keywords: ***Porcine epidemic diarrhea virus*, case–control study, risk factor, endemic, Vietnam**

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *14 April 2020*
Accepted: *16 June 2020*
Published: *29 July 2020*

Citation:
Mai TN, Bui TP, Huynh TML, Sasaki Y, Mitoma S, Daous HE, Fahkrajang W, Norimine J and Sekiguchi S (2020) Evaluating the Risk Factors for Porcine Epidemic Diarrhea Virus Infection in an Endemic Area of Vietnam. Front. Vet. Sci. 7:433. doi: 10.3389/fvets.2020.00433

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) causes enteritis, vomiting, watery diarrhea, and high mortality in < 10-day-old suckling pigs (almost 100%) and significant economic losses in the swine industry (1). PEDV re-emerged in 2013 and caused huge economic losses globally, in many important swine-producing countries in North America (USA, Canada, and Mexico) (2–4) and

Asia (South Korea, Japan, and Taiwan) (3, 5, 6). Direct contact between the pigs within farms is the primary transmission route of PEDV, via the fecal–oral route (7). Improvements in farm hygiene management and avoiding risky practices associated with contact with pig excrement are key factors in preventing the transmission of PEDV to farms (6). Several studies have comprehensively evaluated the risk factors for the spread of PEDV in the early phase of PEDV outbreaks (6, 8, 9). Indirect PEDV transmission through contaminated personal protection equipment occurs rapidly, within 24 h (10). In Japan, large-scale farms, proximity to an infected farm, number of feed trucks, short disinfectant contact time, and visiting veterinarians are factors strongly associated with the PEDV status of farms (11, 12). In the USA, transport is considered the main route of PEDV transmission (13, 14). In Italy, trucks have been shown to play an important role in the spread of PEDV (15). PEDV-contaminated feed was reported to be a significant risk factor for the transmission of PEDV between farms in the USA, Japan, and Canada (8, 9, 11, 16–18). Its transmission by transport vehicles was also reported to be a biological factor causing the rapid spread of PEDV in the USA and Japan (11, 13). Because PEDV is highly infectious and the infectious dose is low, it can be locally transmitted from PEDV-infected farms to neighboring PEDV-free farms through aerosol transmission or contaminated fomites (12, 19, 20). However, no study has analyzed the risk factors for PEDV spread in endemic areas or countries.

In Vietnam, PEDV was first observed in the southern provinces in 2009 (21). Published studies have demonstrated that PEDV is present in all major swine-producing regions in Vietnam (21–26). A descriptive survey recently provided evidence that northern Vietnam is an endemic area for PEDV, with a high proportion of PED-positive farms (30.89%) (Mai et al., unpublished). However, since its first detection, no specific PEDV control measures have been implemented by veterinary services to control the disease. Although vaccination or the feedback method has been applied on some pig farms, PEDV still occurs and frequently recurs. Our hypothesis is that the farm location and poor biosecurity measures for fomites, animals, and humans are the main risk factors for the nationwide transmission of PEDV in endemic areas. Some risk factors related to social and cultural practices in the Vietnamese swine industry could also play important roles in PEDV transmission among the pig population in Vietnam. Therefore, the objective of this study was to evaluate the potential risk factors for the widespread dissemination of PEDV in an endemic area with a case–control study. Theoutcome should extend our understanding of the dynamics of PEDV spread, in an effort to eliminate this economically important disease, which has emerged or re-emerged worldwide.

MATERIALS AND METHODS

Data Collection

In northern Vietnam, most piglets are produced and then transported to southernparts of the country. We have performed a survey between January 2018 and February 2019 to evaluate the proportion of PEDV-positive farms in a high-density pig population in northern Vietnam (**Figure 1**; the map was edited with PowerPoint from a screenshot of Google Maps [Map data©2020 Google]). The geographic location of Vietnam and the study area were mapped with the free, open-source Quantum Geographic Information System (QGIS) version 2.14.14 (https://www.qgis.org/en/site/). The required fecal sample size was estimated as 20 samples per farm. Therefore, on each farm, up to 20 fecal samples were collected from pigs in all ages. Then, 20 individual fecal samples from each farm were combined into two pooled samples (with a pooled size of 10 samples) for the test. Pooled samples were tested with reverse transcription (RT)–loop-mediated isothermal amplification (LAMP) (27). A "PEDV-positive farm" was defined as any swine herd with at least one positive result in two pooled samples from 20 samples collected from all-aged pigs. In total, 6,601 fecal samples were collected from 327 pig farms in northern Vietnam. The proportion of PEDV-positive farms was 30.9% (101 farms) and PEDV-positive farms were distributed throughout the study area. From the results of a PEDV survey of the pig population in northern Vietnam, there were 101 PEDV positive farms. All 101 PEDV-positive farms were selected as case farms, and 101 PEDV-negative farms, which were individually matched to a case farm based on the province in which they were located, were selected as the control farms for this study. The case and control farms were confirmed in 2018 with a PEDV survey to ensure that their management practices had not changed, even on the case farms, since 2018. We conducted a matched case–control study of the herd management practices for a period of 1 year (January—December, 2018). Questionnaires were sent to veterinarians or managers of these farms in July 2019, and the author (MTN) received the responses through the mail by the middle of August 2019.

Questionnaire

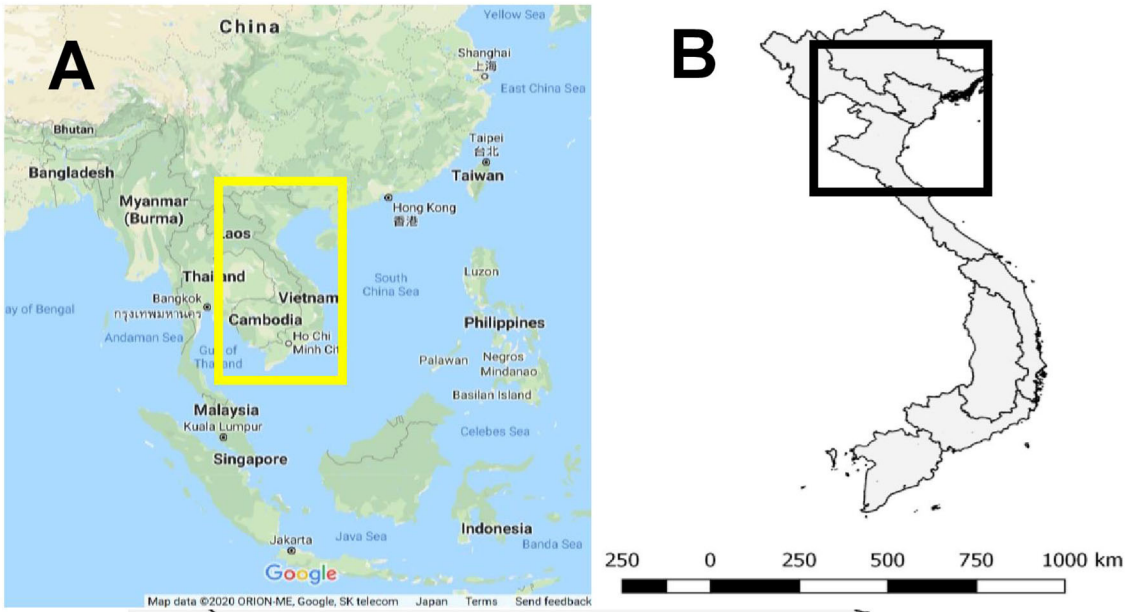
The questionnaire was developed based on known or published risk factors for PED and other infectious diseases of pigs (6, 9, 11, 13, 28). The designed questionnaire was checked by the authors (SS and YS) and then pre-tested with a selected number of veterinarians for the clarity and appropriateness of its content, questions, and responses in terms of the local situation, before the start of the study. Modifications were made if necessary.

Using a postal questionnaire survey, a case–control study was conducted on the PEDV-positive and -negative farms in northern Vietnam. The final version of the questionnaire contained 49 questions focusing on four main categories (**Table S1**).

Based on the actual data in Vietnam, PED frequently recurred in pig farms that had the presence of PEDV outbreaks in the past. To know whether there is a risk of the persistence of PEDV in these farms or not, only two questions applied specifically to the case farms. The first question concerned whether there had been an occurrence of PEDV in the preceding 2 years. If the answer was "yes," they were asked the second question, regarding how they dealt with PEDV on their farms.

Statistical Analysis

The outcome of interest for this study was the binary response variable "PEDV status," which took the value 1 if the farm was



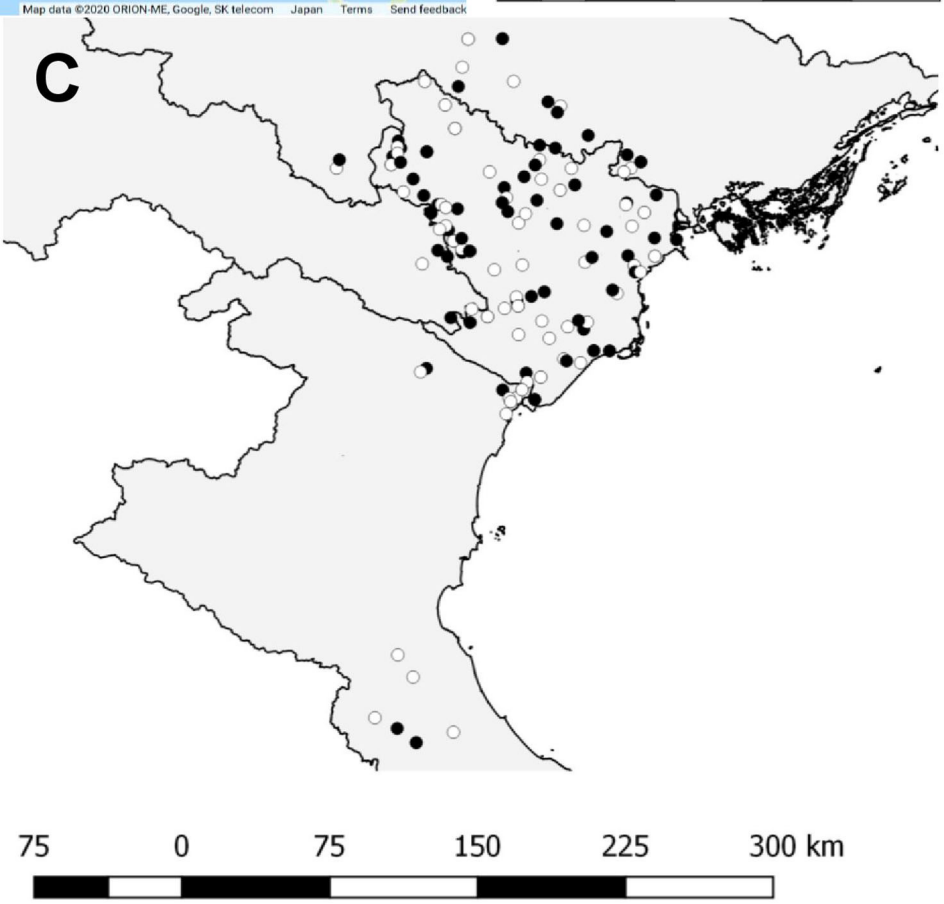


FIGURE 1 | Map of the study area. **(A)** Map is from Google Maps (Map data©2020 Google) and the yellow rectangle shows Vietnam. **(B)** Map of Vietnam; black square indicates the study area. **(C)** Locations of the 187 pig farms in northern Vietnam involved in this study. Black dots indicate PEDV case farms and white dots indicate PEDV control farms. Geographical locations of the farms were mapped with the Quantum Geographic Information System.

PEDV positive and 0 if the farm was PED negative according to RT–LAMP. The data captured with the questionnaire were entered into Microsoft Excel and all statistical analyses were performed with the statistical software R version 3.4.3 (CRAN, 2017). A univariate logistic regression analysis was performed to assess the association between PEDV status and each independent variable in the questionnaire. The significance of each explanatory variable was tested with the Wald test. Any variables significantly associated with PEDV status at the $P < 0.20$ level were subsequently selected for multivariable modeling. Backwards stepwise explanatory variable selection, beginning with the least significant variable, was performed while observing the changes in Akaike's information criterion (AIC). The final model was obtained with P levels for the remaining variables of < 0.05 and minimum AIC. After the main effects were identified, all possible two-way interactions were also examined. In this study, the total number of pigs did not correlate with PEDV status in the regression analysis. Therefore, to distinguish the influence of location (province) and the total number of pigs on the risk of PEDV, both variables were included as random effects in the multivariate model, by fitting generalized linear mixed-effects models. We used the lme4 package in R to analyze the fitted generalized linear mixed-effects models. The strengths of association between all the variables and the outcome were quantified by evaluating the odds ratios (ORs) and the corresponding 95% confidence intervals (CIs). The contribution of each variable in the final model of the risk of PEDV infection was quantified as the population attributable fraction (29). To avoid model convergence, multicollinearity was also tested with the variance inflation factor of explanatory variables (30).

Ethical Considerations

Informed consent was obtained in written form (by signature) or orally from all participants, who agreed to participate after they had received written information about the study. Ethical approval for the study was granted by the Hanoi School of Public Health Institutional Review Board, Hanoi, Vietnam (approval number 019-405/DD-YTCC) and the Animal Ethics Committee of the University of Miyazaki's Faculty of Agriculture, Miyazaki, Japan (approval number 2017–541).

RESULTS

Descriptive Statistics and Univariate Associations

In total, 187/202 (92.6%) respondents from 92/101 (91.1%) case farms and 95/101 (94.1%) control farms, located in a high-density pig-farming area in northern Vietnam, were included in this study (**Figure 1**). Some questionnaires had missing responses, which were excluded from the analysis. These farms were identified in a previous PEDV survey of the pig population in northern Vietnam. The data collected from these 187 farms were included in the analysis. In general, the number of pigs on the case farms was lower than the number on the control farms (mean numbers of pigs on the case and control farms were 1090 ± 1055.4 and 1533 ± 1661.4, respectively, $P > 0.05$). On average, the case farms had fewer vehicles visiting the farm per month than the control farms during the time of the study (mean numbers of vehicles per month on the case and control farms were 8.0 ± 4.3 and 8.4 ± 4.7, respectively, $P > 0.05$). However, the case farms contained more animal species other than pigs than the control farms (mean numbers of different animal species on the case and control farms were 3.6 ± 1.4 and 2.5 ± 1.5 species, respectively, $P < 0.05$). Of the 92 case farms, 52.17% (48/92) had experienced PEDV in the preceding 2 years and these farms used feedback to control the PEDV outbreaks. Of these farms, 27.08% (13/48) reported that the PEDV-infected pigs on their farms were still moved to other farms or sold to an abattoir. Only 5.88% (11/187) of the farms in the study used a PEDV vaccine to prevent infection.

In total, there were 49 variables in the case-control study. The univariate associations of 49 variables that were considered possible risk factors for PEDV infection are presented in **Table 1**. Of these 9 variables of interest, 29 were associated with PEDV status ($P < 0.20$).

Multivariable Associations

Because many variables (29 variables) were eligible for inclusion in the multivariable model, the selected variables were separated and categorized into four groups based on 4 main categories in **Table S1** and then four corresponding models were established. Backward stepwise selection was performed until the P-values of all the remaining variables were < 0.1 in the four corresponding models. A new model was established using the remaining variables in the four models ($P < 0.1$) (6). Further stepwise simplification of the model has applied until the estimated regression coefficients for all the explanatory variables retained in the final model were significant on the Wald test at a $P < 0.05$. **Table 2** shows the results of the final model. Only three of the 29 variables in these four models remained significant risk factors for PEDV infection in the final model: farrow-to-wean production (OR = 3.35, 95% CI: 1.51–7.43), close proximity of the farm to the slaughterhouse (OR = 7.15, 95% CI: 2.36–21.70), and the presence of chickens on the farm (OR = 3.36, 95% CI: 1.84–6.12) (AIC = 124.2).

DISCUSSION

To the best of our knowledge, this is the first study to quantify the risk factors for the spread of PEDV in an endemic area using a case–control strategy based on a questionnaire survey. Only three of the 49 variables tested remained significant risk factors for PEDV spread in the final model. The three main risk factors for the spread of PEDV in an endemic area in Vietnam are the farrow-to-wean production type, close proximity of the farm to the slaughterhouse, and the presence of chickens on the farm. These factors were significantly associated with the PEDV status of the farms.

In this study, there was a strong relationship between the distance from the farm to the slaughterhouse and the PEDV status of the farm. Close proximity to the slaughterhouse (< 1,000 m) increased the risk of PEDV infection 7.15-fold relative to that on farms further from the slaughterhouse. Population attributed fraction is the proportion of disease in the population

TABLE 1 | Results of a univariate analysis of location variables in the risk of *Porcine epidemic diarrhea virus* infection in a case–control study of northern Vietnamese pig farms in 2018.

Groups	Variables	Category	No. of cases	Proportion of response (%)	No. of controls	Proportion of response (%)	OR (95% CI)	*P*-value
Farm location ($n = 8$)	Distance from farm to the closest farm	<200 m	24	26.1	17	17.9	1.96 (0.82–4.67)	0.127
		201–500 m	16	17.4	17	17.9	1.31 (0.52–3.26)	0.565
		501–1,000 m	34	37.0	36	37.9	1.31 (0.61–2.82)	0.487
		>1,000 m	18	19.6	25	26.3	Ref	
	Distance from farm to the main road	<200 m	24	26.1	14	14.7	3.31 (1.34–8.21)	0.009
		201–500 m	26	28.3	15	15.8	3.35 (1.38–8.16)	0.007
		501–1,000 m	27	29.3	37	38.9	1.41 (0.64–3.13)	0.396
		>1,000 m	15	16.3	29	30.5	Ref	
	Distance from farm to the residential area	<200 m	19	20.7	9	9.5	4.10 (1.53–10.98)	0.004
		201–500 m	24	26.1	25	26.3	1.86 (0.83–4.19)	0.13
		501–1,000 m	32	34.8	28	29.5	2.22 (1.02–4.81)	0.042
		>1,000 m	17	18.5	33	34.7	Ref	
	Distance from farm to the irrigation system	<200	54	58.7	40	42.1	2.16 (1.08–4.32)	0.028
		201–500 m	18	19.6	23	24.2	1.25 (0.54–2.88)	0.596
		>500 m	20	21.7	32	33.7	Ref	
	Distance from farm to the slaughterhouse	<=1,000 m	22	23.9	4	4.2	5.35 (1.68–17.07)	0.002
		Unknown	33	35.9	55	57.9	0.58 (0.31–1.10)	0.093
		>1,000 m	37	40.2	36	37.9	Ref	
	Distance from farm to local market	<500 m	12	13.0	2	2.1	9.27 (1.73–49.66)	0.004
		500–1,000 m	26	28.3	21	22.1	1.91 (0.74–4.96)	0.179
		1,001–5,000 m	43	46.7	55	57.9	1.21 (0.51–2.85)	0.665
		>5,000 m	11	12.0	17	17.9	Ref	
	Distance from barn to living room	<10 m	21	22.8	18	18.9	1.71 (0.69–4.25)	0.246
		10–20 m	21	22.8	23	24.2	1.34 (0.55–3.24)	0.517
		21–50 m	35	38.0	32	33.7	1.60 (0.71–3.62)	0.253
		>50 m	15	16.3	22	23.2	Ref	
	Distance from barn to the pig loading/unloading place	<=50 m	62	67.4	49	51.6	1.94 (1.07–3.51)	0.028
		>50 m	30	32.6	46	48.4	Ref	
Farm management ($n = 20$)	Farm status	Private	28	30.4	29	30.5	1.00 (0.53–1.86)	0.989
		Company	64	69.6	66	69.5	Ref	
	Production type	FF	20	21.7	26	27.4	0.99 (0.49–1.98)	0.97
		FW	26	28.3	10	10.5	3.33 (1.46–7.61)	0.003
		WF	46	50.0	59	62.1	Ref	
	Total pigs	<500	28	30.4	28	29.5	Ref	
		500–1,000	31	33.7	24	25.3	1.29 (0.61–2.73)	0.502
		1,000–1,500	18	19.6	15	15.8	1.20 (0.51–2.84)	0.679
		>1,500	15	16.3	28	29.5	0.54 (0.24–1.21)	0.133
	All-in/all-out policy in each barn	No	27	29.3	23	24.2	1.30 (0.68–2.49)	0.427
		Yes	65	70.7	72	75.8		
	Pig movement	Pig addition	10	37.0	4	17.4	Ref	
		Pig removal	9	33.3	11	47.8	0.33 (0.08–1.40)	0.127
		Both	8	29.6	4	17.4	0.8 (0.15–4.24)	0.793
	Separate place for pig movement	No	21	22.8	17	17.9	1.36 (0.66–2.78)	0.402
		Yes	71	77.2	78	82.1		
	Pig movement place is located on farm's property	No	17	18.5	15	15.8	1.21 (0.56–2.59)	0.625
		Yes	75	81.5	80	84.2		
	Truck through the same route at entrance and exit	Yes	89	96.7	87	91.6	2.73 (0.7–10.62)	0.134
		No	3	3.3	8	8.4		

(Continued)

TABLE 1 | Continued

Groups	Variables	Category	No. of cases	Proportion of response (%)	No. of controls	Proportion of response (%)	OR (95% CI)	*P*-value
	Source of trucks for the pig transport to the slaughterhouse	Slaughterhouse trucks	48	52.2	38	40.0		0.095
		Business operator trucks	44	47.8	57	60.0	1.64 (0.92–2.92)	
	Having a separate worker in isolation barn	No	64	69.6	57	60.0	1.52 (0.83–2.79)	0.171
		Yes	28	30.4	38	40.0		
	Opened barn type	Yes	18	19.6	14	14.7	1.41 (0.65–3.03)	0.381
		No	74	80.4	81	85.3		
	Water source	Direct	49	53.3	25	26.3	3.19 (1.73–5.89)	<0.001
		Indirect	43	46.7	70	73.7	Ref	
	Feeding swill to pigs	Yes	4	4.3	0	0.0	Inf	0.04
		No	88	95.7	95	100.0		
	Having workers in farm	Yes	76	82.6	71	74.7	1.61 (0.79–3.27)	0.189
		No	16	17.4	24	25.3		
	Changing workers	Monthly	6	6.5	2	2.1	1.98 (0.37–10.44)	0.414
		6 months	12	13.0	7	7.4	1.13 (0.40–3.19)	0.816
		Yearly	17	18.5	33	34.7	0.34 (0.16–0.71)	0.004
		No	47	51.1	31	32.6	Ref	
	Living place of workers after finishing work on the farm	Staying at farms	46	50.0	45	47.4	Ref	
		Go home	8	8.7	5	5.3	1.13 (0.40–3.19)	0.458
		Both	32	34.8	38	40.0	0.34 (0.16–0.71)	0.543
	Waste treatment applies in your farm	No	9	9.8	3	3.2	3.33 (0.87–12.70)	0.065
		Yes	83	90.2	92	96.8		
	Manure application	Feed for fish	7	7.6	3	3.2	7.58 (1.75–32.78)	0.003
		Applied on land inside farm	25	27.2	17	17.9	4.78 (2.08–10.99)	<0.001
		Mixed type	44	47.8	23	24.2	6.22 (2.93–13.21)	<0.001
		Sold	16	17.4	52	54.7	Ref	
	Share boars with other farms	Yes	10	10.9	11	11.6	0.93 (0.38–2.31)	0.878
		No	82	89.1	84	88.4		
	Addition ingredients in feed	Antibiotic	36	39.1	49	51.6	0.69 (0.29–1.60)	0.381
		Probiotic	4	4.3	4	4.2	0.93 (0.20–4.47)	0.931
		Both	37	40.2	28	29.5	1.23 (0.51–2.97)	0.64
		None	15	16.3	14	14.7	Ref	
Biosecurity practice and health management ($n = 11$)	Disinfection of environment on premises	Monthly	4	4.3	4	4.2	1.03 (0.25–4.26)	0.963
		Weekly	88	95.7	91	95.8		
	Biosecurity practices apply to people inside farm	High	67	72.8	69	72.6	Ref	
		Intermediate	23	25.0	21	22.1	1.13 (0.57–2.23)	0.729
		Low	2	2.2	5	5.3	0.41 (0.08–2.20)	0.285
	Biosecurity practices apply to visitors	High	55	59.8	63	66.3	Ref	
		Intermediate	21	22.8	17	17.9	1.41 (0.68–2.95)	0.353
		Low	16	17.4	15	15.8	1.22 (0.55–2.70)	0.62
	Biosecurity practices apply at pig loading/unloading place	High	54	58.7	50	52.6	Ref	
		Intermediate	26	28.3	22	23.2	1.09 (0.55–2.17)	0.797
		Low	12	13.0	23	24.2	0.48 (0.22–1.07)	0.071
	Time that vehicles must wait after disinfection to get into the farm	≤2 h	71	77.2	82	86.3	0.54 (0.25–1.15)	0.105
		>2 h	21	22.8	13	13.7	Ref	

(Continued)

TABLE 1 | Continued

Groups	Variables	Category	No. of cases	Proportion of response (%)	No. of controls	Proportion of response (%)	OR (95% CI)	*P*-value
	Time that vehicles must wait after disinfection to get into the farm	<30 min	15	16.3	20	21.1	Ref	
		30–60 min	55	59.8	46	48.4	1.59 (0.73–3.46)	0.237
		>60 min	22	23.9	29	30.5	1.01 (0.42–2.41)	0.979
	Diseases happen in farm	High	40	43.5	33	34.7	1.73 (0.59–5.05)	0.311
		Intermediate	45	48.9	52	54.7	1.24 (0.43–3.52)	0.69
		Low	7	7.6	10	10.5	Ref	
	Vaccination applying in farm	High	42	45.7	47	49.5	Ref	
		Intermediate	39	42.4	43	45.3	1.01 (0.56–1.85)	0.961
		Low	11	12.0	5	5.3	2.46 (0.79–7.67)	0.112
	Source of human food	Local market	78	84.8	72	75.8	1.69 (0.69–4.13)	0.25
		Supermarket	5	5.4	9	9.5	0.86 (0.22–3.43)	0.835
		Inside farm	9	9.8	14	14.7	Ref	
	Human food including pig products	Yes	21	22.8	16	16.8	1.46 (0.71–3.02)	0.304
		No	71	77.2	79	83.2		
	Cook human food before entering farm	No	81	88.0	80	84.2	1.38 (0.60–3.19)	0.449
		Yes	11	12.0	15	15.8		
People, animal and vehicle contact ($n = 10$)	Visiting of vet	Daily	6	6.5	5	5.3	1.37 (0.29–6.53)	0.691
		Weekly	29	31.5	28	29.5	1.18 (0.38–3.70)	0.772
		Monthly	50	54.3	54	56.8	1.06 (0.36–3.13)	0.919
		No	7	7.6	8	8.4	Ref	
	Other visitors	Yes	47	51.1	37	38.9	1.64 (0.92–2.93)	0.095
		No	45	48.9	58	61.1		
	Presence of wild birds inside farm	Yes	47	51.1	32	33.7	2.06 (1.14–3.71)	0.016
		No	45	48.9	63	66.3		
	Presence of rodents inside farm	Yes	86	93.5	59	62.1	8.75 (3.57–22.07)	<0.001
		No	6	6.5	36	37.9		
	Presence of chicken in farm	Yes	58	63.0	32	33.7	3.36 (1.84–6.12)	<0.001
		No	34	37.0	63	66.3		
	Presence of ducks in farm	Yes	31	33.7	23	24.2	1.59 (0.84–3.01)	0.152
		No	61	66.3	72	75.8		
	Presence of dog in farm	Yes	78	84.8	80	84.2	1.04 (0.47–2.31)	0.914
		No	14	15.2	15	15.8		
	Presence of cat in farm	Yes	31	33.7	14	14.7	2.94 (1.44–6.0)	0.002
		No	61	66.3	81	85.3		
	Vehicles visit another farm on the same day/trip	Yes	7	7.6	8	8.4	1.01 (0.34–2.97)	0.986
		Unknown	33	35.9	27	28.4	1.41 (0.75–2.65)	0.284
		No	52	56.5	60	63.2	Ref	
	Number of truck vehicles visit to farm/month	High	14	15.2	22	23.2	0.58 (0.27–1.28)	0.177
		Intermediate	29	31.5	28	29.5	0.95 (0.49–1.84)	0.882
		Low	49	53.3	45	47.4	Ref	

TABLE 2 | Results of the final multivariate model of risk factors associated with *Porcine epidemic diarrhea virus* in a case–control study on northern Vietnamese pig farms in 2018.

Variables	OR	95% CI	Coefficient	SE	Z-Statistic	*P*-value
Intercept			−0.9694	0.4587	−2.113	0.0346
Production type (FW)	3.35	1.51–7.43	1.6194	0.6473	2.502	0.0124
Near distance to the slaughterhouse (<1,000 m)	7.15	2.36–21.70	1.9391	0.7085	2.737	0.0062
Presence of chicken	3.36	1.84–6.12	1.1282	0.5646	1.998	0.0457

that is due to expose (29, 31). The population attributable fraction for this risk factor was 20.57% (95% CI 10.26–29.70), so eliminating or completely preventing its effects would reduce the incidence of PEDV by 20.57%. Cross-contamination of farm vehicles is reported to occur at slaughterhouses (11, 13). There are few central slaughterhouses in Vietnam, and pigs are commonly slaughtered at a slaughtering point near the house of a butcher or pig trader to supply meat to traditional markets. This partly explains why 47.06% (88/187) of the farms in this study reported "unknown distance" in response to the question on this variable. In this study, 27.08% (13/48) of the farms that had experienced PEDV in the preceding 2 years still moved or sold infected pigs to slaughterhouses or other farms. Moving the infected pigs to slaughterhouses could be a risk for environmental contamination of facility because of pigs shedding the virus in feces which can be tracked back to farms. Moving infected pigs has been shown to play a role in the spread of PEDV among farms (13, 14). The open transportation of pigs and pig products from the slaughterhouse to the local market on motorbikes, which is a popular transport method in Vietnam, could also explain why variables related to the farm location, such as the distance from the farm to the main road (< 500 m), the distance from the farm to the local market (< 500 m), and the distance from the farm to the residential area (< 200 m), were associated with the risk of PEDV infection in the univariate analysis. PEDV is known to persist on pig transportation vehicles especially if not disinfected post-use for transportation (13). All the reasons cited above could increase the risk of PEDV infection on farms close to slaughterhouses through the cross-contamination between animals, vehicles, fomites, and humans, and through the movement of animals or aerosol transmission. Increases in the risk of PEDV transmission have also been attributed to the movement of animals and aerosol transmission in previous studies (14, 32).

Another finding of the present study was the strong relationship between the presence of chickens on the farms and PEDV status (OR = 3.36, 95% CI: 1.84–6.12). Other animal species were also more frequently present on the case farms than on the control farms (average numbers of other animal species on the case and control farms were 3.6 ± 1.4 and 2.5 ± 1.5, respectively). Movements of animals between farms and other neighborhood features were indicated to be the most important factors associated with PEDV occurrence (33). Previous studies have also demonstrated that other animals on pig farms can transmit PEDV. PEDV was found in the tonsils in 4.2% of cats, suggesting that cats may play a role in the transmission of PEDV on swine farms (28). Experience in the USA suggests that the transmission of PEDV is related to bird traffic (32). Therefore, the transmission of PEDV on the case farms may be attributable to animal contact because animal species other than pigs were recorded on site. For example, 22.2% of the case farms had both cats and dogs, and another 22.2% of case farms had only cats (8). In our study, although the presence of other animal species (including wild birds, rodents, and cats) did not remain a risk factor in the final model, it was significantly associated with PEDV status in the univariate analysis. Furthermore, 31 of 92 (33.7%) case farms reported having ducks and 78/92 (84.78%) reported having dogs. Therefore, the presence of other animal species in general and especially the presence of chickens on the farms could play a role in the transmission of PEDV through animal contact and movement. Animal movement is an important mechanisms of pathogen transmission.

In this study, the farrow-to-wean production type was related to PEDV status, and had a 3.35-fold (95% CI: 1.51–7.43) higher risk of PEDV infection than other production systems. When PEDV infection occurs on a pig farm, it usually spreads among pigs of different ages. However, pigs display age-dependent resistance to pathogenic PEDV infection (34, 35). The virus accumulates and infects pregnant sows, and the subclinically infected sows transmit PEDV to the suckling piglets. The virus is then transferred to pigs of different ages (36). In another study, PEDV was considered to have been introduced in feeder pigs, fattening pigs, and adult pigs, and then spread to piglets (37). In the early phase of PEDV outbreaks, sow farms have had the highest incidence of PEDV (80.0%) (19, 32). However, after an acute outbreak, PEDV may remain in the farrowing unit because of poor biosecurity or persist in pigs in weaning or growing–finishing units, where the virus circulates (32). The number of sows on a farm is thought to play a role in the persistence of PEDV after the original outbreak (37). Of the case farms in the present study, 52.17% (48/92) had experienced PEDV in the preceding 2 years, and 68.75% (33/48) of these were sow farms (farrow-to-finish and farrow to wean production types). These outbreaks could have been caused by PEDV that was still circulating on these farms after the previous outbreak, which acted as the source of the recurrent epidemic outbreaks. This may explain why PED outbreaks occur periodically in endemic regions.

The application of manure as a biosecurity issue did not remain a risk factor for PEDV spread in the final model. However, there was a strong relationship between manure application and PEDV status in the univariate analysis. A previous study indicated that PEDV may persist in a herd long after its clinical impact (32). Previous studies have also provided evidence that PEDV can survive for up to 28 days in manure between -20 and $4\ ^{\circ}$C, and for up to 9 months in infected earthen manure stored at temperatures ranging from -30 to $23\ ^{\circ}$C (32, 38). In the present study, 68/187 farms sold manure from the farm, which could be a risk factor for the wide transmission of PEDV to other farms if the virus was present in the manure. Risk factors based on social or cultural practices, such as water sources (direct from drilled wells or irrigation systems), were not included in the final model, but were associated with PEDV status in the univariate analysis. In Vietnam, water for pig rearing can be taken from the irrigation system surrounding the farm, drilled wells, or fish ponds. Dead pigs are often thrown into the river. Therefore, there is a high risk of introducing PEDV into the irrigation system or groundwater, which could then be dispersed by the water flow. Therefore, to prevent further transmission of PEDV, it is necessary to raise public awareness about the risk entailed by social and cultural practices in pig raising in Vietnam.

Our study had several limitations. The accuracy of the information depended partly on the professional ability of each veterinarian or farm manager, whose knowledge of the epidemiology of PEDV could be limited. Second, bias may

have been introduced by the time lag between the PEDV occurrence on farms and the questionnaire survey. Third, the respondents may have answered questions involving sensitive issues incorrectly. Typical examples are how sick pigs (selling) or dead pigs (throw away) are dealt with. In addition, the selection of PEDV-negative farm could affect the analysis for the identification of risk factors.

CONCLUSIONS

This is the first study to identify the three main risk factors for the spread of PEDV in an endemic area: the presence of chickens on the farm, close proximity to the slaughterhouse, and the farrow-to-wean production type. It is also the first study to show the distance to the slaughterhouse can play an important role in transmitting PEDV and to indicate this was the principal risk factor associated with the endemic area. In addition, the mechanical transmission by the presence of chicken in the farm that caused by the movement of chicken could be some way to explain the widespread of PEDV.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Informed consent was obtained in written form (by signature) or orally from all participants, who agreed to participate after they had received written information about the study. Ethical approval for the study was granted by the Hanoi School of Public Health Institutional Review Board, Hanoi, Vietnam (Approval Number 019-405/DD-YTCC) and the Animal Ethics Committee of the University of Miyazaki's Faculty of Agriculture, Miyazaki, Japan (Approval Number 2017–541). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TM designed the questionnaire, performed the study, analyzed the data, and drafted the manuscript. TB participated in the distribution and collection the questionnaires. YS participated in the design of the questionnaire, reviewed the drafts of the manuscript, and suggested revisions. TH, SM, HD, WF, and JN reviewed the drafts and suggested revisions. SS conceptualized and supervised the study, and analyzed and revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was supported by Japan Society for the Promotion of Science, Grants-in-Aid for Scientific Research—JSPS KAKENHI (Grant Number JP15K18786).

ACKNOWLEDGMENTS

We are grateful to all the veterinarians and farm managers who participated in this study. We thank Janine Miller, Ph.D., from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00433/full#supplementary-material

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Conflict of Interest: TB is an employee of Branch of Cargill Vietnam Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 14 August 2020
doi: 10.3389/fvets.2020.00462

Coinfection and Genetic Characterization of Porcine Astrovirus in Diarrheic Piglets in China From 2015 to 2018

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Porcine astrovirus (PAstV) is broadly distributed globally and exists as at least five distinct genotypes. PAstV, which was recently identified as an important pathogen of diarrhea in piglets, is widely distributed in China. However, few studies have investigated the coinfection and genetic characterization of PAstV in diarrheic piglets in China. In this study, 89 PAstV-positive samples were identified in 543 diarrhea samples in China from 2015 to 2018, of which 75.28% (67/89) were coinfected with three to five different porcine pathogens, while none were positive for PAstV only. Among the 543 diarrhea samples, statistical analysis showed that PAstV-induced diarrhea was potentially associated with coinfection of PEV ($p < 0.01$) and GARV ($p < 0.01$). Phylogenetic analysis showed that the 27 identified PAstV strains belong to three different genotypes and that PAstV-2 (81.48%, 22/27) was predominant in diarrheic piglets in China, followed by PAstV-4 (11.11%, 3/27) and PAasV-5 (7.41%, 2/27). Sequence analysis revealed that the 27 RdRp genes identified in this study had nucleotide homologies of 53.8–99.5%. In addition, the RdRp gene of PAstV-4 strain JL/MHK/2018/0115 harbored a unique insert of three nucleotides (GAA) as compared with other known PAstV-4 strains. Furthermore, the genotypes of PAstV varied among different geographical locations, although PAstV-2 was the most widely distributed in China. These data demonstrate that PAstV coinfection with other porcine pathogens was common and there was genetic diversity of PAstV in diarrheic piglets in China.

Keywords: PAstV, coinfection, genetic characterization, diarrhea, piglet

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *04 May 2020*
Accepted: *23 June 2020*
Published: *14 August 2020*

Citation:
Su M, Qi S, Yang D, Guo D, Yin B and Sun D (2020) Coinfection and Genetic Characterization of Porcine Astrovirus in Diarrheic Piglets in China From 2015 to 2018. Front. Vet. Sci. 7:462. doi: 10.3389/fvets.2020.00462

INTRODUCTION

Porcine astrovirus (PAstV), belonging to the family *Astroviridae*, genus *Mamastrovirus*, is a non-enveloped, single-strand, positive-sense RNA virus (1). The PAstV genome consists of three open reading frames (ORFs): ORF1a, ORF1b, and ORF2. ORF1a and ORF1b encode non-structural proteins and the RNA-dependent RNA polymerase (RdRp), and ORF2 encodes the capsid protein (2). PAstV was first identified in diarrheic piglets in 1980 (1). Since then, PAstV has been isolated worldwide, including Europe, Asia, and the Americas (1, 3–6).

Diarrhea of piglets has long been a problem afflicting the global pig industry. Coinfections with more than one porcine pathogen are common and often more clinically severe (7, 8). A previous surveillance study conducted by our group found a high percentage of coinfection among diarrheic piglets (9). PAstV, which has been identified as an important agent of diarrhea (10), and frequently presents as a coinfection with other porcine pathogens (3, 5, 6, 9, 11). However, data regarding coinfections with PAstV in diarrheic piglets in China are limited. Therefore, coinfections with PAstV and other porcine pathogens should be monitored in China.

To date, five genotypes of PAstV (PAstV-1 to PAstV-5) with different prevalences have been identified worldwide. Although all five PAstV genotypes have been reported in Europe, the most common is reportedly PAstV-4 (4), while PAstV-2 and PAstV-4 are the most common throughout Asia (5, 6, 12). However, information available on the genetic characterization of PAstV in China is fairly limited (2, 11). Therefore, it is necessary to investigate the genetic diversity and evolution of PAstV currently in China.

In this study, 89 PAstV-positive diarrhea samples were collected to investigate the prevalence of PAstV coinfection with 12 other porcine pathogens. The obtained RdRp genes were genetically characterized in order to provide insights into the epidemiology of PAstV circulating among diarrheic piglets in China.

MATERIALS AND METHODS

Sample Collection

In our previous study (9), 89 (16.4%) of 543 diarrhea samples collected from 17 provinces or municipalities in China (Anhui, Fujian, Guangdong, Hebei, Heilongjiang, Hubei, Hunan, Jiangxi, Jilin, Liaoning, Shandong, Shaanxi, Shanxi, Sichuan, Shanghai, and the Inner Mongolia Autonomous Region; **Table S1**) from 2015 to 2018 were confirmed as PAstV-positive by reverse-transcription polymerase chain reaction (RT-PCR) and stored at −80°C.

Sequencing and Analysis of the RdRp Gene of PAstV

RNA extraction and cDNA synthesis were performed as previously described by Wang et al. (13). The PAstV RdRp gene was amplified using the nested RT-PCR method described by Chu et al. (14) and then cloned into the vector pMD18-T (TaKaRa Biotechnology Co., Ltd, Dalian, China) in accordance with the manufacturer's protocol. Three positive clones of each amplicon were subjected to Sanger sequencing. Sequence analysis was conducted using the EditSeq tool included with the Lasergene DNASTAR™ 5.06 software package (DNASTAR Inc., Madison, WI, USA). Multiple-sequence alignments were performed using the multiple-sequence alignment tool included with the DNAMAN 6.0 software package (Lynnon BioSoft, Pointe-Claire, QB, Canada).

Phylogenetic Analysis

Sequences of the PAstV RdRp gene retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) were used for sequence alignments and phylogenetic analyses. Multiple-sequence alignments were generated using the ClustalX alignment program included with the MEGA 6.06 software package (15). A phylogenetic tree was constructed from the aligned nucleotide sequences using the p-distance model and 1000 bootstrap replicates and annotated with Interactive Tree Of Life (iTOL) software (http://itol.embl.de/) (16).

Statistical Analysis

The correlation of PAstV infection with other pathogens was assessed with the use of 2 × 2 contingency tables and the chi-square (χ^2) test with confidence limits of 95%. All analyses were performed using IBM SPSS Statistics for Windows, version 22.0 (IBM Corporation, Armonk, NY, USA). Probability (p) values of <0.05 and 0.01 were considered statistically significant and highly significant, respectively. Data regarding the detection of porcine circovirus type 3, porcine group A rotavirus (GARV), mammalian reovirus, porcine bocavirus, porcine deltacoronavirus, porcine enterovirus 9/10 (PEV), porcine kobuvirus (PKV), porcine sapelovirus, porcine torovirus, porcine teschovirus, porcine transmissible gastroenteritis virus (TGEV), and torque teno sus virus 2 in the 543 diarrhea samples (including the 89 PAstV-positive samples) were published in our previous reports (9, 17).

RESULTS AND DISCUSSION

Coinfection of PAstV With Multiple Porcine Pathogens in Diarrheic Piglets

As reported in our previous study, the PAstV-positive rate in diarrheic piglets was 16.39% (89/543), indicating wide distribution in China (9). In the present study, PAstV coinfection with 12 other porcine pathogens in diarrheic piglets was investigated. Of the 89 PAstV-positive diarrhea samples, the rate of PAstV coinfection with 12 other porcine pathogens ranged from 7.87% (7/89) to 85.39% (76/89) (**Figure 1A**). The average number of viruses detected in each sample was 4.12, while 75.28% (67/89) of samples had three to five different viruses, 3.37% (3/89) had seven to eight different viruses, and none were positive for PAstV only (**Figure 1B**). Coinfections of PAstV with other porcine pathogens, such as rotavirus, PEDV, TGEV, porcine circovirus-2 (PCV2), and porcine hemagglutinating encephalomyelitis virus (PHEV), have been reported previously (3, 5, 6, 11). In the present study, the rate of PKV coinfection in PAstV-positive samples was relatively high (85.39%, 76/89), and there was evidence that u shed more PKV than did healthy individuals (18), indicating that PKV may have a potential role in PAstV-induced diarrhea in piglets. PEDV is the major cause of viral diarrheal disease in swine in China (9). Although there was a high prevalence of PEDV in piglets infected with PAstV in our previous study, statistical analysis indicated that PEDV-induced diarrhea was not associated with PAstV coinfection ($p > 0.05$) (9). The pathogenicity of PEV is typically mild in pigs (19), and GARV is among the most common pathogens of diarrhea

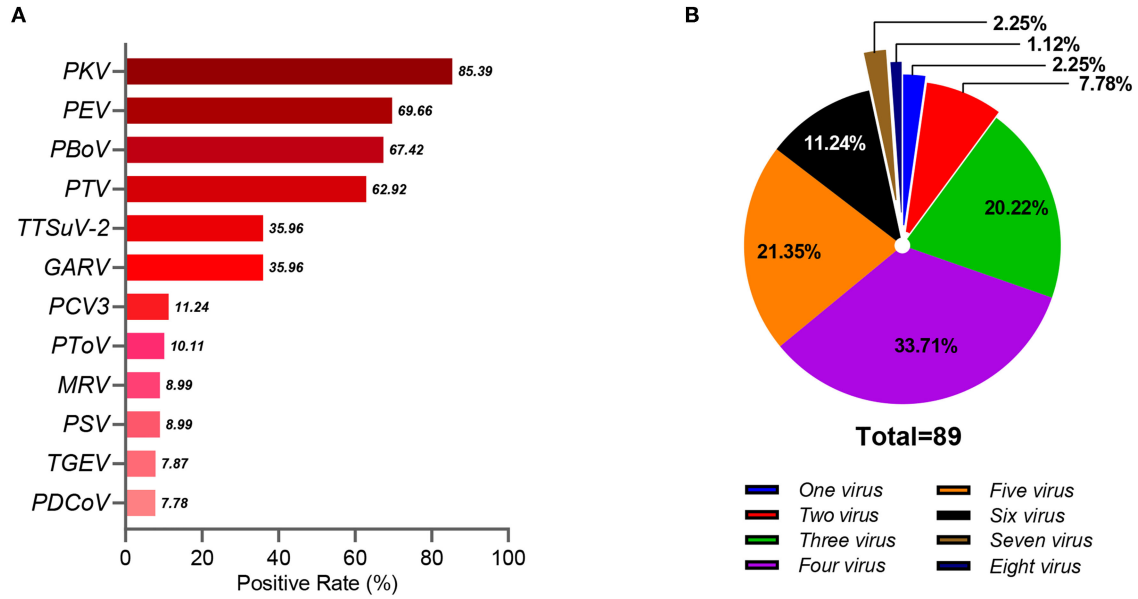

FIGURE 1 | The coinfection of PAstV with multiple porcine pathogens in diarrheic piglets. **(A)** The positive rate of the 12 porcine pathogens in the 89 PAstV-positive samples. **(B)** The coinfection patterns of the 12 porcine pathogens in the 89 PAstV-positive samples.

in piglets (20). In this study, the positivity rates of PEV (69.66%, 62/89) and GARV (35.96%, 32/89) were relatively high in PAstV-positive samples, suggesting a highly significant association of coinfection with PEV ($p < 0.01$) and GARV ($p < 0.01$) in PAstV-positive diarrhea samples of piglets (**Table 1**). However, since evidence of coinfections of PAstV with PEV and GARV causing diarrhea in piglets is somewhat limited, further studies are warranted.

Phylogenetic Analysis of PAstV

In the current study, a total of 27 RdRp genes were successfully sequenced from the 89 PAstV-positive diarrhea samples (**Table S2**). The RdRp gene is widely used to classify the genotype of PAstV (5, 11, 12, 21–23). Here, a phylogenetic tree was constructed based on the RdRp genes of the 27 identified PAstV strains and 115 reference PAstV strains. In the phylogenic tree, 142 PAstV strains were divided into five groups and three distinct genotypes (PAstV-2, PAstV-4, and PAstV-5) (**Figure 2A**). In the phylogenetic analysis, 22 identified PAstV strains and 39 reference strains from nine other countries were placed into the PAstV-2 group and divided into two clusters. With the exception of PAstV strain JX/2015/1224, all other identified PAstV strains and 21 reference PAstV strains formed one cluster in the PAstV-2 group, which shared nucleotide homologies of 84.8–99.5%. PAstV strain JX/2015/1224 and 18 reference PAstV strains shared nucleotide identities of 83.1–90.2% and formed the other cluster in the PAstV-2 group. Two identified PAstV strains, SD/YT/2015/1228b and JX/2015/1221d, which are closely related to a PAstV strain isolated in Croatia, were classified into the PAstV-5 group, which had nucleotide identities of 79.8–94.0%. Three identified PAstV strains (JL/MHK/2018/0115, SX/XZ/2017/1215, and JX/2015/1221a) and 35 reference strains, which shared nucleotide homologies of 62.1–93.6%, were classified to the PAstV-4 group. Most of the 27 identified PAstV strains were classified to the PAstV-2 group (81.5%, 22/27), indicating that PAstV-2 was predominant in diarrheic piglets in China. Similarly, the high prevalence of PAstV-2 in China was reported by Cai et al. (11) and Qin et al. (22). In contrast, the prevalence of PAstV-4 is reportedly higher than that of PAstV-2 in Thailand, South Korea, and India (5, 6, 12).

Sequence analysis of the 27 RdRp genes identified in this study had nucleotide identities of 53.8–99.5%, indicating

TABLE 1 | The statistical analysis of correlations of PAstV with other porcine pathogens.

	P-value	Odds ratio (OR)	95% confidence interval (95% CI)
PKV	0.103	1.778	0.908–3.480
PEV	0.000	4.219	2.582–6.896
PBoV	0.193	1.384	0.855–2.240
PTV	0.722	1.115	0.697–1.783
TTSuV-2	0.623	1.149	0.715–1.848
GARV	0.000	2.563	1.563–4.246
PCV3	0.702	1.150	0.556–2.379
PToV	0.393	1.390	0.642–3.009
MRV	0.583	0.747	0.342–1.631
PSV	0.128	1.939	0.835–4.507
TGEV	1.000	0.908	0.393–2.101
PDCoV	0.182	1.852	0.759–4.522

OR, odd ratio; CI, confidence interval.

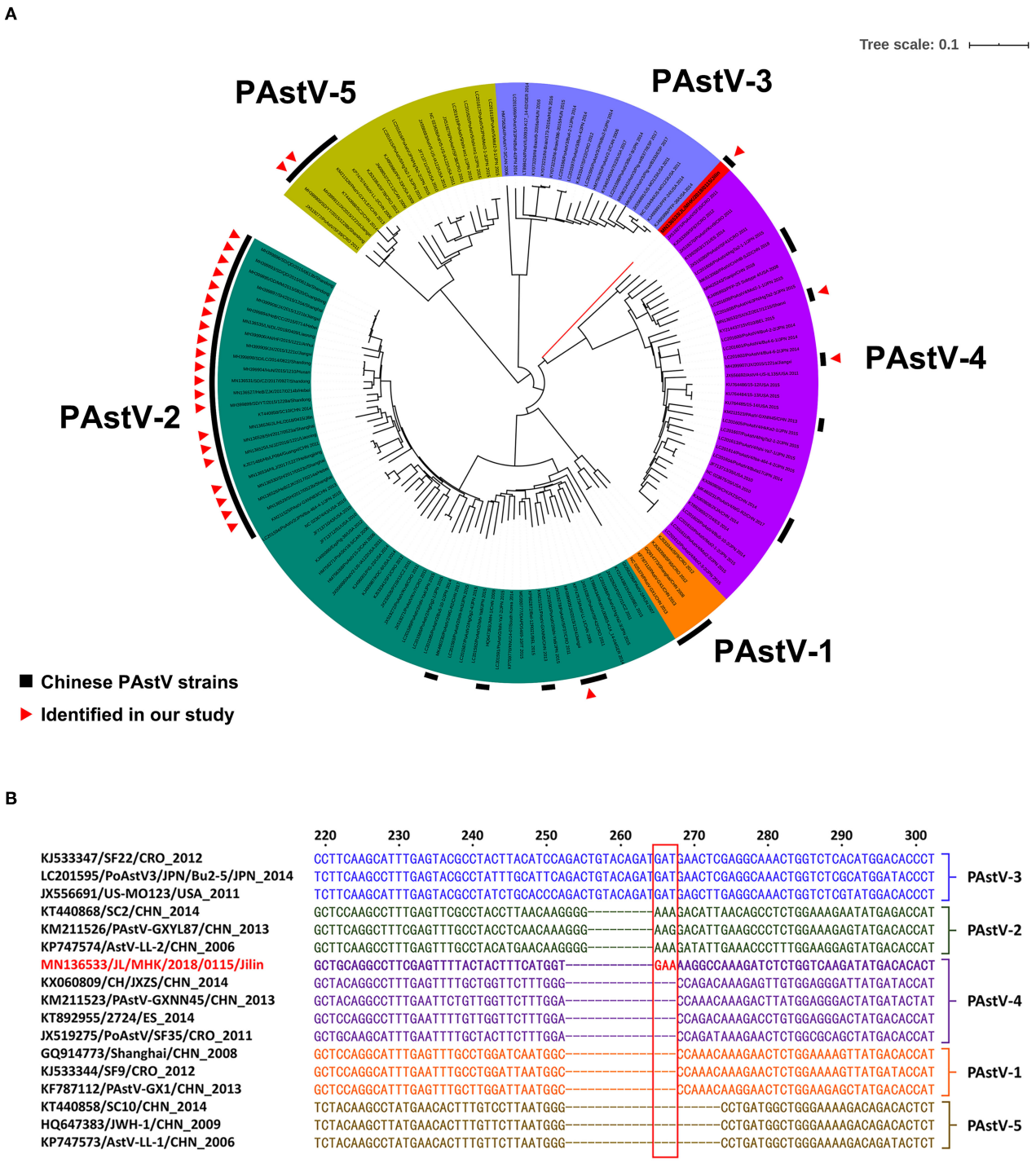

FIGURE 2 | (A) The tree was constructed on the base of 366- to 381-bp (depend on PAstV genotype) nucleotide sequences of RdRp of the PAstV and selected sequences from GenBank. **(B)** Sequence analysis of the RdRp gene of the PAstV strain JL/MHK/2018/0115.

a wide variation at the nucleotide level. PAstV strain JL/MHK/2018/0115 differed genetically from other PAstV-4 strains and formed a unique clade within the PAstV-4 group. Sequence analysis showed that the RdRp gene of strain JL/MHK/2018/0115 had low sequence similarity to the other PAstV-4 strains, with nucleotide identities of 62.1–67.1% and had a unique insert of three nucleotides (GAA) as compared with other PAstV-4 strains (**Figure 2B**). Previous

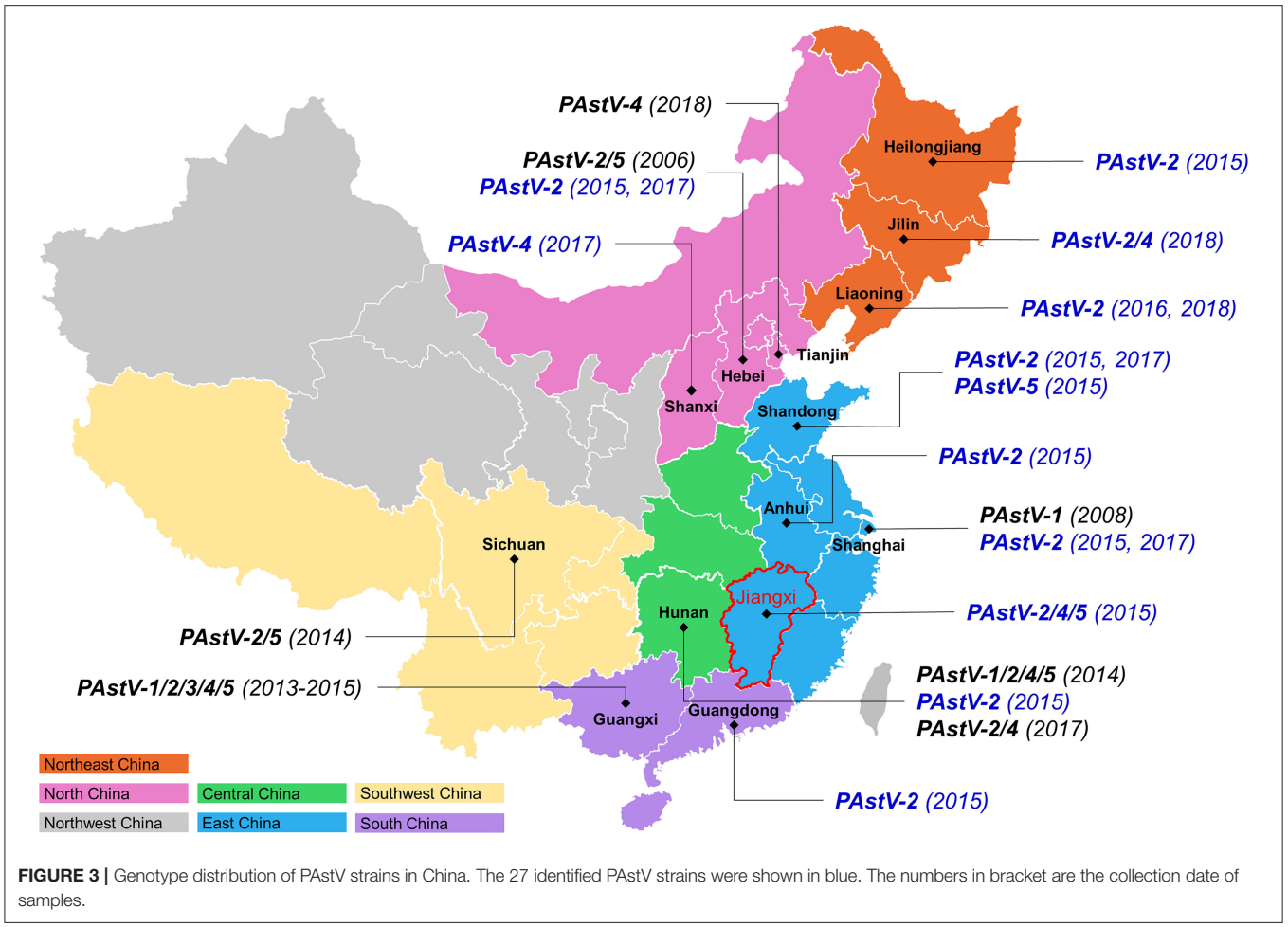

FIGURE 3 | Genotype distribution of PAstV strains in China. The 27 identified PAstV strains were shown in blue. The numbers in bracket are the collection date of samples.

studies have reported a high prevalence of PAstV-4 in other Asian countries, including South Korea (88.46%, 23/26), Thailand (92.00%, 23/25), and India (95.65%, 22/23), as well as European countries (70.4%, 295/419) (4–6, 12). PAstV-4, which was first reported in 2013, is a newly identified genotype in China that has since spread to the provinces of Hunan, Tianjin, Shanxi, Jilin, and Jiangxi (9, 21, 22). In addition, Lv et al. (21) and Zhao et al. (24) reported novel recombinant PAstV-4 strains in China. These data suggest that the Chinese PAstV-4 strains have undergone genetic variations and may have become the predominant strains in diarrheic piglets in China.

Genotype Distribution of PAstV in China

The results of the present study showed that PAstV-2 was circulating in ten different provinces, covering five regions of China (**Figure 3**), suggesting that PAstV-2 is the most widely distributed strain in China, which is supported by recent studies conducted in the provinces of Hebei, Hunan, Sichuan, and Guangxi (11, 21, 22, 25). Previous studies have reported the existence of multiple PAstV genotypes in China. For example, PAstV-1 was identified in Shanghai as well as the provinces of Hunan and Guangxi (22, 25), while PAstV-3 was identified in Guangxi province only (22), PAstV-4 has been reported in the provinces of Tianjin, Hunan, and Guangxi (22, 24, 25), and PAstV-5 has been detected in the provinces of Hebei, Sichuan, Hunan, and Guangxi (11, 22, 24–26). In the present study, three PAstV-4 strains were identified in the provinces of Jilin, Shanxi, and Jiangxi, and two PAstV-5 strains were identified in Shandong and Jiangxi provinces, respectively. These results suggest the presence of various genotypes of PAstV in different regions of China. Moreover, five PAstV strains identified in Jiangxi province exhibited three distinct genotypes (PAstV-2, PAstV-4, and PAstV-5). Previous studies reported the presence of two or more genotypes of PAstV in the same province of China, such as four different genotypes of PAstV circulating in Hunan province (21, 25), while all five genotypes of PAstV were identified in Guangxi province (22), indicating a remarkable diversity of genotypes of PAstV cocirculating among pig farms in China.

In conclusion, results from the present study provide evidence that coinfection of PAstV with multiple porcine pathogens is common in diarrheic piglets in China, and PAstV-induced diarrhea is potentially associated with PEV and GARV coinfection. Phylogenetic analysis revealed that PAstV-2 was predominant in diarrheic piglets in China and

multiple genotypes of PAstV were co-circulating in China from 2015 to 2018. In addition, one PAstV-4 strain was shown to harbor a unique insert within the RdRp gene. These results increase our current understanding of the coinfection and genetic characterization of PAstV in diarrheic piglets in China and provide valuable information for further studies of PAstV.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

This animal study was reviewed and approved by Animal Experiments Committee of the Heilongjiang Bayi Agricultural University (registration protocol 201501003). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

DS conceived the study. MS, SQ, DY, DG, and BY analyzed the data. MS and SQ wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China (Grant No. 31873011), the Outstanding Youth Science Foundation of Heilongjiang province (Grant No. JC2017007), the National Key Research and Development Program of China (Grant No. 2017YFD0501604-5), and the Heilongjiang Bayi Agricultural University Support Program for San Heng San Zong (Grant No. TDJH201804).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00462/full#supplementary-material

Table S1 | The background information of 89 PAstV-positive samples.

Table S2 | The PAstV RdRp genes identified in this study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 28 August 2020
doi: 10.3389/fvets.2020.00554

Spatial Distribution of Trypanosomes in Cattle From Western Kenya

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African Animal Trypanosomiasis (AAT) is a tsetse-transmitted protozoan disease endemic in "the tsetse belt" of Africa. Past studies investigating the epidemiology of the disease rarely focused on spatial distribution when reporting the prevalence. The challenge of understanding the spatial epidemiology of the disease is further confounded by low-sensitive parasitological techniques used in field investigations. This study aimed to identify trypanosome species in cattle and their spatial distribution in western Kenya. Low-sensitive microscopic analysis and highly-sensitive polymerase chain reaction (PCR) techniques were also compared to better understand the epidemiology of *Trypanosoma* infections by use of the geographical information system (GIS). Blood samples from 888 cattle, collected between August 2010 and July 2012, were examined for *Trypanosoma* parasites by light microscopy and PCR. The spatial distribution of *Trypanosoma* positive cases by species were mapped and overlaid on the map for tsetse distribution. The estimated prevalence was 4.17% by PCR compared to 2.48% by microscopy. Trypanosomes were detected in tsetse free areas. *Trypanosoma vivax* and *Trypanosoma b. brucei* were identified, but not the zoonotic *Trypanosoma b. rhodesiense*. This study demonstrated the importance of geospatial data analysis to understand the epidemiology of the parasite, to inform future research and formulate control strategies.

Keywords: trypanosomiasis, tsetse, cattle, Kenya, spatial distribution

OPEN ACCESS

Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

Reviewed by:
Md. Aminul Islam, Tohoku University, Japan
Dongbo Sun, Heilongjiang Bayi Agricultural University, China

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *03 February 2020*
Accepted: *14 July 2020*
Published: *28 August 2020*

Citation:
Kivali V, Kiyong'a AN, Fyfe J, Toye P, Fèvre EM and Cook EAJ (2020) Spatial Distribution of Trypanosomes in Cattle From Western Kenya. Front. Vet. Sci. 7:554. doi: 10.3389/fvets.2020.00554

INTRODUCTION

Trypanosomiasis is a disease of humans and animals caused by protozoan parasites of the genus *Trypanasoma* and is transmitted by tsetse flies of the genus *Glossina.* It is endemic in 38 countries in sub-Saharan Africa (1). The disease exists in two forms; Human African Trypanosomiasis (HAT), a neglected tropical disease that is fatal if left untreated (2) and African Animal Trypanosomiasis (AAT). AAT is associated with substantial economic losses to farmers and is a major constraint in the fight against poverty in the affected countries (3). *Trypanasoma* species that affect cattle are *Trypanasoma vivax, Trypanasoma congolense*, and *Trypanasoma brucei.* Trypanosomiasis has a zoonotic aspect whose etiological agents are *Trypanasoma b. rhodesiense* and *Trypanasoma b. gambiense.* Cattle and other domestic animals are the reservoirs for these zoonotic trypanosomes which are transmitted cyclically between humans and animals by tsetse flies (4).

Diagnosis of trypanosomiasis can be clinical, parasitological, serological, or by use of molecular techniques (5). Control of trypanosomiasis is through the combination of both vector control, to reduce the tsetse fly population, and prophylactic chemotherapy (6). Trypanocidal drugs are also used to treat clinical cases of bovine trypanosomiasis (7).

Kenya lies partially within the tsetse belt of sub-Saharan Africa. According to Machila et al. (8) trypanosomiasis is endemic to the western and coastal regions of Kenya. Epidemiological studies of both HAT and AAT have been conducted in different parts of the country (8–11).

Previous studies have shown that AAT is a major constraint to cattle production in western Kenya (10). Epidemiological studies in this area in 2004 estimated a prevalence of 20.1% of trypanosomiasis in cattle, 8.4 and 17.4% in pigs from two different sites in the region and a prevalence of <5% in small ruminants (11). A study conducted in Teso and Suba Districts in western Kenya in 2006 investigated the spatial distribution of AAT and showed the majority of *Trypanasoma* infections were caused by *T. vivax* outside the tsetse belt (12).

This study aimed to identify the different trypanosome species infecting cattle in Busia county and surrounding areas and map their distribution. Additionally, it sought to establish whether human infective *T. brucei*, i.e., *T. b. rhodesiense* was present in cattle in this region.

MATERIALS AND METHODS

Cattle samples used in this study were collected between August 2010 and July 2012 as part of a cross-sectional study conducted in Busia region of the former Western province of Kenya (13). Sampling was conducted within a 45 km radius of Busia town spreading into the current Siaya, Kakamega, and Bungoma Counties. The sample size was determined and adjusted using the Survey package in the R program version 3.1.1 (http://cran.r-project.org/) and the study design powered by an estimated lowest prevalence of 5% of disease in cattle with a standard error of 2%. The primary sampling unit was a homestead defined as the place where family members shared an evening meal. Eight hundred and eighty eight cattle samples were collected from 416 randomly selected homesteads. These were apparently healthy animals on farms included in a cross-sectional survey of zoonotic infections. Sampling was done after seeking consent from the head of all eligible homesteads.

Structured questionnaires were administered prior to sampling to capture animal characteristics, such as age, sex, and breed. Venous blood was collected from the jugular vein of each animal into ethylenediaminetetraacetic acid (EDTA) coated tubes which were barcoded and labeled with the animal identity. The samples were transported in upright position to the project laboratory in Busia and stored at −40°C, before being shipped to Nairobi for subsequent analysis. All samples were examined by both microscopy and PCR and the results interpreted in parallel.

Thin and thick blood smears were prepared in the field at the point of collection from the marginal ear veins of the study cattle. Microscopic diagnosis on thick and thin blood smears was used to determine presence or absence of the trypanosomes. Thick smears were examined using X10 objective lens and the number of parasites counted in each of 100 fields. Thin smears were also examined at X10 and then at X100 under oil immersion and number of parasites in every 100 fields was recorded. The results were interpreted as per the OIE recommendations, i.e., one parasite in 10 fields examined was interpreted as low, one parasite in 5 fields was interpreted as medium and more than one parasite in 1 field was interpreted as high.

DNA was extracted from all EDTA blood samples using the MagNa Pure LC DNA Isolation Kit I (Cat. No. 03 003 990 001) and the MagNa Pure LC 2.0 Instrument (Roche Applied Science, Mannheim, Germany). Conventional species specific PCR testing was performed on extracted DNA for detection of *T. b. brucei*, *T. congolense*, *T. vivax*. All *T. b. brucei* positive samples were further analyzed to determine if they were zoonotic, i.e., *T. b. rhodesiense*. Previously described PCR protocols (14–16) were used for:

- *T. b. brucei*

forward primer sequence GAATATTAAACAATGCGCAG
reverse primer CCATTTATTAGCTTTGTTGC,

- *T. congolense*

forward primer sequence CGAGAACGGGCACTTTGCGA
reverse primer GGACAAACAAATCCCGCACA,

- *T. vivax*

forward primer sequence CAGCTCGGCGAAGGCCACTTCG CTGGGGTG
reverse primer sequence TCGCTACCACAGTCGCAATCG TCGTCT CAAGG,

- *T. b. rhodesiense*

forward primer sequence ATAGTGACAAGATGCGTACTC AACGC
reverse primer sequence AATGTGTTCGAGTACTTCGGT CACGCT.

Annealing temperatures for the primers were determined using gradient PCR. Reaction volume for each sample consisted of 1X DreamTaq Green PCR Master Mix (ThermoFisher Scientific; Waltham, Massachusetts), 10 μM of forward and reverse species-specific primers, DNA and nuclease-free water. Thermocycling was done on the C1000 touch Thermo cycler (BIO-RAD, Hercules, California, United States) and amplicons were detected using gel electrophoresis.

Descriptive analysis was performed to determine the distribution of the results obtained based on age, sex, and breed of the animals. This was achieved using the "frequency" tool of IBM SPSS® version 20. A Cohen's Kappa test was run (17) to compare the level of agreement between microscopy and PCR.

ESRI ArcGIS 10.3.1 software was used to spatially map all the positive cases using the x and y coordinates. Additional data layers' describing the tsetse distribution in the study area was obtained from a GIS database available at the International Livestock Research Institute website (http://www.ilri.org/GIS). A positive case was defined as positive using either microscopy, PCR or both diagnostic techniques.

A spatial risk map was generated to identify areas with the greatest relative risk for trypanosome infection. The density of positive cases of trypanosomes was assessed using Kernel smoothing in the *spatstat* package (18) in R. This was done

with a fixed bandwidth of 5 km and correlation for edge effects. The kernel intensity of positive homesteads was divided by the kernel intensity of the all homesteads in the study area creating a risk surface.

RESULTS

Out of the 888 cattle analyzed, 65.3% were female and almost all of them belonged to the shorthorn Zebu and Zebu breeds (**Supplementary Table**).

The estimated prevalence of trypanosome species detected by both microscopy and PCR was 4.17% (95% CI 3.931–4.409). Thirty four animals were infected with a single trypanosome species while three animals had mixed trypanosome infections of *T. vivax and T. b. brucei. T. congolense* constituted the smallest proportion of all trypanosomes found in cattle in the study site (**Table 1**).

Spatial Distribution and Relative Risk

The locations of all positive cases were mapped as shown in **Figure 1**. Most of the infected cattle were located in the western and south-western parts of the study area (**Figures 1A–C**). The addition of the tsetse fly distribution layer to the map revealed that there were trypanasome positive cattle in tsetse-free areas in the eastern part of the study area. The distribution of positive cattle was the same for both diagnostic tests used (**Figures 1A,B**).

Spatial relative risk maps (**Figure 2**) also demonstrated that areas of greatest relative risk for trypanosome infection were the south western parts of the study area around Lake Victoria.

Comparison of Microscopy and PCR

T. vivax was the most common infection detected by microscopy (**Figure 1A**) and *T. brucei* was the most common infection detected by PCR (**Figure 1B**). Only *T. vivax* results provided a statistically reasonable number of positives hence possible to determine the level of agreement between the PCR and microscopy results. A Cohen's Kappa test was run and the results revealed a moderate agreement between the two tests, i.e., $K = 0.403$ (95% CI 0.098–1.521), $p < 0.0005$.

TABLE 1 | Prevalence of trypanosoma species identified based on the test used.

Trypanosome species (Test used)	Positive/ sample size	Apparent prevalence (95% confidence interval)
All species tested	37/888	4.17 (3.931–4.409)
T. brucei (thick smears)	1/888	0.11 (0.131–0.089)
T. brucei (thin smears)	1/888	0.11 (0.089–0.131)
T. brucei (PCR)	8/888	0.90 (0.920–0.880)
T. congolense (thick smear)	4/888	0.45 (0.483–0.417)
T. congolense (thin smear)	2/888	0.23 (0.258–0.202)
T. congolense savannah (PCR)	0/888	0.00 (0.000–0.000)
T. vivax (thick smear)	13/888	1.46 (1.514–1.406)
T. vivax (thin smear)	13/888	1.46 (1.514–1.406)
T. vivax (PCR)	12/888	1.35 (0.468–0.332)

DISCUSSION

This study found a prevalence of 4.17% of *Trypanosoma* infection which is lower compared to earlier studies in cattle in the same region that established a prevalence of 41% and 29% in Suba and Teso regions of western Kenya, respectively in 2006 (12) and 20.1% in the former Busia district in 2004 (11). This might suggest the impact of improved control strategies against trypanasomiasis, improved local knowledge of the disease or a change in disease dynamics since the other studies were conducted. Between 1999 and 2012 different control strategies were implemented in the region with funding from the European Union for Farming in Tsetse Controlled Areas (FITCA) and Pan African Tsetse And Trypanosomiasis Eradication Council (PATTEC) projects. A study conducted in 2008 demonstrated that 61% of people in western Kenya were aware of tsetse and trypanosome control methods and bush clearing was recently used by almost half of respondents as a control method (19). These interventions might have greatly contributed to the decrease in prevalence of trypansomiasis. However, it is important to determine the current prevalence status as there has been less focus, support and funding for trypanosomiasis in recent years (20) and hence a possibility of an increase in prevalence.

Mapping the positive cases by microscopy (**Figure 1A**) and by PCR (**Figure 1B**) showed a similar geographical distribution of trypanasome infection with most cases located in the south western parts of the study area. Spatial mapping of the distribution of *Trypanasoma* species overlaid with the tsetse infested layer showed a number of trypanosomes in the tsetse-free areas which was previously demonstrated in the former Teso and Suba districts (12). Most infections in the tsetse-free areas were of *T. vivax*, which is known to be a mechanically-transmitted parasite by both biting flies and tsetse flies (12, 21). There is a need for further studies to establish whether this is due to mechanical transmission or a change in the distribution of tsetse flies resulting from land use changes due to habitat transformation, degradation and encroachment into the tsetse free areas. The geo-spatial data maps demonstrating the distribution of tsetse flies were developed in the year 1998, using data collected in 1973 (22). There is a likelihood that urbanization and changes in landuse could have modified and even interfered with the tsetse habitats and their distribution.

The risk of trypanasome infection in the study area is similar regardless of the diagnostic test used (**Figures 2A,B**). The spatially-smoothed risk maps showed the area with the highest risk of trypanasome infection to be the south western region of the study close to Lake Victoria. This may indicate a localized hot-spot for trypanosome transmission in this region which could be targeted for future control initiatives.

Microscopic analysis of trypanosomes is a cheap and simple technique and is easily employed in the field in the detection of trypanaomiasis. It has been shown to have low sensitivity (23). However, in this study microscopy provided an important pre-screening step and enabled comparative assessment of the results obtained. Earlier studies have shown that PCR has a

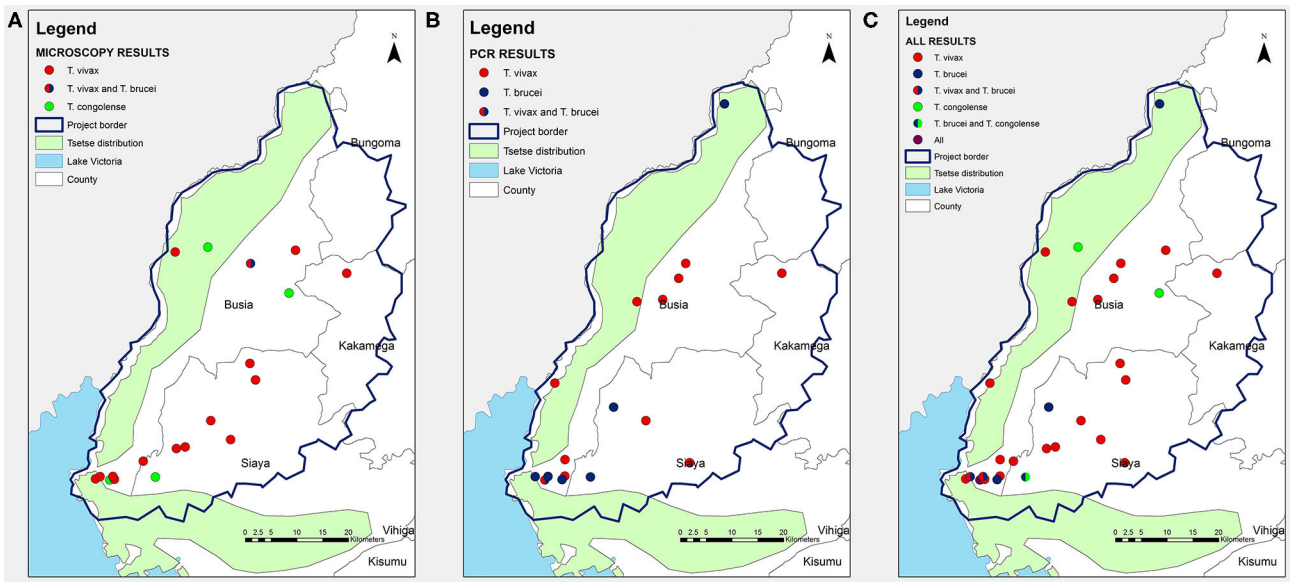

FIGURE 1 | Map of the distribution of homesteads with trypanosome positive cattle in Busia region detected by **(A)** Microscopy, **(B)** PCR, and **(C)** Microscopy and PCR.

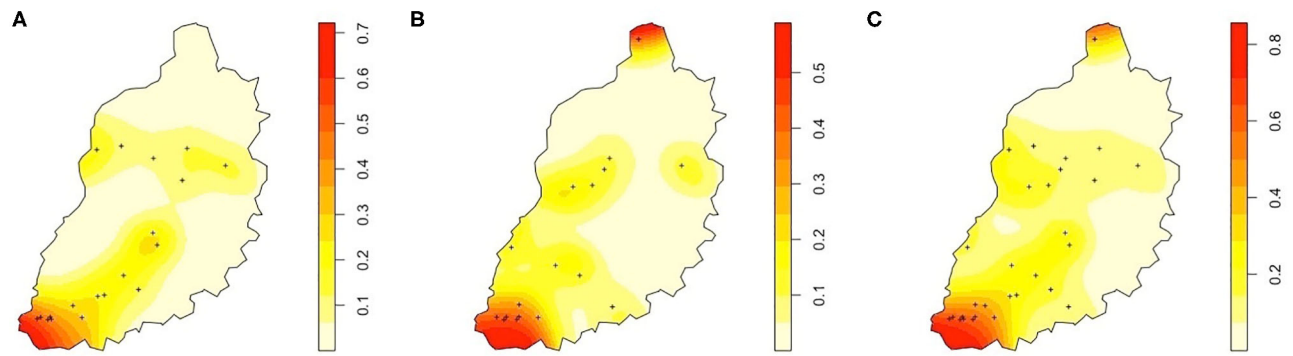

FIGURE 2 | Spatially-smoothed risk map for trypanosomes in cattle in Busia region detected by **(A)** Microscopy, **(B)** PCR, and **(C)** Microscopy and PCR. The points indicate locations of hometeads with positive trypanasome samples. Risk is measured as a ratio between 0 and 1 with results closest to 1 having the highest risk and demonstrated by increasing color intensity.

high specificity and sensitivity when diagnosing trypanosome infections (15, 24). Using PCR increased the number of total positive cases identified from 22 to 37 with the diagnosis of *T. brucei* cases being greatly improved as demonstrated in **Figure 1B**. The predominant *Trypanasoma* infection was *T. vivax* by both tests which agrees with a study conducted in other parts of western Kenya (former Teso and Suba district) (12). There were no positive *T. congolense* cases on PCR and this could be attributed to the primers used which were only specific for *T. congolense* savannah and hence there is a possibility that other sub-species of *T. congolense* could be present but were not detected.

It is important to note that PCR is a very sensitive technique for detecting parasite DNA and not every PCR-positive animal will be clinically ill. This must be considered when relating the prevalence recorded to impacts on animal health and production as these will not be directly proportional. We were not able to make conclusions regarding the clinical status of positive animals in this study. It has been suggested that asymptomatic carrier cattle may impact the success of control programmes targeting the treatment of symptomatic cattle (11).

Human infective *T. b. rhodesiense* infections were not detected even though this part of western Kenya has previously been reported to be a sleeping sickness focus with sporadic cases of HAT (11). Earlier studies reported the prevalence of *T. b. rhodensiense* in cattle to be 21.5% (11). However, the implementation of the PATTEC project in the Lake Victoria region from 2005 to 2012 may have reduced the prevalence of *T. b. rhodesiense* and Kenya has not reported a sleeping sickness case in more than 10 years (25).

CONCLUSION AND RECOMMENDATIONS

This study has demonstrated the crucial role that geospatial data analysis has in improving the understanding of disease status. This will greatly inform future research and policy formulation as it will provide evidence crucial in spearheading informed control strategies to mitigate this disease. The evidence of multiple species co-infections of trypanosomes warrants the need for change of research outlook from single-species to multiple-species investigations to better understand how the different pathogens co-exist in the targeted host. Additionally, it is crucial for continuous studies to be carried out to establish whether cattle in this region are reservoirs for zoonotic trypanosomes which is of great public health importance. This information may help Kenya with its move toward declaring the elimination of HAT as a public health problem.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Welfare and Ethical Review Body of the Roslin Institute, University of Edinburgh, UK (AWA004). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

VK and EF: conceptualization. VK and EC: formal analysis. EF: funding acquisition. VK and AK: investigation. EF, EC, and VK: methodology. EF and VK: project administration. EF, PT, and JF: supervision. VK, PT, and EC: writing-original draft. All authors: writing, review, and edit.

FUNDING

The Wellcome Trust (085308) supported the People, Animals and their Zoonoses (PAZ) project. Support was also received from the CGIAR Research Program on Agriculture for Nutrition and Health (A4NH), led by the International Food Policy Research Institute (IFPRI). We acknowledge the CGIAR Fund Donors (https://www.cgiar.org/funders/). This work was part-funded by the Global Challenges Research Fund (GCRF) One Health Regional Network for the Horn of Africa (HORN) Project, from UK Research and Innovation (UKRI) and Biotechnology and Biological Sciences Research Council (BBSRC) (project number BB/P027954/1).

ACKNOWLEDGMENTS

We were grateful to all the members of the PAZ field and laboratory teams in Busia in particular James Akoko, Omoto Lazarus, Maseno Cleophas, Jenipher Ambaka, Fredrick Amanya, Lorren Alumasa, Daniel Cheruiyot, George Omondi, John Mwaniki, Hannah Kariuki, and Lilian Achola.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00554/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 02 September 2020
doi: 10.3389/fvets.2020.00526

Epidemiology and Associated Risk Factors for Brucellosis in Small Ruminants Kept at Institutional Livestock Farms in Punjab, Pakistan

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OPEN ACCESS

Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

Reviewed by:
Sidharath Dev Thakur, Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya, India
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *13 January 2020*
Accepted: *08 July 2020*
Published: *02 September 2020*

Citation:
Ullah Q, Jamil T, Melzer F, Saqib M, Hussain MH, Aslam MA, Jamil H, Iqbal MA, Tahir U, Ullah S, Qureshi ZI, Schwarz S and Neubauer H (2020) Epidemiology and Associated Risk Factors for Brucellosis in Small Ruminants Kept at Institutional Livestock Farms in Punjab, Pakistan. Front. Vet. Sci. 7:526. doi: 10.3389/fvets.2020.00526

Brucellosis is reportedly endemic in ruminants in Pakistan. Both *Brucella abortus* and *B. melitensis* infections have been documented in domestic animals and humans in the country. This study aimed to identify the burden of anti-*Brucella* antibodies in small ruminants as well as associated potential risk factors with its occurrence at nine institutional livestock farms in Punjab, Pakistan. The sera collected from equal number of sheep and goats (500 from each species) were screened by indirect-ELISA for anti-smooth-*Brucella* antibodies followed by a serial detection by real-time PCR. Overall, 5.1% (51/1000) seropositivity was registered corresponding to 5% (25/500) prevalence in goats and 5.2% (26/500) in sheep. *Brucella*-DNA could not be detected in any of the tested sera by real-time PCR. Multiple logistic regression model indicated that farm location (OR 34.05), >4 years of age (OR 2.88), with history of reproductive disorders (OR 2.69), and with BCS of ≤3 (OR 12.37) were more likely to test positive for brucellosis at these farms. A routine screening, stringent biosecurity, and quarantine measures are warranted for monitoring and eradication of the infection. Similarly, isolation and molecular investigation of the etiologic agent(s) are needed to understand the relationship of epidemiology and out-breaks of brucellosis in the country.

Keywords: sheep, goats, brucellosis, risk factors, Pakistan

INTRODUCTION

Brucellosis is a bacterial zoonosis with worldwide distribution, which is caused by bacteria of the genus *Brucella*. This genus comprises; *B. melitensis, B. abortus, B. suis, B. canis, B. ovis*, and *B. neotome* (classical *Brucella* species), *B. ceti* and *B. pinipedialis* from marine mammals, *B. microti* from voles, *B. inopinata* from human females, *B. papionis* from baboons and recently *B. vulpis* from red foxes (1–6). Based upon host preference; *B. abortus* predominantly infects bovines, *B. melitensis*

small ruminants, *B. canis* dogs, *B. suis* pigs, and *B. ovis* rams, however, infection in non-prefered hosts is transmissible (7–9). In developing countries, a higher prevalence rate is observed where it causes abortion and retention of fetal membranes (10). The infection may stay undiagnosed due to its asymptomatic form and the infected animals may conceive subsequently, but remain carriers for their life. The infection is of economic importance, especially in developing countries (11). Direct or indirect contact with infected animals and consumption of contaminated raw milk and products are the main routes of transmission, respectively, in animals and humans (12). Brucellosis is an established occupational health hazard (13–16). Diagnosis remains a challenge and is based primarily on serology [e.g., Rose Bengal Test (RBT) and Milk Ring Test (MRT)]. Molecular detection of *Brucella*-DNA (e.g., PCR) in clinical/biological samples, is coupled with serology to identify the etiology precisely where necessary. The bacterial isolation is a gold standard for the diagnosis, but requires specific growth conditions. Moreover, owing to fastidious nature of the organism (*B. abortus* for one), the turn-around-time for the samples is beyond a week. Vaccination is recommended but practiced mostly in elite herds in developing countries including in Pakistan (17). Treatment of brucellosis in ruminants is also not very popular in the country hence, test and slaughter/culling policy remains a sole solution for eradication of the infection in farm animals.

Pakistan is an agriculture-based country in south-Asia, where livestock plays a vital role in the national economy. The total livestock population in the country is 142.8 millions, where small ruminants (sheep and goat) share 80.27 million heads (18). In the past, brucellosis has been reported in both large and small ruminants in Punjab, Pakistan (19–23). This study was aimed to ascertain the current status of brucellosis in small ruminants at institutional livestock farms located in Punjab. Additionally,

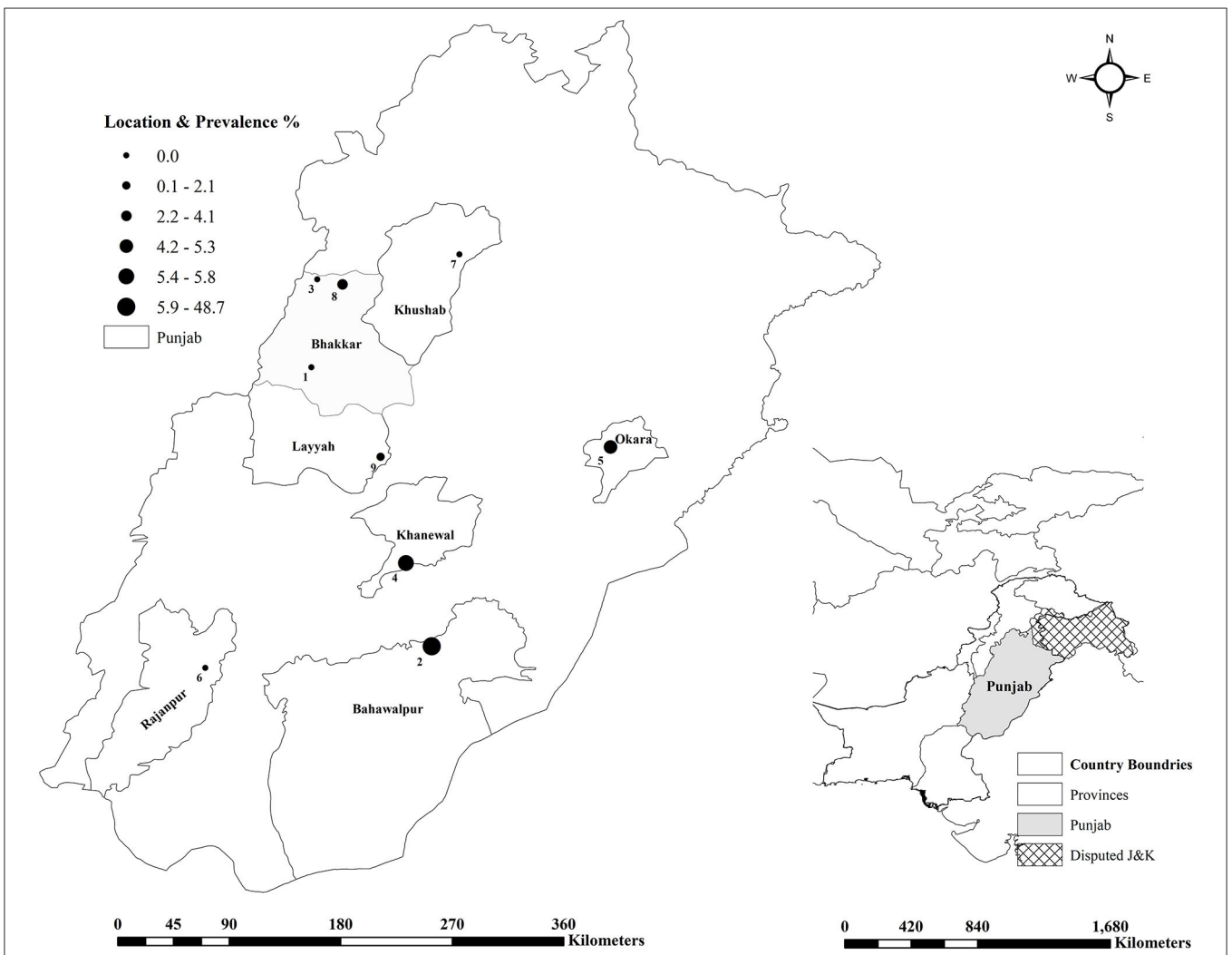

FIGURE 1 | Frontiers Media SA remains neutral with regard to jurisdictional claims in published maps and institutional affiliations. Gegraphical representation of the small ruminant farms tested for brucellosis in Punjab, Pakistan.

we determined the risk factors associated with the occurrence of the disease.

MATERIALS AND METHODS

A total of 1,000 sera (500 each from sheep and goats) were collected from nine different institutional livestock farms maintained under the Livestock and Dairy Development Department (L&DD), Government of Punjab, Lahore, Pakistan (**Figure 1**) (24). The sample size was calculated for an estimated disease prevalence of 50% at a 95% confidence interval, and 5% desired absolute precision (**Table 1**) (25). A minimum of 384 samples from each species were required by this method. The sample size was further inflated to accommodate for the potential losses during the transportation. The final sample size was proportionally allocated to each farm according to the population of the animals at each farm. Available identification record was used at each farm, to randomly select animals by using a random number generator and to collect the animal level data. Individual animals were restrained and blood was collected in a 9 mL vacutainer tube without anticoagulant through the jugular vein. No animals were harmed during this process. The animals had no prior history of brucellosis vaccination.

Sera were screened by ID Screen® Brucellosis Serum Indirect Multi-species (IDVet, Grabels, France), an indirect-ELISA for detection of anti-smooth-lipopolysaccharide (LPS) (*B. abortus, B. melitensis,* and *B. suis*). The samples were tested at the National Reference Laboratory (NRL) for brucellosis, Friedrich-Loeffler-Institut (FLI), Jena, Germany as per manufacturer's recommendations. DNA was extracted from sera by using the High Pure Template Kit (Roche, Rotkreuz, Switzerland) and molecular detection was serially done by real-time PCR as described by Probert et al. (26). The DNA extraction was run along with *E. coli* controls. The real-time PCR was run along with *B. abortus* (ATCC 23448) and *B. melitensis* (ATCC 23456) as positive controls. In tandem with positive controls, nuclease-free water was run as negative control (NTC).

Brucellosis prevalence at species level was calculated by dividing the number of positive animals (numerator) by the total number of animals sampled (denominator). The statistical analysis was performed in two parts. In the first part, univariate and multivariate analysis were conducted to determine the association of the risk factors with the seroprevalence. The univariate analysis was conducted for farm related and animal level variables. Seroprevalence of brucellosis was considered as an outcome or dependent variable while biological plausible variables [e.g., farm location, species, sex, age/parity status, breed, history of reproductive disorders, and body condition score (BCS)] were considered as explanatory or independent variables. A $p \leq 0.05$ was considered as a level of significance. A backward stepwise approach was used for the binary logistic regression analysis (27). Nagelkerke R^2 (NR^2) and Hosmer and Lemeshow test (HLT) were used to assess the model-fitness. The statistical analysis was conducted using the IBM SPSS Statistics (IBM Corporation, Armonk, New York, USA).

The second part of statistical analysis was performed using R software and each of the variable was tested one by one alone in a mixed effect model approach with "farm" variable as random factor and using "lmer" function from lme4 package, and logistic binary model function (28). The results of these models showed that five variables were significantly associated with seroprevalence of brucellosis, i.e., species, age, parity status, reproductive disorders, and body condition score (see **Table 4**). To check if any of these variables showing significance association were confounded, all the five variables were tested in one single model and stepwise backward regression was performed (i.e., least significant variables were taken out in the next model). After running the model, collinearity and confounding behavior was tested by determining variance inflation factor using "vif" function from "car" package. Those variables were taken out of the model which showed high *p*-value and high variance

TABLE 1 | Seroprevalence in small ruminants of Punjab, Pakistan.

Farm[a]	Goats[b]		Sheep[c]		Total	
	Pos./Tested	Prev.%(95% CI)	Pos./Tested	Prev.%(95% CI)	Pos./Tested	Prev.%(95% CI)
1	0/0	-	0/41	0 (0–8.6)	0/41	0 (0–8.6)
2	0/0	-	18/37	48.7 (31.9–65.6)	18/37	48.7 (31.9–65.6)
3	0/0	-	0/22	0 (0–15.4)	0/22	0 (0–15.4)
4	13/203	6.4 (3.5–10.7)	1/40	2.5 (0.1–13.2)	14/243	5.8 (3.2–9.5)
5	7/44	15.9 (6.6–30.1)	0/88	0 (0–4.1)	7/132	5.3 (2.2–10.6)
6	0/43	0 (0–8.2)	0/9	0 (0–33.6)	0/52	0 (0–6.8)
7	0/0	-	0/45	0 (0–7.9)	0/45	0 (0–7.9)
8	0/0	-	6/145	4.1 (1.5–8.8)	6/145	4.1 (1.5–8.8)
9	6/210	2.9 (1.1–6.1)	0/73	0 (0–4.9)	6/283	2.1 (0.8–4.6)
Total	**26/500**	**5.2 (3.4–7.5)**	**25/500**	**5 (3.3–7.3)**	**51/1,000**	**5.1 (3.8–6.7)**

[a] *The seroprevalence varied significantly among sampled farms;* $\chi^2 = 159.281$, $p < 0.001$.
[b] *The seroprevalence in sheep varied significantly among sampled farms;* $\chi^2 = 163.790$, $p < 0.001$.
[c] *The seroprevalence in goats varied significantly among sampled farms;* $\chi^2 = 15.530$, $p = 0.001$.

TABLE 2 | Univariable analysis of the seroprevalence of brucellosis in small ruminants sampled from nine institutional livestock farms of Punjab, Pakistan.

Variable	Category	Pos. / tested	Prev. % (95% CI)	Odds ratio	95% CI	*p*-value*
Farm	Farm 2	18/37	48.7 (31.9–65.6)	25.7	12.84–55.52	<0.001
	Others	33/963	3.4 (2.4–4.8)	Ref	-	
Species	Sheep	26/500	5.2 (3.4–7.5)	1.042	0.593–1.831	0.886
	Goats	25/500	5 (3.3–7.3)	Ref	-	
Sex	Females	47/893	5.3 (3.9–6.9)	1.43	0.51–4.05	0.5
	Males	4/107	3.7 (1–9.3)	Ref	-	
Age	Above 4Y	35/440	7.9 (5.6–10.9)	2.94	1.60–5.38	<0.001
	Below 4Y	16/560	2.9 (1.6–4.6)	Ref	-	
Parity Status	Multiparous	40/594	6.7 (4.9–9.1)	2.59	1.31–5.12	0.006
	Nulli/Primi	11/406	2.7 (1.4–4.8)	Ref	-	
Breeds	Buchi	18/37	48.7 (31.9–65.6)	26.7	12.84–55.52	<0.001
	Others	33/963	3.4 (2.4–4.8)	Ref	-	
Reproductive disorders	Yes	25/178	14.0 (9.3–20.0)	5.00	2.81–8.89	<0.001
	No	26/822	3.2 (2.1–4.6)	Ref	-	
BCS	<underline<>3	34/172	19.8 (14.1–26.5)	11.74	6.39–21.62	<0.001
	>3	17/828	2.1 (1.2–3.3)	Ref	-	

**Statistical value of significance: $p \leq 0.05$.*

inflation factor. In the next model if the *p*-value and variance inflation factor of the other remaining variables changed by a factor of 20%, then the taken-out variable was considered to be confounded with other variables. The maps were generated by using ArcGIS version 10.5.1 (ESRI, Redlands, CA, USA).

RESULTS

Anti-*Brucella* antibodies were detected in 51 (5.1%, CI 3.8–6.7) samples from sheep and goats. The farm-herd based and univariate analysis showed the seroprevalence almost identical in goats (5.2%) and sheep (5.0%), $p = 0.886$ (**Tables 1**, **2**). Seropositive animals were detected at the five of nine sampled farms, and the prevalence varied from 2.1% (Farm 9) to 48.7% (Farm 2), $p < 0.001$. In goats, the highest seroprevalence was recorded in the small ruminants at Farm 5 (15.9%) and the lowest at the Farm 9 (2.9%), $p = 0.001$. In sheep, the seropositivity ranged from 2.5% (Farm 4) to 48.7% (Farm 2), $p < 0.001$ (**Figure 1**). None of the samples contained *Brucella* DNA as confirmed by negative real-time PCR results.

The univariable analysis indicated that sheep at Farm 2 were significantly ($p < 0.001$) more likely to test positive for anti-*Brucella* antibodies (OR 25.7, CI 12.84–55.52). In females, the seropositivity (5.3%) and odds for testing positive (OR 1.43, 0.51–4.05) were higher as compared to males (3.7%), $p = 0.5$. The small ruminants; above 4 years of age (7.9%, OR 2.94 CI 1.60–5.38), of multiparous status (6.7%, OR 2.59 CI 1.31–5.12), belonging to Buchi breed (48.7%, OR 26.7 CI 12.84–55.52), with history of reproductive disorders (13.6%, OR 3.19 CI 1.29–7.95) and having BCS ≤3 (19.8%, OR 11.74 CI 6.39–21.62) were found significantly ($p < 0.05$) more likely to test seropositive (**Table 2**).

The multivariable analysis indicated that small ruminants; kept at Farm 2 (OR 34.05 CI 13.47–86.10), above 4 years of age (OR 2.88 CI 1.39–5.94), with history of reproductive disorders (OR 2.69 CI 1.33–5.42), and BCS ≤3 (OR 12.37 CI 5.98–25.57) were significantly ($p < 0.01$) more likely to test positive for anti-*Brucella* antibodies (**Table 3**). The values of Nagelkerke R^2 (0.407) and Hosmer and Lemeshow test (Ci-square value; $\chi 2 = 3.092$, $p = 0.543$) indicated that it was a reasonable model to predict seroprevalence of brucellosis at the sampled farms.

In the second part of statistical analysis, using mixed effects model approach while testing each variable one by one in each model, the following were significant, i.e., species, age, parity status, reproductive disorders, and body condition score while sex and breed were non-significant (**Table 4**). Using backward regression analysis, testing all these five significant variables together, species and body condition score were found significant while age, parity status, and reproductive disorders were non-significant, with age showing least significant *p*-value (0.82) and high vif value (3.50) (**Table 5**). Variable "age" was taken out in the next model, and species, parity status, and body condition score were significant while reproductive disorders was non-significant (0.33) in this model and all variables showed lower vif values. Variable "reproductive disorders" was taken out in the next model, and all the remaining three variables (i.e., species, parity status, and body condition score) were significant and displayed low vif values. Low vif values in the last model pointed out that all the three variables were not confounded (**Table 5**).

TABLE 3 | Multivariable analysis of the seroprevalence of brucellosis in small ruminants sampled from nine institutional livestock farms of Punjab, Pakistan.

Variable	Exposure variable	Comparison	OR	95%CI	*p*-value*
Farm	Farm 2	Others	34.05	13.47–86.10	<0.001
Age group	>4 years	<4 years	2.88	1.39–5.94	0.004
Reproductive disorders	Yes	No	2.69	1.33–5.42	0.006
BCS	<underline<>3	> 3	12.37	5.98–25.57	<0.001

Model Fit: Nagelkerke $R^2 = 0.407$, Hosmer and Lemeshow Test ($\chi^2 = 3.092$, $p = 0.543$).
**Statistical value of significance: $p \leq 0.05$.*

TABLE 4 | Each independent variable was tested separately in Mixed effect logistic regression model with farm as random factor.

Dependent variable	Model Sr. No	Independent variable	Estimate	*z*-value	*p*-value*
Brucella-iELISA outcome	1	Species	−2.546	−2.903	0.003
	2	Age	0.4379	2.563	0.01
	3	Sex	−0.1153	−0.203	0.83
	4	Parity	−1.1371	−3.033	0.002
	5	Breed	−0.1660	−0.995	0.31
	6	Reproductive disorder	0.3344	2.814	0.004
	7	BCS	−2.8795	−7.739	1e−14

**Statistical value of significance: $p \leq 0.05$.*

TABLE 5 | Stepwise backward regression models with starting model containing five independent variables and farm as random factor*.

Dependent variable	Model Sr. No	Independent variables tested together in one model	*p*-values*	Variance inflation factor (vif) value
Brucella-iELISA outcome	1	Species	0.003	1.01
		Age	0.82	3.50
		Parity status	0.16	3.44
		Reproductive disorders	0.35	1.07
		BCS	6.63e−14	1.06
	2	Species	0.003	1.01
		Parity status	0.005	1.09
		Reproductive disorders	0.33	1.06
		BCS	5.48e−14	1.04
	3	Species	0.002	1.01
		Parity status	0.001	1.03
		BCS	1.83e−14	1.04

**Variable showing least significance and high variance inflation factor (vif) value were taken out in next model; (Statistical value of significance: $p \leq 0.05$).*

DISCUSSION

Brucellosis remains an endemic infection in livestock in Pakistan (17, 29). Serology is a preferred and handy choice for diagnosis of brucellosis. ELISA is a sensitive test and is useful for diagnostic screening on larger scale but is unable to differentiate precisely between vaccinated and infected animals (30, 31). Molecular biological tests e.g., PCR, focus on the presence of DNA in the sample and are potentially able to differentiate the vaccine and field strains of *Brucella* (32). Real-time PCR can even detect and differentiate at lower amounts of DNA in a clinical sample when compared to conventional PCR. However, it requires the presence of bacterial DNA in the sample, which may not be present at every time during and after an infection and might be affected by laboratory procedures (33). Hence, a proper validation process is needed for every test. We used indirect-ELISA as a single screening test and real-time PCR for confirmation of the etiology.

Among variables, the odds for testing positive varied significantly depending upon the farm location and were significantly higher in the animals kept at Farm 2 (**Tables 1**, **3**). These findings are supported by previous reports (20, 22, 34).

This could be related to the environmental factors including herd management system at these farms. Furthermore, small ruminants had a close contact with bovines at Farms (2, 5, 6, 7, and 8), where brucellosis was reported previously (21, 23, 35). Moreover, common grazing and watering areas, use of brucellosis positive males for breeding and introduction of new animals without testing could be the factors responsible for brucellosis incidence at these locations (36, 37).

Age (>4 years) and parity status (multiparous) were found significantly associated ($p < 0.05$) with higher odds as compared to younger (<4 years) and null/primiparous (≤1 parturited) animals, respectively. Furthermore, age was also found significantly associated ($p < 0.05$) with seroprevalence (OR 2.88) by multivariate analysis (**Table 3**). A similar trend was reported in both sheep and goats with significant association (21, 38), non-significant association (22), and without determination of association (39, 40). This may be ascribed to increased frequency of contact with other animals with respect to age, higher coital chances, and sexual maturity as compared to younger animals (12, 41).

Reproductive disorders showed significant association (OR 2.69, $p = 0.006$) with brucellosis in the current study (**Tables 2**, **3**). It is understandable as late abortion and retention of fetal membranes are characteristic signs of brucellosis. These findings are supported by similar results reported previously by others investigators (19, 34, 42). However, a non-significant association ($p > 0.05$) in sheep has also been documented (22). Furthermore, animals having BCS ≤ 3, were more likely to test positive (OR 12.37, $p < 0.001$) in our study which is concordance with findings of Ethiopian workers (43). A possible reason could be the higher susceptibility of animals already infected with brucellosis to other infections or the loss in BCS caused by the brucellosis itself.

CONCLUSION AND RECOMMENDATIONS

In conclusion, we found anti-*Brucella* antibodies in sheep and goats at these livestock farms in Punjab, Pakistan. Farm location, age, and species of the animals, history of reproductive disorders and BCS were found to play a significant role for brucellosis seropositivity in these animals. Although vaccination is recommended and treatment is possible for brucellosis, they are not considered safe for human health, hence regular screening and culling of the reactor animals remain the only choice to monitor and eradicate brucellosis. Introduction of the new stock at these farms should be carried out only after screening and quarantine. Furthermore, farm workers should be advised to adopt protection measures as a routine. Abortion at these farms should not go unnoticed and must be investigated to confirm its cause to adopt recommended control measures. If abortions occur, disinfection of the area should be ensured along with strict biosecurity measures to restrict chances of dissemination of infection through the dogs, cats, other domestic animals, visitors, and farm equipment/supply movement. Standardization and validation of the diagnostic tests are required based on the local conditions. Isolation and molecular investigations of the etiological agents might be helpful for future understanding of the epidemiology of the infection and the relationship of the outbreaks.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

Blood and serum samples were collected as per bio-safety, ethical, and animal welfare guidelines defined by Research Board of the University of Agriculture, Faisalabad, Pakistan (letter No. 3253/ORIC, dated: 25.11.2015). Permission was granted by the Livestock and Dairy Development Department (LNDD), Government of Punjab, Pakistan to collect samples at the farms (vide letter No. SO (I&C)/L&DD/2-6/2016, dated: 15.02.2016).

AUTHOR CONTRIBUTIONS

QU and TJ: Conceptualization. FM and MS: methodology. MH and MA: formal analysis. TJ and QU: investigation. UT, MI, and QU: data curation. TJ: writing-original draft preparation. SU, ZQ, HJ, SS, and HN: writing-review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

Authors would like to thank German Federal Foreign Office for financially supporting the study under the project German Biosecurity Program. Funding agencies had no role in designing, performing, and publication of the results.

ACKNOWLEDGMENTS

Authors are thankful to Higher Education Commission (HEC)-Pakistan to support the stay of QU under the project International Research Support Program (IRSP) and TJ under the project 90% Overseas Scholarship Scheme at Friedrich-Loeffler-Institut (FLI), Jena, Germany. Authors would also like to thank the Livestock and Dairy Development (L&DD), Government of Punjab, Pakistan for their cooperation in the provision of samples. The agencies had no role in designing, performing, and publication of the results.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 10 September 2020
doi: 10.3389/fvets.2020.00608

Detection and Molecular Characterization of Bovine Leukemia Virus in Egyptian Dairy Cattle

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Bovine leukemia virus (BLV) causes enzootic bovine leukosis (EBL), the most common neoplastic disease in cattle worldwide. The first EBL outbreak in Egypt was reported in 1997. To date, there are few studies regarding BLV diagnosis using only serological detection and no studies investigating the distribution of BLV provirus, which is the retroviral genome integrated into the host genome, in Egypt. The genetic characteristics of Egyptian BLV strains are also unknown. Therefore, we aimed to detect BLV provirus and determine BLV genetic variability among dairy cattle in Egypt. We collected 270 blood samples of dairy cattle from 24 farms located in five provinces in Egypt. Out of the 270 samples, 58 (21.5%) were positive for BLV provirus. Phylogenetic analysis based on 18 420-bp selected sequences out of 50 isolates of the BLV *env*-gp51 gene demonstrated that Egyptian BLV isolates were clustered into genotype-1 and-4, among 11 genotypes detected worldwide. Furthermore, phylogenetic analysis and alignment of the 501-bp sequence of the *env*-gp51 gene revealed that at least six genetically different strains are present in Egypt. Genotype-1 isolates comprised four different strains (G1-a, G1-b, G1-c, and G1-d) and genotype-4 isolates included two different strains (G4-x and G4-y). Moreover, in one farm with 100% infection rate, we identified three isolates of G1-a strain, 35 isolates of G4-x strain, and two isolates of G4-y strain. Overall, this study provides the new report on molecular prevalence of BLV in Egypt and records the coexistence of BLV genotype-1 and-4 in Egyptian cattle.

Keywords: bovine leukemia virus, Egypt, dairy cattle, prevalence, BLV genotype

OPEN ACCESS

Edited by:
Van Giap Nguyen, Vietnam National University of Agriculture, Vietnam

Reviewed by:
Marzena Rola-Łuszczak, National Veterinary Research Institute (NVRI), Poland
Semmannan Kalaiyarasu, ICAR-National Institute of High Security Animal Diseases (ICAR-NIHSAD), India

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *30 May 2020*
Accepted: *28 July 2020*
Published: *10 September 2020*

Citation:
Hamada R, Metwally S, Polat M, Borjigin L, Ali AO, Abdel-Hady AAA, Mohamed AEA, Wada S and Aida Y (2020) Detection and Molecular Characterization of Bovine Leukemia Virus in Egyptian Dairy Cattle. Front. Vet. Sci. 7:608. doi: 10.3389/fvets.2020.00608

INTRODUCTION

Bovine leukemia virus (BLV), an oncogenic member of the family *Retroviridae*, genus *Deltaretrovirus*, is closely related to the human T-cell leukemia virus types 1 and 2 (1, 2). It causes enzootic bovine leukosis (EBL), the most common neoplastic disease of cattle globally, and imposes a severe economic loss to the dairy industry (1–3). Most BLV-infected cattle are apparently healthy in the aleukemic stage and approximately one-third can enter the persistent lymphocytosis stage characterized by non-malignant polyclonal expansion of B-lymphocytes; however, only 1–5%

develop B-cell leukemia, manifesting clinical signs of lymphoma after a long latency (1, 2). BLV infection can be transmitted both vertical route and horizontal route in addition to the other iatrogenic procedures involving the transfer of infected blood between animals (i.e., dehorning, ear tattooing, rectal palpation, and needle reuse), and is responsible for disease propagation in a herd (4). BLV has spread worldwide via the continual trade of breeding cattle between several countries (3). However, vaccines and effective treatments are not yet available for practical application (1).

BLV proviral load (PVL), which represents the retroviral genome integrated into the host genome (5, 6), correlates strongly with not only BLV infection capacity as assessed by syncytium formation (7, 8), but also EBL progression (7, 9, 10). Additionally, it is a useful index for estimating transmission risk (11, 12). For example, previous reports have posited that as determined by the BLV-CoCoMo-quantitative polymerase chain reaction (qPCR)-2 method, the quantitative measurement of PVL (7, 13), BLV provirus was detected in the milk, nasal mucus, and saliva samples from dairy cattle with PVL >10,000, 14,000, and 18,000 copies/10^5 cells in blood samples, respectively (11, 12). It has been suggested that these infected cattle may increase the risk of BLV transmission via direct contact with healthy cattle. In contrast, cattle with low PVL are known to prevent natural BLV infection (14).

BLV genome consists of four structural and enzymatic regions that encode genes namely, *gag*, *pro*, *pol*, and *env,* along with the pX region that encodes two regulatory, *tax* and *rex,* and two accessory genes, *R3* and *G4*, and is flanked by two identical long terminal repeats (LTRs) at the 5′ and 3′ termini (1, 2). The BLV *env* gene is transcribed as an envelope glycoprotein (Env) protein complex that comprises the surface (gp51) and transmembrane (gp30) proteins (1), which play a major role in the virus lifecycle and entry into the host cell (15). gp51 is the target for neutralizing antibodies (16). The conformational epitopes (F, G, and H) located in the N-terminal half of gp51 are strongly involved in syncytium formation and viral infectivity (15, 16). The C-terminal half contains linear epitopes (A, B, D, and E) mapped with antipeptide antibodies (17). In contrast to gp51, gp30 is poorly immunogenic (1) and therefore, BLV *env*-gp51 sequencing is mainly used for BLV phylogenetic studies (3). At least 11 BLV genotypes have been identified worldwide based on the phylogenetic analysis of *env*-gp51 sequences of BLV after identifying BLV genotype-11 in China (3, 18). Three genotypes of BLV, namely genotype-1,-4, and-6, has been mainly detected worldwide (3).

Egypt is a transcontinental country spanning the northeast corner of Africa and southwest corner of Asia. The bovine sector in Egypt is well-integrated with cropland since there are limited natural pastures. Female cattle and buffaloes are used for milk production, while male animals and infertile females are fattened for meat (19). Native cattle in Egypt are called "Baladi," literally meaning "local" without genetic subdivisions. They are reared throughout the country, acclimatized to Egyptian conditions, and have a high tolerance to endemic diseases. Due to the low milk productivity of these animals, genetic improvement schemes have involved crossbreeding with exotic high producing cattle breeds such as Holstein, Brown Swiss, and Simmental (20–22), resulting in a large scattered population raised in small- or medium-sized herds by local farmers under the breed name "Mixed," as described and characterized by the Ministry of Agriculture and Land Reclamation. Egyptian livestock import has increased over the last 10 years, improving the dairy industry performance. Germany, Netherlands, and the United States of America remain the top suppliers of live dairy cattle to Egypt (23).

Sero-epidemiological surveys have shown that BLV infection is widespread in all continents except Europe, where the disease is present only in the eastern European countries (3). However, reports indicate that BLV infection prevalence in the Middle Eastern countries is somewhat lower than that in the other regions of the world (3). In Egypt, the first EBL outbreak occurred in 1997 in Assiut province, Upper Egypt with typical clinical signs of leukosis in a closed herd of Holstein-Friesian cattle imported from Minnesota, USA 8 years earlier (24). Thus, the authors assumed that these cattle were imported as clinically healthy BLV-infected cattle during the herd construction period and the disease progressed to lymphoma after the latent period passed (24). It was believed that this outbreak introduced BLV in Egypt (25). After completely eradicating the infected animals in the herd (24), Egypt was reported to be BLV-free (3, 26). In 2012, a seroprevalence study for BLV infection among dairy cattle in Egypt was conducted, which reported that 15.83% of the tested dairy cattle were BLV-infected (27). Recently, 20.8% BLV seropositivity was reported during the survey for BLV infection among Egyptian cattle (28). These findings indicate that BLV still infects Egyptian cattle. However, to date there has been no reports on BLV genotyping in Egypt.

In this study, we investigated BLV infection prevalence among dairy cattle in five different regions of Egypt to cover representative area of the country to include the northern (Beheira and Damietta provinces), the central (Fayoum province), and the southern (Qena and Luxor provinces) parts using CoCoMo-qPCR-2 assay. Furthermore, we demonstrated that the BLV PVL level varied among different regions of Egypt. Moreover, by conducting the phylogenetic analysis of BLV *env*-gp51 gene sequence, we investigated the six genetically distinct strains present in Egyptian cattle. Overall, this study provides a new evidence for the molecular detection of BLV in Egypt and records the coexistence of BLV genotype-1 and-4 among Egyptian cattle.

MATERIALS AND METHODS

Sample Collection and DNA Extraction

Blood samples were collected from 270 cattle of 24 different dairy farms located in five provinces in Egypt, namely Luxor, Qena, Fayoum, Beheira, and Damietta, between November 2018 and January 2019 (**Figure 1** and **Table 1**). These cattle comprised various breeds of dairy cattle, such as the Egyptian Native breed, some foreign breeds (Holstein and Simmental), and the Mixed breed, the crossbreed between foreign breeds and Native cattle, and included cattle of both sexes aged 3-months to 12-years (**Table 1**).

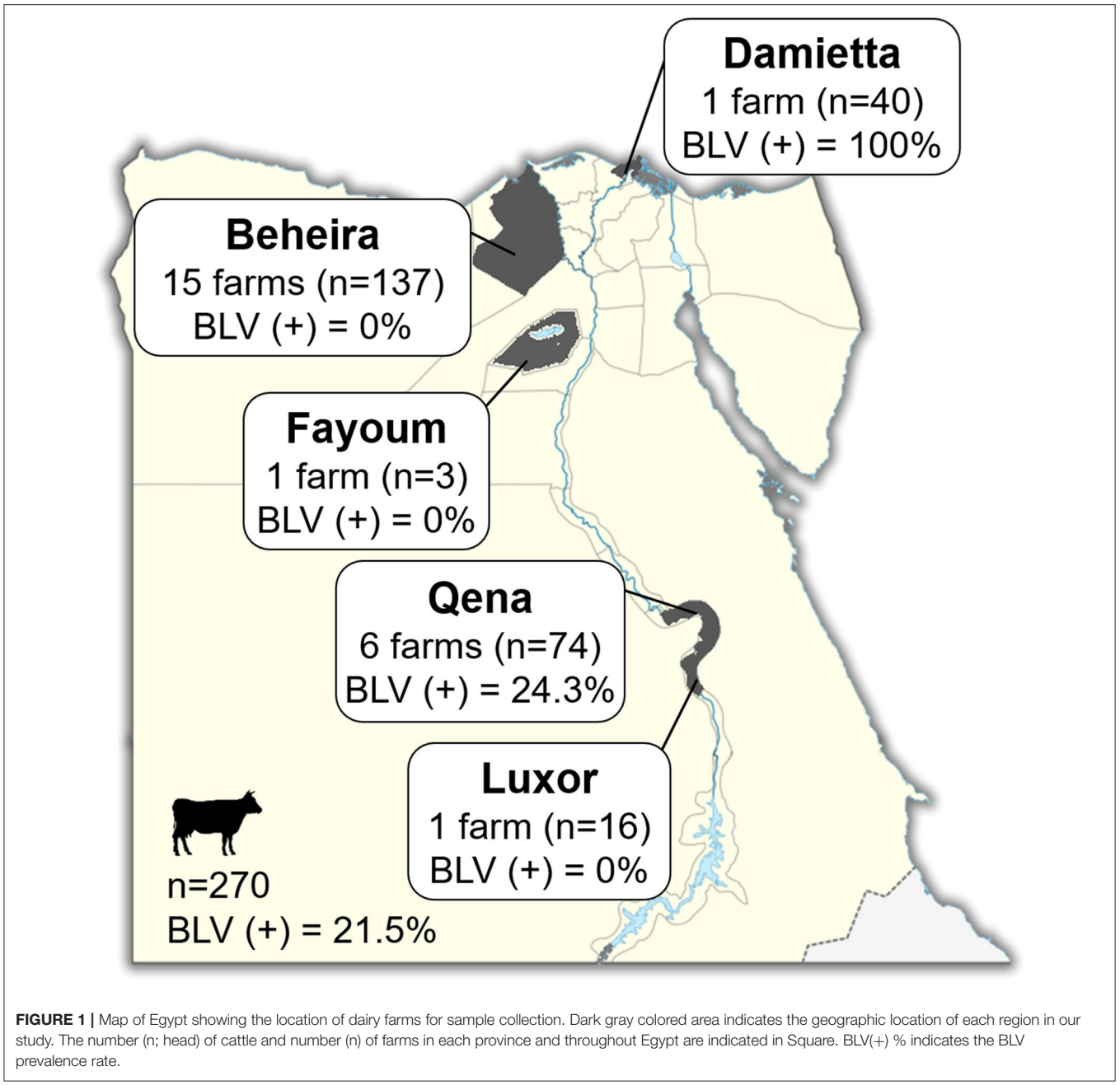

FIGURE 1 | Map of Egypt showing the location of dairy farms for sample collection. Dark gray colored area indicates the geographic location of each region in our study. The number (n; head) of cattle and number (n) of farms in each province and throughout Egypt are indicated in Square. BLV(+) % indicates the BLV prevalence rate.

Genomic DNA was extracted from 300 μl whole blood using the Wizard Genomic DNA Purification Kit (Promega; Madison, WI, USA), following manufacturer's instructions. The concentration of extracted DNA samples was measured using NanoDrop One Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA). DNA samples were diluted in nuclease-free water to a final concentration of 30 ng/μl for PCR experiments.

Evaluation of BLV PVL Using CoCoMo-qPCR-2

BLV PVL was estimated using CoCoMo-qPCR-2 (RIKEN Genesis; Kanagawa, Japan), as described previously (7, 13). Briefly, a 183-bp sequence of the BLV LTR regions was amplified in a reaction mixture containing THUNDERBIRD Probe qPCR Mix (Toyobo; Tokyo, Japan), using the degenerate primer pairs, CoCoMo FRW and CoCoMo REV, and a 15 bp 6-carboxyfluorescein (FAM)-labeled LTR probe. To normalize the viral genomic DNA level within the host cellular genome, a 151-bp sequence of *BoLA-DRA* was amplified using the primer pairs, DRA-FW and DRA-RW, and a FAM-labeled DRA probe, as previously described (13). The PVL was calculated using the equation (number of BLV-LTR copies/number of *BoLA-DRA* copies) × 10^5 cells.

TABLE 1 | Detection of BLV infection in five provinces in Egypt as determined by CoCoMo-quantitative PCR (qPCR).

Province	Town	Farm ID[a]	Breed	(Positive no. /Tested samples no.) Positive %
Luxor	EL-Qurna	LQ1	Mixed	(0/14) 0.0
			Native	(0/ 2) 0.0
Qena	EL-Brahma	QB1	Mixed	(3/10) 30.0
		QB2	Mixed	(1/ 4) 25.0
		QB3	Mixed	(3/ 5) 60.0
		QB4	Native	(2/ 5) 40.0
			Mixed	(0/ 2) 0.0
		QB5	Native	(2/ 4) 50.0
			Mixed	(2/ 5) 40.0
	Qena-city	QC6	Holstein	(5/39) 12.8
Fayoum	Tamia	FT1	Holstein	(0/ 3) 0.0
Beheira	Abu-Elmatamir	BA1	Mixed	(0/ 9) 0.0
		BA2	Mixed	(0/ 2) 0.0
		BA3	Mixed	(0/ 7) 0.0
		BA4	Mixed	(0/ 2) 0.0
		BA5	Mixed	(0/15) 0.0
		BA6	Mixed	(0/ 4) 0.0
			Native	(0/ 1) 0.0
		BA7	Mixed	(0/ 6) 0.0
		BA8	Mixed	(0/ 3) 0.0
		BA9	Mixed	(0/ 2) 0.0
		BA10	Mixed	(0/19) 0.0
		BA11	Mixed	(0/ 6) 0.0
		BA12	Mixed	(0/ 2) 0.0
		BA13	Mixed	(0/ 7) 0.0
		BA14	Mixed	(0/ 5) 0.0
		BA15	Holstein	(0/43) 0.0
			Simmental	(0/ 4) 0.0
Damietta	El-Zarqa	DZ1	Holstein	(40/40) 100.0
Total farms		24	Total	(58/270) 21.5

[a]*List of farm ID tested for BLV infection: 17 farms (QB1–QB5, BA1–BA4, BA6–BA9, and BA11–BA14) are small holder breeders, having less than 10 heads in each farm, 3 farms (LQ1, BA5, and BA10) are semi-intensive farms (over10 to 50 heads) and both of these small farms and the semi-intensive ones raring cattle of Native and Mixed breeds, and the other 4 farms (QC6, FT1, BA15, and DZ1) are intensive farms, having a cattle herd of more than 300 heads, rearing Holstein cattle except for farm BA15 that has few number of Simmental cattle rearing together with Holstein cows.*

Nested PCR Amplification of BLV *env*-gp51 Gene Fragment and Nucleotide Sequencing

A 598-bp fragment of BLV *env*-gp51 gene was amplified via nested PCR using Prime STAR GXL DNA Polymerase (Takara Bio Inc.; Otsu, Japan) as described previously (29–31). The primer set External Forward (5′-ATGCCYAAAGAACGACGG-3′) and External Reverse (5′-CGACGGGACTAGGTCTGACCC-3′) resulted in the first-round amplification of the 913-bp fragment of full length BLV *env* gene corresponding to the nucleotide positions 4826 to 5738 of the whole BLV genomic sequence recorded in GenBank (accession No. EF600696) (32), and then internal ENV_{5032} (5′-TCTGTGCCAAGTCTCCCAGATA-3′) and internal ENV_{5608r} (5′-AACAACAACCTCTGGGAAGGGT-3′) resulted in the second-round amplification of the 598-bp fragment of the BLV *env*-gp51 gene corresponding to the nucleotide positions 5037 to 5634 of the whole BLV genomic sequence.

Positive second-round PCR products were purified using 5 × Exo-SAP IT (USB Corp.; Cleveland, OH, USA) and directly sequenced on an ABI3730x1 DNA Analyzer using ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems; Foster City, CA, USA) using the primers ENV_{5032} and ENV_{5608r}. The resulting sequences included a 501-bp region of the *env* gene, corresponding to nucleotide positions 5084 to 5584 of the whole BLV genomic sequence. The editing, alignment, and identification of nucleotide sequences were performed using MEGA 7 software (33).

Phylogenetic Analysis and Phylogenetic Tree Construction

The partial BLV *env*-gp51 sequences from the Egyptian isolates were aligned with the 51 partial BLV *env*-gp51 sequences from GenBank (representative of the 11 BLV genotypes distributed worldwide) using MEGA 7 software. Phylogenetic analysis of the partial BLV *env*-gp51 sequences from 50 BLV positive samples successfully amplified using nested PCR were also conducted using MEGA 7 software (33). For robust and accurate phylogenetic analysis of the BLV *env*-gp51 sequence, the "find best DNA/Protein models" tool of MEGA 7 software was used to choose the best fit model. The Kimura-2 parameter model was chosen as the model with the best fit to analyze the BLV *env*-gp51 sequence with the smallest Akaike information criterion (AIC) value. Two phylogenetic trees were constructed using the maximum likelihood (ML) algorithm based on 420 and 501 bp sequences with the K2+I and K2 models of nucleotide substitution in MEGA 7, respectively. The reliability of the phylogenetic relationships was evaluated using non-parametric bootstrap analysis with 1,000 replicates. The deduction of protein sequence through translation of nucleotide to amino acid sequence was performed using MEGA 7 (33). The sequences of isolates obtained in this study were deposited in GenBank under accession numbers: LC498589 (DZ1.1), LC498590 (DZ1.2), LC498591 (DZ1.3), LC498592 (QB1.1), LC498593 (QB3.1), LC498594 (QB5.1), LC498595 (QC6.1), LC498596 (QB2.1), LC498597 (QB4.1), LC498580 (DZ1.4), LC498581 (DZ1.5), LC498582 (DZ1.6), LC498583 (QB1.2), LC498584 (QB3.2), LC498585 (QB5.2), LC498586 (DZ1.39), LC498587 (DZ1.40), LC498588 (QB5.3), and LC500799∼LC500830 (DZ1.7∼DZ1.38).

Statistical Analysis

The significance of PVL difference between the cattle from different geographic regions was tested using multiple comparison by Tukey's test after the analysis of variance. $P < 0.05$ was considered significant.

RESULTS

Prevalence of BLV Among Egyptian Cattle

Blood samples were collected from 270 dairy cattle of 24 farms located at five provinces of different geographic location in Egypt (**Figure 1**). Cattle samples were classified as Native Egyptian

(12 samples), Holstein (125 samples), Mixed (129 samples), and Simmental (4 samples) cattle (**Table 1**). All samples were tested for BLV infection using BLV-CoCoMo-qPCR-2 assay, which detects the two copies of BLV LTRs present per provirus. Our results showed that BLV infection rate varied among the cattle tested from farms in different regions (**Table 1**). Interestingly, BLV infection was detected only in the cattle from two provinces (Qena and Damietta), while those from the other three provinces (Beheira, Luxor, and Fayoum) tested BLV-negative. In Qena province, 74 blood samples were collected from the cattle of six farms, namely, QB1, QB2, QB3, QB4, QB5, and QC6. Five (QB1, QB2, QB3, QB4, and QB5) are small farms (<10 cattle) located at El-Brahma town, harboring either Native or Mixed breed cattle, while the sixth (QC6) is a large farm (∼300 cattle) located at the provincial capital town, harboring only Holstein breed cattle. It was interesting that the cattle from all 6 farms were BLV-positive and the infection rate for each distinct breed was 0–60% (**Table 1**). In Damietta province, 40 blood samples were collected from the cattle of a large intensive farm (DZ1) located at El-Zarqa harboring ∼500 cattle of Holstein breed. Surprisingly, all the tested samples were BLV-positive, thus having 100% infection rate (**Table 1**). A total of 137 samples were collected from the cattle of the 15 farms (BA1–BA15) located at Abu-Elmatamir in Beheira province, among which 14 are small or semi-intensive farms harboring Native or Mixed breeds and BA15 is a large intensive farm harboring Holstein and Simmental cattle. All the tested samples were BLV-negative, thus having 0% infection rate for each farm. In Luxor province, the 16 samples collected from Native and Mixed cattle in one small farm (LQ1) located at EL-Qurna were BLV-negative. A limited number of available samples from Holstein cattle in one large dairy farm (FT1) from Fayoum province was tested, but none of them were BLV-positive.

Our results demonstrated that 58 samples among the 270 tested samples were BLV-positive (21.5%; **Table 1** and **Figure 1**) and BLV infection was distributed only in two provinces; thus, BLV infection in Egypt may be limited to particular regions.

Phylogenetic Analysis of the 420-bp Sequence of BLV *env*-gp51 Region of the Selected 18 Typical BLV Strains in Egypt and Known Strains From Different Geographic Locations Worldwide

To analyze the genetic variability among the BLV strains in Egypt, phylogenetic characterization was carried out after sequencing the BLV *env*-gp51 gene. Out of the 58 BLV-positive samples collected from the cattle in six different farms in Qena province and one farm in Damietta province as shown in **Table 2**, 50 samples were successfully amplified by nested PCR for partial BLV *env*-gp51 gene and a 501-bp sequence corresponding to the nucleotide positions 5084 to 5584 of the full-length BLV genome of the reference strain FLK-BLV subclone pBLV913 (accession number EF600696) were obtained.

For phylogenetic analysis of BLV genotypes in Egypt, the 420-bp sequences corresponding to the nucleotide positions 5126 to 5545 of the full-length BLV genome were used in order to be shared with all the currently known 11 genotypes of BLV in the world to our references. The 18 selected BLV isolates out of the 50 PCR-amplified samples were aligned with those from BLV strains representing all known 11 different BLV genotypes (genotype-1 to-11) deposited in GenBank. An ML phylogenetic tree was constructed using the best K2+I model of nucleotide substitution (33). The results of phylogenetic analysis were similar to those published in previous studies and BLV strains were classified into 11 genotypes (3, 18, 29, 34–36), as shown in **Figure 2**. Interestingly, our phylogenetic analysis also showed that the Egyptian BLV isolates were clustered into genotype-1 or -4.

Identification of the Six Genetically Distinct Strains Present in Egyptian Cattle by Phylogenetic Analysis Based on the 501-bp Sequence of BLV *env*-gp51 Region

To gain insight into the genetic variability of BLV strains present in Egyptian cattle, we classified all the 50 BLV isolates via another

TABLE 2 | Distribution of the six genetically distinct BLV strains circulating in Egypt cattle throughout the infected dairy farms.

Province	Town	Farm ID	BLV infectivity by CoCoMo-qPCR (%)	BLV isolated strains						
				G-1				G-4		No. of detected isolates by nested PCR
				a	b	c	d	x	y	
Qena	El-Brahma	QB1	3/10 (30.0)	–	1[m]	–	–	1[m]	–	2
		QB2	1/4 (25.0)	–	–	1[m]	–	–	–	1
		QB3	3/5 (60.0)	–	1[m]	–	–	1[m]	–	2
		QB4	2/7 (28.6)	–	–	–	1[n]	–	–	1
		QB5	4/9 (44.4)	–	1[m]	–	–	1[n]	1[m]	3
	Qena-city	QC6	5/39 (12.8)	–	1[h]	–	–	–	–	1
Damietta	El-Zarqa	DZ1	40/40 (100.0)	3[h]	–	–	–	35[h]	2[h]	40
Total				3	4	1	1	38	3	50

[m]*Mixed breed;* [n]*Native breed;* [h]*Holstein breed.*

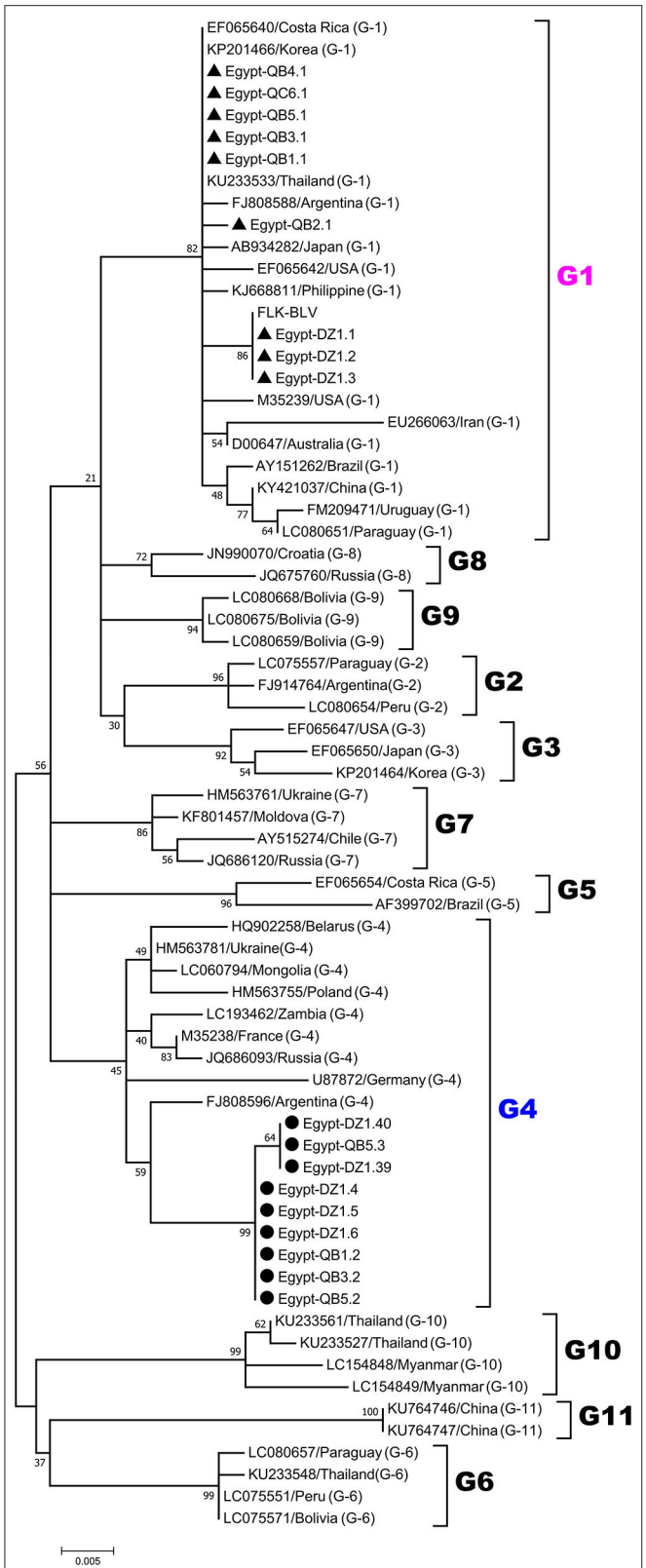

FIGURE 2 | Maximum likelihood (ML) phylogenetic tree based on 420-bp nucleotide of partial BLV *env*-gp51 gene sequences from the selected 18

(Continued)

FIGURE 2 | typical BLV isolates in Egypt and 51 BLV isolates from other countries worldwide. The Egyptian isolates are indicated by the country name together with the sample ID and farm origin. Other isolates are indicated by the accession number and country name. Egyptian BLV isolates are aligned as shown in **Figure 4**, and those clustered into genotypes-1 and-4 are marked by filled triangles (▲) and circles (•), respectively. BLV genotypes are indicated by numbers on the right side of the tree. The bottom bar of the tree denotes distance.

ML phylogenetic tree based on the 501-bp sequence of BLV *env*-gp51 region (**Figure 3**). In this tree, all the 50 BLV isolates were aligned with the reference strain. It clearly showed that nine out of 50 BLV sequences belonged to genotype-1 and were further classified into four distinct strains (G1-a, G1-b, G1-c, and G1-d). In contrast, the remaining 41 BLV isolates belonged to genotype-4 and were further classified into two distinct strains (G4-x and G4-y). Thus, our results indicate that six genetically distinct BLV strains are present in Egyptian cattle.

Nucleotide and Amino Acid Sequence Analysis of the 501-bp Sequence of BLV *env*-gp51 Region of the Six Genetically Distinct Strains Present in Egyptian Cattle

The similarity of the 501-bp sequence of BLV *env*-gp51 region of all 50 Egyptian isolates ranged from 96.7 to 100% (data not shown). The sequence from the nine Egyptian BLV isolates belonging to G1-a, G1-b, G1-c, and G1-d strains exhibited the highest similarity (96.7–100%) to that from all BLV strains representing genotype-1 from various geographic locations worldwide used as references for the phylogenetic analysis (data not shown). In contrast, the sequence from the 41 Egyptian BLV isolates belonging to G4-x and G4-y strains were 97.7–99.1% similar to that from all BLV strains representing genotype-4 (data not shown).

Nucleotide sequences for the 18 typical selected BLV isolates representing the six genetically distinct strains (G1-a, G1-b, G1-c, G1-d, G4-x, and G4-y) were aligned with that of the reference strain (**Figure 4**). The three sequences for G1-a strain showed 100% similarity with that of the reference strain. The four sequences for G1-b strain exhibited two silent nucleotide substitutions: one located in the third base of residue 149 (nt 447) and the other in the third base of residue 203 (nt 609). We found only one sequence for G1-c strain, which exhibited three nucleotide substitutions: two of them were similar to those in G1-b strain sequences, while the third was located in the third base of residue 112 (nt 336); however, all of them were silent substitutions. Similarly, we detected one sequence for G1-d strain, which exhibited three nucleotide substitutions: two of them were silent and at the same positions as those in G1-b strain sequences, while the third one was a G to T substitution in the second base of residue 99 (nt 296), which changed the deduced amino acid located in the neutralizing domain (ND1) and the $CD4^{+}$ epitope region of gp51 protein from glycine to lysine. We found 15 nucleotide substitutions for G4-x strain. Among them, 13 were silent and included

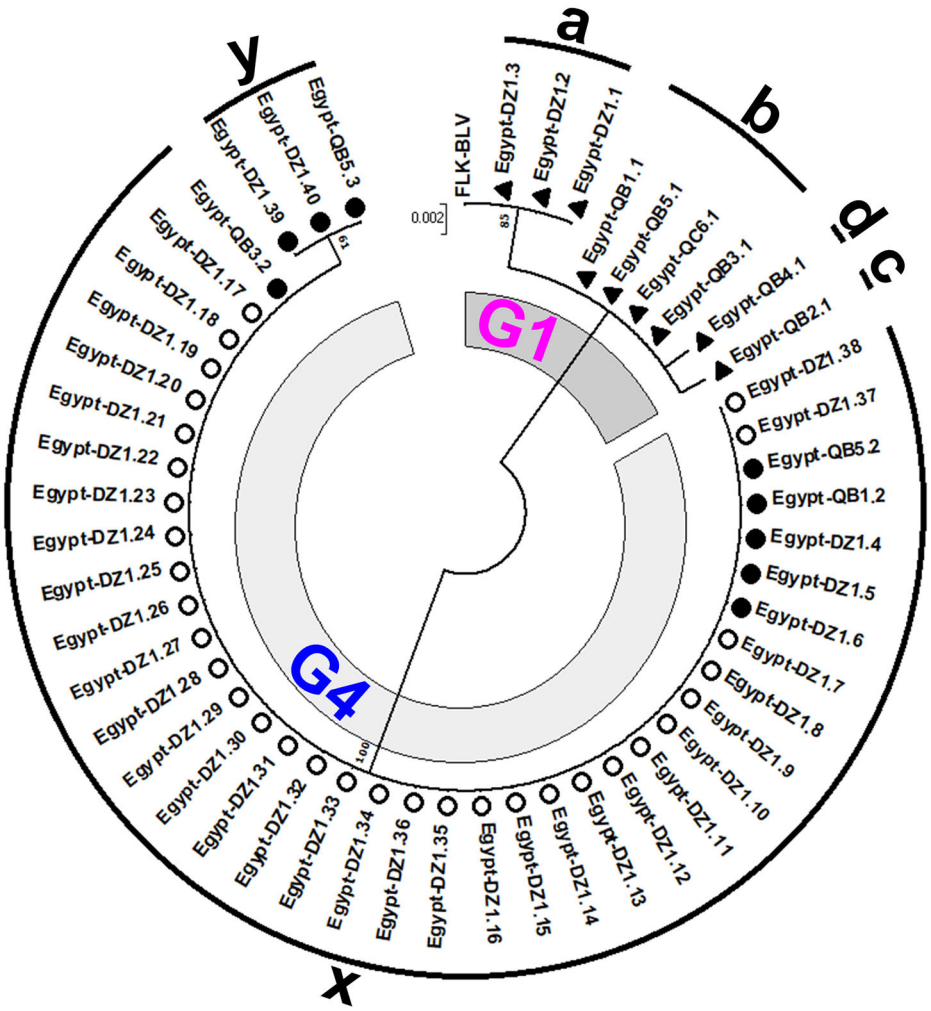

FIGURE 3 | Maximum likelihood (ML) phylogenetic tree based on 501-bp nucleotide of BLV *env*-gp51 gene from 50 BLV isolates classified into the six genetically distinct strains identified in Egyptian cattle (G1-a, G1-b, G1-c, G1-d, G4-x, and G4-y). This tree was constructed with the sequences of 50 BLV isolates detected in the cattle from different farms at two geographic locations in Egypt, including the 18 typical BLV Egyptian isolates as shown in **Figure 2** and other Egyptian isolates detected in different locations, and the reference strain FLK-BLV subclone pBLV913 (accession number EF600696). Egyptian BLV sequences on the tree are indicated by farm ID, and sample number. BLV isolates that belong to genotype-1 are indicated using filled triangles (▲), while those that belong to genotype-4 are indicated using open (○) and filled (•) circles. Sequences for each distinct BLV strain are indicated via alphabets around the circumference of the figure. The 18 typical Egyptian BLV isolates from identical samples were aligned as shown in **Figure 4** and are indicated by filled circles (•) and triangles (▲). Other isolates are indicated using open circles (○). The genotypes are shown as G1 and G4. The bar at the above portion denotes distance.

residues 90 (nt 270), 125 (nt 375), 136 (nt 408), 149 (nt 447), 161(nt 483), 175 (nt 525), 185 (nt 555), 187 (nt 559), 196 (nt 588), 197 (nt 591), 203 (nt 609), 205 (nt 615), and 239 (nt 717), but the other two substitutions were not silent. The first substitution (G to A) occurred in the second base in residue 121 (nt 362), changing the deduced amino acid located in the epitope G of gp51 protein from arginine to histidine, while the second substitution (A to G) in the first base in residue 233 (nt 697) changed the amino acid located in the B epitope region from isoleucine to valine. The substitutions for G4-y strain were identical to those for G4-x strain; however, one additional non-silent substitution (G to A) in the first base of residue 239 (nt 715) was detected, which changed the deduced amino acid from glycine to serine, which is a non-synonymous residue of gp51 protein.

Distribution of the Six Genetically Distinct Strains Throughout the Infected Farms in Egypt

We summarized the distribution of the six genetically distinct strains at different farms to specify the strain present in the cattle from each BLV-infected farm (**Table 2**). Strain G1-a was detected in three different cattle from DZ1 farm located at El-Zarqa in Damietta province. Strain G1-b was found in four different cattle: three isolates were detected as identical in the cattle from QB1,

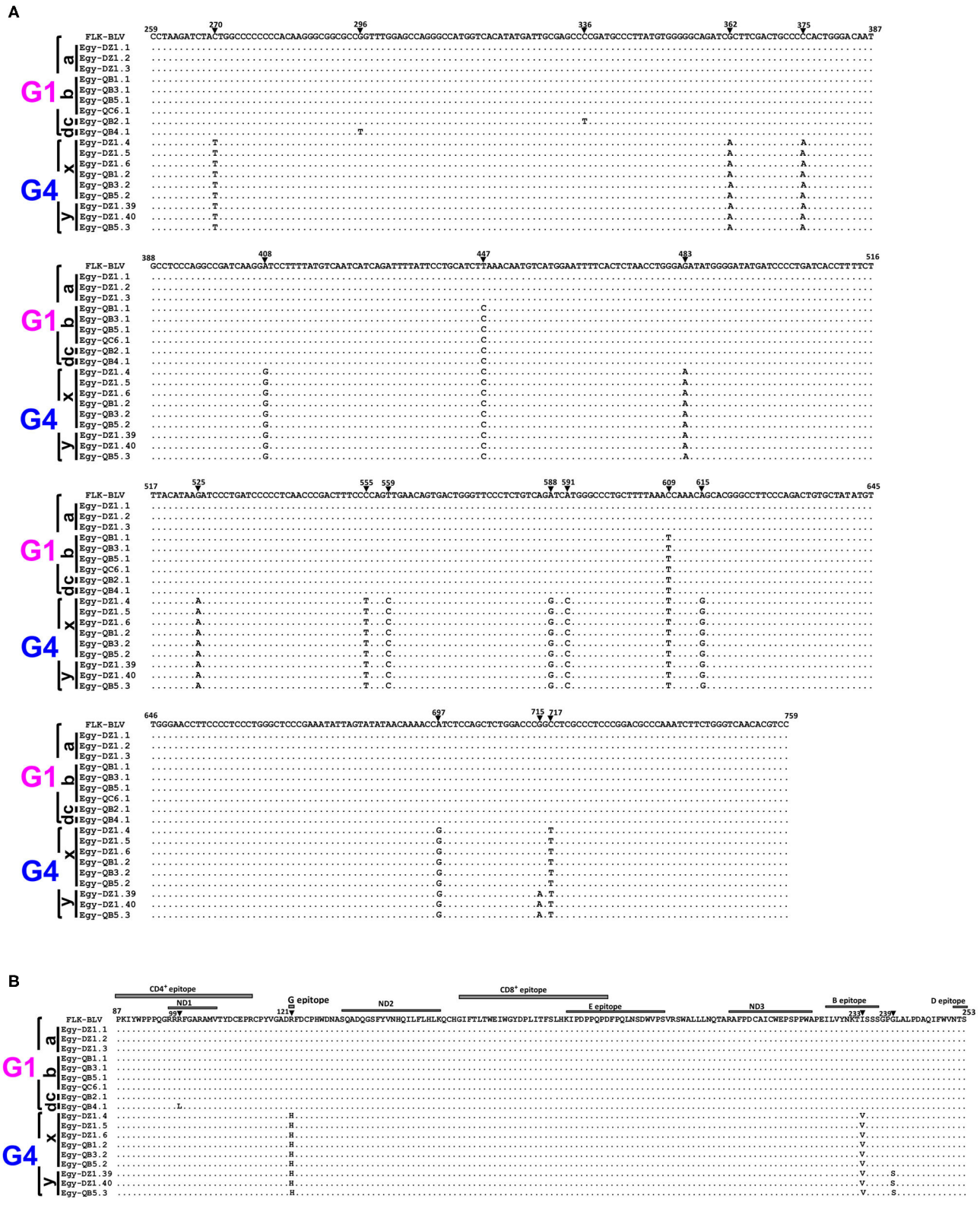

FIGURE 4 | Alignment of nucleotide sequences and deduced amino acid sequences of 501-bp nucleotide of BLV *env*-gp51 gene from the six genetically distinct strains identified in Egyptian cattle. Alignment was performed for the selected 18 typical Egyptian BLV isolates representative for the six genetically distinct strains *(Continued)*

FIGURE 4 | circulating in Egyptian cattle (G1-a, G1-b, G1-c, G1-d, G4-x, and G4-y). Egyptian BLV isolates are shown by the country name abbreviation (Egy), farm ID, and sample ID. **(A)** Nucleotide sequence alignment showing the nucleotide substitutions are indicated using numbers and filled triangles above the sequences. **(B)** The deduced amino acid alignment showing the amino acid substitutions are indicated using numbers above the sequences; the labeled gray rectangles refer to coding sequences of the antigenic determinants of the gp51 protein. The black bars at the left side of the figure indicate Egyptian BLV isolates that belong to each particular strain. Genotypes (G1 and G4) are indicated using black braces at the far-left side of the figure. Dots indicate identity with FLK-BLV subclone pBLV913 (accession No. EF600696), which was used as a reference in a reference in this work.

QB3, and QB5 farms located very close to each other at El-Brahma in Qena province, while the fourth isolate was detected in the cattle from QC6 farm located in the provincial capital town. One isolate of strains G1-c and G1-d each were detected in the cattle from QB2 and those from QB4 farm, respectively, both located at El-Brahma. Among the 38 isolates classified as G4-x strain, 35 were detected in the cattle from DZ1 farm located at El-Zarqa in Damietta province and one each were detected in cattle from QB1, QB3, and QB5 farms. Among the three isolates classified as G4-y strain, two were detected in the cattle from DZ1 farm and the third was detected in those from QB5 farm.

Regarding the distribution of the six genetically distinct strains present in Egyptian cattle, our results concluded that genotype-1 and-4 strains coexisted in most BLV-infected farms.

Estimation of BLV PVL Among Egyptian Cattle

PVL is an important risk factor of BLV-associated disease progression and transmission risk (7–12). Therefore, BLV PVL was calculated by CoCoMo-qPCR2 assay and its level was summarized with BLV infection rate in each tested province of Egypt, as shown in **Figure 5**. In Qena province, 18 out of the 74 tested samples were BLV-positive, their PVL ranging from 7 to 125 copies/10^5 cells with a mean value of 41 copies/10^5 cells. In DZ1 farm from Damietta province, all 40 tested samples were BLV-positive and the PVL ranged from 45 to 98,725 copies/10^5 cells with a mean value of 22,522 copies/10^5 cells. Our previous reports (11, 12) suggest that a PVL of around 10,000 copies/10^5 cells in cow blood might be an indicator of efficient BLV spreading within the whole body, thereby easily detecting BLV proviruses into milk, nasal mucus, and saliva. Interestingly, 17 cattle from DZ1 farm (42.5%) had high PVL ($>10^4$ copies/10^5 cells) ranging from 16,985 to 98,725 copies/10^5 cells, indicating that they may be high-risk transmitters. Indeed, all tested samples from DZ1 farm were BLV-positive with infection rate of 100% (**Table 2**). In addition, 15 of these 17 cattle were infected with G4-x strain and 2 with G4-y strain, as shown in **Table 2**. In contrast, all 18 BLV-positive cattle from 5 different farms in Qena province had low PVL ($\leq 10^4$ copies/10^5 cells).

DISCUSSION

This study provides a new molecular evidence for BLV infection in Egyptian cattle. First, we successfully detected BLV provirus, which integrates into the host genome, in the blood of BLV-infected cattle with a prevalence rate of 21.5%, since the previous studies focused only on the serological detection of BLV in Egyptian cattle. BLV infection was detected in cattle from dairy farms in two of the five tested provinces, suggesting that BLV infection might exist in only particular regions. However, it is possible that the absence of BLV infection in the other three regions resulted from the small number of samples investigated. Therefore, further studies are required to accurately determine the prevalence of infection through a nation-wide survey. Second, the phylogenetical analysis and alignment of the partial BLV *env*-gp51 sequences clearly showed at least six genetically distinct strains, which belong to genotype-1 and-4 among the 11 globally detected genotypes, are present in Egyptian cattle. This is the new report of BLV genetic diversity in Egypt through molecular characterization. Third, we concluded that BLV PVL level is associated with viral horizontal transmission under field conditions in Egypt. Interestingly, in DZ1 farm, where all collected samples were BLV-positive, 35 among the 40 cattle were infected with G4-x strain and the PVL in 15 among these 35 BLV-infected cattle was >10,000 copies/10^5 cells, indicating they may be high-risk transmitters. We demonstrated that BLV-infected cattle with high PVL are a source of infection in BLV-free cattle in Egyptian dairies, which supports the results of previous studies (7–12, 37). In addition, this result supported the previous report that major strain was the source spread at the farm, which existed several BLV strains (38).

Egypt is located between the three continents of Africa, Asia, and Europe. Most of the neighboring countries of Egypt are BLV-infected, including Greece (3), Turkey (39), Jordan (40), Syria (41), and Saudi Arabia (42), however there is no information for the disease in several neighboring countries including Libya and Sudan (43). The total percentage of BLV prevalence determined in our study was 21.5%. This result is in agreement with the results of the recent seroprevalence study reporting BLV infection rate to be 20.8% among Egyptian cattle (28) and similar to the previously reported BLV infection prevalence rate of 15.83% (27). Likewise, BLV infection rate reported in some of the neighboring countries include 20.2% in Saudi Arabia (42) 7.75% in Iraq (44), despite being high (48.3% in herd level) in Turkey (39). In contrast, the BLV infection rate among cattle of Egypt reported in this study was close to or lower than that in India (27.9%) (45). However, the BLV infection rate reported here compared with that reported in some other Asian countries was being higher than that reported in Philippines (4.8–9.7%) (29) and markedly lower that in Japan (40.9%) (46), Korea (42.16%) (47), and Myanmar (37.04) (36). The Egyptian livestock import has increased over the last 10 years and the Egyptian dairy industry is completely dependent on imported cattle for herd construction. Germany, Netherlands, and the United States of America represent the major cattle

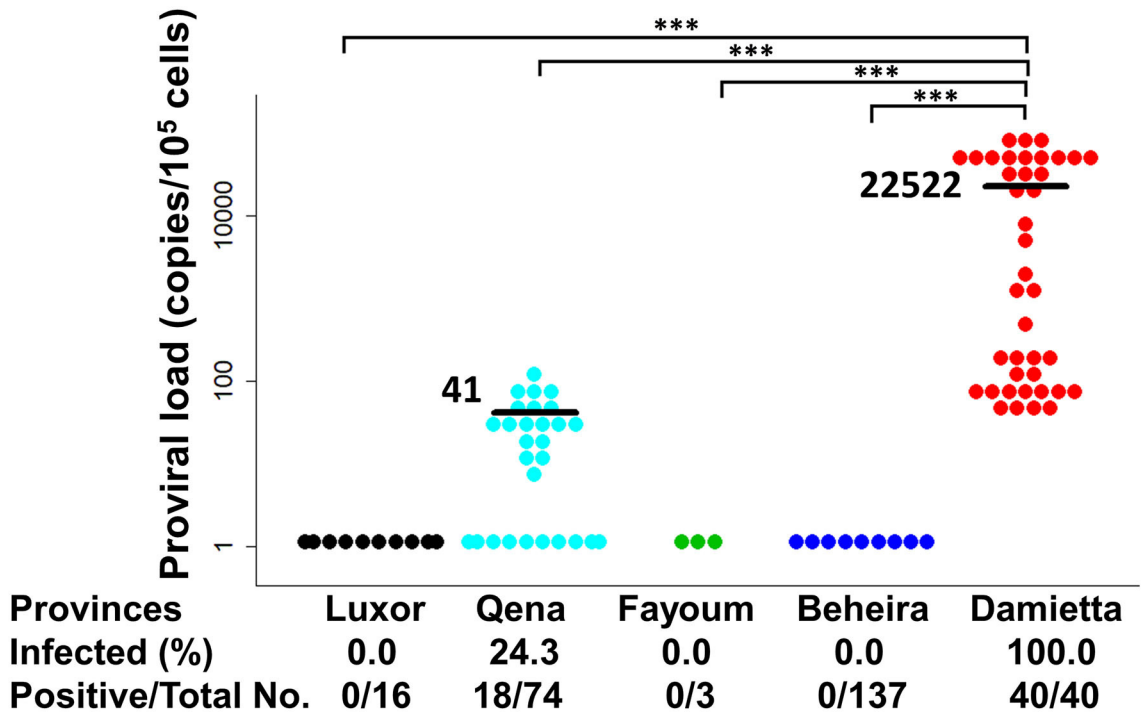

FIGURE 5 | Estimation of BLV proviral load (PVL) among Egyptian cattle. BLV PVL calculated using CoCoMo-qPCR-2 among cattle samples were tested in five different provinces in Egypt. Black bars show the main BLV PVL values. The actual number (No.) of cattle reported positive via BLV-CoCoMo-qPCR-2 per total No. of tested cattle and BLV-infected rate (%) are indicted at the bottom of each block. Significant differences between different geographic regions were calculated using Tukey's test. $P<0.05$ was considered significant. ***$P < 0.00$.

suppliers in Egypt (23). Indeed, phylogenetic analysis of the total 50 partial *env*-gp51 sequences detected here clearly showed that Egyptian BLV isolates were clustered into genotype-1 and-4, which have been detected worldwide. Genotype-1 is the most dominant genotype of BLV, distributed across almost all continents, including Europe, America, Asia, and Australia. Genotype-4 is the second most widely distributed genotype, primarily detected in Europe and some American countries (3). Noticeably, genotype-1 and-4 cover large geographic areas from Europe to America, suggesting the possibility of extensive trading between countries (47). Therefore, it is recommended to determine the risk factors for BLV infection that might have been introduced to Egypt from the imported cattle breeds or be specific for the management system employed for cattle in the field.

The phylogenetic analysis of the 420-bp sequences of *env*-gp51 region in this study showed that Egyptian BLV isolates were assigned to genotype-1 and-4 and alignment of the 501-bp sequences of *env*-gp51 region resulted in the identification of 18 nucleotide substitutions, of which 14 were silent and four were amino acid substitutions. The substitutions varied according to genotype. For example, phylogenetic tree based on the 501-bp sequence of *env*-gp51 region demonstrated four different strains (G1-a, G1-b, G1-c, and G1-d) with genotype-1 and two different strains (G4-x and G4-y) with genotype-4. Similarly, Marawan et al. (37) performed phylogenetic analysis and demonstrated the existence of 20 different BLV subgenotypes in Miyazaki prefecture of Japan based on their nucleotide sequences. Interestingly, the substitutions in genotype-1 strains were highly conserved and only one unique non-synonymous substitution was analyzed in the G1-d strain sequence, which is located at the residue 99 and changed an amino acid in the neutralizing domain (ND1) and the CD4+ epitope region of gp51 protein from glycine to lysine. Moreover, in the genotype-4 strains, G4-x strain exhibited two amino acid substitutions in residues 121 and 233, which were located in G and B epitopes, respectively, and G4-y strain exhibited three substitutions, two of them were identical to the aforementioned substitutions in G4-x strain and the third was an unique amino acid substitution in residue 239, which is located in the region between B and D epitopes of gp51 protein.

In this study, we noticed that, in a single farm DZ1, all the 40 samples collected were BLV-positive, with 35 of these BLV isolates being identical and classified as the same BLV strain (G4-x) with genotype-4. Two isolates were classified as G4-y strain having a 99.8% similarity with G4-x strain, but the remaining three isolates were classified as G1-a strain with genotype-1. Interestingly, as calculated by CoCoMo-qPCR2, BLV PVL of these BLV-infected cattle ranged from 45 to 98,725 copies/10^5 cells with a mean value of 22,522 copies/10^5 cells. Regarding horizontal BLV transmission, we previously detected BLV provirus in the nasal secretions and saliva of cattle with PVL >14,000 and >18,000 copies/10^5 cells in blood, respectively,

and suspected that BLV-infected cells were present in nasal secretions and saliva, respectively (11). Furthermore, the BLV provirus was detected in milk samples from dams when the PVL in blood samples were approximately >10,000 copies/10^5 cells (12). It might infect healthy cattle via licking, sneezing, rubbing of noses, or milking. In this study, the PVL in 15 among the 35 infected cattle harboring G4-x strain in farm DZ1 appeared to be >10,000 copies/10^5 cells, indicating that they may be high-risk transmitters. Therefore, we hypothesized that the infected cattle with high PVL act as virus spreader and transmit genetically identical provirus to other cattle during long-time physical contact. Thus, the G4-x strain is widely spread in cattle from DZ1 farm. This result harmonized with those of Murakami et al. (38), who showed that three genetically distinct BLV strains co-existed among the infected cattle in one farm and described that the major viral strains were the source of infection spread in that farm. In contrast, BLV-infected cattle with low PVL are not a source of infection for BLV-free cattle (14). Therefore, it is recommended to isolate and monitor the BLV-infected cattle with high PVL to prohibit virus spread horizontally and vertically.

Here we detected BLV infectivity in two provinces: Qena and Damietta. We examined cattle from six dairy farms in Qena province and found cattle from all of them to be BLV-infected. Five of these farms (QB1, QB2, QB3, QB4, and QB5) are small-sized, but farm QC6 is large. The infection rate was higher in the small-sized farms than that in the large farm (**Table 2**). These findings concurred with those of other studies demonstrating that average BLV seroprevalence in small farms (less than 20 cattle) was higher than that in medium or large farms in Miyazaki prefecture, Japan (48). Several studies have described the risk factors for BLV transmission within herd and considered that physical contact (49), loose housing system (50), use of common sleeves during rectal palpation (51), blood sucking insects, blood-contaminated devices, and needle reuse (4) are the most dominant factors for virus transmission. Therefore, it is possible that in the small-sized farms, loose cattle housing system, presence of hematophagous insects, and poor managemental procedures may increase the chance for virus transmission from infected to uninfected cattle.

In contrast, in DZ1 farm located in Damietta province, we demonstrated the highest BLV infection rate (100%). It is a large dairy farm (∼500 cattle) and depends on natural breeding for reproduction. Interestingly, the milking system of this farm depends on using a limited number of common portable milking machines, thus lacking sufficient cleaning during milking between individual cows. Therefore, the likelihood of bovine milk pathogen transmission is higher in herds using mobile milking machines than that in herds using fixed milking machines, as previously demonstrated (52). In addition, BLV has a broad tropism to the mammary tissue (53) and the milk or milk cells of infected cattle could be a source of infection (12, 54, 55). However, the role of milk in virus transmission is considered to be little (56). Thus, we hypothesized that there is a possibility for BLV transfer from infected to healthy receptive cattle during the milking process.

To gain insight into the fundamental nature of existence of each genetically distinct virus strain, we checked the virus strain spreading the infection between animals in each particular farm. In the five small-sized BLV-infected farms (QB1, QB2, QB3, QB4, and QB5) harboring Native and Mixed breed cattle located in El-Brahma, Qena, the BLV infection rate was ranged from 25 to 60%. These farms were neighbors and located very close to each other. Shettigara et al. (57) have proposed that at least 200 m distance should be maintained between farms to prevent transmission among herds. It is interesting that the cattle from three of these farms (QB1, QB3, and QB5) harbored genetically identical strains (G1-b and G4-x), as shown in **Table 2**. This result might be a good example for horizontal BLV transmission from one farm to its adjoining farm in the field. On the contrary, we identified two genetically different strains G1-c and G1-d in farms QB2 and QB4, respectively. The sixth farm (QC6) located in the capital town of Qena province was a large-sized dairy farm (∼300 cattle) harboring Holstein breed cattle. Although the BLV infection rate in this farm was 12.8% (5/39) as determined by CoCoMo-qPCR, we successfully identified a single BLV isolate classified as G1-b strain. This might be due to the difference in sensitivity between CoCoMo-qPCR assay used for virus detection and nested PCR technique used for gp51 amplification (29).

In conclusion, the phylogenetic analysis based on partial BLV *env*-gp51 sequence revealed that there are at least six genetically distinct BLV strains present in Egyptian cattle, which belong to genotype-1 and-4 among the 11 globally detected genotypes. This study provides a new molecular characterization of BLV in Egyptian dairy cattle and calculated the PVL among BLV-infected cattle in Egypt. This study also showed that the BLV infection prevalence was 21.5% (58 out of 270 head) in five provinces located in the northern, central, and southern parts of Egypt. Further research should focus on determining the impact of BLV genetic diversity on viral pathogenicity and disease progression in dairy cattle on a large scale.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

All animals were handled by the regulation of the Animal Ethics Committee at the Faculty of Veterinary Medicine, South Valley University, Qena, Egypt, and by the regulation of RIKEN, Japan in strict accordance with good animal practice following the guidelines of RIKEN. The study was reviewed and approved by Research Code of Ethics (RCOE-SVU) at the South Valley University, and by the RIKEN Animal Experiments Committee (approval number H29-2-104).

AUTHOR CONTRIBUTIONS

YA conceived of and designed the study. SM, AA, AA-H, and AM collected the samples. RH, SM, MP, LB, SW, and YA acquired, analyzed, and interpreted the data. YA contributed reagents,

materials, and analysis tools. RH and YA drafted and revised the manuscript. All authors agree to be accountable for the content of the work.

FUNDING

This work was supported by a Grant-in-Aid for Scientific Research (A) from the Japan Society for the Promotion of Science (JSPS) (Grant no. 16H02590) and by a grant from Embassy of Egypt Culture, Education and Science Bureau.

ACKNOWLEDGMENTS

We thank the veterinarians and our collaborators for kindly assisting with sampling from many farms in Egypt. We are grateful to members of the Viral Infectious Diseases Field, RIKEN, for their technical assistance, help, and suggestions, and Support Unit at the Bio-material Analysis, RIKEN BSI Research Resources Center, for help with sequence analysis. We would like to thank Editage (http://www.editage.com) for English language editing.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 11 September 2020
doi: 10.3389/fvets.2020.00636

Association Between Infectious Agents and Lesions in Post-Weaned Piglets and Fattening Heavy Pigs With Porcine Respiratory Disease Complex (PRDC)

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OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
Christelle Fablet, Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail (ANSES), France
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *07 April 2020*
Accepted: *04 August 2020*
Published: *11 September 2020*

Citation:
Ruggeri J, Salogni C, Giovannini S, Vitale N, Boniotti MB, Corradi A, Pozzi P, Pasquali P and Alborali GL (2020) Association Between Infectious Agents and Lesions in Post-Weaned Piglets and Fattening Heavy Pigs With Porcine Respiratory Disease Complex (PRDC). Front. Vet. Sci. 7:636. doi: 10.3389/fvets.2020.00636

Porcine Respiratory Disease Complex (PRDC) is a multifactorial syndrome that causes health problems in growing pigs and economic losses to farmers. The etiological factors involved can be bacteria, viruses, or mycoplasmas. However, environmental stressors associated with farm management can influence the status of the animal's health. The role and impact of different microorganisms in the development of the disease can be complex, and these are not fully understood. The severity of lesions are a consequence of synergism and combination of different factors. The aim of this study was to systematically analyse samples, conferred to the Veterinary Diagnostic Laboratory (IZSLER, Brescia), with a standardized diagnostic protocol in case of suspected PRDC. During necropsy, the lungs and carcasses were analyzed to determine the severity and extension of lesions. Gross lung lesions were classified according to a pre-established scheme adapted from literature. Furthermore, pulmonary, pleural, and nasal lesions were scored to determine their severity and extension. Finally, the presence of infectious agents was investigated to identify the microorganisms involved in the cases studied. During the years 2014–2016, 1,658 samples of lungs and carcasses with PRDC from 863 farms were analyzed; among them 931 and 727 samples were from weaned piglets and fattening pigs, respectively. The most frequently observed lesions were characteristic of catarrhal bronchopneumonia, broncho-interstitial pneumonia, pleuropneumonia, and pleuritis. Some pathogens identified were correlated to specific lesions, whereas other pathogens to various lesions. These underline the need for the establishment of control and treatment programmes for individual farms.

Keywords: porcine respiratory disease complex (PRDC), pig, lung lesion, multifactorial disease, diagnostic protocol

INTRODUCTION

Porcine Respiratory Disease Complex (PRDC) is a multifactorial disease that affects growing pigs in different stages of production, causing economic losses. This complex syndrome is influenced by the presence of several types of pathogens (porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus (PCV)-type 2, *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Pasteurella multocida*, *Haemophilus parasuis,* etc.), along with environmental conditions, as temperature, dust, ammonia, carbon dioxide and airborne bacteria and farm management (1–4).

PRDC is a major burden in piggeries worldwide because of the consequent economic losses. In affected farms, considerable costs are associated with high percentages of mortality (2–20%) and morbidity (10–40%), therapy, and limited growth performances (5, 6). Reportedly, infection with *M. hyopneumoniae* causes major economic losses to the pig industry, mainly because of reduced performance, uneven growth, increase in the number of days to reach slaughter weight, treatment and control, and increase in mortality rate when complicated infections occur (7). However, the economic impact of *M. hyopneumoniae* subclinical infection was inferred only once based on the difference in average daily weight gain (ADWG) (38 g/day) between seropositive and seronegative pigs from 18 different cohorts (8). Various studies have demonstrated the economic impact of lung lesions on growth performances. The results were mostly based on the relationship between lung lesions observed at the slaughterhouse and ADWG (9). Some authors have reported a reduction of 6–16% in the growth rate of finishing pigs (10, 11). The main study on respiratory diseases in Italian piggeries started with the observation of lung and pleural lesions (and correspondent scores) while slaughtering. The results demonstrated that diseases affecting the respiratory tract greatly prevail, and they are very likely underestimated in live animals (12, 13). Inspection while slaughtering is a valid tool to estimate the incidence of PRDC in pigs. The most commonly recorded lesion corresponds to catarrhal bronchopneumonia mainly affecting cranial lobes. This is frequently associated with interstitial pneumonia and pleuritis (14). In Italy, the percentage of catarrhal bronchopneumonia associated with enzootic pneumonia is 46.4% and that of pleuritis is 47.5% (13). These lesions indicate the evolution or exacerbation of respiratory diseases affecting pigs during the farrow-finishing period.

On the contrary, the evaluation of lesions in piglets or pigs during PRDC outbreaks is a valid tool to estimate the type of acute lesions, their extension, and the possible involvement of serosa and nasal mucosa. Furthermore, the isolation of etiological pathogens is easier in acute lesions than in chronic ones. As a consequence, treatment or the control of each outbreak should be addressed according to the specific farm situation to limit the generic use of antimicrobials.

The aim of this study was to investigate the association between lesions and infectious agents and to assess the association between nasal, pleural, and lung scores, in order to gain insights about the etiological agents associated with PRDC. The novelty of this study is to assess the etiology and lesions in samples from dead pigs with clinical suspect of respiratory disease.

TABLE 1 | Data on samples analyzed during 2014–2016, presented as number of animals, number of investigated cases, and production stage.

Year	N° of animals	N° of investigated cases	Weaned piglets	Growing/fattening pigs
2014	510	257	295 (57.8%)	215 (42.2%)
2015	622	320	334 (53.7%)	288 (46.3%)
2016	524	286	301 (57.5%)	223 (42.5%)
Total	1,656	863	930 (56.2%)	726 (43.8%)

MATERIALS AND METHODS

Samples

A standardized diagnostic protocol was applied to growing pigs that died because of respiratory diseases, conferred to the Veterinary Diagnostic Laboratory (IZSLER, Brescia), during 2014–2016. **Table 1** shows the total number and the production stage of the sampled pigs. A further distinction per year was made to carry out a temporal assessment.

The protocol used for the qualitative and quantitative evaluation of the lung, pleura, and nasal lesions is associated with a systematic monitoring of pathogens. It was applied to carcasses or organs (lungs) submitted to the Diagnostic Laboratory of Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna with the suspicion of respiratory disease, which was then confirmed during necropsy.

Lung, pleural, and nasal lesion scores were registered during necropsy with other information such as that on the productive stage and laboratory investigations performed.

Classification of Lung Lesions

The scheme for the classification of lesions was adapted from published methods (15).

The lesions of the organs were identified to be associated with the following diseases/conditions: catarrhal bronchopneumonia (CBP), purulent bronchopneumonia (PBP), interstitial pneumonia (IP), interstitial bronchopneumonia (BIP), pleuropneumonia (PP), pleuritis (PL), pericarditis (PE), and pleuro-pericarditis (PL-PE).

CBP is characterized by lesions of parenchyma and bronchi affecting principally the cranial, cardiac, and anterior portions of diaphragmatic lobes. It is characterized by mucus and catarrhal exudate in the lumen of the bronchus tree, by parenchymal consolidation and by interstitial space thickening.

Abbreviations: PRDC, Porcine Respiratory Disease Complex; PRRSV, Porcine Reproductive and Respiratory Syndrome Virus; PCV, type 2: Porcine Circovirus Type 2; ADWG, Average Daily Weight Gain; CBP, Catarrhal Bronchopneumonia; PBP, Purulent Bronchopneumonia; IP, Interstitial Pneumonia; BIP, Interstitial Bronchopneumonia; PP, Pleuropneumonia; PL, Pleuritis; PE, Pericarditis; PL-PE, Pleuro-pericarditis; SD, standard deviation; SPES, Slaughterhouse Pleuritis Evaluation System; SIV, Swine Influenza Virus; PCR, Polymerase Chain Reaction; CI, Confidence Interval; OR, Odds Ratio.

The appearance of the lungs varies from red to light brown and changes to a grayish color during chronic infection.

PBP is characterized by lesions of lung parenchyma and bronchi, which are characterized by mucus and catarrhal-purulent exudate in the lumen of the bronchus tree. Generally, it is a totally disseminated complication of CPB or BIP. Hence, cellular detritus and stagnated exudates favor the replication of pyogenic bacteria. These lesions develop abscess formations detectable with palpation.

IP is characterized by serosal exudation into alveolar walls and by interstitial oedema. Parenchyma suffers from incremented consistency, and the color changes from light red to purple red during the acute phase and to light pink during the chronic phase. These lesions are commonly associated with viral infections and involve the entire affected lung. Unfortunately, this lesion can be masked (by other lesions) or be complicated, and it can evolve into broncho-interstitial pneumonia. BIP is a complication of IP because it also involves the bronchus tree. In particular, serosal exudation is found in the lumen of bronchi and bronchioles.

PP is a fibrinous/necrotising pneumonia associated with pleuritis and affects the dorso-caudal portions of the diaphragmatic lobes. A hyperacute lesion is characterized by an increased consistency and by a color variation of the parenchyma that ranges from brown to red. An acute lesion is characterized by an increased consistency of the affected portions of parenchyma and by an alternation of the red area with a lighter pink area, which is a result of fibrin deposition. The chronic evolution is characterized by abscess formations and sequestrum, complicated by fibrous adherences between lung lesions and chest wall.

PL, PE, and PL-PE are characterized by inflammation of the pleura and pericardium (or both) and by fibrinous exudation that can evolve in adherences.

Lung, Pleural, and Nasal Scores

Here, irrespectively. to the character of the lung lesion (as above assessed), we scored (0–4 points) the extension of the lesion in each lobe, according to Madec and Derrien (16) and Madec and Kobish (17). SPES (Slaughterhouse Pleuritis Evaluation System) was applied to score (0–4 points) pleural lesions (18). Nasal lesions were scored on a 6-point scale (0–5 points) according to the system described by de Jong (19).

Laboratory Investigations

The lung, heart, pericardium, lymph nodes, pericardial and pleural fluids, and tracheobronchial swabs were processed to conduct, histology, microbiological examinations, and molecular identification.

For bacteriological examination, we inoculated the processed samples into blood agar and Gassner agar (Reparto Produzione Terreni – IZSLER, Brescia) and incubated the culture plates for 24–48 h at 37°C and 5% CO_2 to identify *Actinobacillus pleuropneumoniae, P. multocida, Streptococcus* spp., and *Actinomyces pyogenes*, the bacterial cultures were subjected to Gram's staining and confirmation with biochemical identification and serotyping. Furthermore, to isolate NAD-dependent pathogens (*A. pleuropneumoniae* and *H. parasuis*), blood agar cultures were cross-streaked with a *Staphylococcus intermedius* to evaluate colony-satellitism. Furthermore, DNA extraction was applied to pure cultures of *A. pleuropneumoniae* in order to serotype each isolated strain by an end-point PCR, as described below.

Viral isolation of SIV (Swine Influenza Virus) was performed. A lung fragment was homogenized with Minimum Essential Medium Eagle (Sigma-Aldrich) containing Streptomycin, Penicillin G, and Sulfate Streptomycin. The solution was centrifuged at 1,500 rpm for 5 min. An aliquot of supernatant was used to infect cells (MDBK or Caco-2).

Molecular identification of PRRSV, PCV-type 2, *M. hyopneumoniae*, *M. hyorhinis*, *H. parasuis,* and *A. pleuropneumoniae* was performed.

Several commercial kits were used for Nucleic Acid Extraction: NucleoMag Vet 200 (Macherey-Nagel) for PRRSV and PCV-type 2; Rneasy mini kit (Qiagen) for SIV; Dneasy Blood & Tissue kit (Qiagen) for *M. hyopneumoniae*, *M. hyorhinis,* and *H. parasuis*. Finally, DNA boiling extraction was applied to a pure cultures of *A. pleuropneumoniae* (98°C for 10 min with 1,050 rpm oscillation).

An end-point PCR was performed to confirm the presence of *H. parasuis* according to the protocol described by Oliveira et al. (20), and *A. pleuropneumoniae* serotyping was performed according to the protocol described by Xie et al. (21).

A Real Time RT-PCR for PRRSV detection from blood and lung tissue homogenate was performed using the[1] following the manufacturer's instructions.

A Real Time PCR for PCV-type2 detection from lung tissue homogenate and inguinal lymph node homogenate was performed in accordance with the protocol described by Olvera et al. (22).

A Real Time RT-PCR for SIV detection was performed from lung tissue homogenate in accordance with the protocol described by Spackman et al. (23).

A Real Time PCR for *M. hyopneumoniae* detection was performed from lung tissue homogenate in accordance with the protocol described by Marois et al. (24).

A Real Time PCR for *M. hyorhinis* detection was performed from lung tissue homogenate in accordance with the protocol described by Tocqueville et al. (25).

Finally, a Real Time PCR for *A. pleuropneumoniae* detection from trachea-bronchial swabs and lung tissue homogenate was performed in accordance with the protocol described by Tobias et al. (26).

Statistical Analysis

The proportion of each type of lesion was calculated, and the binomial exact method was used to compute 95% confidence intervals (95% CI). The association of the production stage and pathological lesions with pathogens was assessed by Fisher's exact test (FET). For *A. pyogenes, A. pleuropneumoniae, Streptococcus spp*., and PCV-type 2, multivariate logistic regression models were employed, with pathogens as dependent variables and production stage and pathological lesions as covariate. To

[1]LSI VetMAX (TM) PRRSV EU/NA Real-Time PCR kit (Thermo Fisher).

identify a possible association among different scores and to a verify score distribution, Spearman's correlation coefficient (r) was calculated between different scores (lung vs. pleural scores, lung vs. nasal scores, and pleural vs. nasal scores). Furthermore, a Chi-square test (3 × 2) was performed to compare the scores according to the different classes. The classes identified were: slight (0–9), moderate (10–19), and severe (20–28) for the lung score; slight (0–2) and severe (3, 4) for the pleural score; and slight (0–2) and severe (3–5) for the nasal score. The Chi-square test (2 × 2) was also performed to compare pleural and nasal scores. For each test a $p < 0.05$ was considered statistically significant. All analyses were performed using R software (R version 3.3.1, R Core Team, R Foundation for Statistical Computing. R: A language and environment for statistical computing, http://www.R-project.org/; 2016 [accessed 31/05/2017]).

RESULTS

The proportions of weaned piglets and growing/fattening pigs with PRDC were 57.8 and 42.2%, respectively, in 2014; 53.7 and 46.3%, respectively, in 2015; and 57.5 and 42.5%, respectively, in 2016 (**Table 1**). Four hundred sixty-one farms conferred pigs 863 times for diagnostic investigations, with a cumulative number of 1,656 pigs. The mean number of pigs conferred for each farm was 3.6 (SD 4.4) and the mean of times each farm conferred pigs was 1.9 (SD 1.9).

Pleuritis Is the Most Frequently Observed Lesion in Both Groups of Animals Affected by PRDC

The frequencies of lesion distribution in the lungs, pleura, and pericardium are depicted in **Figure 1** (number of pigs with lesions). PL was recorded in the highest number of samples (469; 28.3%), followed by pleuropneumonia (PP) (286; 17.2%), catarrhal bronchopneumonia (CBP) (273; 16.5%), and BIP (265; 16.0%).

The frequencies of lesion distributions in the organs of weaned piglets and growing/fattening pigs are depicted in **Table 2**. PL was the most frequent lesion observed in both the groups (253 and 216 cases, respectively). Other frequent lesions corresponded to CBP (184), BIP (122), PP (125), PE (119), and PL-PE (77) in weaned piglets. On the contrary, PP and BIP were frequently observed in fattening pigs (161 and 143, respectively). The association between the production stage and pathological lesions was statistically significant for IP, PP, PBP, PL, and PE. The post-weaning group showed a lower probability of developing pathological lesions associated with PP, PBP, and PE and a higher probability to developing pathological lesions associated with IP and PL than did the growing group.

Detection of Respiratory Pathogens

The proportions of respiratory pathogens in the samples collected from weaned piglets or fattening pigs are depicted in **Table 3**. The association between the production stage and the isolated pathogen was statistically significant for *Streptococcus suis, P. multocida, A. pleuropneumoniae*, PRRSV, PCV-type 2, *M. hyopneumoniae, M. hyorhinis,* and *H. parasuis*. The post-weaning group was more likely to show *S. suis*, PRRSV, *M. hyorhinis,* and *H. parasuis* than was the growing group. *M. hyorhinis* was the most commonly detected pathogen in the lungs of weaned piglets with lesions (98.7%). In the lungs of weaned piglets with and without lesions, PRRSV (75 and 59.6%), *H. parasuis* (61.3 and 38.2%), and *Streptococcus* spp. (45.3 and 34.8%) were the most commonly detected pathogens.

Differently, the growing group was more likely to show *P. multocida, A. pleuropneumoniae*, PCV-type 2, and *M. hyopneumoniae*.

M. hyopneumoniae (68.5%) and PCV-type 2 (43.7%) were the most commonly detected pathogens in the organs of fattening pigs with lesions, whereas, in the organs of fattening pigs with and without lesions, PRRSV (55.1 and 41.3%), *Streptococcus* spp. (41 and 18.7%), and *P. multocida* (26.3 and 14.7%) were the most frequently detected pathogens.

Distribution Between a Single Lesion and Respiratory Pathogen Detection in Weaned Piglets and Fattening Pigs

The distribution between pathogen detection and each single lesion in samples collected from weaned piglets and fattening pigs are depicted in **Figure 2**.

P. multocida was detected in the organs of weaned piglets affected by the lesions associated with the following diseases/conditions: PBP (28.6%); PL (26%); CBP (25.9%); and PE (25%). This pathogen was detected in different proportions in the organs of fattening pigs with the lesions associated with the following diseases/conditions: CBP (37.8%); PL (29.9%); BIP (28%); PBP (28%); and IP (23.3%). *A pleuropneumoniae* was detected in the organs of weaned piglets affected by the lesions associated with the following diseases/conditions: PP (64.4%); PL (16.7%); and PBP (14.3%). This pathogen was detected in different proportions in the organs of fattening pigs with the lesions associated with the following diseases/conditions: PP (81%); PL (38.10%); PBP (28%); BIP (24%); and PE (20.8%).

H. parasuis was detected in the organs of weaned piglets affected by the lesions associated with the following diseases/conditions: BIP (76.9%); PP (73.3%); PE (70.4%); and PL (60.7%). This pathogen was detected in different proportions in the organs of fattening pigs with the lesions associated with the following diseases/conditions: PBP (66.3%); CBP (58.3%); BIP (52.8%); IP (50%); PL (50%); PE (50%); and PP (25%).

Streptococcus spp. was detected in the organs of weaned piglets affected by the lesions associated with the following diseases/conditions: IP (83%); BIP (59%); PL (61%); and PE (50%). The pathogen was also detected in high proportions in the organs of fattening pigs with the lesions associated with the following diseases/conditions: IP (63%); CBP (60%); PBP (52%); BIP (51%); PE (63%); and PL (57%).

A. pyogenes was detected in the organs of both groups affected by purulent bronchopneumonia (21% in weaned piglets and 20% in fattening pigs). The pathogen was also detected in the organs with the lesions associated with the following

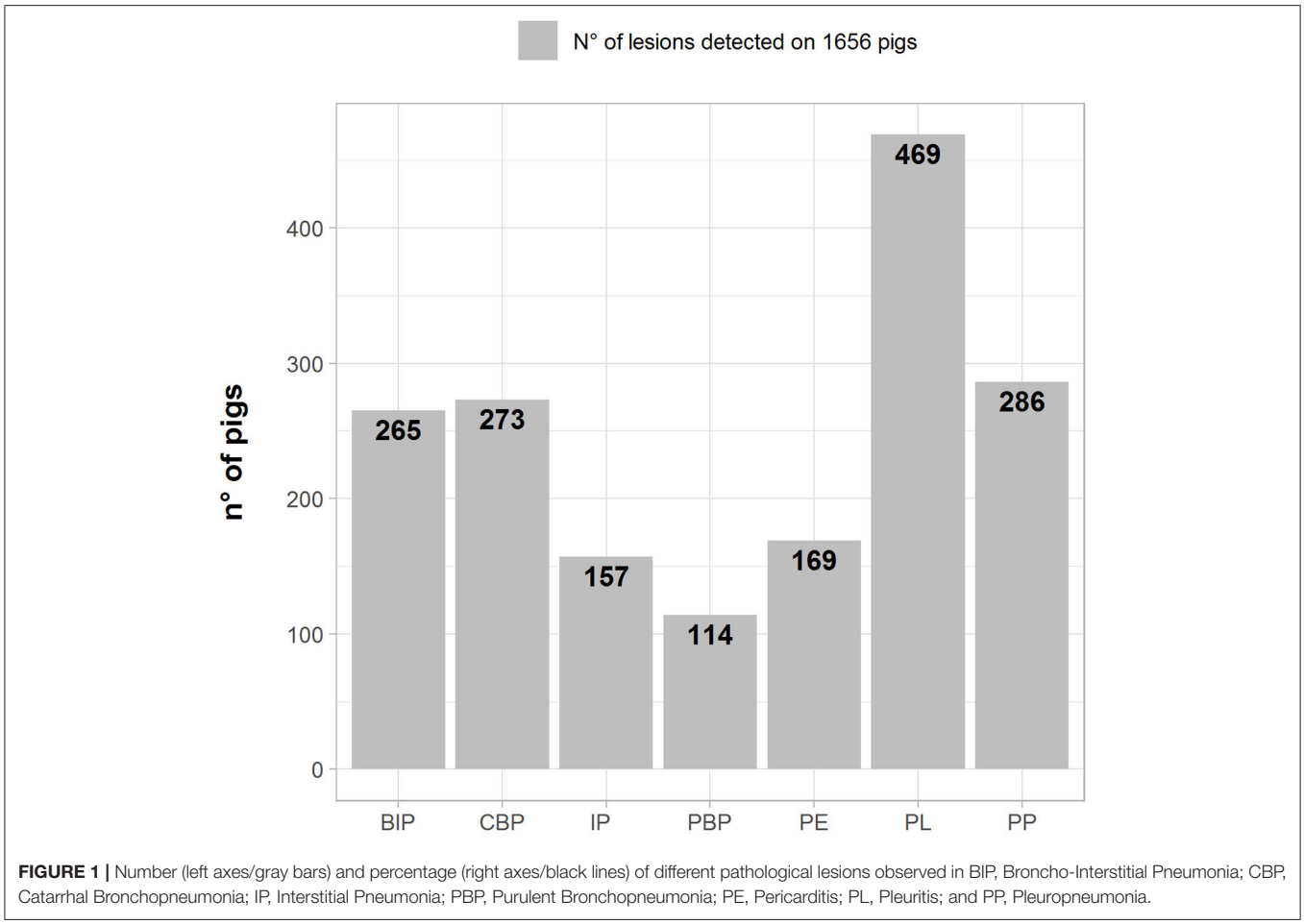

FIGURE 1 | Number (left axes/gray bars) and percentage (right axes/black lines) of different pathological lesions observed in BIP, Broncho-Interstitial Pneumonia; CBP, Catarrhal Bronchopneumonia; IP, Interstitial Pneumonia; PBP, Purulent Bronchopneumonia; PE, Pericarditis; PL, Pleuritis; and PP, Pleuropneumonia.

TABLE 2 | Number, percentage (%), and 95% CI of pathological lesions detected in pigs according to production stage.

Pathological	*N°*	Growing/fattening			Post-weaning			*p*-value	OR	OR CI 95%
Lesions		*N°*	%	CI95%	*N°*	%	CI 95%			
CBP	273	89	32.6	27–38.1	184	67	61.8–72.9	0.0000	1.76	1.33–2.35
IP	157	83	52.8	45–60.6	74	47	39.3–54.9	0.0179	0.67	0.47–0.94
BIP	265	143	53.9	47.9–59.9	122	46	40–52	0.0003	0.62	0.47–0.81
PP	286	161	56.2	50.5–62	125	44	37.9–49.4	0.0000	0.55	0.42–0.71
PBP	114	59	51.7	42.5–60.9	55	48	39–57.4	0.0792		
PL	469	216	46	41.5–50.5	253	54	49.4–58.4	0.2717		
PE	169	50	29.5	22.7–36.4	119	70	63.5–77.2	0.0001	1.98	1.39–2.86

The association between the pathological lesions and the production stage was calculated by Fisher's exact test; when the association was statistically significant ($p < 0.05$), the odds ratio (OR) was calculated using the growing group as the source of baseline data.

diseases/conditions: CBP (14% in weaned piglets and 18% in fattening pigs); BIP (12% in weaned piglets and 11% in fattening pigs); and PL (12% in weaned piglets and 10% in fattening pigs).

M hyorhinis was detected in high proportions in the organs of weaned piglets (percentage range 70–90%) and in low proportions in the lungs affected by BIP (53.8%). The pathogen was detected in low proportions in the organs of fattening pigs showing lesions associated with the following diseases/conditions: CBP (66.7%); PBP (60%); PL-PE (85.7%); and PE (38.9%).

M. hyopneumoniae was scarcely detected in the lung and serosal lesions of weaned piglets (38.9% in PBP and 30.9% in CBP). On the contrary, it was detected in high proportions in the lung and serosal lesions of fattening pigs (percentage range 65–90%) with the exception of those associated with IP (44.4%) and pleuropneumonia.

TABLE 3 | Pathogens detected, proportion and 95% CI according to production stage.

Respiratory pathogen	N° isolated	Growing/fattening			Post-weaning			*p*-value	OR	OR CI 95%
		N°	%	CI 95%	*N°*	%	CI 95%			
Streptococcus spp	372	160	43	38.0–48.0	212	57	52.0–62.0	0.722		
Streptococcus suis	17	3	18	0.5–35.8	14	82	64.2–100	0.046	3.68	1.02–20.05
A. pyogenes	72	31	43	31.6–54.5	41	57	45.5–68.4	0.904		
P. multocida	213	109	51	44.5–57.9	104	49	42.1–55.5	0.021	0.71	0.53–0.96
A. pleuropneumoniae	264	167	63	57.4–69.1	97	37	30.9–42.6	0.001	0.39	0.29–0.52
PRRS	946	344	36	33.3–39.4	602	64	60.6–66.7	0.001	2.24	1.79–2.80
PCV-type2	455	251	55	50.6–59.7	204	45	40.3–49.4	0.001	0.47	0.37–0.60
SIV	192	94	49	41.9-56.0	98	51	44.0-58.1	0.16		
M. hyopneumoniae	141	99	70	62.7–77.8	42	30	22.2–37.3	0.001	0.15	0.09–0.25
M. hyorhinis	217	43	20	14.5–25.1	174	80	74.9-85.5	0.001	23.85	6.54–131.94
H. parasuis	185	58	31	24.7–38.0	127	69	62.0–75.3	0.016	1.75	1.09–2.82

The association between the pathogens isolated and the production stage was calculated using Fisher's exact test; when the association was statistically significant ($p < 0.05$), OR was calculated using the growing group as the source of baseline data.

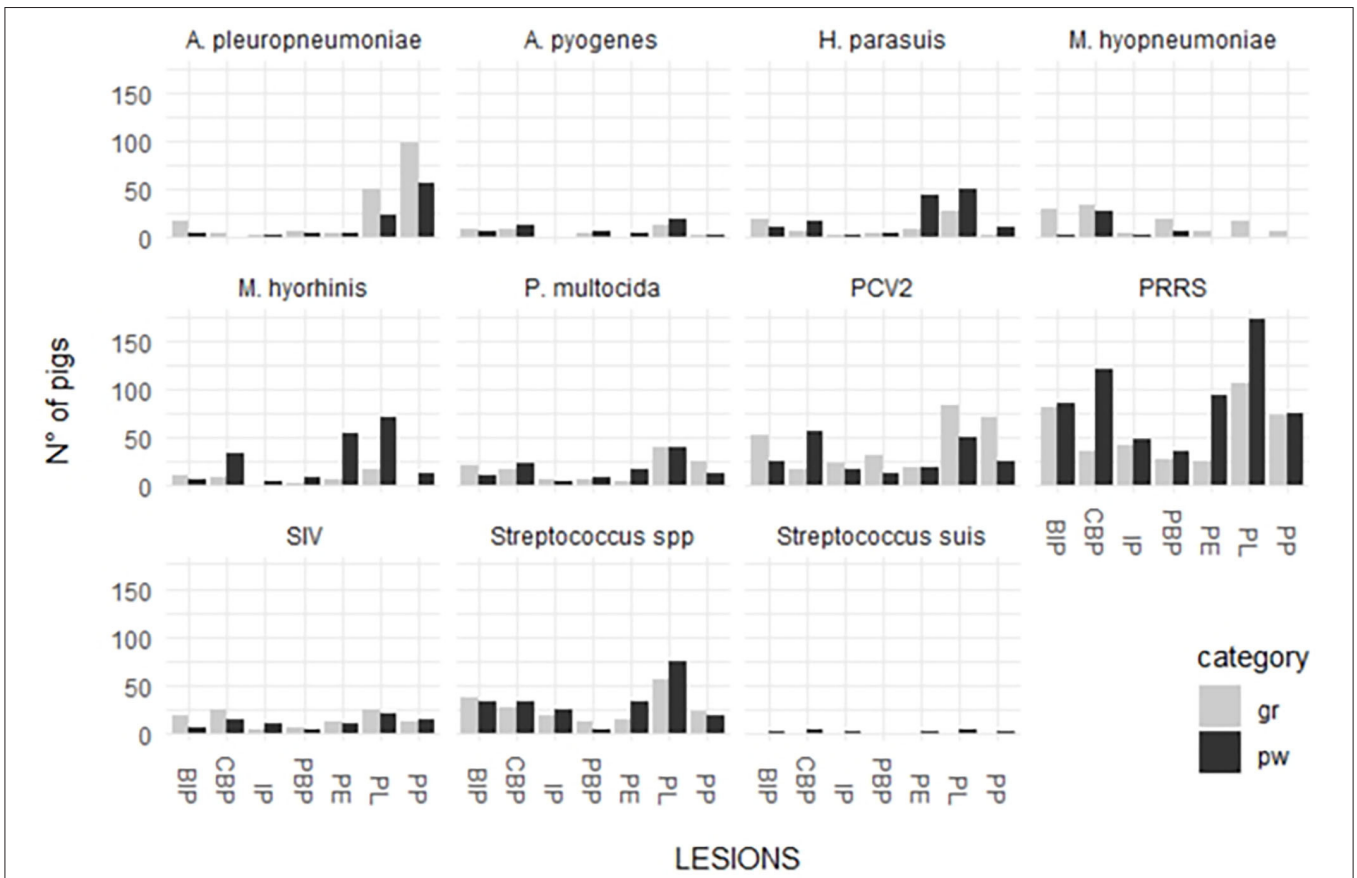

FIGURE 2 | Distribution of detected respiratory pathogens in lesions recorded in weaned piglets and fattening pigs.

PRRSV was highly associated with lung and serosal lesions of both the groups. The detection percentages of different lesions were higher in weaned piglets than in fattening pigs. The rate of detection in weaned piglets varied from 65.8% (PP) to 84.7% (PE) and that in fattening pigs varied from 45.6% (CBP) to 66.7% (PL). The highest percentages of detection were associated with PL (66.7%) and BIP (62.5%).

SIV was associated with PL in weaned piglets (30.8%) and CBP (30.4%), PE (26.1%), and PL (33.3%) in fattening pigs.

TABLE 4 | Number of pathogens isolated from pathological lesions detected in weaned piglets and fattening pigs.

Pathogens	Isolated	CBP	IP	BIP	PP	PBP	PL	PE
Streptococcus spp.	372	58	44	72	**41**^{*I}	18	**129**^{*I}	**49**^{*I}
Streptococcus suis	17	4	1	1	1	0	4	3
A. pyogenes	72	**20**^{*I}	**1**^{*D}	15	**5**^{*D}	**11**^{*I}	**32**^{*I}	4
P. multocida	213	39	**11**^{*D}	31	39	15	**79**^{*I}	21
A. pleuropneumoniae	264	**5**^{*D}	**4**^{*D}	**23**^{*D}	**153**^{*I}	11	74	**10**^{*D}
PRRSV	946	157	89	165	147	62	279	**118**^{*I}
PCV-type2	455	73	38	78	**94**^{*I}	**45**^{*I}	132	**38**^{*D}
SIV	192	39	14	24	26	12	46	23
M. hyopneumoniae	141	62	6	32	7	**26**^{*I}	18	6
M. hyorhinis	217	41	4	**17**^{*D}	13	11	86	60
H. parasuis	185	23	4	29	13	8	79	52

The association between the detected pathogen (yes/no) and pathological lesions (yes/no) was calculated by Fisher exact test.

** Statistically significant association. I = presence of pathogens increased the probability of detecting a lesion. D = the presence of pathogens decreased the probability of detecting a lesion.*

PCV-type 2 was more associated with lung and serosal lesions in fattening pigs than with those in weaned piglets. The rate of detection in weaned piglets varied from 17.6% (PE) to 37.3% (CBP) and that in fattening pigs varied from 25.4% (CBP) to 65.3% (PBP).

As reported in **Table 4**, *A. pyogenes* was associated with the following: CBP (FET: $p < 0.05$; OR, 2.02; 95% CI, 1.12–3.52), IP (FET: $p < 0.05$; OR, 0.13; 95% CI, 0.003–0.754), PP (FET: $p < 0.05$; OR, 0.35; 95% CI, 0.11–0.86), PBP (FET: $p < 0.05$; OR, 2.6; 95% CI, 1.20–5.17), and PL (FET: $p < 0.001$; OR, 2.1; 95% CI, 1.3–3.5). The detection of *A. pyogenes* made the detection of PP and IP lesions twice less probable and CBP, PBL, and PL lesions it twice more probable.

A. pleuropneumoniae was associated with the following: CBP (FET: $p < 0.0001$; OR, 0.08; 95% CI, 0.03–0.2), IP (FET: $p < 0.0001$; OR, 0.13; 95% CI, 0.03–0.33), BIP (FET: $p < 0.0001$; OR, 0.45; 95% CI, 0.28–0.72), PP (FET: $p < 0.0001$; OR, 13.0; 95% CI, 9.53–17.85), and PE (FET: $p < 0.0001$; OR, 0.31; 95% CI, 1.20–5.17). The detection of *A. pleuropneumoniae* made the detection of CBP, IP, BIP, and PE lesions 13 times less probable and that of PP lesions 13 times more probable.

M. hyopneumoniae was associated with PBP (FET: $p < 0.001$; OR, 2.91; 95% CI, 1.38–6.45). The detection of *M. hyopneumoniae* doubled the probability of detection of PBP lesions.

M. hyorhinis was associated with BIP (FET: $p < 0.0001$; OR, 0.14; 95% CI, 0.05–0.45). The detection of *M. hyorhinis* decreased the probability of detection of BIP lesions.

P. multocida was associated with IP (FET: $p < 0.05$; OR, 0.49; 95% CI, 0.23–0.91) and PL (FET: $p < 0.01$; OR, 1.59; 95% CI, 1.16–2.17). The detection of *P. multocida* decreased the probability of detection of IP lesions and nearly doubled the probability of detection of PL lesions.

PCV-type 2 was associated with pathological lesions of PP (FET: $p < 0.01$; OR, 1.47; 95% CI, 1.08–1.99), PBP (FET: $p < 0.001$; OR, 1.92; 95% CI, 1.23–3.01), and PE (FET: $p < 0.05$; OR, 0.65; 95% CI, 0.43–0.96). The detection of PCV-type 2 decreased the probability of detection of PE pathological lesions and increased the probability of detection of PP and PBP pathological lesions.

PRRSV was associated with PE pathological lesions (FET: $p < 0.001$; OR, 1.84; 95% CI, 1.24–2.76). The detection of PRRSV nearly doubled the probability of detection of BIP lesions.

Streptococcus spp. was associated with pathological lesions of PP (FET: $p < 0.0001$; OR, 0.52; 95% CI, 0.36–0.75), PL (FET: $p < 0.001$; OR, 1.47; 95% CI, 1.14–1.90), and PE (FET: $p < 0.05$; OR, 1.47; 95% CI, 1.01–2.12). The detection of *Streptococcus* spp. decreased the probability of detection of PP lesions and increased the probability of detection of PL and PE lesions by one and an half times (**Table 4**).

Multivariate Analysis

Multivariate regression logistic models were applied, with pathogens (*A. pyogenes, A. pleuropneumoniae, Streptococcus spp.,* and PCV-type 2) as dependent variables and production stage and pathological lesions as covariates. The results of the multivariate regression analysis are shown in **Table 5**. For *A. pyogenes*, the best model (LRT, 33.99; df = 3; $p < 0.0001$) included CBP, PBP, and PL lesions. The detection of *A. pyogenes* was four times more probable with CBP, PBL, and PL lesions.

For *A. pleuropneumoniae,* the best model ($p < 0.0001$) included all factors that were found to be statistically significant by FET, as well as production stage. The presence of *A. pleuropneumoniae* was more likely to occur in the growing group than in the post-weaning group with PP lesions and without CBP, IP, BIP, and PE lesions.

For PCV-type 2, the best model (LRT, 48.3; df = 2; $p < 0.0001$) included PCP lesion and production stage. The presence of PCV-type2 was more likely to occur in the growing group than in the post-weaning group with PCP lesions.

Finally, for *Streptococcus* spp. the best model (LRT, 14.3; df = 1; $p < 0.0001$) included PP lesion. The presence of *Streptococcus* spp. was more likely to occur in the absence of PP lesions.

TABLE 5 | Factors that were found to be statistically significant by multivariate regression analysis for *A. pyogenes*, *A. pleuropneumoniae*, *Streptococcus* spp., and PCV-type 2 pathogens.

Pathogens	Factor	Baseline	OR	OR 95% CI		*p*-value
A. pyogenes	CBP	Lesion detected	4.40	2.27	8.61	<0.0001
	PBP	Lesion detected	3.77	1.77	7.46	<0.0001
	PL	Lesion detected	3.55	2.01	6.44	<0.0001
A. pleuropneumoniae	CBP	Lesion not detected	7.61	3.36	21.89	<0.0001
	IP	Lesion not detected	6.62	2.67	21.95	<0.0001
	BIP	Lesion not detected	1.70	1.05	2.85	0.03
	PP	Lesion detected	7.21	5.16	10.14	<0.0001
	PE	Lesion not detected	2.51	1.33	5.28	<0.0001
	Production stage	Growing	2.27	1.66	3.10	<0.0001
PCV-type2	PBP	Lesion detected	1.82	1.18	2.80	0.001
	Production stage	Growing	2.08	1.65	2.62	<0.0001
Streptococcus spp.	PP	Lesion not detected	1.90	1.35	2.74	0.001

OR and 95% CI were calculated by multivariate logistic regression.

TABLE 6 | Frequency (n) and percentage (%) of detection of respiratory pathogens in samples without lesions are shown at global level (overall) and by production stage (growing/post-weaning).

Pathogen	Overall			Growing		Post-weaning		*p*-value
	Without lesions	Positive	%	*n*	%	*n*	%	
Streptococcus spp.	234	46	19.7%	11	23.9%	35	76.1%	0.288
Streptococcus suis	234	6	2.6%	3	50.0%	3	50.0%	0.379
A. pyogenes	234	4	1.7%	2	50.0%	2	50.0%	0.591
P. multocida	234	20	8.5%	6	30.0%	14	70.0%	1.000
A. pleuropneumoniae	**234**	**19**	**8.1%**	**11**	**57.9%**	**8**	**42.1%**	**0.017**
PRRSV	204	133	65.2%	35	26.3%	98	73.7%	0.058
PCV-type2	185	45	24.3%	15	33.3%	30	66.7%	0.572
SIV	202	40	19.8%	12	30.0%	28	70.0%	1.000
M. *hyopneumoniae*	**18**	**7**	**38.9%**	**6**	**85.7%**	**1**	**14.3%**	**0.049**
M. *hyorhinis*	**56**	**51**	**91.1%**	**7**	**13.7%**	**44**	**86.3%**	**0.000**
H. parasuis	66	41	62.1%	7	17.1%	34	82.9%	0.535

p-value was calculated using Fisher's exact test to assess the association between the occurrence of pathogens and the production stage.

Isolation of Single Pathogens in Organs Without Lesions

Among 1,656 lung samples, 234 non-lesioned samples were observed (14.1%, 95% CI, 12.5–15.9%); in particular, among these 234 samples, 161 (68.8%, 95% CI, 62.4–74.7%) and 73 (31.2%, 95% CI, 25.3–37.6%) were identified in the post-weaning and growing groups. The association between the production stage and the lesions was statistically significant (FET: $p < 0.0001$), with the post-weaning group showing a lower probability of presenting lesions (OR, 0.53; 95% CI, 0.39–0.73). **Table 6** shows the frequencies and percentages of the pathogens detected in the lungs without lesions according to the production stage. The *p*-value obtained by FET indicates the association between the pathogen and the production stage. Statistically significant associations of the production stage were found with *A. pleuropneumoniae* (FET: $p = 0.017$), *M. hyopneumoniae* (FET: $p = 0.049$), and *M. hyorhinis* (FET: $p < 0.001$). The post-weaning group showed lower probabilities of detection of *A. pleuropneumoniae* (OR, 0.3; 95% CI, 0.1–0.8) and *M. hyopneumoniae* (OR, 0.1; 95% CI, 0.1–0.9) than did the growing group. A different pattern was observed for *M. hyorhinis*; it was mostly detected in the post-weaning group (OR, 3.14; 95% CI, 4.4–∞).

Relation Between Nasal, Pleural, and Lung Scores

The mean lung score was 13.53 in weaned piglets and 12.14 in fattening pigs With 14.7% of the samples being non-lesioned (equaly distributed between weaned and fattening pigs). The mean pleural score was 1.46 and 1.48, in the weaned and fattening pigs, respectively, with 42.71 and 39.10% of the samples, respectively, being non-lesioned. The mean nasal score was 0.50 and 0.78 in the weaned and fattening pigs, respectively, with 61.82 and 42.71% of the samples, respectively, being non-lesioned.

Data distributions and correlations between the different scores are shown in **Figure 3**. The results indicate that the difference was statistically significant among scores ($p < 0.0001$, lung score vs. pleural score; $p = 0.0039$, lung score vs. nasal score; $p = 0.0023$, pleural score vs. nasal score). However, the Spearman r value was low, indicating a discrete/limited correlation ($r = 0.39$; $r = 0.14$; $r = 0.15$, respectively). On the contrary, Chi-square test showed that there was a statistically significant relationship between lung and pleural scores (3×2; $p < 0.0001$) and that there was no statistically significant relationship between the lung and nasal scores (3×2) and between the pleural and nasal scores (2×2); however, p was 0.0572 in the case relationship between the lung and nasal scores (**Supplementary Material**).

DISCUSSION

The systematic application of the standardized protocol during 2014–2016 allowed the identification of the principal lesions and pathogens associated with PRDC outbreaks in growing pigs and the comparison of different situations. Our results indicate that the PRDC percentages recorded during the 3 years of study were almost constant between the weaned and the fattening pigs and that PRDC principally affects the weaned pigs (53–58 vs. 42–47%) causing high mortality. The proportion of lung lesions was high with values comparable to those reported by previous investigations at slaughterhouses (12, 13, 27–29). Nevertheless, it is important to highlight that the frequency of pleuritis, BIP, and pleuropneumonia along with that of IP and purulent bronchopneumonia was similar between piglets and pigs. Conversely, catarrhal bronchopneumonia, pericarditis, and pleuro-pericarditis affected principally weaned piglets. Generally, pleuritis lesions were more frequently detected in weaned piglets. The comparison between these results and the data obtained from slaughtered pigs with catarrhal bronchopneumonia and pleuropneumonia (12, 13), highlighted an evolution of the lesions from the growing stage to the slaughtering stage.

The analysis of the association between lesions and the pathogens involved showed that the presence of *A. pleuropneumoniae* is largely associated with pleuropneumonia in fattening pigs. *A. pleuropneumoniae* is the agent associated with porcine pleuropneumonia, a contagious respiratory disease capable of causing significant economic losses to the swine industry worldwide (30).

Frequently, pigs that overcome acute diseases remain chronically infected, showing no clinical signs, but likely harboring chronic lung alterations, such as fibroblastic pleurisy and lung tissue sequesters surrounded by fibrotic tissue (13, 31). In this study, in fact, *A. pleuropneumoniae* was strongly associated with lesions characteristic of pleuropneumonia with a minor involvement of pleura. In addition, we isolated *A. pleuropneumoniae* from non-lesioned lungs in 8.1% of the cases; this finding suggests that *A. pleuropneumoniae* is unlikely to be associated with a subclinical lung condition.

Streptococcus spp. and *S. suis* were associated with pleural and pericardial lesions, mainly in post-weaning pigs. Fibrinous or fibrinopurulent pleuritis, peritonitis, or polyserositis were identified in pigs infected with both *S. suis* and *H. parasuis*. Pigs in which only *S. suis* was isolated had a more extensive suppurative exudation than that was associated with *H. parasuis*.

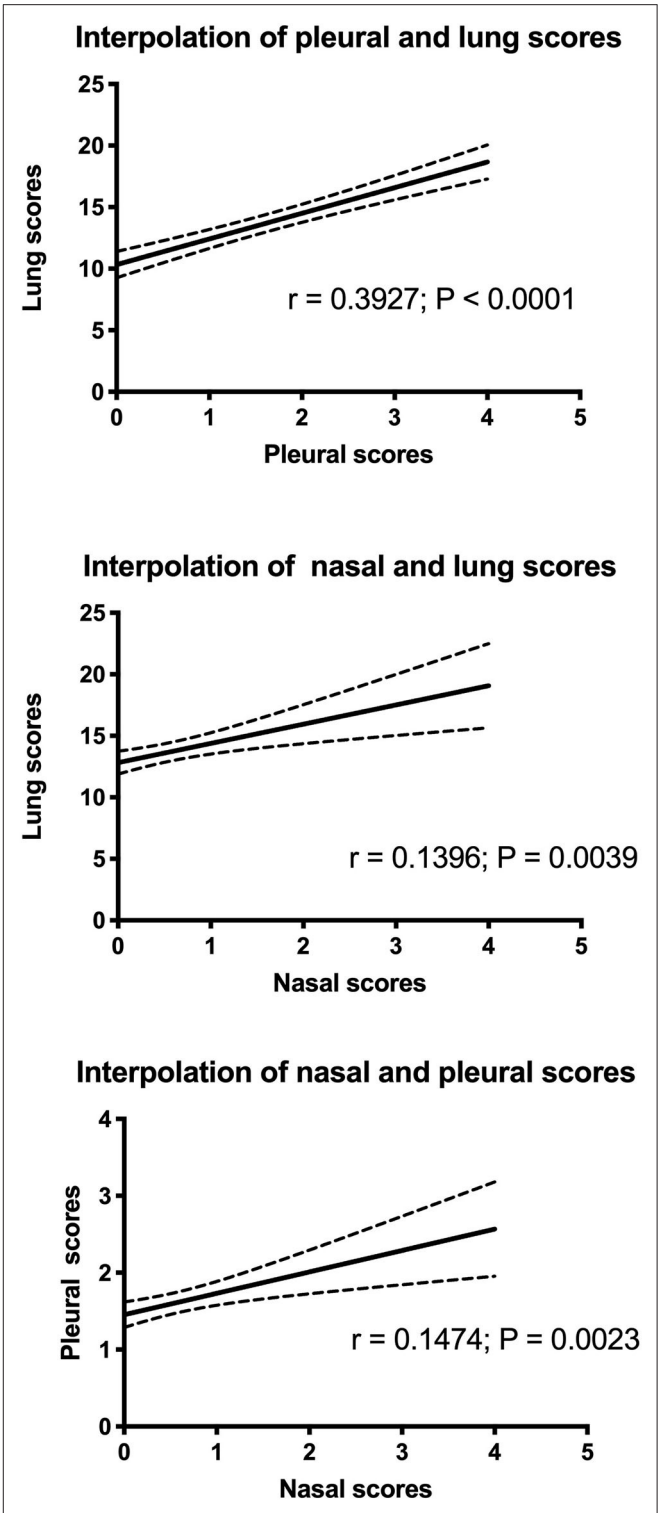

FIGURE 3 | Interpolation of lung, pleural, and nasal scores.

S. suis in pigs with pleuritis, peritonitis, and polyserositis should be considered first during differential diagnosis, especially when the exudate was more suppurative than fibrinous (32). In this study, the frequency of *Streptococcus* spp. and *S. suis* detection in non-lesioned lungs was 19.7 and 2.6%, respectively. This finding is partly in contrast with other published data, which showed that asymptomatic colonization of *S. suis* in the upper respiratory tract as well as in the intestinal and genital tract was common (33).

M. hyopneumoniae was significantly more prevalent in cases of purulent bronchopneumoniae and even more apparently prevalent in catarrhal bronchopneumoniae. The lesions were more frequent in fattening pigs than in weaned piglets. *M. hyopneumoniae* is an important respiratory pathogen capable of causing the disease by itself or in combination with other pathogens (34, 35). A combination was observed between *M. hyopneumoniae* and PRRSV or *S.* suis (36, 37). Different studies have demonstrated that disease severity in growing pigs is correlated with several factors (38, 39). The fact that *M. hyopneumoniae* was frequently found in organs without lesions corroborates the finding that it is a common pathogen with effects that are likely dependent on other triggering factors, however we cannot exclude that *M. hyopneumoniae* has been detected in the first stage of the infection.

The correlation between lung and pleural scores was found to be linear with $p < 0.0001$. However, it was a moderate correlation because Spearman's r-coefficient was low ($r < 0.4$). Probably, this aspect is associated with a wide distribution of lung scores in each class of pleural scores. For this reason, a further comparison between scores was performed using the Chi-squared test. Lung scores were divided into three categories, while pleural scores were divided into two categories. The results indicated that the frequency of severe lesions in lungs corresponded to a high frequency of severe pleural lesions. Therefore, the results suggest that the proposed scoring approach could be a reliable system to evaluate lesions in the respiratory tracts during pathological investigations.

In conclusion, these data shed light on the impact of different pathogens on the respiratory disease of pigs and highlight the need for a systematic diagnostic approach to manage the respiratory disease in pig farms.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Animal approval was not required according to national/local legislation as we exclusively analyzed the remains of pigs that died naturally. Written informed consent was obtained from the owners of the animals studied.

AUTHOR CONTRIBUTIONS

JR, PPa, and GA contributed to the conception and design of the study, acquisition of data, analysis and interpretation of data, and drafting of the article. CS, SG, and NV contributed to the acquisition of data, analysis and interpretation of data, and drafting of the article. BB contributed to the acquisition of data, analysis and interpretation of data, and revise revisions of the article. AC and PPo contributed to the analysis and interpretation of data and revision of the article. All authors contributed to the revision of the manuscript and furthermore they read and approved the submitted version.

ACKNOWLEDGMENTS

Special thanks to E. language editing service for the English writing assistance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00636/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 11 September 2020
doi: 10.3389/fvets.2020.00614

Quantitative Understanding of the Decision-Making Process for Farm Biosecurity Among Japanese Livestock Farmers Using the KAP-Capacity Framework

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 16 May 2020
Accepted: 29 July 2020
Published: 11 September 2020

Citation:
Makita K, Steenbergen E, Haruta L, Hossain S, Nakahara Y, Tamura Y, Watanabe T, Kadowaki H and Asakura S (2020) Quantitative Understanding of the Decision-Making Process for Farm Biosecurity Among Japanese Livestock Farmers Using the KAP-Capacity Framework. Front. Vet. Sci. 7:614. doi: 10.3389/fvets.2020.00614

In a globalized world, the frequency of transboundary livestock infectious diseases is increasing, and strengthening of farm biosecurity is vital to stabilize food production. The aim of this study was to understand the decision-making process for farm biosecurity among Japanese livestock farmers. Postal surveys using structured questionnaires were conducted on beef, dairy, pig, and layer farms in Hokkaido and Saitama Prefectures, which represent the principal production area and peri-urban Tokyo, respectively, as well as randomly selected broiler farms across Japan. The question items included the attributes of farms and owners, disease experiences, related associations and sources of hygiene information, attitude toward hygiene management, and compliance with the Standards of Rearing Hygiene Management (SRHM). The compliance rates were compared between livestock sectors. Univariable analyses were conducted using combined data from both prefectures, with the compliance rate as the outcome variable and the questionnaire items as explanatory variables, in generalized linear models. Exploratory factor analyses were conducted using the variables with $p < 0.2$ in the univariable analyses. The factors identified were classified into knowledge, attitude, capacity, practice, and structural equation modeling (SEM) was performed. The questionnaires were completed and returned by 97 and 66 beef cattle, 86 and 136 dairy, 67 and 45 pig, 20 and 39 layer farmers in Hokkaido and Saitama Prefectures, respectively, and 95 broiler farms. The compliance rate was significantly higher among broiler farms (88.9%) compared with the other sectors, followed by pig (77.1%), layer (67.2%), dairy (63.8%), and beef (59.1%) farms in Hokkaido Prefecture, and layer (64.9%), pig (60.0%), dairy (58.5%), and beef (57.6%) farms in Saitama Prefecture. Based on SEM, the decision-making process from greater knowledge to higher attitude, and from higher attitude to greater compliance with the SRHM were significant ($p < 0.01$) in all sectors. Higher capacity was significantly associated with higher knowledge in dairy,

pig,break and layer farms ($p < 0.01$), and with higher compliance in beef, pig, and layer farms ($p < 0.05$). These results suggest that the provision of targeted hygiene knowledge to livestock farmers and the support to smallholder farms would improve biosecurity through elevated attitudes and self-efficacy.

Keywords: KAP analysis, capacity, biosecurity, decision-making, structural equation modeling

INTRODUCTION

Infectious diseases remain a significant threat to the livestock industry in a globalized world. For example, African swine fever rapidly expanded from its original territory in sub-Saharan Africa to Georgia in 2007 (1), with subsequent outbreaks in the Caucasus, eastern and central Europe, and the Baltic countries (2, 3), as well as wide regions of east and southeast Asian countries, since 2018 (4, 5). Moreover, increased travel and trade, which also increases the chance of illegal importation of infected livestock products, pose an elevated risk of long-distance international transmission of infectious agents (6). Infectious animal disease epidemics not only cause severe economic damage, but also affect the mental well-being of farmers, veterinarians, and civilians in affected areas, as seen worldwide in the foot-and-mouth disease (FMD) epidemic (7–9).

Farm biosecurity is an integral part of livestock production (10). It prevents the introduction of infectious agents into farms thereby reducing the economic burden caused by infectious disease outbreaks in animals. In 2004, the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan established the Standards of Rearing Hygiene Management (SRHM), specified by the Act on Domestic Animal Infectious Diseases Control in Japanese law (11, 12). The SRHM incorporated 10 basic on-farm standards for cattle, pig, and poultry farms as minimum hygiene standards, including hygienic rearing, disinfection at farm entrances and vehicles, quarantine upon animal introduction, and the acquisition of knowledge regarding infectious disease prevention. However, in 2010, major outbreaks of FMD and highly pathogenic avian influenza (HPAI) occurred in Japan, during which a total of 1.8 million chickens, 230,000 pigs, and 70,000 cattle were culled, causing serious losses to the livestock sectors (13, 14). After these major outbreaks, in 2011, the MAFF revised the SRHM by setting species-specific standards for (1) cattle, buffaloes, deer, sheep, and goats, (2) pigs and domesticated wild boars, (3) poultry, and (4) horses, and by increasing the number of hygiene standards from 10 to 22–25 items (15). Hereby, more emphasis was placed on the establishment of hygienic zones, farm entrance restrictions, the securing of land for burying carcasses after emergency culling, and the early detection and reporting of infectious diseases (12). Although substantial efforts were made to implement the SRHM on livestock farms, in 2013, porcine epidemic diarrhea (PED), which was causing a global pandemic, occurred in Japan, resulting in the deaths of over 500,000 pigs in the country (16). Reflecting on the findings of epidemiological investigations on PED (16) and critical discussions by experts (12), the SRHM were revised to include the prevention of contact between animal carcasses and wildlife, the establishment of a minimum temperature to heat human food waste for animal feeding, and the avoidance of leakage during the transportation of carcasses and animal excrement (17). The SRHM provided clear guidance to Japanese livestock farmers regarding biosecurity; however, compliance remains a challenge (18). The recent incursion of classical swine fever into the wild boar population has been causing infections on Japanese pig farms since 2018 (19, 20), and thus, improvements in farm biosecurity are becoming increasingly important.

Several frameworks have been proposed to understand the decision-making process in health-related practices. For example, the Health Belief Model (HBM), which consists of four components—perceived susceptibility to a health threat, severity of the threat, benefits to prevent the threat, and barriers to the preventive behavior (21)—has been reported in a review of 24 studies to affect preventive actions in the order of barriers, susceptibility, benefits, and severity (22). The Protection Motivation Theory (PMT) proposes that two cognitive processes—threat appraisal and coping appraisal—determine the conduct or inhibition of protective actions (23). Threat appraisal process evaluates the factors that increase or decrease the probability of making the maladaptive responses such as smoking or not wearing a seat-belt. Intrinsic (e.g., bodily pleasure) and extrinsic rewards (e.g., social approval) increase the probability of the maladaptive response, while assessed severity of the threat and perceived vulnerability to the threat reduce the probability. Coping appraisal is increased by judgments about the efficacy of a preventive response and one's ability to adapt the response successfully, and is decreased by the response cost (23). PMT has been applied in animal health to determine the factors associated with biosecurity practices during an equine influenza outbreak in Australia (24) and with the perception of vulnerability to future outbreaks (25) using logistic regression analysis. The Theory of Reasoned Action (TRA) states that an individual's behavior may be predicted by the strength of the intention, which depends on a combination of attitudes and subjective norms (26, 27). The Theory of Planned Behavior (TPB) is an extension of the TRA. In the TPB, in addition to attitudes and subjective norms, "perceived behavioral control (PBC)," which accounts for the belief in self-efficacy and perceived difficulties, is assumed to influence behavioral intentions (28). PBC not only affects behavioral intentions, but is also directly related to actual behaviors (27, 28). The TRA and TPB are applied in qualitative studies on the decision-making process of farm disease control (27–29). Another framework to identify knowledge gaps, cultural beliefs, or behavioral patterns that may be obstacles to the control of infectious diseases is

the Knowledge, Attitudes, and Practice (KAP) framework (30). Numerous qualitative studies on the KAP framework have been carried out in animal health worldwide, and some studies, for example, on rabies, have applied this framework using uni- and multivariable linear analyses (31). The limitation of this approach is that linear analysis measures direct and indirect associations between factors and key disease preventive practice(s), but cannot infer complex mechanisms in the decision process. Toma et al. (10) modeled flows from several attitude nodes to the biosecurity behaviors of British cattle and sheep farmers, and Kadowaki et al. presented the flow of sociological factors to the KAP framework as a sequence (better livelihood enhances knowledge, higher knowledge leads to better attitudes, and better attitudes initiate good practices) in voluntary rabies control measures in Vietnam (32) using structural equation modeling (SEM). SEM is not intended to discover causes (33), but rather, to assess the soundness of causal relationships formulated a priori (10). SEM is useful for understanding decision-making mechanisms because it can distinguish latent variables (e.g., KAP) from observed variables (34) and assess the relationships between latent variables using observed variables.

The initial purpose of this study was to gain a better understanding of the influence of socioeconomic factors in the practice of biosecurity measures on livestock farms in Japan in order to further improve the level of biosecurity. During focus group discussions (FGDs) and an analysis of results from a postal survey using linear models, in addition to the KAP framework, the importance of the capacity of farmers, such as the size of the operation and the age of owners in family-owned farms, was perceived. A preliminary analysis on pig farmers, using the same data with the present study, is published in Japanese (35). Therefore, this study assesses the decision-making mechanism of farm owners in regard to biosecurity based on the KAP plus capacity (KAP-Capacity) framework.

MATERIALS AND METHODS

Study Areas

Studies on beef and dairy cattle, pig, and layer farm owners were conducted in Hokkaido and Saitama Prefectures, Japan, to compare livestock production (Hokkaido) and peri-urban areas (Saitama) (**Figure 1**). In Japan, broiler producers are no longer considered farmers, but rather, companies, so a study on these companies was conducted at the national level.

Focus Group Discussions

Between October 2013 and January 2014, at least one FDG was held for each of the livestock sectors, except for the broiler sector, to collect information that could be used to develop questionnaires. For the beef and dairy cattle, pig, and layer hen sectors, FDGs were held with people from both Hokkaido and Saitama Prefectures. For the broiler sector, semi-structured interviews were carried out with hygiene management veterinarians employed in broiler companies in Miyazaki and Hokkaido Prefectures. Each FGD, other than that for the broiler sector, was performed with a small group of people (5–6 persons) and included female farmers (or the wives of farm owners) and employees of livestock associations to gain different perspectives on the topics discussed. The discussions lasted a total of 80 min (two 40-min discussions with 10-min break in between), during which time, topics such as hygiene management, the influence of changes in the SRHM, livestock-related associations, and requirements for a questionnaire that could be easily interpreted and completed by farmers were discussed. All the FGDs were facilitated by an epidemiologist who was trained in participatory epidemiology and had 5-years' experience of the application in research.

Postal Surveys

Based on the results of the FDGs and semi-structured interviews, livestock sector-specific questionnaires were constructed and then pretested on farmers and veterinarians. **Table 1** shows a list of the question items with respect to farmer attributes, farm information, experience with animal diseases, cooperating associations, source of hygiene information, attitude toward hygiene information and management, and compliance with the SRHM. The questionnaire for the broiler sector was slightly different from that used in the other livestock sectors, as these are considered business companies owning several farms.

In Hokkaido Prefecture, questionnaires were sent to all beef cattle farmers belonging to Japan Agricultural Cooperatives (JA) in the Engaru and Yubetsu areas, those who attended technical workshops held in JA in the Tokachi area and the Hokkaido Dairy and Livestock Association (questionnaires were filled out at the workshops), all dairy farmers belonging to JA in the Engaru and Yubetsu areas, all pig farmers belonging to the Hokkaido Pig Farm Producers Association, and all layer farmers in Chitose, Ebetsu, Eniwa, and Ishikari cities, with assistance from the respective organizations. In Hokkaido, there were no integrated beef and dairy producers associations which can directly send questionnaires to farmers, and several intensive production areas were purposively selected. For layer farming, there were a few large scale companies, and there was no integrated farmers' association. Therefore, reachable cities from Rakuno Gakuen University were visited to enroll study participants. In Saitama Prefecture, questionnaires were sent to all beef and dairy cattle, pig, and layer farmers belonging to the Saitama Livestock Association, with assistance from the organization. For the broiler sector, sets of five questionnaires were sent to 50 randomly selected broiler-producing companies from a list published by the Japan Chicken Association, which has 81 member producers (36). The sample size for broiler companies was determined based on the available resources.

SRHM Compliance Rates

The SRHM compliance rates were calculated as a proportion of SRHM items complied out of all the SRHM items for livestock species. **Table 2** shows the SRHM items in the categories prepared following careful discussions by the authors: (1) prevention of disease incursion from fomites and animals, (2) limitation of access to a farm, (3) prevention of infection from wildlife, (4) prevention of within-farm spread, and (5) maintenance of preparedness. Some items such as provision of drinkable water and rearing animals with suitable density were associated with

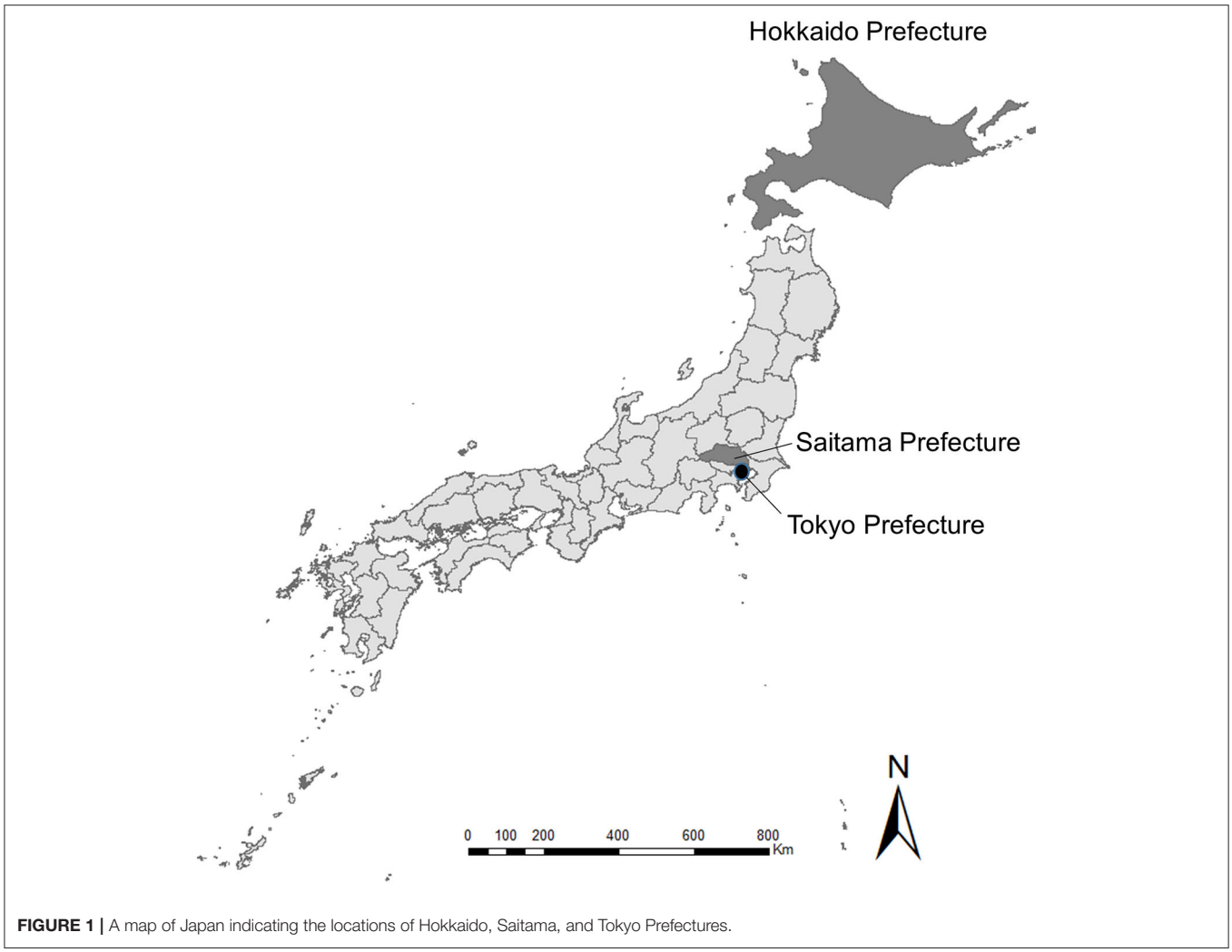

FIGURE 1 | A map of Japan indicating the locations of Hokkaido, Saitama, and Tokyo Prefectures.

TABLE 1 | Content of the questionnaire for farmers.

Category	Questionnaire item
Farmer's attributes	Age, gender, household size, schooling years, working time, and farming years of the farmer.
Farm information	Level of urbanization, year of farming since establishment, successor availability, number of livestock, housing facilities, management type, number of farm workers, whether the farm is adjacent to the main road, the density of neighboring farms, distance to the nearest farm, and the source of feed
Experience of diseases	Occurrence and impact of disease symptoms, a list of diseases experienced in last decade, and number of disease occurrences
Associations and information sources	Related associations, sources of hygiene information, and attendance of lectures and seminars about animal health
Attitudes toward hygiene information and management	Priority of information sources and activity, presence of change in attitude toward SRHM after its revision, and the reasons for this change, satisfaction level for hygiene management on the own farm and by the government, and the level of change in communication with other farmers after the revision of SRHM
Compliance with the Standards of Rearing Hygiene Management (SRHM)	Compliance with each standard described in the SRHM, categorized under five topics: the prevention of introduction with fomites, limitation of access to the farm, prevention of infection from wildlife, prevention of within-farm spread, and maintenance of preparedness

animal ethics and not necessarily with biosecurity; however they were classified into the most suitable categories. The SRHM included a few items specific to large-scale farms (adult cattle and buffalo: >300 heads; heifer, deer, sheep, and goats: >3,000 heads; poultry and quail: >100,000 birds; and ducks: >10,000 birds); however, for the purpose of analysis, in terms of compliance

TABLE 2 | Standards of rearing hygiene management.

Category	Hygiene standards
Prevention of disease incursion with fomites and animals	Disinfection of vehicles; Disinfection of hands and shoes of those who enter to the farm building; Provision of clothes and shoes only for hygiene control area (*only for pig and poultry farmers*); Cleaning or disinfection of materials directly used for animals when carry them in hygiene control area; Prohibition of carrying clothes and shoes used abroad into the farm; Quarantine of animals under segregation from other animals for certain period when introducing into the farm; Heat treatment of recycled feed (*only for pig farmers*)
Limitation of access to the farm	Segregation of hygiene control area from the other areas; Placement of a signboard indicating the hygiene control area; Limit of access for those who entered other farms or recently returned from abroad
Prevention of incursion from wildlife	Prevention of wildlife feces entering to feeding and water facilities; Provision of drinkable water; Placement of nets preventing entrance of wild birds (*only for poultry farmers*); Pest control, repair of damaged roof and walls (*only for poultry farmers*)
Prevention of within-farm spread	Change (disposal) or disinfection of materials to which body fluid of animals got attached, at each use; Cleaning and disinfection of a barn or cage after being emptied; Rearing animals with suitable density
Maintenance of preparedness	Collecting up-to-date information on prevention of animal infectious diseases; Immediate report of specific symptoms by law to the Livestock Hygiene Service Centre (LHSC) and restriction of animal movement; Immediate call of veterinarians when animals are sick without specific symptoms by law; Daily health check of animals; Removal of dirt and health check at selling out animals; Securing a land to bury culled animals; Record keeping for early identification of source of infection
Items for large scale farms (excluded from the analyses)	Designate one veterinarian or clinic, who tightly communicate with LHSM and regularly guide animal health management per farm, for all farms belonging to the company; Farm employees are aware that LHSC should be called without seeking permission of farm owner or manager immediately after the symptoms of specific diseases are detected

rate, these items for large-scale farms were excluded from the denominator to deal with all the respondents unweighted, regardless of farm size.

The compliance rates were compared among livestock species in Hokkaido and Saitama Prefectures separately using a generalized linear model (GLM) with quasi-binomial errors, as overdispersion was observed in the compliance rates. As broiler farms were sampled from the entire country, all broiler farm responses were included in the models for both prefectures. For dairy, beef, pig, and layer hen farmers, the compliance rates were compared between Hokkaido and Saitama Prefectures using GLMs.

Comparisons of Farm Capacity and Density Between Hokkaido and Saitama Prefectures

To understand the differences in farm capacity and farm density between Hokkaido and Saitama Prefectures, capacity-related factors—the age of the owners, number of animals raised, number of farm workers, including the owner, the availability of successors, and the shortest distance to a farm raising the same species were compared between the two prefectures for beef and dairy cattle, pig, and layer farms. Capacity related factors were identified during above mentioned FGDs; as the owner gets older physical ability would be weak; large-scale farms with large numbers of animals and workers have greater work force; and availability of successor would be a good motivation of investments. The age of the owners was compared using *t*-tests when the data were normally-distributed, and the Wilcoxon rank sum test otherwise. The total numbers of animals in dairy and layer farms were compared using GLMs with quasi-Poisson errors. Beef cattle and pig farms were categorized according to management types (farrow-to-finisher, fattening, growing, and reproduction for beef cattle farms; and farrow-to-finisher, fattening, and reproduction for pig farms), and the total numbers of animals within these types were compared using the Wilcoxon rank sum test. The numbers of farm workers were compared using GLMs with quasi-Poisson errors. The proportions of farms with a successor were compared using chi-squared tests. The shortest distance to a farm raising the same species was also compared using the Wilcoxon rank sum test.

Univariable Analysis of the Factors Affecting Compliance With the SRHM

We assumed that a decision making framework for implementing biosecurity measures would be common between Hokkaido and Saitama Prefectures, and as the sample sizes of the two prefectures were not so large, univariable analyses were conducted after combining both prefectures in terms of dairy and beef cattle, pigs, and layer farmers. For beef and dairy cattle, pigs, layer hen, and broiler producers, univariable analyses were conducted using GLMs with quasi-binomial errors, as overdispersion was commonly observed, selecting the compliance rate as the outcome variable and questionnaire items as the explanatory variables.

Exploratory Factor Analysis

For each livestock species, factors with $p < 0.2$ were used for the exploratory factor analysis. When variables with similar socioeconomic meanings were identified by the investigators, those with the most direct meaning were selected to keep the number of variables as small as possible. For the practice of the SRHM, category-specific compliance rates were used for the analysis. According to our hypothesis, higher knowledge (K) would foster a better attitude (A), and a better attitude would determine the decision to conduct biosecurity practices (P). In

addition, we hypothesized that higher capacity (Capacity) would facilitate gaining animal health knowledge, and that capacity would be needed to implement practices. Using parallel analysis scree plots plotted with the fa.parallel() function in the package "psych" (37) in R, the validity of the number of factors—four for the KAP-Capacity framework—was checked. Exploratory factor analyses specifying four factors were performed using the fa() function in the "psych" (37) and "GPArotation" packages in R (38), and the factors were checked based on the variables grouped together in terms of whether they were representative of K, A, P, and Capacity.

Structural Equation Modeling

SEM was performed using the "lavaan" package in R (39). A path graph, which shows the structural part of the model, was designed selecting K, A, P, and Capacity as latent variables, and the remaining questionnaire variables from the exploratory factor analysis and category-specific SRHM compliance rates were used as the explanatory variables. To improve the model fit, in model tuning, non-significant variables in the exploratory factor analysis were either removed from the model or allocated to other factors based on the meanings of the variables. As the data sets included categorical and dichotomous variables and missing values, SEM was performed using mean-adjusted weighted least squares (WLSM) estimation, which is the robust version of weighted least squares (WLS) estimation (34). The fit indexes used were the significance of model chi-square (χ^2), Tucker–Lewis Index (TLI), root mean square error of approximation (RMSEA), and standardized root mean square residual (SRMR). For a satisfactory fit, a model should have a non-significant model-χ^2 ($p > 0.05$), TLI > 0.900, RMSEA < 0.08, and SRMR < 0.100 (40, 41). For all analyses, R statistical software version 3.6.1 (42) was used.

RESULTS

Questionnaire Responses

The response rates for beef cattle, dairy, pig, and layer farmers in Hokkaido and Saitama Prefectures were 33.3% (97/291) and 41.5% (66/159), 29.7% (86/290) and 44.2% (136/308), 34.5% (67/194) and 36.0% (45/125), and 33.3% (20/60) and 39.8% (39/98), respectively. The response rate for broiler farms belonging to commercial companies was 38.0% (95/250).

Compliance With the SRHM

Table 3 shows a comparison of SRHM compliance rates between livestock species in Hokkaido and Saitama Prefectures. In both prefectures, the mean compliance rates for beef cattle farms were the lowest among all livestock species (59.1 and 57.6% in Hokkaido and Saitama Prefectures, respectively). In Hokkaido Prefecture, the mean compliance rates of dairy (63.8%) and layer farms (67.2%) were not significantly different from that of beef cattle farms ($p > 0.05$), but the mean compliance rate of pig farms (77.1%) was significantly higher ($p < 0.001$). Conversely, in Saitama Prefecture, the mean compliance rate of layer farms (64.9%), but not those of dairy (58.5%) and pig farms (60.0%), was significantly higher than that of beef cattle farms (57.6%, $p = 0.001$). The compliance rate of broiler farms (88.9%) was significantly higher than any other species (the 95% confidence interval of the compliance rate did not overlap with any other species) in both prefectures.

When the SRHM compliance rates of the same livestock species were compared between the two prefectures, only pig farms showed a significant difference (Hokkaido > Saitama, difference in logit = 0.811, standard error [SE] = 0.214, $p < 0.001$); no significant differences were seen for beef, dairy, or layer farms ($p > 0.05$).

Figure 2 shows the category-specific SRHM compliance rates of the different livestock species in both prefectures and broiler. The beef cattle and dairy farms in both Hokkaido and Saitama Prefectures had particularly poor compliance with the SRHM items for the prevention of disease incursion from fomites compared with other categories. By contrast, the compliance rate associated with the SRHM category to maintain preparedness was high for both beef and dairy cattle farms in both prefectures. The SRHM categories to limit access and to prevent disease incursion with wildlife were particularly high for pig farms in Hokkaido Prefecture. A tendency toward poor prevention of within-farm

TABLE 3 | Comparison of the Standards of Rearing Hygiene Management compliance rates between livestock species in Hokkaido and Saitama Prefectures.

Livestock species	Compliance rate (%) (95% CI)	Estimate	Standard error	*p*-value
Hokkaido Prefecture				
Beef cattle farm	59.1 (54.6–63.5)	Reference	0.095	-
Dairy farm	63.8 (57.4–69.7)	0.196	0.136	0.150
Pig farm	77.1 (71.5–81.9)	0.846	0.151	<0.001
Layer farm	67.2 (57.5–75.7)	0.350	0.212	0.100
Broiler farm (entire Japan)	88.9 (85.4–91.7)*	1.714	0.160*	<0.001
Saitama Prefecture				
Beef cattle farm	57.6 (54.9–60.3)	Reference	0.056	-
Dairy farm	58.5 (55.2–61.7)	0.036	0.069	0.601
Pig farm	60.0 (55.8–64.0)	0.098	0.088	0.264
Layer farm	64.9 (60.7–68.8)	0.306	0.091	0.001
Broiler farm (entire Japan)	88.9 (87.1–90.5)*	1.776	0.088*	<0.001

**Note that standard errors of the same dataset were estimated differently according to the models.*

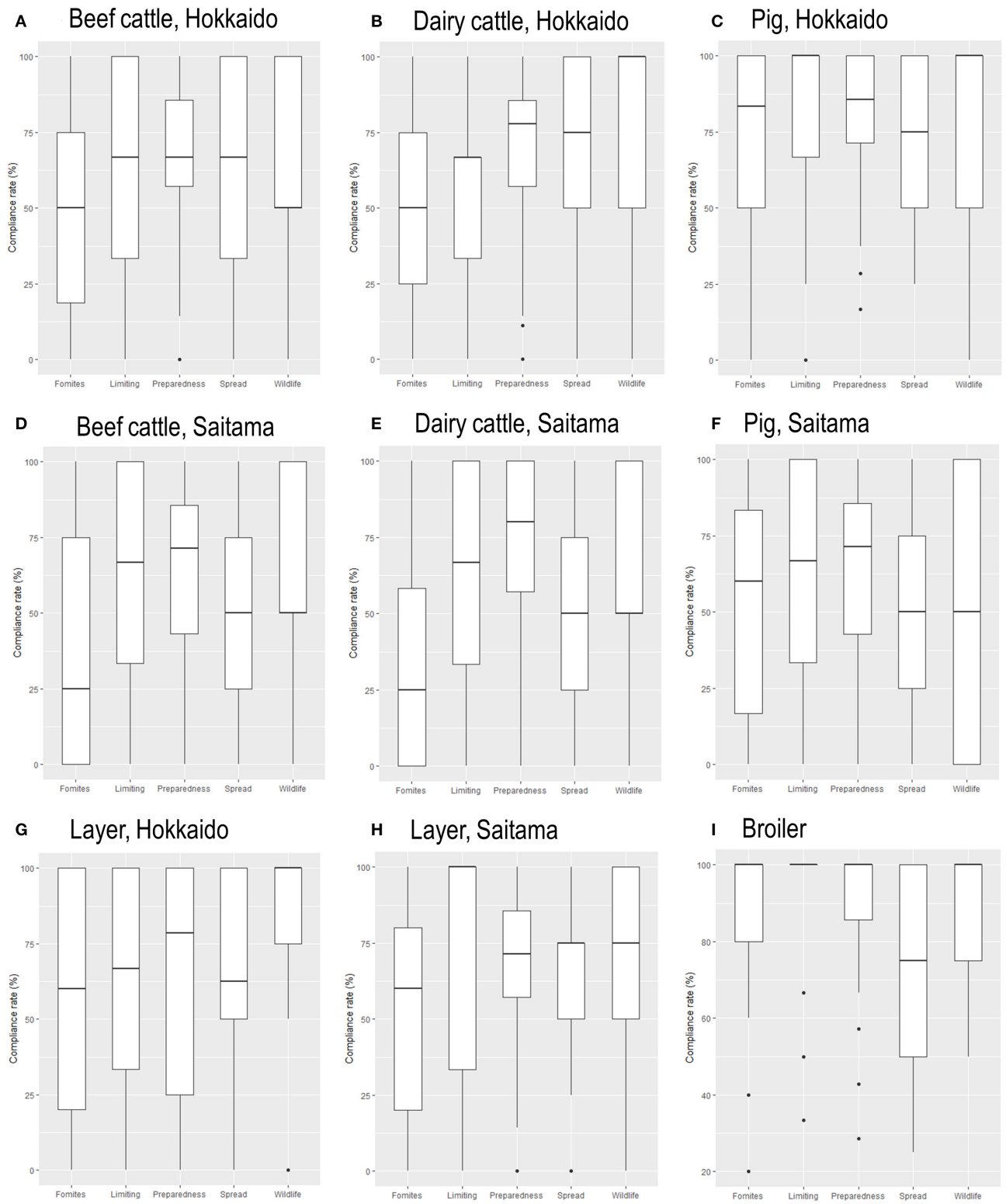

FIGURE 2 | Box plots of category-specific Standard Rearing Hygiene Management compliance rates for **(A,D)** beef cattle, dairy **(B,E)**, pig **(C,F)**, and layer farms **(G,H)** in Hokkaido and Saitama Prefectures, and broiler farms **(I)**. Categories include the prevention of disease incursion from fomites and animals, limitation of access to a farm, maintenance of preparedness, prevention of within-farm spread, and prevention of incursion from wildlife, from left to right.

TABLE 4 | Comparison of livestock farm capacity and density between Hokkaido and Saitama Prefectures.

Items	Hokkaido	Saitama	Statistics	*p*-value
Beef Cattle Farm				
Age of owner	48.5	63.6	$t = -7.4$, df = 139.2	<0.001
Number of animals (farrow-to-finisher)	161.4 ($n = 44$)	98.7 ($n = 10$)	W = 306	0.027
Number of animals (fattening)	64.0 ($n = 2$)	317.9 ($n = 28$)	-	-
Number of animals (growing)	44.3 ($n = 4$)	212.6 ($n = 7$)	W = 13	0.927
Number of animals (reproduction)	71.2 ($n = 44$)	20.6 ($n = 21$)	W = 813	<0.001
Total number of workers	3.5	2.9	Log = 0.18, se = 0.09	0.051
Availability of successor	61.8%	43.1%	$x^2 = 3.7$, df = 1	0.055
Nearest distance to other beef farm	1.6 km	2.3 km	W = 1893.5	0.026
Dairy Cattle Farm				
Age of owner	53.4	61.5	W = 2967.5	<0.001
Number of animals	113.6	44.7	Log = 0.93, se = 0.02	<0.001
Total number of workers	3.3	2.6	Log = 0.22, se = 0.08	0.005
Availability of successor	37.0%	31.9%	$x^2 = 0.4$, df = 1	0.528
Nearest distance to other dairy farm	1.4 km	2.1 km	W = 3747	0.026
Pig Farm				
Age of owner	55.8	60.0	W = 1055.5	0.028
Number of animals (farrow-to-finisher)	4,109.0 ($n = 59$)	1,024.5 ($n = 41$)	W = 1711.5	<0.001
Number of animals (fattening)	432.9 ($n = 7$)	774.3 ($n = 3$)	W = 17	0.628
Number of animals (reproduction)	62 ($n = 1$)	-	-	-
Total number of workers	6.4	3.0	Log = 0.77, se = 0.24	0.002
Availability of successor	46.2%	41.9%	$x^2 = 0.1$, df = 1	0.809
Nearest distance to other pig farm	7.0 km	2.5 km	W = 1883.5	0.013
Layer Farm				
Age of owner	56.5	62.3	$t = -1.24$, df = 14.3	0.235
Number of animals	242,146.0	40,375.6	Log = 1.79, se = 0.59	0.004
Total number of workers	10.1	8.4	Log = 0.18, se = 0.41	0.674
Availability of successor	23.1%	45.7%	$x^2 = 1.2$, df = 1	0.274
Nearest distance to other layer farm	8.4	4.1	W = 140	0.389

disease spread was observed for pig farms in Saitama Prefecture. While the compliance rate associated with preventing disease incursion with wildlife was high in layer farms in Hokkaido, the other category specific compliance rates varied greatly in layer farms in both prefectures. For broiler farms, the compliance rate associated with the prevention of within-farm spread was lower than any other category. The item-specific compliance rates for beef cattle, dairy, pig, layer, and broiler farms are described in **Supplementary Tables 1–5**.

Comparison of Farm Capacity and Density Between Hokkaido and Saitama Prefectures

Table 4 shows the results of comparisons of farm capacity and density between Hokkaido and Saitama Prefectures. For all livestock species, the farms in Hokkaido Prefecture were significantly larger than those in Saitama Prefecture in terms of the numbers of animals/birds. The numbers of workers were significantly larger in Hokkaido than in Saitama Prefecture for all livestock sectors except for in layer farms ($p = 0.674$). Farm owners were significantly younger in Hokkaido than in Saitama Prefecture for all livestock sectors except for layer farms ($p = 0.235$). The availability of a successor tended to be higher in beef cattle farms in Hokkaido than in Saitama Prefecture, but no difference was seen in the other sectors. Beef cattle and dairy farms were geographically closer to each other in Hokkaido than in Saitama Prefecture, but pig farms were more distant from each other in Hokkaido Prefecture.

Structural Equation Modeling (SEM)

Parallel analysis scree plots generally supported the number of factors (four factors) needed to consider the decision-making process structure, and the default SEM structure included the KAP-Capacity framework. **Figures 3–7** show the SEM path graphs on the structures of the decision-making process to practice the SRHM items for beef, dairy, pig, layer, and broiler farms, respectively, and **Supplementary Tables 6–10** show the detailed statistics. After excluding data with missing responses, 95, 192, 97, 36, and 84 responses from beef, dairy, pig, layer, and broiler farms were used in the SEM. The ellipses and rectangles in the figures indicate latent and measured variables, respectively. The measured variables connected to each latent variable were

selected using exploratory factor analysis beforehand. The round arrows connecting to each of the latent and measured variables are disturbances, or in other words, variances. The values on the arrows in the figures show the SEM coefficients in tables, which are standardized factor loadings.

Regarding beef farms, flows in which better knowledge enhanced higher attitudes, and higher attitudes and greater capacity improved biosecurity practices, were significant ($p < 0.05$, **Figure 3** and **Supplementary Table 6**, respectively). Knowledge was explained by learning behavior (source of information and attendance to seminars), disease experience, and increased understanding of law and vigilance after the revision of the SRHM. Attitude was explained by the availability of a successor and the prioritization of hygiene. Capacity was explained by a younger owner, longer working hours, higher education levels, and in-house production of concentrate feed. Compliance with all SRHM categories was significantly associated with practice ($p < 0.05$, **Supplementary Table 6**).

Regarding dairy farms, flows were significant in which knowledge was enhanced by greater capacity, better knowledge enhanced higher attitudes, and higher attitudes induced biosecurity practices ($p < 0.001$, **Figure 4** and **Supplementary Table 7**). Capacity was explained by larger farm size (numbers of workers and buildings) and registration as a corporation, as compared with family-owned farms. Knowledge was explained by learning behavior (source of information and attendance to seminars) and disease experience. Attitude was explained by the availability of a successor, diligence (longer working hours), and increased understanding of the law after the revision of the SRHM. Practice was explained by compliance with all SRHM categories ($p < 0.001$, **Supplementary Table 7**).

Regarding pig farms, the decision-making process flow from knowledge and attitude to practice was similar to that for beef and dairy cattle farms, but capacity enhanced both knowledge and practice (**Figure 5** and **Supplementary Table 8**). Knowledge was explained by learning behavior (source of information and attendance to seminars) and relation with the Hokkaido Pig Producers' Association. Attitude was explained by lower satisfaction with own hygiene management, availability of a successor, and prioritization of hygiene among activities. Capacity was explained by registration as a corporation as compared with a family-owned farm, and located in a rural as compared with a peri-urban area. Practice was again explained by compliance with all SRHM categories ($p < 0.001$, **Supplementary Table 8**).

Regarding layer farms, the decision-making process showed exactly the same structure as that for pig farms (**Figure 6** and **Supplementary Table 9**). Knowledge was explained by learning behavior (source of information and attendance to seminars) and the number of related organizations regarding hygiene management. Attitude was explained by increased hygiene awareness and vigilance after the revision of the SRHM, as well as diligence (longer working hours and increased perceived workload). Capacity was explained by larger farm size (numbers of workers and buildings), registration as a corporation as compared with a family-owned farm, and located in rural area as compared with a peri-urban area. Practice was explained by compliance with all SRHM categories ($p < 0.001$, **Supplementary Table 9**).

Regarding broiler farms, the decision-making process flow from knowledge and attitude to practice was consistent with the other livestock sectors. However, capacity did not remain in the structure model (**Figure 7** and **Supplementary Table 10**). Knowledge was explained by the hygiene manager being a source of hygiene information and satisfaction with the animal health policy of the Japanese government. The categorical variable selection of a hygiene management planner was included because the model did not pass the fitness criteria when it was excluded. Attitude was explained by increased hygiene awareness, vigilance, and understanding of the law after the revision of the SRHM. Practice was explained by the prevention of incursion from fomites, introduced animals, and wildlife, the limitation of access to a farm, and the maintenance of preparedness ($p < 0.001$); however, the prevention of within-farm spread was not a significant factor (coefficient = 0.22, SE = 0.13, $p = 0.076$, **Supplementary Table 10**).

All the final models passed the criteria for the chi-square p-value, TLI, RMSEA, and SRMR (**Supplementary Tables 6–10**).

DISCUSSION

This study aimed to provide a better understanding of the decision-making process in biosecurity practices on Japanese livestock farms in order to develop an extension program to improve biosecurity engagement and compliance. Although the response rates were acceptable for a postal survey, both the rates and the actual number of respondents varied between species and areas. The response rates were higher in Saitama Prefecture than Hokkaido Prefecture for all the species (beef cattle, dairy, pig, and layer farms), and this might be due to the closer relationship with the Saitama Livestock Association, which covered all the sectors. The number of farmers responding was the smallest in layer farms in Hokkaido Prefecture, and this reflected the difficulty of access without farmers' association.

Comparisons between livestock sectors found that the already highly commercialized broiler sector had tighter biosecurity than any other sector. In comparisons between Hokkaido and Saitama Prefectures, the SRHM compliance rate was significantly different only for pig farms (higher in Hokkaido Prefecture), which may be explained by factors such as capacity, including a younger owner and larger numbers of workers and animals on a farm in Hokkaido, or potentially more active engagement by the Hokkaido Pig Farmers' Association (as shown in the SEM results). Hokkaido Prefecture is characterized by intensive food production; among Japan's 47 prefectures, it has the largest numbers of beef and dairy cattle, the fifth largest number of pigs, and ninth largest number of layer birds, compared with Saitama Prefecture, which had the 29th, 21st, 21st, and 16th largest numbers, respectively, according to the 2013 livestock census (43); however, the compliance rates did not differ between the two prefectures in other sectors. Although the age of the owner and farm size were significantly different, the causality

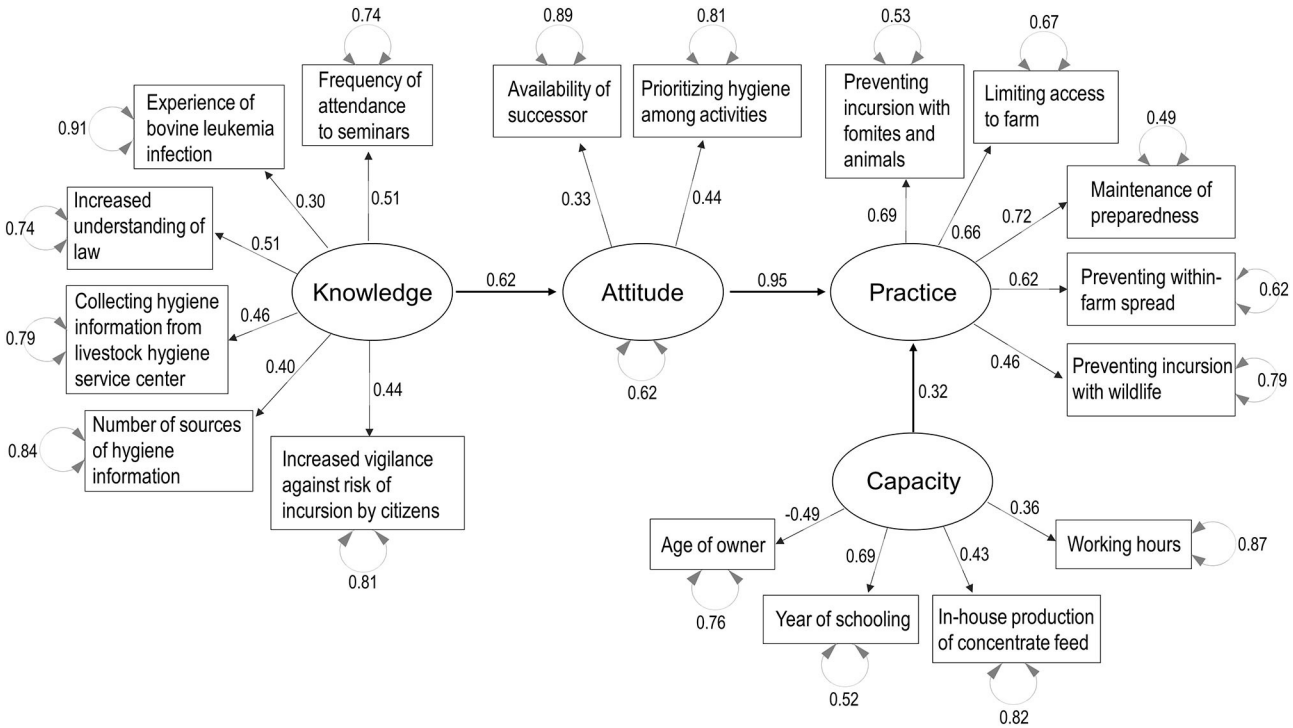

FIGURE 3 | Structural equation modeling path graph for beef cattle farms. The ellipses and rectangles indicate latent and measured variables, respectively, and the values on the arrows are standardized factor loadings. The round arrows connected to latent and measured variables indicate disturbances.

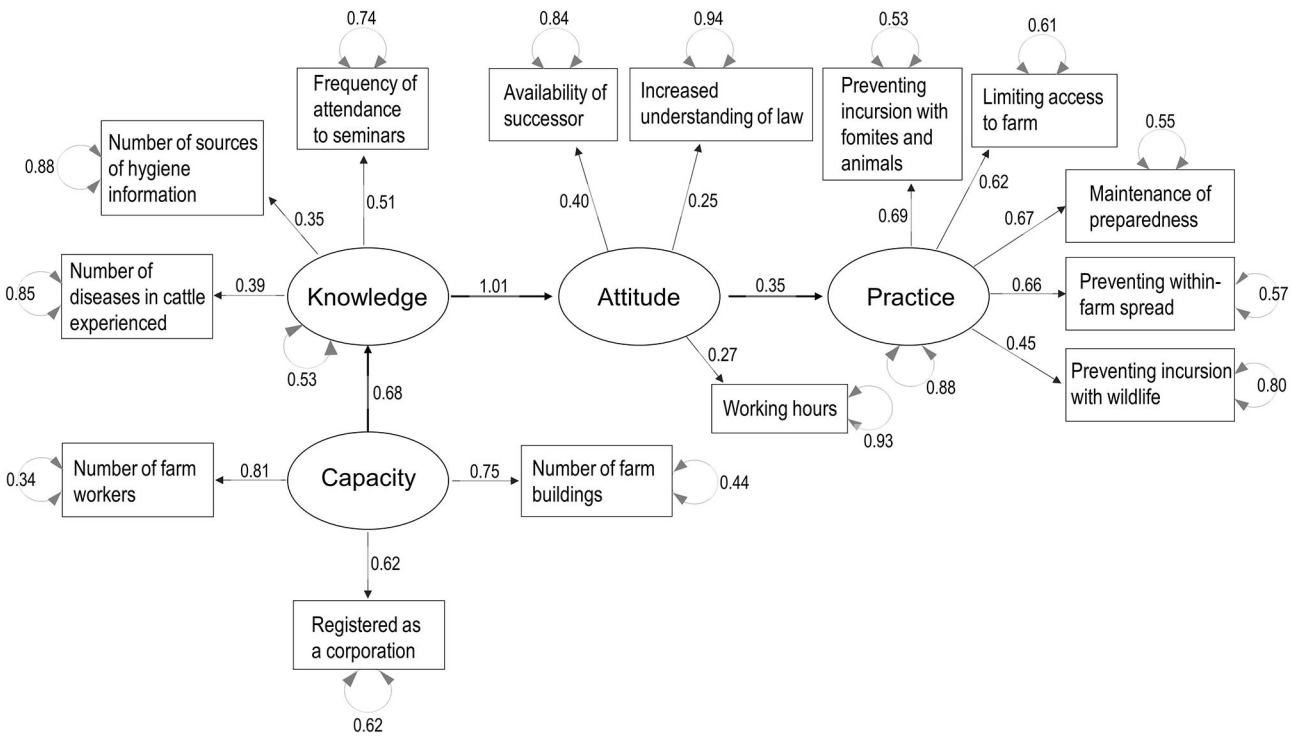

FIGURE 4 | Structural equation modeling path graph for dairy farms. The ellipses and rectangles indicate latent and measured variables, respectively, and the values on the arrows are standardized factor loadings. The round arrows connected to latent and measured variables indicate disturbances.

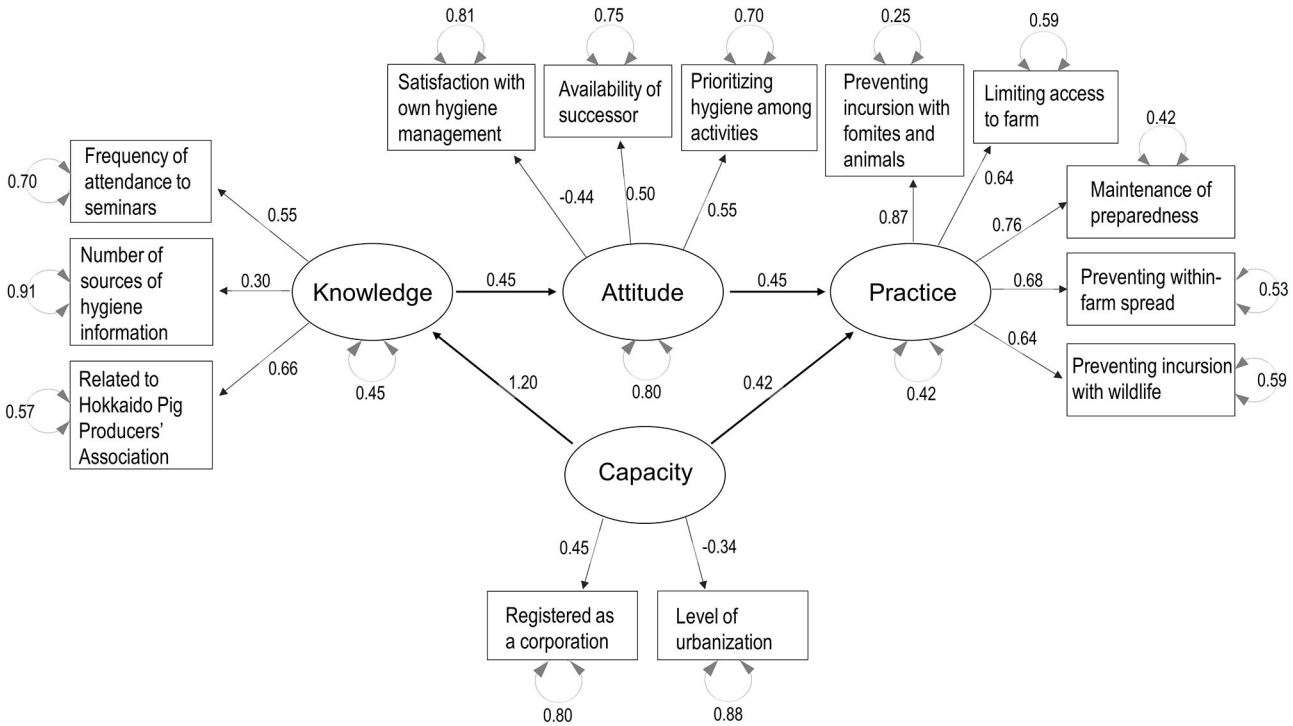

FIGURE 5 | Structural equation modeling path graph for pig farms. The ellipses and rectangles indicate latent and measured variables, respectively, and the values on the arrows are standardized factor loadings. The round arrows connected to latent and measured variables indicate disturbances.

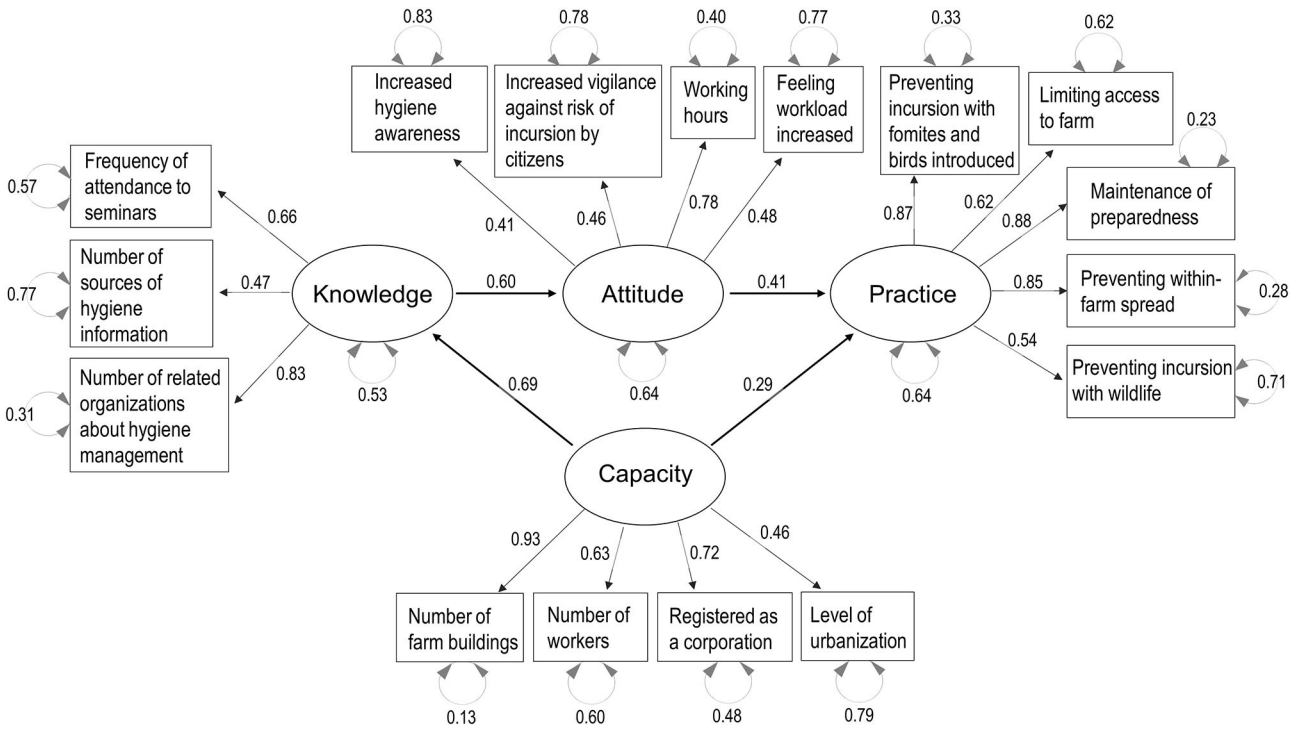

FIGURE 6 | Structural equation modeling path graph for layer farms. The ellipses and rectangles indicate latent and measured variables, respectively, and the values on the arrows are standardized factor loadings. The round arrows connected to latent and measured variables indicate disturbances.

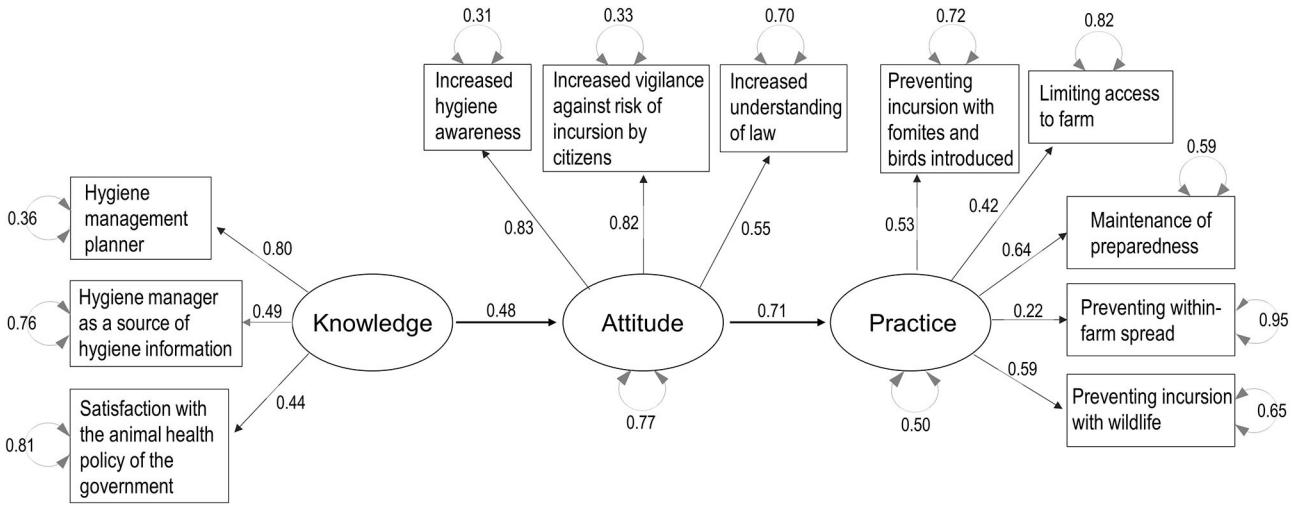

FIGURE 7 | Structural equation modeling path graph for broiler farms. The ellipses and rectangles indicate latent and measured variables, respectively, and the values on the arrows are standardized factor loadings. The round arrows connected to latent and measured variables indicate disturbances.

of the effect of socioeconomic factors on the difference in SRHM compliance rates between the two prefectures cannot be discussed further based on only these comparisons.

Comparisons between SRHM categories in each livestock sector suggested differences in biosecurity strategies between sectors. In beef cattle and dairy farms, while the preparedness for a disease outbreak was well-maintained, practices to prevent disease incursion from fomites and introduced animals, such as through the disinfection of vehicles, hands, and shoes of visitors, and the quarantining of introduced animals, were the most poorly conducted. Beef and dairy farmers complained about a lack of land and facilities to segregate animals for the quarantine period during FGDs. However, beef and dairy farmers should be aware that disease introduction can result in a greater economic burden than preparing an adequate quarantine facility. Intensive swine farms in Hokkaido made substantial efforts to prevent disease incursion from fomites and animals through biosecurity practices such as limiting access to a farm. This may be because farmers are aware of the apparent economic losses that can be incurred due to viral infectious diseases such as PED and porcine reproductive respiratory syndrome (44). Conversely, compliance with the SRHM category of preventing within-farm spread was weak, even for the intensive pig farms in Hokkaido Prefecture, which elucidated the challenges remaining in securing adequate space to reduce animal density and conduct all-in–all-out practices in intensive production systems. Layer farms in both prefectures did not have particularly high or low SRHM compliance categories, except preventing disease incursion from wildlife in Hokkaido, and this might have been due to their smaller sample size compared with other sectors. In Japan, the avian influenza vaccine is not used for layer hens, and thus, outbreaks of HPAI have been occurring in layer farms (14). As shown in **Figure 2**, the high compliance rate for the category of preventing incursion with wildlife in intensified layer farms in Hokkaido Prefecture was comparable to that of broiler farms. For broiler farms, systematic biosecurity strategies to prevent disease incursion from outside the farm are well-observed, but similar to pig farms, challenges remain in preventing within-farm spread, probably because of the intensity of the farming system.

The results of the SEM in all sectors studied suggest that greater knowledge of farm hygiene enhances positive attitudes toward hygiene, which determines the conduct of hygiene practice. In reality, attitude in itself may influence both knowledge uptake and behavior. However, our model assured the significant flow from knowledge *via* attitudes to practice. The KAP model has been applied in many countries for a wide variety of health and animal health problems (32, 45); however, during our FGDs, several farmers in different sectors described that even though they wanted to, they could not tighten biosecurity because farm labor and facilities are limited. We conceptualized that capacity, which is equivalent to self-efficacy in PMT (23) and PBC in the TPB (28), can be a prerequisite to high biosecurity performance. Although we hypothesized that knowledge, attitude, and capacity would be required to conduct biosecurity practices, these four components were not purposively selected in our statistical analyses. Parallel analysis scree plots indicated that four factors could generally explain the variance and covariance for livestock farming, and exploratory factor analyses identified the measured variables in the questionnaires to explain these factors; therefore, the KAP-Capacity decision-making structure was plausible, even statistically. Observation of the latent variable, capacity was useful in understanding the characteristics of the industries. The SEM results for the pig and layer farms suggested that in the default biosecurity decision-making structure, capacity is a driving force to increase the knowledge and feasibility needed to conduct the practices. A structure containing the flow from attitude to capacity, meaning that high motivation can increase capacity, was also tested (not indicated in the results), but the model fit was unacceptable, indicating that

the SEM model cannot explain such a long-term effect of motivation on capacity development. For broiler farms, capacity did not remain in the model, probably because all the broiler producing companies studied had sufficient capacity to maintain high biosecurity. For dairy farms, even with sufficient capacity, the conduct of biosecurity measures may depend on better attitudes toward hygiene, as there was no significant linkage between capacity and practice. However, capacity enhanced knowledge, which would lead to better attitudes toward hygiene, and strengthening capacity would contribute to the higher SRHM compliance. Conversely for beef farms, limited capacity may be a physical obstacle to conduct these practices. Interestingly, the exchangeability of measured variables explaining different latent variables was observed between livestock sectors. For example, increased understanding of the law and vigilance after the revision of the SRHM explained knowledge in beef cattle farms, and attitude in other sectors, which suggests that knowledge, attitude, and even capacity are related and an experience or a response may constitute a personality that determines the strength of the KAP-Capacity process flow.

When planning policies to upgrade farm biosecurity, the provision of knowledge through networking with prefectural livestock hygiene centers, and of hygiene training by these centers, was identified as the most important step. The cost (46) and effectiveness (47) of biosecurity measures influence the motivation of farmers, and the provision of such evidence-based information should increase attitudes toward the SRHM. Policies to strengthen capacity, such as intensification through a shift to a corporate farm and support for smallholder farms, can also be effective. The standardized factor loads in the SEM models are useful for planning detailed intervention strategies and developing monitoring schemes for knowledge, attitude, and capacity; the variables with factor loads distant from zero have a strong relationship with the latent variables, and may be important factors for interventions and monitoring. However, the factor loads can be used as a guidance when developing monitoring schemes, but not as a direct manual or checklist, as each production system and biosecurity program must be addressed individually with practical knowledge. In addition, decision-making process frameworks for protective practices in health and animal health, such as the HBM, PMT, TRA, and TPB, have already described detailed qualitative factors, and thus, it is advisable that veterinary officers at both the national and local government levels understand these theories when carrying out detailed planning for hygiene guidance.

This study had three limitations: (1) the representativeness of farmers, (2) the use of self-administered questionnaires, and (3) the use of binary responses in the analyses. Regarding (1), due to budget constraints and the expected time required for coordination, postal surveys other than for broiler farms were conducted in only two prefectures. Among pig and beef cattle farms, there are reproduction farms, fattening farms, and farrow-to-finisher farms; however, due to the limited numbers of farmers who were reached to participate in this study, all farm types were analyzed together. In Japan, there are small numbers of sheep and goat farmers and those for indigenous breeds of chicken for eggs and meats, and these farmers were excluded from the present study. This study was initially designed to conduct statistics using GLMs, so a sample size calculation for the SEM was not conducted. However, the SEM models had adequate degrees of freedom and passed the model fit criteria, and thus, the results can be considered reliable. Regarding (2), our study used self-administered questionnaires and the results may be affected by information bias. Regarding (3), the default estimation method in SEM, maximum likelihood, assumes multivariate normality in between-variable relationships, and normal distributions of these variables (34). Therefore, in the SEM analyses, the WLSM was used. However, future studies should collect data using a Likert-type scale rather than binary responses. In addition, the postal surveys were conducted between 2013 and 2014, and the SRHM compliance rates in 2018, enumerated by the veterinarians at the prefectural livestock hygiene centers, were much higher (48).

CONCLUSIONS

The study results support the hypothesis that the decision-making process for conducting farm biosecurity starts from the acquisition of good hygiene knowledge, which enhances attitudes, and better attitudes are a trigger to conduct enhanced biosecurity practices. Capacity is an important factor to improve both hygiene knowledge and biosecurity practice. Intensification is a key factor for achieving tighter biosecurity, but well-designed facilities and management plans are needed to ensure the prevention of within-farm disease spread. SEM is a potentially powerful tool for collecting data to support the design of effective and well-targeted intervention programs to improve farm biosecurity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KM planned, coordinated stakeholders, conducted fieldworks, analyses, supervised, and wrote the manuscript. ES and LH conducted analyses including SEM and wrote the manuscript. SH conducted univariable and multivariable analyses on poultry sectors. YN, YT, TW, and SA conducted fieldworks and analyses. HK conducted and supervised univariable and multivariable analyses. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a research project for improving food safety and animal health of the Ministry of Agriculture, Forestry and Fisheries of Japan. SH was supported by Poultry Research Institute, Chittagong Veterinary and Animal Sciences University, Bank Asia Bangladesh, Dutch Bangla Bank, and PHP Group for research internship at Rakuno Gakuen University.

ACKNOWLEDGMENTS

ES participated in this study in the Wageningen University and Research MSc Animal Sciences International Internship. Many thanks go to the Japan Agricultural Cooperatives (JA) and beef cattle farmers in the Engaru, Tokachi, and Yubetsu areas, the Hokkaido Dairy and Livestock Association, dairy farmers in the Engaru and Yubetsu areas, pig farmers belonging to the Hokkaido Pig Farm Producers Association, layer farmers in Chitose, Ebetsu, Eniwa, and Ishikari cities in Hokkaido Prefecture, and beef and dairy cattle, pig, and layer farmers and the Saitama Livestock Association in Saitama Prefecture. We also thank the broiler farmers and Japan Chicken Association for the great facilitation and participation. We cordially thank Dr. Satoko Kubota and Professor Manabu Sawada at Obihiro University of Agriculture and Veterinary Medicine for the critical advice regarding the SEM.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.00614/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor declared a past co-authorship with one of the authors KM.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 September 2020
doi: 10.3389/fvets.2020.00538

Laboratory Diagnosis of a NZ7-like Orf Virus Infection and Pathogen Genetic Characterization, Particularly in the *VEGF* Gene

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Orf is a widespread contagious epithelial viral disease found particularly in most sheep breeding countries in the world. Recently, an orf virus (ORFV) strain OV-HLJ05 was isolated from an outbreak in northeast China. Three genes of interest including ORFV011 (B2L), ORFV059 (F1L), and ORFV132 (VEGF) of ORFV, were recruited to identify and genetically characterize this newly isolated virus. Amino acid (aa) sequence compared with the ORFV references listed in GenBank, both B2L and F1L of OV-HLJ05 showed less microheterogeneity from their references. In contrast, the VEGF gene was included in the NZ7-VEGF like group as previously considered by Mercer in 2002. Unexpectedly, further multiple VEGF matches were made, using 34 published sequences from China and India, resulting in 27 strains of the NZ7 members. Based on Karki's report in 2020, NZ7-VEGF like viruses are emerging more and more frequently in these two countries, damaging the Asian sheep industry. Obvious heterogeneity with the NZ2, insertion of two oligopeptides TATI(L)QVVVAI(L) and SSSS(S) motif were found in the NZ7-like VEGF protein. These VEGFs are divided mainly into two types and a significant increase in the number of hydrogen bonds within the NZ7-like VEGF dimers was observed. The NZ7-like ORFV apparently favors the goat as a host and an emphasis on this in future epidemiological and pathological studies should be considered, focusing on the NZ7-like virus.

Keywords: orf virus, isolation, identification, genetic characterization, VEGF genotype

OPEN ACCESS

Edited by:
Satoshi Sekiguchi,
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *23 May 2020*
Accepted: *10 July 2020*
Published: *17 September 2020*

Citation:
Yu Y, Duan X, Liu Y, Ma J, Song B, Lian Z and Cui Y (2020) Laboratory Diagnosis of a NZ7-like Orf Virus Infection and Pathogen Genetic Characterization, Particularly in the VEGF Gene. Front. Vet. Sci. 7:538. doi: 10.3389/fvets.2020.00538

INTRODUCTION

Orf is an animal pustular dermatitis and an epitheliotrophic contagious disease directly caused by the orf virus (ORFV) with a worldwide distribution (1, 2). This viral skin disease commonly affects sheep, goats, and some other ruminants and has a zoonotic potential in humans who are exposed to a contaminated workplace (3–6). Clinically, orf disease progresses from erythema to macule, papule, and vesicle formation and then from pustules to thick scabs. Severely affected animals may lose weight and become more susceptible to secondary bacterial infections (7). Prolonged infection and increased severity are associated with often severe secondary bacterial infection. More usually, minor staphylococcal infection is a frequent occurrence, but mortality rates can be over 5% in infected herds (8). Higher mortality occurs frequently in lambs or kids during the lactation period due to dehydration and starvation, as the pain and distortion of the lips and mouth reduces

sucking (8, 9). Because orf has serious economic and environmental impacts in most sheep-feeding countries in the world, it is important to characterize the pathogen of any outbreak in breeding livestock. It is also especially important to determine regional ORFV strains, to predict the risk of outbreak in affected developing countries such as India and China, to improve prevention and control management.

ORFV is a prototype member of genus *Parapoxvirus* (PPV) with a G+C content about 64 percent in the genome (10). The virus has a linear double-stranded closed DNA of nearly 150 kbp in genome length containing 130 to 132 putative genes, with 88 genes conserved in PPVs (1, 10). These genes are responsible for viral replication and the composition of the fixed asset in the center of the genome, while two highly variable regions are located in the closed terminal ends of the viral genome, which encode proteins required for viral invasion (11) or immune evasion (12).

At present there are abundant ORFV isolate sequences published in GenBank, with some full length genome data available, with six of them extensively researched previously such as ORFV-NZ2 (10, 13), ORFV-NZ7 (14, 15), ORFV-SA00, and ORFV-IA82 (16), ORFV-D_{1701} (11), a human biopsy-derived virus ORFV-B029 (partial genome) (17) and eight new ORFV strains from China including ORFV-NA1/11 (18); ORFV-GO, -NP, -YX, and -SJ1 (19); NA17 (*Accession number*:*MG674916*) (20), Shanxi (*Accession number*:*AEN14425*) and Fujian-XP (*Accession number*:*AIZ05258*). These strains may provide many references for evaluating an emerging pathogen from any orf epidemic.

For more accurate diagnosis of orf in the lab, both conventional PCR and real-time PCR methods are used for higher specificity and sensitivity in the detection of viral ORFV pathogens. These techniques have been developed based on the major membrane glycoprotein gene *B2L* (ORFV011) (6, 21, 22) or on the DNA polymerase gene (23). Generally, *B2L* with conserved quality in different PPV species is used as a common and precise marker for examining a virus with its genetic stability, such as ORFV, bovine popular stomatitis virus (BPSV), pseudocowpox virus (PCPV) and parapoxvirus of red deer in New Zealand (PVNZ) (21). Parapox virus can therefore be confirmed by the *B2L* gene on a molecular level in the laboratory, because GenBank can provide abundant *B2L* reference information for researchers (24). Though the *B2L* gene is adopted for the genetic phylogenetic investigation of ORFV (25–28), *B2L* gene data alone is not sufficient to confirm a viral species.

The secondary gene of interest for pathogen investigation is the *F1L* gene (ORFV059) that encodes an envelope protein to exploit a subtle interaction between virus and host, then initiates viral invasion by binding to heparan-sulfate sensors outside the host cells (29). *The* F1L protein, as the main immunogenic protein of ORFV, is transcribed in the mid-late stage of the viral infection period and can bind to glycosaminoglycan (GAG) on the mammalian cell (30). Several functional regional and amino acid motifs are also found in F1L proteins, including a proline-rich region (PRR) and KGD motif, unique motifs in ORFV, and some conservative motifs such as GAG, D/ExD, and Cx3C among the *Poxviridae* family, which are apparent in sequence alignment of ORFVs (31).

The ORFV132 gene has been of interest because it encodes a vascular endothelial growth factor (VEGF) of ORFV which has a direct responsibility for the extensive vascular hyperplastic lesions (32). As a result, the ORFV132 gene is expressed early during infection of ORFV (15); but it has not been found in other poxviruses. The *VEGF* gene therefore plays a unique role in virulence analysis, although it is not the only virulence factor that has been identified. The *VEGF* genes among PPVs show numerous variants which can share only 41 to 61% amino acid sequence identity (16). For ORFVs, two genotype groups were classed by the NZ2- and NZ7-VEGF like genes which show little DNA homology to each other, whereas the flanking sequences are over 98% homologous (15). The reported sequence variations might reflect the genetic drift of the *VEGF* gene although the rate of drift seems greater than generally seen in poxvirus genes (33). More recently, Karki et al., reported that the majority of Indian ORFV isolates showed 78.4 to 99.3% amino acid identity with each other in the VEGF gene, even like the NZ7-like VEGF (34). Given that different ORFV isolates from the world show these two genotypes in VEGF, this study places the emphasis on the regional distribution of VEGF genotype, to explain its genetic characteristics and clinical features related to the environment and species.

This paper reports on a new ORFV isolate from the northeast of China. Genetic studies of three genes mentioned above and the VEGF molecular structure observation with high resolution have been performed following the virological identification.

MATERIALS AND METHODS

Clinical Case and Virus Isolation

During an outbreak in the autumn of 2017, a local farmer reported that five young Boer goats were found to be affected by a contagious skin disease with obvious lesions in the oral cavity or lips, but lesion material had only been collected from a 6-month-old kid, that died in an isolation area.

In this flock of over 200 Boer goats, there were no other domestic mammals. The sheep pen was simple, with only guardrails and a roof and sanitary conditions were poor. It was speculated that the outbreak may have been related to stock bought in from other provinces in China several months ago. According to the farmer, none of the animals in this flock had been given orf vaccine before this outbreak but they had been treated with externally applied agents such as gentian violet. The majority of affected animals had recovered spontaneously except the single death.

For virus isolation, Madin-Darby bovine kidney (MDBK) and human keratinocyte cell line (HaCaT) cells (both of these cells are cryopreserved in liquid nitrogen in our laboratory) were cultured separately in DMEM containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2 (35). As per Yu's protocol, a confluent monolayer of MDBK cells were inoculated with some viral supernatant (36). When 70 to 80% cytopathic effect (CPE) was reached, the cells were harvested followed by freezing at

−80°C. The virions were further purified by sucrose gradient ultra-centrifugation. A major virus band was obtained after centrifugation of virus infected MDBK cells in the 32–36% sucrose gradient. Electron microscopy (EM) investigation was completed by negative staining.

EM for Ultrastructural Analyses

The viral samples were assayed immediately as described by Yu et al. (36). Lead citrate was used to make the contrast background, to distinguish ORFV virion with outline geometrical characters and some surface structures.

Immunofluorescence Microscopy

Cells were fixed with 4% paraformaldehyde for 20 min, then incubated in PBS containing 0.2% Triton-X100 for permeabilizing. After three washes in PBS, the cells were incubated with 1% bovine serum albumin (BSA) solution for 30 min. The fixed cells were incubated with 2E4 monoclonal antibody (mAb) (anti-B2L) (hybridoma cells of 2E4 mAb are cryopreserved in liquid nitrogen in our laboratory) for 1 h at 37°C. After three washes with PBS, secondary antibodies were introduced to bind 2E4 mAb at a 1:500 dilution in PBS for 30 min. Anti-mouse Ig conjugated with fluorescein isothiocyanate (FITC) (Sigma-Aldrich) was used as the secondary antibody and images were taken using a Leica fluorescence microscope.

DNA Clone, Sequencing, and Phylogenetic Analysis

Viral DNA was prepared based on commercial kits protocols for polymerase chain reaction (PCR) amplification. Primers used in this study involving in B2L, F1L, and VEGF genes, were designed referencing the ORFV-NZ2 strain (*Accession number: DQ184476*). In addition, the alternative primers of VEGF gene were designed according to the ORFV-NZ7 strain (*Accession number: S67522*) (**Table 1**). After DNA amplification and purification, the target genes were each inserted into PMD18T vectors and the recombinant plasmids of positive clone were sent to Sangon Biological Engineering Technology and Services Co. Ltd. (China), for sequencing. For genetic relationship analysis of B2L, F1L, and VEGF genes among some reference strains available on GenBank at the amino acid (aa) level, a series of aa composition comparisons of the isolates were conducted using the DNAStar program (DNAStar, Inc. USA). All different source sequences in the world were included in **Table 2** and molecular phylogeny and the genetic relationship of this ORFV strain with others were calculated as referenced by Yu et al. (36).

Homologous Modeling Analyzes on the Present VEGFs

Prediction of the three dimensional structure of the VEGF-variants of ORFVs was modeled using SWISS-MODEL online program (*http://swissmodel.expasy.org)*. The structure of the VEGF-variants, including protein subunits A and B, chain A and chain B, were viewed and aligned using the *UCSF Chimera version 1.1.* where the function of Iterative Magic Fit was used for energy minimization and the alignments were manually optimized. Ramachandran plots for the viral VEGF models were compared to determine if the viral models contained residues that did not conform to acceptable ϕ and/or ψ angles (33).

RESULTS

A Case of Orf

For descriptive epidemiology of this outbreak in Daqing city of Heilongjiang province of China (**Figure 1A**), this case was briefly reported in the materials and methods. Affected animals had visible scars from clinical lesions of contagious ecthyma in their lips and angulus oris. The subject kid had developed severe anabrosic lesions in its mouth region, prior to death (**Figure 1B**). Clinical material such as scabs were gathered from the dead Boer kid for laboratory virus isolation.

ORFV Isolation and Identification

In the laboratory, a sterile suspension was prepared using the clinical material to inoculate MDBK and HaCaT cell monolayers, until the CPE was observed on day 3 or 4. The CPE of infected cells was obvious by their appearance and in contrast, there was no pathological change in the mock infected cells (**Figure 1C**).

The PPV virion with an ovoid shape and spiral crisscross pattern was easily identified by morphological features using EM (**Figure 1D**). Virus particles in ultrathin sections were observed in the cytoplasm of infected cells at 72 h post inoculation (pi) (**Figure 1D**).

Immunofluorescence was used to determine the causative agent responsible for CPE, with the virus recognized by 2E4 mAb during cell infection. The images were taken using a Leica fluorescence microscope (**Figure 1E**).

The target genes in viral DNA samples were detected successfully by PCR. Although all of the target bands appeared, before that there was an interlude during this period. Initially, 3 pairs of synthetic oligonucleotide primers namely B2L-F/R, F1L-F/R, and VEGF-F/R designed according to the NZ2 strain were used for PCRs. Both B2L and F1L were successful but absence of VEGF band was shown. It is not surprising that application of PCR primers (VEGF-F'/R') designed according to the NZ7 strain allows us to detect the VEGF gene. Together, these three bands were corresponding to our expectation with the full-length genes as 1,137, 1,029, and 447 bp, respectively, in a 1.0% agarose gel electrophoresis (**Figure 1F**). The PCR products were purified and cloned for direct sequencing, and sequence analysis confirmed this ORFV isolate, which was named OV-HLJ05.

Genetic Characterization of OV-HLJ05

The three genes *B2L*, *F1L,* and *VEGF* were sequenced to analyze the genetic characterization of OV-HLJ05. Using the Jotun Hein Method in MegAlign program (DNAStar, Inc. USA), a rough outline of the genetic factors of the virus was confirmed.

For the *B2L* gene, a total of twenty-four aa sequences from different sources in the world, including the NZ2 strain (*Accession number*: *AAA50479*, *ABA00527*); OV-IA82 (*AAR98106*); OV-SA00(*AAR98236*); OV-D1701 (*ADY76795*); OV-B029 (*AHH34200*); OV-HLJ04 (*KU523790*); and OV-HLJ05 (*MK317955*), were used for alignment in this study. These B2Ls

TABLE 1 | PCR primers designed referencing to the popular strains of ORFV.

Name	Nucleotide	Endonuclease	Reference
B2L-F	CG*GGATCC*ATGTGGCCGTTCTCCTCCATC	*BamH* I	OV-NZ2(*DQ184476*)
B2L-R	CCC*AAGCTT*TTAATTTATTGGCTTGCAGAACTC	*Hind* III	OV-NZ2(*DQ184476*)
F1L-F	CG*GAATCC*ATGGATCCAC CCGAAATCACG	*EcoR* I	OV-NZ2(*DQ184476*)
F1L-R	CCC*AAGCTT*TCACACGATGGCCGTGACC	*Hind* III	OV-NZ2(*DQ184476*)
VEGF-F	CGC*GGATCC*ATGAAGTTGCTCGTCGGCATA	*BamH* I	OV-NZ2(*DQ184476*)
VEGF-R	CCC*AAGCTT*CTAGCGGCGTCTTCTGGGCG	*Hind* III	OV-NZ2(*DQ184476*)
VEGF-F'	GC*GGATCC*ATGAAGTTAACAGCTACCATA	*BamH* I	OV-NZ7(*S67522*)
VEGF-R'	CCC*AAGCTT*TCGTCTAGGTTCCCTAGT	*Hind* III	OV-NZ7(*S67522*)

TABLE 2 | Part of VEGF genes published in GenBank recent years were used in this study.

No.	Name of strain or isolate	Country	Host	Collection date	GenBank Accession No.	Target gene
1	OV-SA00▲	USA	Goat	2004	AY386264	VEGF
2	OV-NZ2▲	New Zealand	Sheep	2006	DQ184476	VEGF
3	OV-NZ7▲	New Zealand	Sheep	2016	S67522	VEGF
4	ORFV Mukteswar/09	India	Sheep	2010	GU139358	VEGF
5	Cam/09	India	Camel	2010	GU460373	VEGF
6	ORFV/Mukteswar/59/05/Goat/P51	India	Goat	2018	MF414681	VEGF
7	ORFV/Mukteswar/59/05/Goat/P6	India	Goat	2018	MF414682	VEGF
8	ORFV/Meghalaya/SP45/Goat/2003	India	Goat	2018	MF414683	VEGF
9	ORFV/Shahjahanpur/82/Goat/2004	India	Goat	2018	MF414684	VEGF
10	ORFV/Jalandhar/SP41/Goat/2007	India	Goat	2018	MF414685	VEGF
11	ORFV/Bangalore/89/05/Goat	India	Goat	2018	MF414686	VEGF
12	ORFV/Hyderabad/25/Sheep/2006	India	Sheep	2018	MF414687	VEGF
13	ORFV/Gujarat/SP26/Goat/2006	India	Goat	2018	MF414688	VEGF
14	ORFV/Assam/LK/Goat/2014	India	Goat	2018	MF414689	VEGF
15	ORFV/Bhopal/Goat	India	Goat	2018	MF414690	VEGF
16	NP	China	Goat	2015	KP010355	VEGF
17	NA17	China	Goat	2015	MG674916	VEGF
18	Shanxi	China	Ovis aries	2016	AEN14425	VEGF
19	Fujian-XP	China	Goat	2016	AIZ05258	VEGF
20	NA1/11	China	Sheep	2014	JQ663432	VEGF
21	Xinjiang1	China	Goat	2013	KF666562	VEGF
22	SY17	China	Sheep	2018	MG712417	VEGF
23	OV-HN3/12	China	Sheep	2018	KY053526	VEGF
24	Shihezi2/SHZ2	China	Goat	2013	KF726849	VEGF
25	Shihezi3/SHZ3	China	Goat	2013	KF726850	VEGF
26	DG	China	Goat	2016	KM675376	VEGF
27	YX	China	Goat	2016	KM675382	VEGF
28	XD	China	Goat	2016	KM675377	VEGF
29	FQ	China	Goat	2016	KM675383	VEGF
30	GT	China	Goat	2016	KM675384	VEGF
31	SL	China	Goat	2016	KM675385	VEGF
32	DS	China	Goat	2016	KM675386	VEGF
33	GS	China	Goat	2016	KM675387	VEGF
34	SJ2	China	Goat	2016	KM675388	VEGF
35	GO	China	Goat	2016	KM675380	VEGF
36	SJ1	China	Goat	2016	KM675381	VEGF
37	OV-HLJ05★	China	Goat	2019	MK317956	VEGF

Black pentastar means the orf virus isolate in this paper; Black triangle means the important reference strain.

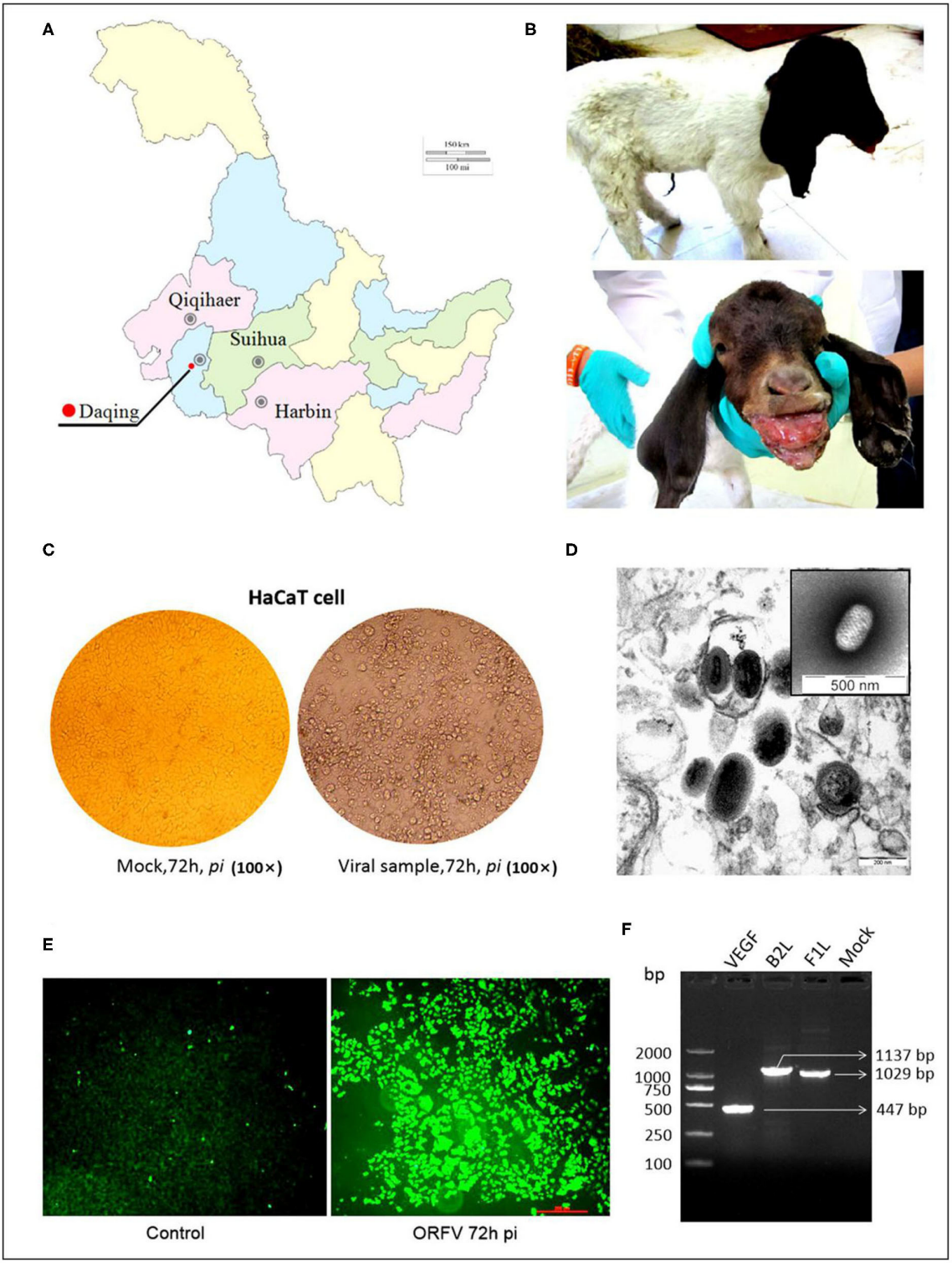

FIGURE 1 | ORFV isolation from an orf outbreak in the northeast of China. **(A)** Picture indicates the geographical location of the outbreak in China, Sep 2017. **(B)** Typical clinical lesions of orf in a Boer goat kid. The severe tumid lesions in his mouth region showed more pejorative anabrosis nidus. **(C)** Cytopathic effect on HaCaT cells infected by ORFV sample. Mock-infected HaCaT cells appeared an ordered fashion after 72 h, while HaCaT cells infected with the supernatants became ragged, appearing rounded and pyknotic, with retraction of the cell membranes from surrounding cells at 72 h *pi* (×200). **(D)** The virions in MDBK cell revealed the typical ovoid shape when observed by electron microscopy. **(E)** The result of the immunofluorescence assay. Anti-B2L monoclonal antibody (mAb) (2E4, 1:200) was used as the primary antibody. **(F)** Amplification of *B2L* gene, *F1L* gene, and *VEGF* gene. Lane 1:DL2000 DNA Marker (bp); Lane 2: *VEGF* gene (447 bp); Lane 3: *B2L* gene (1137 bp); Lane 4: *F1L* gene (1029 bp); Lane 5: Mock.

were employed from the GenBank datasets for aa sequence multiple comparison and revealed that all B2Ls were like each other, sharing 93.1 to 98.4% amino acid identity (**Figure 2A**). It is worth noting that OV-HLJ05 shared 98.4% aa identity with the SA00 strain (*AY386264*) in B2L protein and 97.4% identity with NZ2, so it should be closely related to the SA00 strain genetically.

On comparison of F1L homologs in ORFVs, a total of 18 aa sequences, including NZ2 (*Accession number*: *ABA00576*), IA82 (*AAR98154*), SA00 (*AAR98284*), B029 (*AHH34248*), Chinese OV-HLJ05 (*MK317957*). and OV-HLJ04 (*MK317958*), FJ-MH2015(*KU199840*), SDLC(*AKL79702*), NP (*AKU76812*), SJ1(*AKU76936*), YX (*AKU76548*), GDQY(*AIY55506*), Jilin(*FJ808075*), Nongan (*JQ271535*), NA1-11(*AHZ33756*), GO (*AKU76680*), Hubei (*KJ619840*), and Xinjiang (*KC291656*), were aligned in batches. This study found that the *F1L* gene was highly conserved as well within the ORFV group, except for the proline rich region with a repetitive character in the N-terminal of F1L protein. In addition, several highly conserved motifs mentioned by Scagliarini et al. (37) and Yogisharadhya et al. (31) such as the GAG motif, KGD (Lys-Gly-Asp) motif, KTR motif, D/ExD motif and a Cx3C motif of interest all remained stable in their basic amino acid composition (**Figure 2B**).

Taken together, the *B2L* gene and the *F1L* gene in all isolates from around the world were relatively conservative in viral genomes.

For the *VEGF* gene, it was also found during the multiple alignment that two clustering groups known as the NZ2- and the NZ7-VEGF like isolates between aa sequences were used in this study. The isolates involved in comparison were ORFV-NZ2 (*DQ184476*), SA00 (*AY386264*), NZ7 (*S67522*), IA82 (*AY386263*), D1701 (*AF106020*), B029 (*KF837136*), NA1/11 (*JQ663432*), NA17(*MG674916*), Shanxi (*AEN14425*), Fujian-XP (*AIZ05258*), GO (*KM675380*), NP (*KM576379*), YX (*KM675382*), and SJ1(*KM675381*). Among them, OV-HLJ05 (*MK317956*) was shown to share 100% identity with the NA17, Shanxi and Fujian-XP strains, which all came from the Jilin, Shanxi and Fujian provinces in China, 94.6% identity with the SA00 strain and 89.2% identity with the NZ7 strain (**Figure 3A**). The phylogenetic tree showed that the OV-HLJ05 has a highly homologous relationship to SA00 compared with NZ7 despite coming from the same group (**Figure 3B**). In contrast, the inconsistent amino acid residues in the NZ7 strain occurred about 16 times, compared with eight times in the SA00 strain (data not shown). According to the current alignments, these two groups formed immediately by the program possessed obvious differences in VEGF sequence length between each other. The NZ7-VEGF like group had approximately 150 more residues, but the NZ2-VEGF like group had about 130 residues. Those additional residues in the NZ7-VEGF like individuals were shown as a TATI(L)QVVVAI(L) motif (IR1) and a SSSSS or SSSS motif (IR2) and they occupied two positions front and back in the protein respectively (**Figure 5A**). Insertion of TATI(L)QVVVAI(L) made the first two cysteine positions move back, but the other eight cysteine residue positions have not been impaired by insertion mutation. Residue substitution mutation on the first cysteine residue position, meant that cysteine was replaced by glycine in some NZ2-VEGF like strains including NZ2, D1701, B029, and IA82 (**Figure 5A**), but no mutation on this position was observed in the NZ7-VEGF like individuals. In the NZ7-VEGF like individuals like OV-HLJ05, the serine level has been raised because of the additional SSSSS or SSSS motif (**Figure 5**).

In aa composition, the 37 VEGFs published in GenBank including NZ2, NZ7, SA00, and 22 isolates from China and 12 isolates from India showed two separate parts in the phylogenetic tree map (**Table 2**). The percentage of the NZ7-like VEGFs have about 79.4% of all sequences derived from China and India, while only 20.6% of sequences have the NZ2-like VEGFs (**Figure 4**).

Structural Modeling Implied Heterogeneity Between the Current VEGFs

The predicted structures of the VEGF-variants of ORFVs were determined by comparison to the solved crystal structure of subunits A and B of 2gnn.1 (orf Virus NZ2 Variant of VEGF-E in SWISS-MODEL). Homologous modeling showed maximum heterogeneity at loop three (**Figure 5C**) and the contact points of chain A and chain B (**Figure 6**) between the VEGFs. There was more heterogeneity between the NZ2-like VEGFs and the NZ7-like VEGFs, but the essential structure was conservative (**Figure 5**). In addition, the residues involved in dimerization of chain A and chain B of VEGF protein monomer were from $ST^{34}NE\mathbf{W}^{\mathbf{37}}MRT\mathbf{L}^{\mathbf{41}}DK^{43}S^{44}G^{45}$ of chain B in the OV-HLJ05 strain, compared with $NT^{24}KG\mathbf{W}^{\mathbf{27}}SEV\mathbf{L}^{\mathbf{31}}K^{32}G^{33}S^{34}$ in the NZ2 strain. Among these two motifs, the "Txx**W**xxx**L**(x)KSG (GS)" motif was a relatively conservative pattern that was probably responsible for dimerization of chain A and chain B and the motifs **W**xxx**L**. Another two motifs, **T**xx**R** in NZ2 and **T**xx**Q** in NZ7, were responsible for binding VEGFR-2 according to Mercer's report, with x representing any amino acid residue. For the dimerization, H-bonds between chain A and chain B, particularly at the binding site, were labeled in different lengths ranging from 2.7 to 3.4 Å (**Figure 6**).

DISCUSSION

Orf epidemics are common in the world particularly in developing countries such as China, India, and South Africa but this disease has been largely ignored, due to relatively low mortality rates, or spontaneous recovery (38). In this study, a small outbreak of orf involving a suburban livestock farm in northeast China was investigated, where the Boer goat kid presented for evaluation died. The ORFV was suspected to be responsible for this outbreak and it was confirmed and identified in our laboratory.

As a primary subject of study, the B2L protein of ORFV is a F13L homolog of Vaccinia virus (VACV). The F13L is purportedly required for the efficient formation of enveloped VACV virions (39) and contains the variant HKD (His-Lys-Asp) motif of phospholipases and phospholipid synthases (40, 41), leading to a report of associated lipase activity (42). The B2L protein owns the same HKD variant as in F13L which has an NKD pattern, where the His in HKD is substituted by the Asn both in F13L and B2L, while the detailed biochemical lipase function of B2L protein remains unclear. The *B2L* gene is used

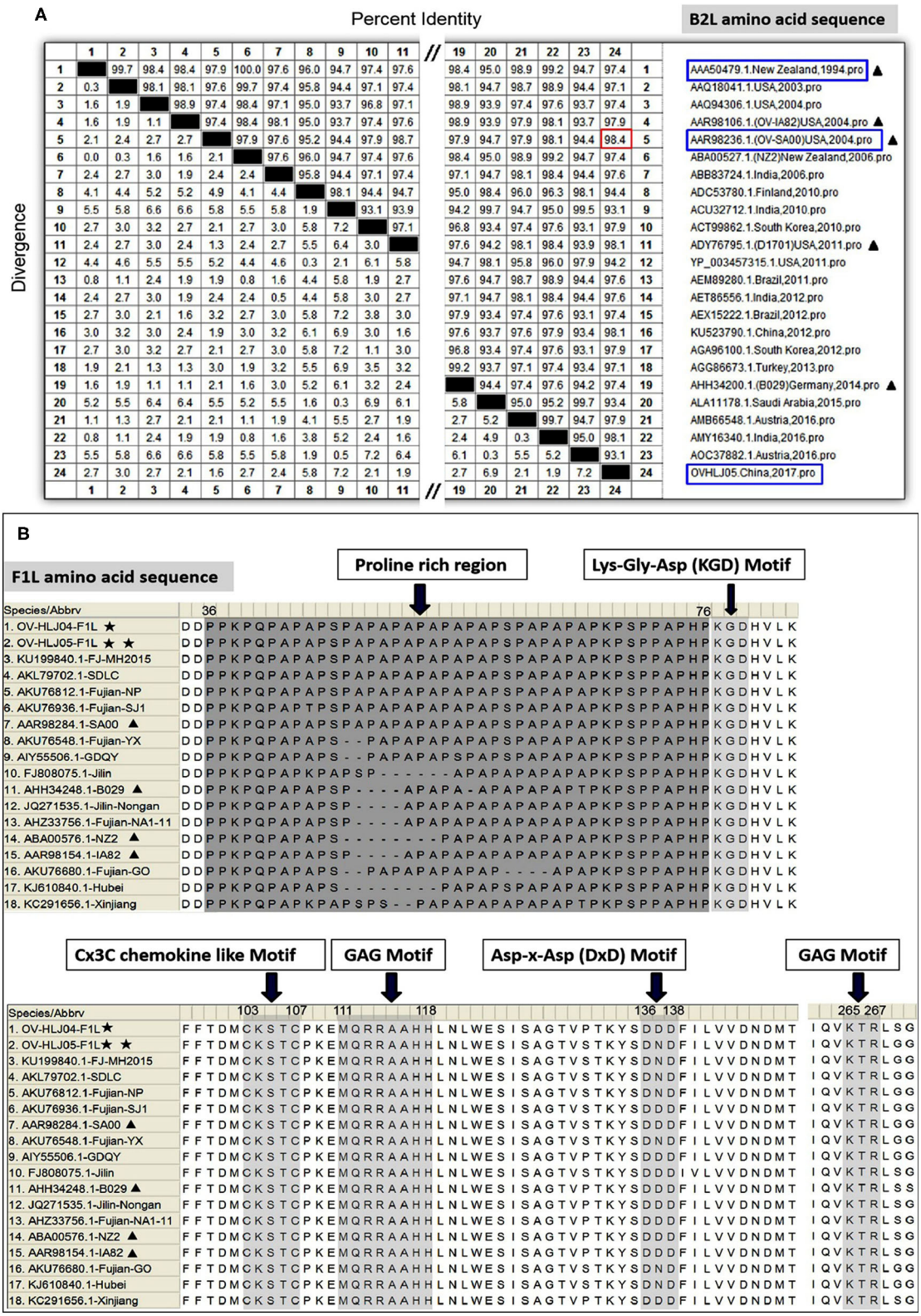

FIGURE 2 | Multiple alignments on the *B2L* genes and on the F1L genes. **(A)** Twenty-four *B2L* gene sequences from the world were used in multiple comparisons and their aa identity was displayed in **(A)**. Black triangles represent five important isolates published previously. Blue boxes represent the isolates which this study investigated. Red boxes represent the maximum among these subjects. **(B)** Fourteen *F1L* gene sequences from China together with NZ2, IA82, SA00, and B029, were used in multiple alignment and the obvious difference was showed at the proline rich regions of N-terminal. Other motifs were essentially conservative (31). One black pentastar indicates the orf virus isolate of previous; Two-black-pentastar indicates the orf virus isolate in this paper.

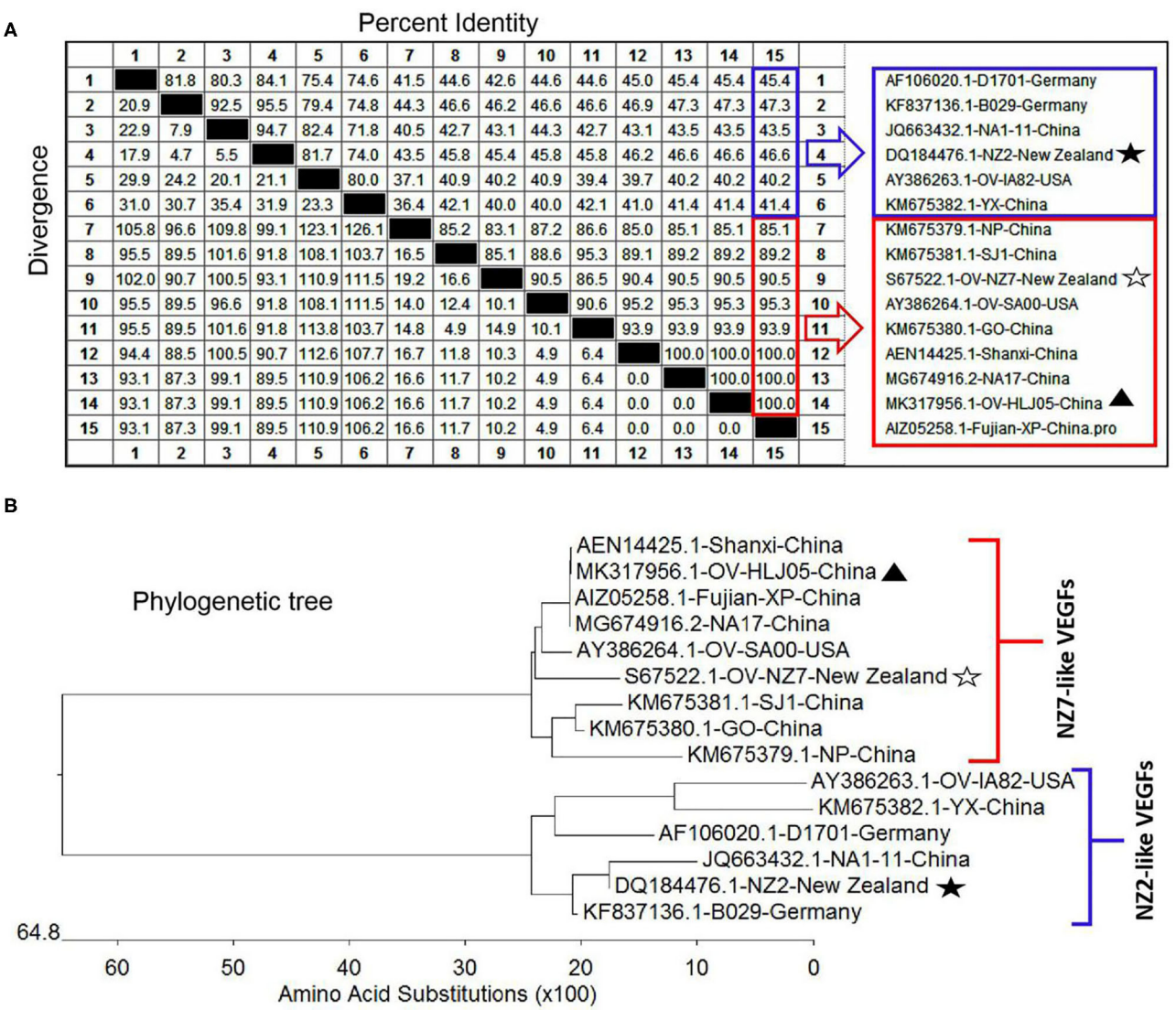

FIGURE 3 | Comparison of different VEGFs with the OV-HLJ05 strain and related sequences published in GenBank. Solid black pentacle denotes the NZ2 strain; hollow black pentacle denotes the NZ7 strain; solid black triangle denotes the OV-HLJ05 strain in this study. **(A)** An alignment of the deduced amino acid sequences of the VEGFs from various sources was generated using MegAlign and Clustal W Method. The aligned sequences are assembled in blue box (low identity to OV-HLJ05) or red box (high identity to OV-HLJ05). **(B)** Predicted evolutionary relationships between the OV-HLJ05 strain and the references in the world mentioned above. They include OV-HLJ05, divided into two groups as the NZ2-like VEGFs (blue half-lattice frame) or the NZ7-like VEGFs (red half-lattice frame).

for phylogenetic analysis of ORFV (25–28) and PCR by *B2L* has been described previously as an available tool to amplify target DNAs within the PPV genus (21). However, merely to investigate molecular characterization of ORFV isolate, information of the *B2L* gene combined with the *F1L* and *VEGF* genes is necessary for understanding of the virus. Comparative analysis resulted in OV-HLJ05 owning an extensive homologous relationship with the SA00 and the NZ7 rather than the NZ2 in these three genes. Unsurprisingly, OV-HLJ05 has some divergence from other candidates in the *B2L* gene product constitution (see **Figure 2A**), but this was not enough to affect their conservative nature due to their over 93% identity.

Another evidence of stability is for the F1L protein. Beside the proline rich regions, during the sequence alignment, the study found the functional motifs, which was mentioned by Yogisharadhya's team (31). The similar quality suggested that the F1L was maintaining its multiple roles with intra- and extra-cellular activity during ORFV infection and the largest heterogeneity between these F1L targets was found to be only located in the proline-rich regions. This event was initiated by the natural deletion or loss of individual proline residues in viral generation, but in fact it hardly impairs F1L's functions (37).

Previously, all VEGFs were shown as only 41 to 61% aa sequence identity among PPVs by Delhon et al. (16). In ORFV,

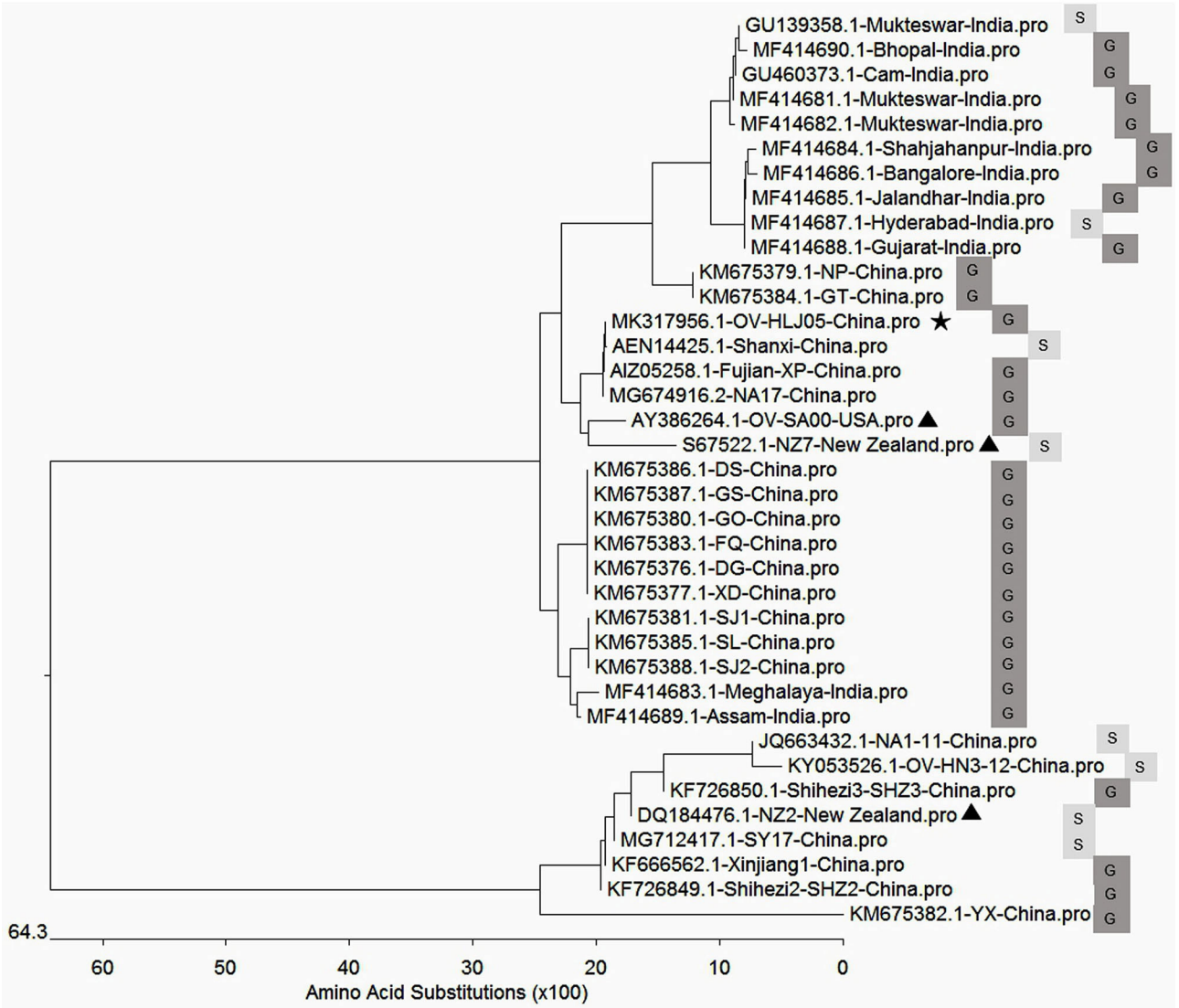

FIGURE 4 | Phylogenetic relationship of VEGFs encoded by Orf virus between the reference strains worldwide and the isolates from China and India. The black triangle indicates reference strains; the black pentacle symbol indicates the OV-HLJ05 strain in this study. Letter S indicates sheep host, and the letter G indicates goat host.

genetic consistency presents a polarized distribution, therefore, two typical genotype groups also known as the NZ2- and the NZ7-like VEGFs were presented by Mercer (33). The VEGFs were used as representatives of the diversity analysis within the ORFVs even though this study did not know the scale and distributed situation of the two groups (14, 15, 33). Genetically, it was possible that the NZ7-like VEGF was acquired by ORFV independently of the NZ2 acquisition event and from a different source. The virus with NZ7 VEGF genotype can be found around the world, particularly in India as described by Karki et al. (34) and in China as shown by the results of this study, so the virus may have been selected by adaptation or from host species from distinct environments. Both these VEGF-like ORFVs can stabilize the inheritance of the genome, with the remaining critical issues studied by epidemiology and pathology.

The data from this study suggested that OV-HLJ05 strain was closer to the SA00 strain at the aa level particularly in the *VEFG* product (**Figures 3A,B**). Except for three Chinese isolates including NA17, Shanxi and Fujian-XP, OV-HLJ05 was found to share 95.3% identity of VEGF with the SA00 strain and to exceed 90.5% identity with the NZ7 (**Figure 3A**). There was increasing evidence that clinical symptoms of the affected kid in this outbreak were similar to reports from Guo et al. (26) with the SA00 strain affecting North American and Texan Boer goat flocks, Hosamani et al. (43), involving the Muk5905 strain in a Mukteswar goat in India, Charles (44) with TZ/BB/13 strain in a Tanzanian goat and Zhang et al. (45) who reported on three strains SDLC, SDTA, and SDJN identified from a Shandong goat in East China, as relevant examples. None of the quoted studies provided any VEGF information.

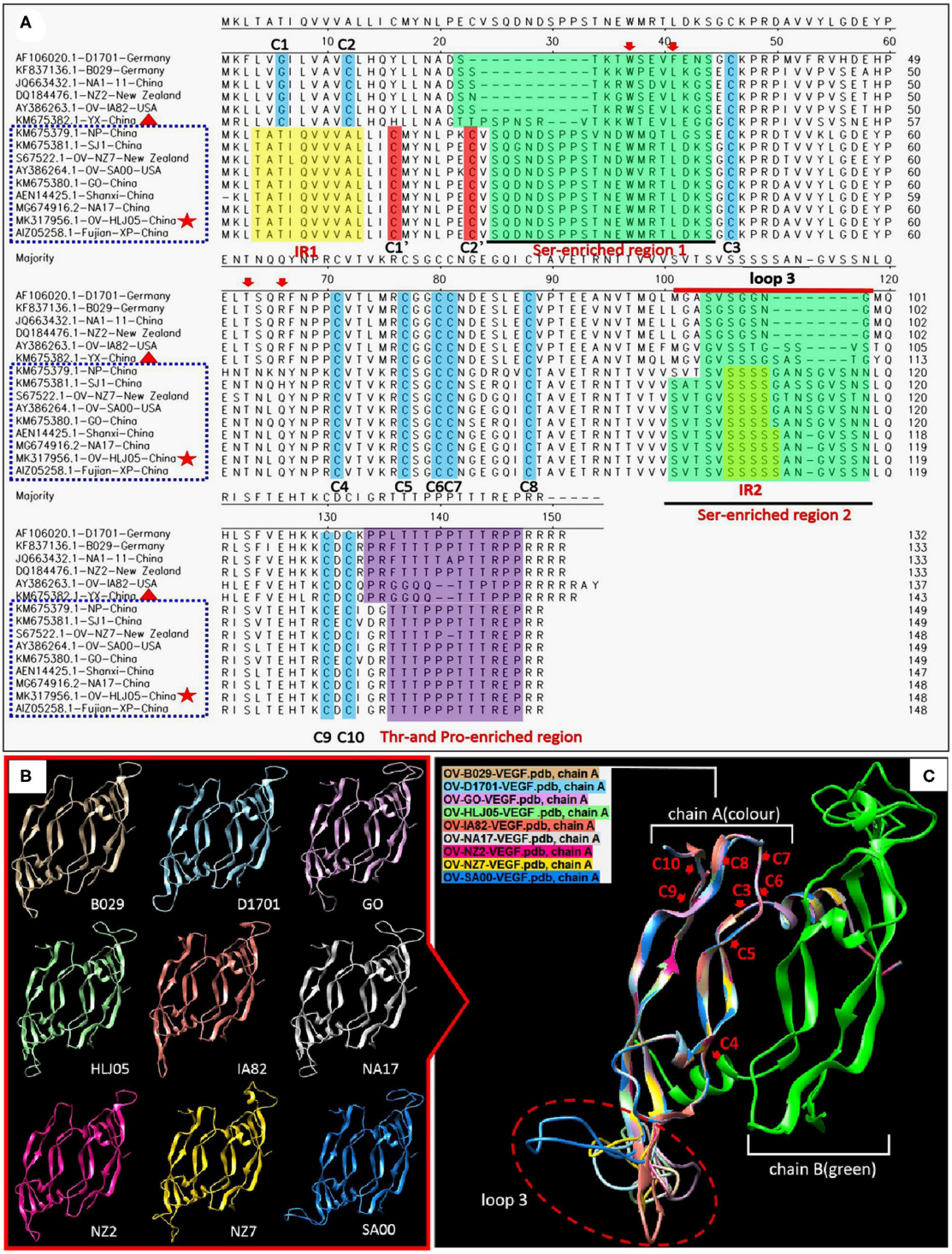

FIGURE 5 | Comparison of amino acid levels between primary and secondary structures of VEGFs. **(A)** Alignment in primary structure of VEGFs. VEGF references of the whole length genome published from GenBank were employed to reflect the structural homology with OV-HLJ05. Important residues matching the consensus of the alignment of the viral VEGFs are shaded in various colors. The two green regions indicate Ser-enriched region I and II; the two yellow regions indicate "insertion mutation" [IRI:TATI(L)QVVVAI(L) motif and IRII:SSSSS or SSSS motif]; the Thr- and Pro-enriched region is shaded in purple (potential *O*-linked glycosylation sites) (33); two cystine knot motifs C1' and C2' are shaded in red whereas the other constitutionally stable ones (C1 to C10) are shaded in blue color. These include the eight cysteines of the cystine-knot motif (15, 33). Loop3 is indicated by a red line. The red arrows indicate binding site of VEGFR-2 (33); dotted blue frames represent the NZ7-VEGF like members, red pentacles represent OV-HLJ05 and red triangles represent YX from China. **(B)** Ribbon representations of the predicted structures of dimer of selected members of the VEGF family are shown, respectively. **(C)** Superimposed structures by members from **(B)**. On the **(A)** chain, cystine residues (C3 to C10) are labeled by red letters, and the loop3-regions in **(A)** are shown by an oval dotted red frame.

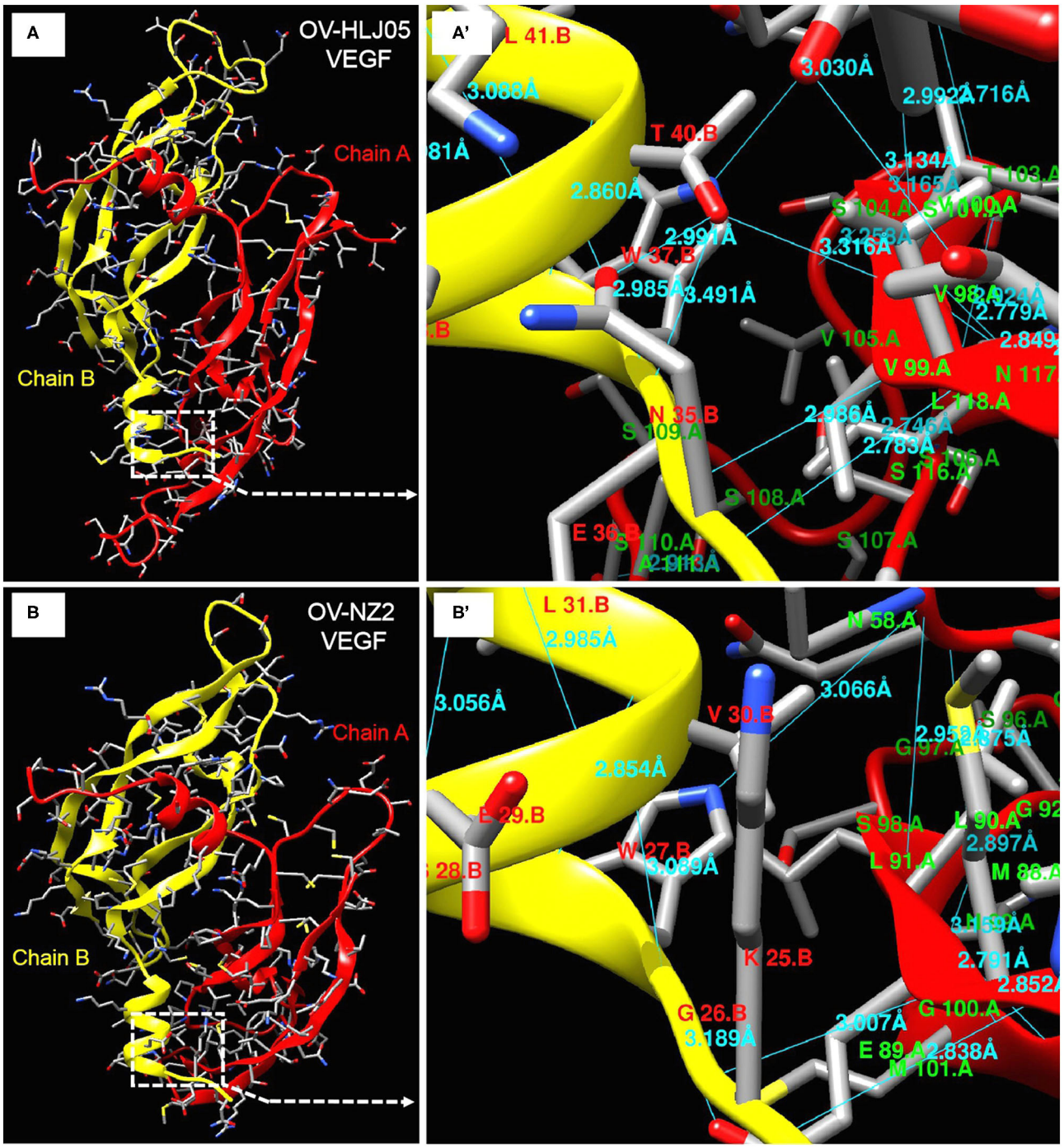

FIGURE 6 | Deeper insight on dimer structures of OV-NZ2 VEGF and OV-HLJ05 VEGF. **(A)** The dimer structure of OV-HLJ05 VEGF is composed of chain A (red) and chain B (yellow). **(A')** showed the enlarged outline of the contact sites of chain A and chain B with more hydrogen bonds. **(B)** The dimer structure of NZ2 VEGF is composed of chain A (also in red) and chain B (also in yellow). **(B')** showed the enlarged outline of the contact sites of chain A and chain B with several hydrogen bonds. The contact parts from **(A',B')** were intuitively compared for finding the inner differences of OV-HLJ05 VEGF and NZ2 VEGF, resulting in distinction on amino acid residue configuration with various hydrogen bond distance in the contact regions.

For investigating the essential divergence between the NZ7-VEGFs and the NZ2-VEGFs, the full length of VEGF primary structures were elaborately arranged in a pool, of course, the region rich in threonine (T) and proline (P) (15) is retained in the C-terminal of all the 33 VEGFs (**Figure 5**). Despite showing little DNA homology to each other, such

as insertion of a TATI(L)QVVVAI(L) motif and a SSSS(S) motif; besides deficiency or substitution, whereas the flanking sequences are over 98% homologous. Depending on the huge homologous nature, the PPVs may be favorably characterized and distinguished with the VEGF-like gene (15). Theoretically, despite the surprising extent of sequence variation among the viral VEGFs, key motifs of structural and functional importance were conserved (33). As both NZ2- and NZ7-like VEGFs have been shown to bind and activate VEGFR-2 functionally, then in the short term their clinical manifestations are nearly indistinguishable (46–49) and structural modeling more objectively reflected heterogeneity between the current VEGFs. The dimerization, contribution of residues for chain B binding to chain A was measured and the Txx**W**xxx**L**(x)KSG (or GS) motif was shared by these two VEGFs. The significant difference found was the number of hydrogen bonds (**Figure 6**), which implied *in vivo* that their biological activities are not exactly consistent.

An additional discovery during alignment using OV-HLJ05 with the fourteen representative ORFV strains, such as NZ2, NZ7, SA00, IA82, D1701, and B029 and another eight strains from China namely NA1-11, GO, NP, SJ1, YX, NA17, Shanxi and Fujian-XP, showed that the YX strain seemed to be a mid-transition type virus variant between the NZ2 and the NZ7 in VEGF, but currently it still belongs to the NZ2 camp due to the C1 and C2 locations at the N-terminal of protein. This finding is based on observation on the two Ser-enriched regions in VEGF sequences (**Figure 5**).

Genetic evidence on the *VEGF* genes has already been used to explain the ORFV scenario. An extreme example of application for the *VEGF* gene was to generate a recombinant ORFV known as D1701, a VEGF deletion mutant, by which the influence of ORFV genes in attenuation and virulence were successfully evaluated (50). Available sequence heterogeneity in the *VEGF* gene is likely to be ubiquitous or show individual features of each ORFV isolate from various geographic areas. This variant OV-HLJ05 genetically appears to be consistent to the Shanxi isolate, Jilin-NA17 isolate and Fujian-XP isolate with 100% identity. This condition suggested that the NZ7-VEGF like strain has spread throughout the mainland of China in recent years mainly due to transportation spread and a similar scenario is also presenting in India (34) (**Figure 5**). Accordingly, most isolates from these two countries have highly homologous VEGF profiles to the NZ7 strain, which seems to favor goats as hosts rather than sheep (**Figure 4**). This issue needs to be clarified worldwide by extensive epidemiological and statistical investigation.

The animal inoculation experiment showed that GO (NZ7 member) had the strongest virulence, second was YX, but NP and SJ1 showed low virulence (19). The outcome of an experiment sometimes was not consistent with the clinical feature. Previously, ORFV isolated from a goat severely affected by orf had not led to similar severe symptoms in susceptible kids (51). Consideration of the virulence determinants of a virus should not neglect the impacts of the host health status, age, and lifestyle. The impact of endogenous and exogenous factors on susceptibility to ORFV for some goats will probably reflect the host's specific susceptibility toward a certain individual strain, but the fact that NZ7-like viruses mostly come from goats suggests that the species factor should not be neglected in the clinical investigation.

In summary, an elaborated investigation can be used to diagnose the genetic characterization, molecular epidemiology and likely emerging pathogenicity of any new ORFV variant in the field and it is vital that developing countries control such any orf endemic initiated by either of these two genotypes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

AUTHOR CONTRIBUTIONS

YY, ZL, and YC conceived and designed the experiments. XD, YL, JM, and BS performed the experiments. YY, XD, and YC analyzed the data. YY and XD wrote the manuscript and prepared the Figures. YY, XD, and YC checked and finalized the manuscript. YY and ZL provided resources. All authors read and approved the final manuscript.

FUNDING

The Nature Science Foundation of Heilongjiang project, General Program (No. c2017052); the Science Research Team Support Plan of Heilongjiang Bayi Agricultural University (No. TDJH201810); the project of NTCBGP (National Transgenic Creature Breeding Grand Project) (No. 2016ZX08008-003) and the NSFC (National Nature Science Foundation of China) project (No. 31172353).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 22 September 2020
doi: 10.3389/fvets.2020.573480

Epidemiology of Classical Swine Fever in Japan—A Descriptive Analysis of the Outbreaks in 2018–2019

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OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
Shankar Yadav, European Commission for the Control of Foot and Mouth Disease (EuFMD), Italy
Katja Schulz, Friedrich Loeffler Institute, Germany

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *17 June 2020*
Accepted: *18 August 2020*
Published: *22 September 2020*

Citation:
Shimizu Y, Hayama Y, Murato Y, Sawai K, Yamaguchi E and Yamamoto T (2020) Epidemiology of Classical Swine Fever in Japan—A Descriptive Analysis of the Outbreaks in 2018–2019. Front. Vet. Sci. 7:573480. doi: 10.3389/fvets.2020.573480

This study describes the epidemiological characteristics of classical swine fever (CSF) outbreaks in Japan. The first case was confirmed in September 2018, 26 years after the last known case. Outbreaks occurred on 39 farms, 34 commercial farms, and 5 non-commercial farms, between September 2018 and August 2019. In this study, a descriptive analysis was conducted of the epidemiological data on the characteristics of the affected farms, clinical manifestations, intra-farm transmission, association with infected wild boars, and control measures implemented on the farms. Twenty-eight of the 34 affected commercial farms were farrow-to-finish farms. It was assumed that the major risk factors were frequent human-pig interactions and the movement of pigs between farms. Fever and leukopenia were commonly observed in infected pigs. In 12 out of 18 farms where clinical manifestations among fattening pigs was the reason for notification, death was the most frequent clinical manifestation, but the proportion of dead animals did not exceed 0.5% of the total number of animals at most of the affected farms. Therefore, the clinical form of CSF in Japan was considered to be sub-acute. Twenty-three of the 29 farms (79%) with pigs at multiple stages (i.e., piglets, fattening pigs, and sows), had infection across the multiple stages. Many of these farms were within 5 km of the site where the first infected wild boars had been discovered, suggesting that infected wild boars were the source of infection. Infections still occurred at farms that had implemented measures at their farm boundaries to prevent the introduction of the virus into their farms, such as disinfection of vehicles and people, changing boots of the workers, and installation of perimeter fences. It is necessary to continue to strengthen biosecurity measures for farms located in areas with infected wild boars and to continue monitoring the distribution of infected wild boars so that any abnormalities can be reported and inspected at an early stage.

Keywords: classical swine fever (CSF), domestic pig (Sus scrofa), epidemiology, outbreak investigation, Japan

INTRODUCTION

Classical swine fever (CSF) is among the most devastating contagious diseases in pigs. Due to its impact on pig production, the prevention and control of the disease has been a major priority in pig producing countries. In Japan, 9.2 million heads of pigs are reared at about 4,300 farms as of 2019 and pork is produced mainly for domestic consumption (about 900,000 ton/year) and partially for export (about 2,000 ton/year). Although the export of pork is not a major industry in Japan, since the domestic demand for pork in Japan is increasing in recent years to more than 1.8 million ton/year, the protection of domestic pig industry from CSF and the maintenance of productivity is also a major issue.

The disease is caused by the CSF virus (CSFV), a single-stranded RNA virus of the *Pestivirus* genus of the *Flaviviridae* family. Pigs and wild boars are the virus hosts. Infected animals experience non-specific clinical symptoms due to immunosuppression (1, 2). Clinical forms of the disease vary depending on the virulence of the virus, age of host animals, hygiene management at the farm, and the presence of secondary infections; the clinical forms that have been seen in wild boars are similar to those in pigs (2). CSF can be divided into the following forms: acute, chronic, and persistent. The acute form is characterized by atypical clinical signs such as high fever, anorexia, gastrointestinal symptoms, general weakness, and conjunctivitis. This is followed by neurological signs and skin hemorrhages or cyanosis in different locations of the body 2 to 4 weeks after infection, known as the "typical" CSF signs. Animals with this form usually die 10 to 30 days after CSFV infection. In the chronic form, animals show various non-specific symptoms including fever, listlessness, loss of appetite, decreased growth, and death after 1 month from infection. The persistent form is observed in piglets infected as fetuses through vertical transmission (2). These piglets can become immunotolerant to the virus and can be a constant source of infection. They are able to constantly excrete the virus, even without any clinical symptoms, and are a dangerous virus reservoir until the late onset (2).

CSF outbreaks in pigs have been reported in Central and South America, Europe, Asia, and Africa. In the 1990s, large outbreaks occurred in the Netherlands, Germany, Belgium, and Italy but the disease has now been contained in these Western European countries. These countries are now officially recognized as CSF free, according to the World Organization for Animal Health (OIE) Terrestrial Code (3). Japan suffered from CSF since from the 1880s until the development of a live vaccine using the GPE- strain in the 1960s. The live vaccine was used since 1969, resulting in a sharp decline in the number of outbreaks to zero. The last reported case was recorded in 1992 (4). In 2000, the use of the live vaccine began being restricted before totally ceasing in 2006. Japan was officially recognized as CSF free in 2015, when the OIE began officially recognizing CSF disease status (5). This status was subsequently suspended in September 2018 due to the re-occurrence of CSF in central Japan.

On August 24, 2018, a fallow-to-finish pig farm in Gifu Prefecture, located in the central part of Japan, reported an increase in the number of dying animals to their local veterinary service. At the farm, clinical signs such as fever, loss of appetite, and abortion were more frequently observed prior to the deaths. The farm manager consulted the farm veterinarian, who considered the signs to be caused by heatstroke. On September 9, 2018, CSF viral infection was confirmed by laboratory tests conducted at the National Institute of Animal Health, National Agriculture and Food Research Organization (NIAH-NARO), after an absence of 26 years.

By January 2019, six more outbreaks had been reported near the first infected farm, in the southern area of Gifu Prefecture. In February 2019, the first outbreak in Aichi Prefecture was confirmed in Toyota City, located in the northern area of Aichi. The second outbreak in Aichi Prefecture was reported within the same month, in Tahara City, located in the southern peninsula, almost 47 km away from the infected farm in Toyota City. From March to June 2019, a total of 18 outbreaks had been reported in Gifu and Aichi. In July 2019, the first outbreak in Mie Prefecture and the first outbreak in Fukui Prefecture were reported. By August 2019, 39 outbreaks had been confirmed in Gifu, Aichi, Mie, and Fukui Prefectures (**Figures 1**, **2**).

The virus strain causing the outbreaks in Japan from 2018 to 2020 was found to be the subgenotype 2.1d (6, 7). Subgenotype 2.1d was firstly isolated from the outbreaks in China in 2014 to 2015 (8) and is reported to cause a chronic or moderate form of infection (9–11). It has also been detected in South Korea (12, 13). A phylogenetic study showed that the CSF virus isolated from the 2018 Japan outbreak index case was different from the strains that had caused previous CSF-outbreaks in Japan (6). It has, therefore, been considered that the virus causing the 2018–2020 CSF outbreaks in Japan was newly introduced from the surrounding Asian countries, though the route of introduction or origin of the virus is unclear.

Control measures implemented to contain the CSF outbreaks were based on the Guideline to Control Classical Swine Fever (hereinafter referred to as "the Guideline." A specific national guideline and the last revised version was published on February 5, 2020 under the Act on Domestic Animal Infectious Disease Control of Japan.) (14). These measures included: stamping-out of all animals in the affected farms, control of movement of animals to a radius of 3 km of the affected farms, control of animal shipment in the area of 3 to 10 km from the affected farms, and disinfection of vehicles at control-points set-up at roads inside the movement control areas. All farms identified as having a relationship with the affected farms were investigated, and all animals confirmed to be infected with CSF virus at these farms were also stamped-out. Killed animals were buried at the affected farms or at burial sites near the affected farms, except for one epidemiologically related farm, whose animals were rendered for incineration due to unavailability of burial sites.

Active surveillance was implemented at farms and in wild boars. Surveillance at farms was implemented within the movement-control areas, within a 3 km radius of the affected farms. Clinical investigation, polymerase chain reaction (PCR), and enzyme-linked immunosorbent assay (ELISA) tests were conducted on blood samples of randomly-selected pigs from the affected farms within 24 h of confirmation of an infection. At

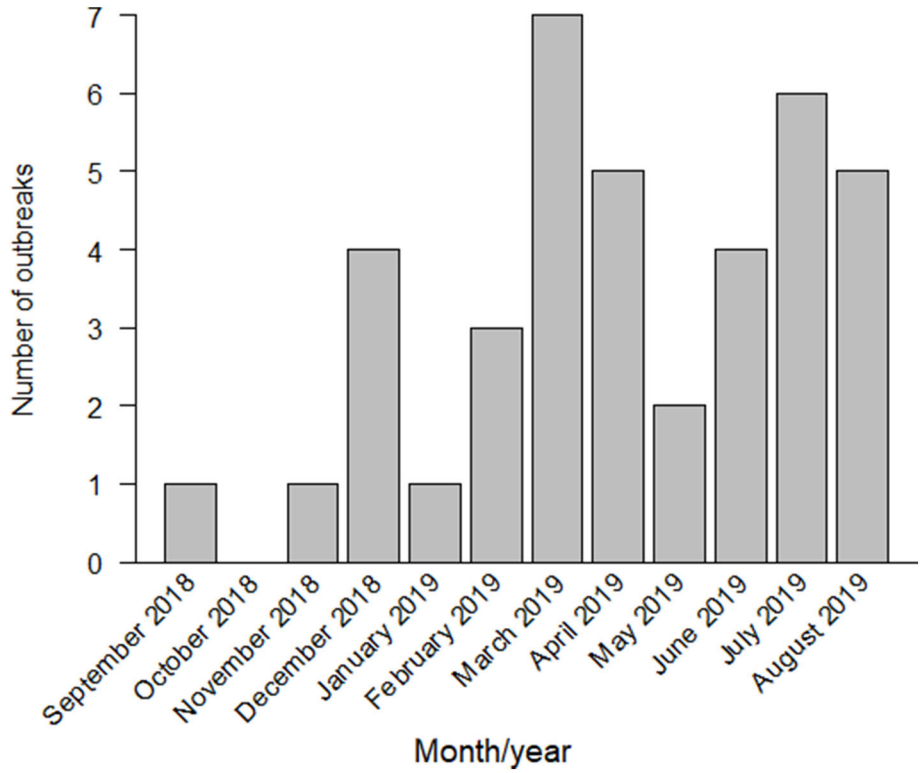

FIGURE 1 | Classical swine fever (CSF) outbreaks reported in Japan from September 2018 to August 2019.

least 30 animals were tested per farm to detect a prevalence of 10% with 95% confidence. When a farm had more than two pig houses, at least five animals per pig house were tested even when the total sample size exceeds 30. In these samplings, pigs with clinical symptoms were sampled with priority. The same set of tests was conducted to all farms present within the movement-control area from 17 days after the completion of all control measures at the affected farm. Shipment restrictions in the area of 3 to 10 km from the affected farms were lifted when all the farms within the movement-control area were confirmed to be CSF free by the second round of tests, while movement controls were lifted 28 days after the completion of all control measures on the affected farms.

A nationwide surveillance in wild boars had been implemented since 2006, after the cessation of the use of vaccine in pigs, and after the official recognition of CSF freedom in 2015, 273, and 389 wild boars were tested in 2016 and 2017, respectively with negative results. After the first pig case was confirmed at a pig farm in Gifu Prefecture in September 2018, intensive CSF surveillance in wild boars targeting the area within 10 km of the affected farm started according to the Guideline. As a result of the intensive surveillance, the first positive case of wild boar was found dead within 10 km of the farm with the first pig case. After the first case of wild boar, hunters captured wild boars within a 10 km radius of the sites where infected wild boars were found, in addition to the area around affected farms. The dead or captured wild boars in the surveillance areas were then tested for CSF by local veterinary services. Additionally, all prefectures were requested to test dead wild boars found in their jurisdiction.

The vaccination of wild boars, using bait vaccine, started in March 2019 in Gifu Prefecture and in the adjacent prefectures with CSF-positive wild boars. By October 2019, given that the spread of the disease had not been controlled by improving biosecurity measures at farms, preventive vaccination at pig farms using the live CSF-vaccine began in Gifu and in the eight adjacent prefectures. By the end of December 2019, vaccination expanded to the additional 11 surrounding prefectures.

Descriptive epidemiological analyses provide an overview of the epidemic and shed light on the characteristics of the outbreaks, including the possible factors related to the occurrence of the disease. There are descriptive epidemiological studies on CSF outbreaks that have occurred in the Netherlands, Germany, and Belgium (15–18). These are all good references for countries needing to control the disease.

Regarding the epidemic of CSF caused by subgenotype 2.1d virus strain, the isolation of the virus has been reported (9–13), but the features of outbreaks caused by the specific subgenotype virus strain have not been fully described. As for the re-occurrence of CSF in Japan since 2018, there were studies describing genetic characteristic of the virus (6, 7), pathogenicity in experimental infection (19), and estimating the risk of infection from wild boars (20). However, an overall description of the outbreaks and analyses of clinical manifestations observed during outbreaks have not been reported.

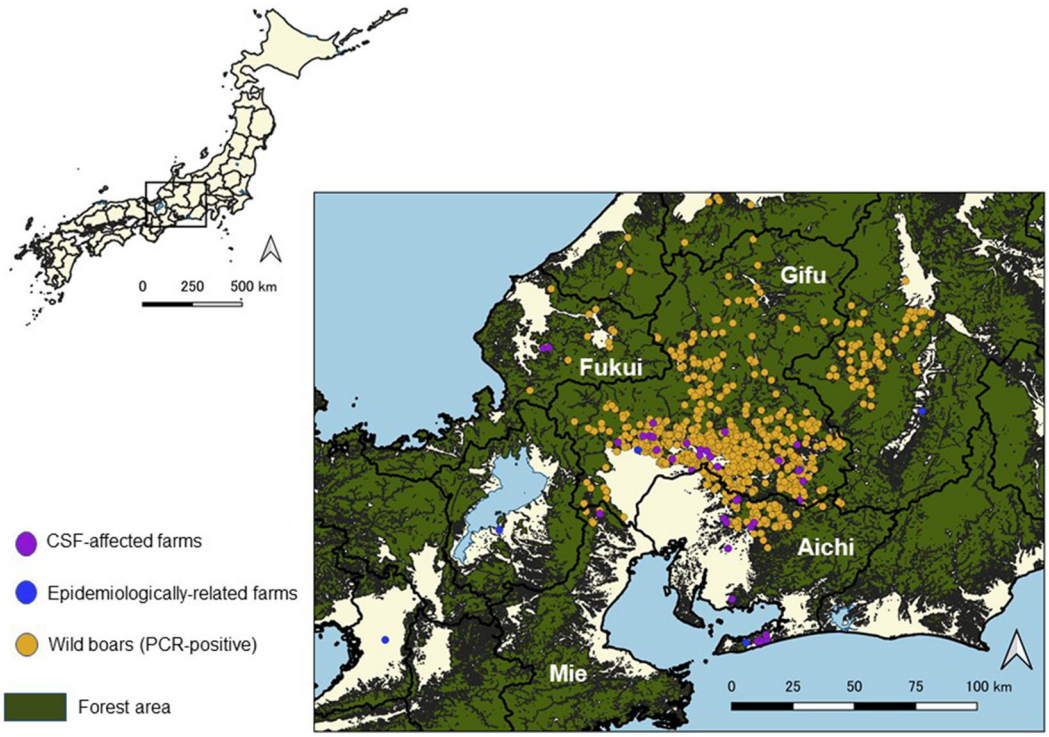

FIGURE 2 | Location of classical swine fever (CSF)-affected farms and virus-positive wild boar cases in Japan from September 2018 to August 2019.

This study is the first report that gives an epidemiological overview of the CSF outbreaks in pig farms by the virus strain of subgenotype 2.1d, which occurred in Japan, for the period from September 2018 to August 2019. The characteristics of the symptoms observed from infected animals and the measures taken at the affected farms described in this study will be a good reference for the countries affected by the epidemic of CSF caused by subgenotype 2.1d, which causes a chronic or moderate form of infection.

MATERIALS AND METHODS

Data Collection

Epidemiological data from the 39 farms where CSF outbreaks occurred in the period from September 2018 to August 2019 were collected using the epidemiological investigation reports. These reports also included information on the preventive measures implemented at the farms. Each epidemiological investigation on an affected farm was conducted by epidemiological investigation team (EIT) from the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan. The EIT consisted of veterinary officials of MAFF and veterinary epidemiologists of NIAH-NARO. Most of the investigation activities were implemented on the date or the next date of confirmation of CSF infection and before starting of the stamping-out at the farm. During the epidemiological investigation, managers of affected farms were interviewed and asked about the biosecurity measures implemented at their farms, and about flows of workers and pigs inside and outside of pig houses. Information on structures of affected pig houses and feedstuff was also collected at the on-site investigation. When fences and/or bird-proof nets were installed at affected farms, the way they were installed was checked by the EIT and the EIT confirmed if there were any possibilities of intrusion of wild animals. Brief list of questions used in the EIT investigations are shown in **Supplementary Table 1**.

Farm locations were extracted from the Domestic Animal Disease Control Map Database of Japan. All farms which did not rear pigs or boars for marketing purposes were classified as non-commercial farms. These included farms being managed by municipalities for breeding or education. Information regarding the number of animals being reared at the affected farms and the duration from infection confirmation to completion of stamping-out was collected from publicly available data from MAFF and prefectural governments.

Data on CSF-positive wild boars, including their location and laboratory test results, were also provided by MAFF.

Data Analysis

All statistical analyses were conducted using R (R Core Team, 2020). The Fisher's exact test was applied for univariate analyses. CSF-cumulative incidence rates by types of farms and clinical symptoms by types of pigs were compared by applying the test to 2×2 contingency tables. The association between the infection in sows and the status of transmission between pig houses was analyzed in the similar method. For the multiple comparisons of CSF-occurrence among farrow-to-finish farms, fattening farms

and breeding farms, a 2 × 3 contingency table was prepared and the Fisher's exact test was applied by the "fisher.multcomp" function of the RVAideMemoire package. For the comparison of the number of animals at farms and the number of leucocytes by categories of farms and pigs, the Wilcoxon rank sum test ("wilcox.test" function) was applied.

Clinical Manifestations at Affected Farms

Information on the clinical manifestation, as observed at disease notification, and the type of pigs showing symptoms (piglets, sows, or fattening pigs) was collected from the following sources: (i) the epidemiological investigation reports completed by EIT (provided by MAFF), (ii) the CSF-EIT meeting reports (published on the MAFF web-site), (iii) the emergency notification reports submitted to the MAFF by the prefectural governments (provided by MAFF), and (iv) the verification reports prepared by Gifu Prefecture on their response to the CSF outbreaks (available on the Gifu prefectural government web-site).

The type of pigs affected, that is, piglets, sows, and fattening pigs, were classified as stated in the above sources. However, some farms only provided data on the age of the affected pigs. In such cases, pigs <3 months (or 90 days) of age were classified as piglets; pigs aged 3 months (or 90 days) and above were classified as fattening pigs, and pigs for breeding purposes were classified as sows. Observed clinical manifestations were classified into eight symptoms; loss of appetite, listlessness, respiratory disorders, fever, cyanosis, diarrhea, death, and neurological symptoms.

The association between the type of pigs (fattening/sows/piglets) and the development of any of the major four symptoms, that is, loss of appetite, listlessness, respiratory disorders, and death, was analyzed for each combination.

Results of Laboratory Tests of Pigs at Affected Farms

Farms with confirmed CSF infections, following tests conducted after receiving notification, had five or more pigs from each pig house randomly sampled before being stamped-out. The basic sample size was at least 30 animals per farm, to detect a prevalence of 10% with 95% confidence, and as additional conditions to detect the infection more efficiently, at least five animals per pig house, from all the pig houses, with priority in sampling from pigs with clinical symptoms were sampled in accordance with the Guideline. Investigations were conducted to measure the number of leucocytes, and PCR and ELISA tests performed to determine the infection status and spread of the virus at the farm. Blood sampling was conducted by the prefecture's local veterinary service, based on the Guideline, and antigens and antibodies against CSF were tested using PCR and ELISA, respectively. Following infection by CSF virus, the viral antigen is detected in the blood and/or organs of pigs where it grows, before any antibodies can be detected. Accordingly, the status is indicated as PCR(+)/ELISA(−). As the course of infection proceeds, antibodies against the CSF virus can be detected and the status becomes PCR(+)/ELISA(+). After the virus is eliminated from the pigs, the pigs become immune to CSF virus infection and the status is indicated as PCR(−)/ELISA(+). Experimental infections using the virus strain isolated from the cases in Gifu Prefecture indicated that antibodies against the virus are developed on or after 14 days from infection, and that antigen detection lasts for more than 28 days after infection (19).

Proportion of Dead Animals at Farms

Obligatory daily reporting of the number of dead animals was imposed on farms located within a 3 km radius of an affected farm, a 10 km radius of an infected wild boar, and that had shipped pigs to the common slaughterhouses shared by affected farms, starting from February 2019, following the detection of the 8th case (the first outbreak in Aichi Prefecture). Reports were collected from the affected farms by the local veterinary service who reported the number to the MAFF. The daily proportion of dead animals was calculated by dividing the number of dead animals per day by the total number of animals at the farm on that day. When the total number of animals at the farm on each day was not available, the number of animals at the farm on the date of stamping-out was used as the denominator.

Data on Geographical Information

Geographical data on administrative divisions (as of 2018) and forested areas (as of 2015) was downloaded from the National Land Numerical Information download service, provided by the Ministry of Land, Infrastructure, Transport and Tourism of Japan, and was used to draw maps. The maps were drawn, and distance measured, by quantum geographic information system (QGIS) version 3.10.

We recorded the distance between the affected farms and the nearest site where a PCR-positive wild boar was found before the farm notified the outbreak. In addition, the shortest distance between affected farms was measured as the distance between an affected farm and the nearest affected farm with a confirmed infection, in which the infection was confirmed before that of the farm in question. This was measured using the distance matrix of the geoprocessing tools of QGIS.

RESULTS

Details of the Affected Farms

Classification and Comparison by Types of Farms

Pig/boar

Out of 39 outbreaks confirmed between September 2018 until August 2019, 38 outbreaks occurred at pig farms and one outbreak at a boar farm. Boars are not common livestock in Japan but there are boar farms where several or a few dozen boars are reared for training hunting dogs or for meat, or often without any particular purpose, as in the affected boar farm. In Gifu Prefecture, 21 out of 44 pig farms and one out of five boar farms were affected. The cumulative incidence rate was 48% (=21/44) for pig farms and 20% (=1/5) for boar farms, and there was no significant difference between the cumulative incidence rate of pig farms and boar farms ($p > 0.1$, Fisher's exact test).

Commercial/non-commercial

Five outbreaks occurred at non-commercial farms; four in Gifu Prefecture from November to December 2018, and one in

TABLE 1 | Number of classical swine fever (CSF) outbreaks at commercial pig farms in Gifu Prefecture from September 2018 to August 2019, by production type.

Production type	Number of farms		
	Affected	Not affected	Total
Farrow-to-finish	13	1	14
Fattening	4	10	14
Multiplier	1	11	12
Total	18	22	40

Aichi Prefecture in August 2019. Affected non-commercial farms included: the livestock research institutes of Gifu and Aichi Prefectures, the Gifu Prefectural College of Agriculture, the Gifu Prefectural Park of Livestock, and a boar farm. Thirty-four outbreaks occurred at commercial pig farms. In Gifu Prefecture, 18 out of 41 commercial pig/boar farms were affected and four out of eight non-commercial pig/boar farms were affected. The cumulative incidence rates were, therefore, 44% (=18/41) in commercial farms and 50% (=4/8) in non-commercial farms, with no significant difference between them ($p > 0.1$, Fisher's exact test).

Farrow-to-finish/fattening/breeding

The affected 34 commercial pig farms consisted of 28 farrow-to-finish farms, 5 fattening farms, and 1 breeding farm. Two out of five fattening farms were group farms comprising a farrow-to-finish and a breeding farm. In Gifu Prefecture, at commercial pig farms, the cumulative incidence rate of the farrow-to-finish farms was 93% (=13/14 farms), fattening farms was 29% (=4/14 farms), and breeding farms was 8.3% (=1/12 farms) (**Table 1**). Comparing the rates among the types of farms, the cumulative incidence rate of the farrow-to-finish farms was significantly higher than that of the fattening and breeding farms ($p < 0.05$, multiple comparison).

Number of Animals

More than half of the affected farms reared <2,000 animals, with a median number of 1,271 (25–75th percentile: 625–3,622) animals (**Figure 3**). The median size of these farms was not significantly different from that of all farms in Japan ($p > 0.1$, Wilcoxon rank sum test) (21). For non-commercial farms, the livestock research institutes of Gifu and Aichi Prefectures reared about 500 and 700 animals each, respectively. The other non-commercial farms reared 10 to 20 animals per farm. For commercial farms, the median number of animals reared per farm was 1,556 (25–75th percentile: 976–4,007) animals, ranging between 250 and 10,000 animals.

In Gifu Prefecture, 22 out of 49 pig/boar farms were affected and 27 farms were not affected. The median number of animals reared at the 22 affected farms was 1,277 (25–75th percentile: 594–2,916) animals, while the median number of animals reared at the 27 non-affected farms was 519 (25–75th percentile: 127–1,584) animals. Therefore, the number of animals reared at affected farms was significantly higher ($p < 0.05$, Wilcoxon rank sum test).

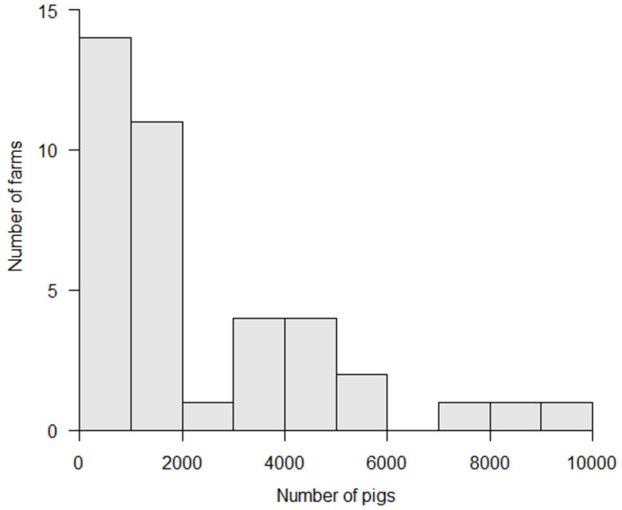

FIGURE 3 | Herd-size (number of pigs) at classic swine fever (CSF)-affected farms.

Number of Days From Diagnosis to Completion of Stamping-Out

The time between definitive diagnosis and the completion of stamping-out ranged between 1 and 5 days, with a median of 2 days (25–75th percentile: 1–3 days) (**Figure 4**). There were 11/39 farms that required 3 days or more to complete stamping-out, of which 10 of these had either more than 4,000 animals or constituted a pig farm complex, resulting in multiple farms needing to be slaughtered simultaneously. The median number of animals at these 11 farms was 4,189 (25–75th percentile: 3,520–5,215) animals and was significantly larger than that of the other 28 farms ($p < 0.01$, Wilcoxon rank sum test).

Clinical Manifestations and Transmission of Virus Within Farms

Common Clinical Manifestations

Fever

Eleven of the 38 pig farms suspected CSF by fever and made notification. After including results from the on-site inspections following notification, pigs with a fever over 40°C were observed at 30/38 pig farms.

Decrease in the number of leucocytes

Pigs from all affected farms were noted to have a decreased leucocyte count to <10,000 cells/μl. Leucocyte counts were not measured at the wild boar farm because of their aggressiveness. The leucocyte level was measured on 939 CSF-positive pigs [PCR(+) and/or ELISA(+)] and 3,005 CSF-negative pigs [PCR(−) and ELISA(−)]. The median leucocyte level within CSF-positive pigs was 8,490 (25–75th percentile: 5,900–13,000)

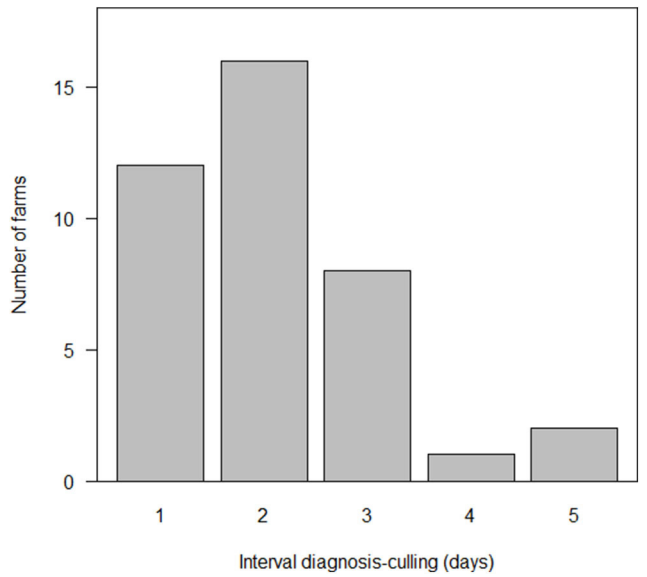

FIGURE 4 | Distribution of the interval between laboratory confirmed diagnosis and the completion of culling at 39 farms affected by classical swine fever (CSF) in Japan, 2018–2019.

cells/μl, which was significantly lower than that of CSF-negative pigs ($p < 0.01$, Wilcoxon rank sum test).

Clinical Manifestations Leading to the Notification and the Results of Laboratory Tests

Clinical manifestations of fattening pigs were the reason for notification at 18 out of 34 commercial farms. Among these 18 farms, death was the common reason for notification in 12 farms.

In 9 out of 34 commercial farms, clinical manifestations of sows led to notification. At all nine farms, the reason for notification was loss of appetite, with death also cited as a reason in one of these nine farms.

In 6 out of 34 commercial farms, clinical manifestations in piglets led to notification. The death of weaning pigs was the reason for notification in four out of six farms while loss of appetite in sows was also cited as a reason in one of the four farms. As for the other two farms, fever and listlessness of weaning pigs were the reasons for notification at one farm, and listlessness of piglets as well as loss of appetite and listlessness of sows were the reasons in the other.

Table 2 shows the observed clinical manifestations and the results of laboratory tests according to farm level.

Other than the farms that notified CSF by clinical manifestations, in 4 out of 34 commercial farms, the clinical manifestations were not observed by farm managers, where the infection was detected by PCR and ELISA tests applied as a part of the movement/shipment-control areas. After the detection by the laboratory tests, pigs with fever were confirmed at two farms, but pigs at the other two farms remained asymptomatic. Pigs tested ELISA(+) at two out of four farms, PCR(+)/ELISA(+) at one farm without fever, and PCR(−)/ELISA(+) at one farm with fever. In the other two farms, pigs only tested PCR(+)/ELISA(−) but one of the farms had pigs with fever.

As a result of analyses on the association between the type of pigs and the development of symptoms, it was indicated that respiratory disorders and loss of appetite significantly led to notification more frequently in fattening pigs and in sows, respectively ($p < 0.05$ and $p < 0.01$, Fisher's exact test). Death was significantly less frequently cited as a reason for notification in sows ($p < 0.05$, Fisher's exact test).

Abnormal Birth

Abnormal births, including abortion and stillbirth, were reported only by three farms during the epidemiological investigation interviews, while stillbirths were recorded in the daily reports from 10 other farms traced up to 60 days before confirmation of infection. In 9 out of these 13 farms, the pig houses with recorded stillbirths were confirmed to be affected with CSF afterwards. However, none of these farms suspected that the abnormal births were a clinical manifestation of CSF infection.

Death and Proportion of Dead Animals

Based on the records of the daily number of dead animals at the farms traced up to a maximum of 60 days before infection was confirmed, 30 out of 39 affected farms had observed their animals dying before notification. In 12 out of 30 farms, the cause of the death was considered to be due to weakness or being crushed (in suckling pigs), diarrhea (in weaning pigs), streptococcus's infection, pneumonia, gastroenteritis, and stress or growth insufficiency (in weaning and fattening pigs). CSF was not suspected as the cause according to both the local veterinary service and the supervising veterinarian since the other pigs being reared in the same pens or pig houses as the dead pigs did not have any abnormal symptoms. In the other 18 farms, CSF was suspected, and the death led to notification.

Data on the daily number of dead animals before notification were available on 31 of the affected farms. In 6 out of the 31 farms, the proportion of dead animals on the date of notification was more than 0.5% (Case no. 9, 17, 27, 28, 30, 31). Three of these six farms notified due to the increase in the number of dead weaning pigs, and the other three farms due to the death of their fattening pigs. Other than those six farms, there was no observed increased number of dead animals from the other farms on the date of notification (**Figure 5**). For the other 25 farms, temporary increases in the proportion of dead animals to more than 0.5% were observed before the dates of notification in six farms (Case no. 21, 24, 29, 33, 35, 37), but the cause of the increase was crushing death in suckling pigs or abortion.

Transmission Between Pig Houses by Pig Flow (Intra Farm Pig Movement)

In the 34 commercial farms, including the two farms without any clinical manifestations, infection was limited to one pig house in eight farms and confirmed in all the pig houses in the other eight farms. In the other 18 farms, infection was confirmed in more than two, but not all, pig houses. Transmission of the infection between pig houses by pig flow was assumed to have occurred when there was a record of the infected pigs having moved

TABLE 2 | Reported clinical signs by types of pigs and the results of serological tests on CSF at the 34 affected commercial farms.

Type of pigs	Reported clinical manifestation	No. of farms with clinical signs	Results of serological tests on CSF at farm level[a]		
			PCR-positive/ELISA-negative	PCR-positive/ELISA-positive	PCR-negative/ELISA-positive
Fattening pig		18	1	6	11
	Death	12	1	3	8
	Fever	7	0	3	4
	Listlessness	8	0	4	4
	Loss of appetite	7	0	3	4
	Respiratory disorders	5	0	0	5
	Diarrhea	2	0	1	1
	Cyanosis	2	0	0	2
	Decreased growth	1	0	0	1
Sow		9	2	3	4
	Loss of appetite	9	2	3	4
	Fever	4	2	1	1
	Listlessness	2	0	1	1
	Death	1	1	0	0
Piglet		6	1	3	2
	Death	4	1	2	1
	Listlessness	3	0	2	1
	Cyanosis	2	1	0	1
	Fever	2	0	2	0
	Loss of appetite	1	0	1	0
	Neurological symptom	1	0	1	0
	Diarrhea	1	0	0	1

[a]*Classification of farms by the results of serological tests. PCR-positive/ELISA-negative, all animals tested at the reporting and before culling were PCR-positive but without CSF-virus-specific antibodies; PCR-positive/ELISA-positive, at least one animal tested at the reporting or before culling was PCR-positive and with CSF-virus-specific antibodies; PCR-negative/ELISA-positive, at least one animal tested at the time of reporting or before culling was with CSF-virus-specific antibodies but PCR-negative.*
PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

between pig houses, and when both pig houses were confirmed to be affected. Based on the epidemiological investigations and movement records of the infected pigs, transmission by pig flow was strongly suspected in 7 of the 26 farms, with infection confirmed in multiple pig houses. In five of the farms, the viral spread by pig flow was refuted since the farms were fattening farms and/or the pigs had not moved between pig houses. In the other 14 farms, the transmission routes between pig houses remained unclear because either there were no records of the movement of pigs between pig houses or because almost all of the pig houses in the farm had been found to be infected with no trace as to the source of the infection within the farm.

Transmission Between Stages of Pigs

The infection was not limited to a single stage in 23 out of 29 commercial farms rearing multiple stages of pigs (28 farrow-to-finish farms and one breeding farm). In one the other six farms, infection was confirmed only in fattening pigs (five of the six farms) and in sows (one of the six farms).

The association between the infection in sows and the status of transmission between pig houses is shown in **Table 3**. Infection in multiple pig houses was observed more frequently when there was infection of sows ($p < 0.01$, Fisher's exact test).

Surrounding Environment of the Affected Farms

Distribution of Infected Wild Boars

In Gifu Prefecture, wild boars confirmed as PCR-positive were frequently detected near the affected farms. On the other hand, PCR-positive wild boars were not found near the affected farms located at the southern peninsula of the Aichi Prefecture (**Figure 2**).

Twenty-eight out of 38 affected farms, excluding the first affected farm, were located within 5 km from PCR-positive wild boars detected before the notification of an outbreak at each farm. Out of the 28 farms, 23 farms were located in the southern area of Gifu Prefecture or in the adjacent northern area of Aichi Prefecture, two farms were located in the central part of Aichi Prefecture, and the other three farms were located in Mie and Fukui Prefectures.

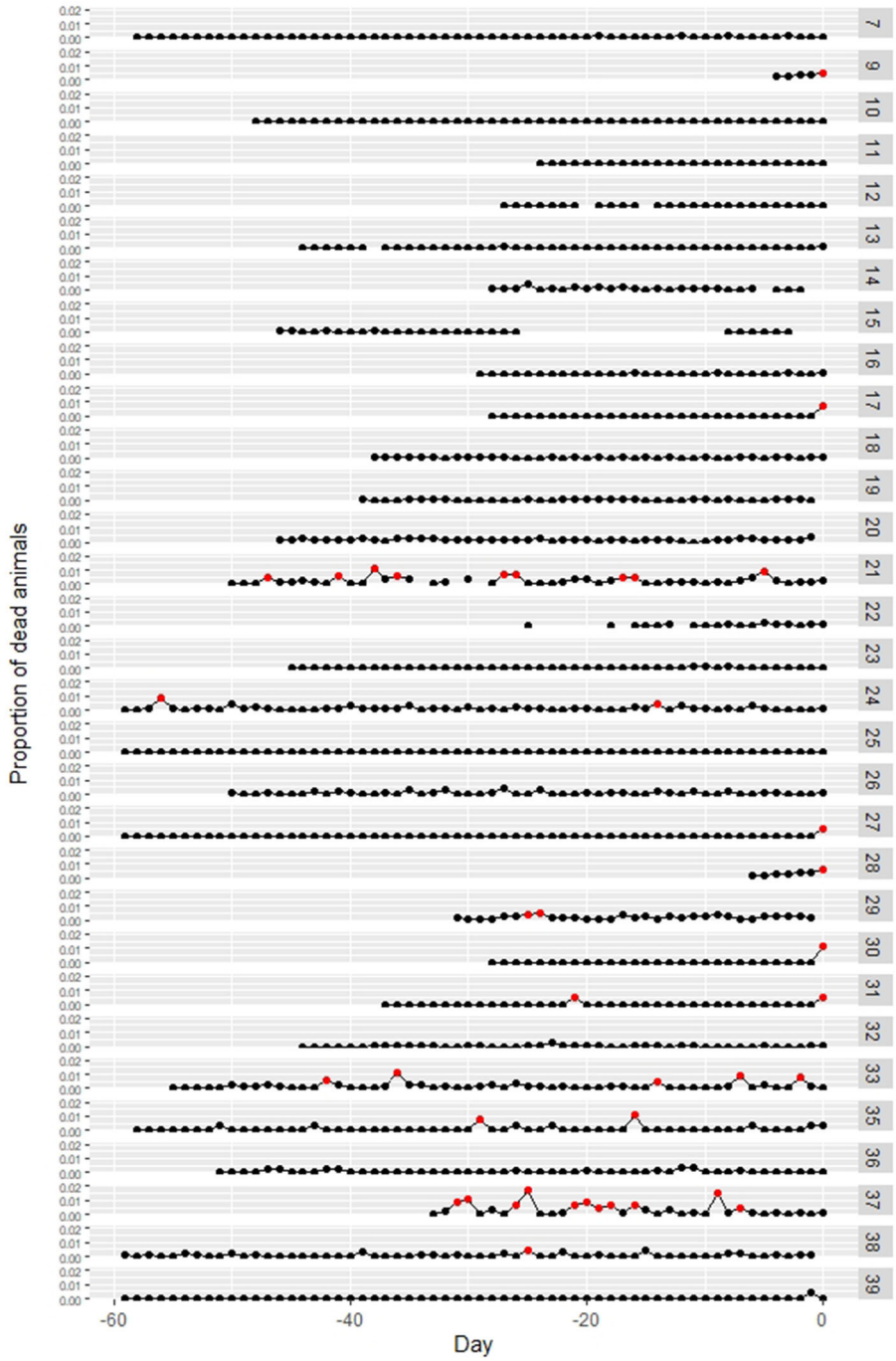

FIGURE 5 | Changes in the proportion of dead animals at classical swine fever (CSF)-affected farms. Numbers at the right side indicate case numbers. The proportion over 0.5% is marked as red point. Day 0 = report date. Data were not available for Case no. 1–6, 8, and 34.

Distance Between Affected Farms

The median distance between affected farms and the nearest other affected farm was 6.95 km (25–75th percentile: 2.35–11.38 km). Four farms were located within a distance of 1 km, with one of these four farms was located in a pig farm complex in the southern peninsula of Aichi Prefecture. The other three farms were located in the northern area of Aichi Prefecture, with two of the three farms adjoining each other and the nearest affected farm being the one in common. One of these farms was an affiliated farm of one of the other two farms.

TABLE 3 | CSF-infection among sows and the spread of classical swine fever (CSF) viruses between pig houses.

Infection in sows	Number of CSF-affected farms rearing multiple stages of pigs		
	Spread of CSF viruses in each farm		Total
	Limited to a single pig house	Observed in multiple pig houses	
Yes	2	19	21
No	5	3	8
Total	7	22	29

TABLE 4 | General characteristics of the 39 farms affected by classical swine fever.

	Number of farms
Structure of affected pig houses	
Windowless	0
Semi-windowless[a]	18
With open windows[b]	21
Feedstuff	
Commercial feed only	33
Other than commercial feed	6

[a] *One of the 18 semi-windowless pig-houses had open-air paddocks.*
[b] *Two of the 21 pig-houses with open windows had open-air paddocks.*

Livestock Health Management at Affected Farms

Feed

In 33 out of 39 farms, only commercial feed was used. The other six farms used feed other than commercial feed (**Table 4**), with one of these six farms being the boar farm which used rice bran, wasted rice, breadcrumbs discarded from food factories, and vegetable scraps discarded by neighboring farmers. The other five farms using non-commercial feed were commercial pig farms using confectionery residues such as biscuit crumbs (two farms), breadcrumbs (one farm), weeds around the farm (one farm), and liquid feed made with food wastes including table leftovers and cooking residues (one farm) as feed.

Measures to Prevent Intrusion of Wild Boars

Fences around farms were installed at 26 of the 39 farms, but only 14 farms had complete fences protecting them against intrusion by wild boars. In addition, 15 of the 39 farms had installed electric fences, but the fences were complete at only 12 farms (**Table 5**).

Biosecurity Measures at Farm Boundaries

Disinfection of vehicles, such as feed transporters, at farm boundaries was implemented at 28 of the 39 farms. At 5 of the remaining 11 farms, the ground surface at the entrance of the farms was covered with lime. To prevent farm workers from bringing in the virus, changing of boots and clothes at the farm entrances were implemented in more than half of the affected farms (**Table 5**).

Biosecurity Measures at the Border of Pig Houses

As for the structure of the pig houses, there were no windows or filters installed on the openings of the pig houses at 21 of the 39 farms. Openings were covered with curtains at the other 18 farms. There were three farms with open-air paddocks, one of which was a commercial farm (**Table 4**). To prevent entry of the virus into the pig houses, more than half of the affected farms installed bird-proof netting on the openings of the pig houses, but some of them had gaps or breakages and complete netting was installed at only 15 farms. Changing of boots at each entrance of the pig houses was implemented at more than half of the affected farms (**Table 5**).

DISCUSSION

Details of the Affected Farms

Regarding the type of management, 80% of the affected commercial farms were farrow-to-finish farms. In Gifu Prefecture, the incidence at farrow-to-finish farms was significantly higher than in other farms. This may be due to the fact that in a farrow-to-finish farm, the management of both piglet production and shipment of fattening pigs requires frequent movements of pigs within the farm, with frequent handling, which increases the risk of introducing the virus into the farm. In a case-control study of the outbreaks of foot-and-mouth disease (FMD) in Japan, it was indicated that the risk of infection was higher in farrow-to-finish farms than in fattening farms (22). The reason is considered that sows and piglets in farrow-to-finish farms require more frequent care than pigs in fattening farms and that the disease transmission via direct contact with animals and contaminated fomites tends to occur more frequently in farrow-to-finish farms. Other studies on African swine fever (ASF) outbreaks in Estonia (23) and CSF in the Netherlands (24) have also indicated that the incidence tended to be higher in farms rearing sows with piglets and fattening pigs.

The number of animals at affected farms was significantly larger when comparing affected and non-affected farms in Gifu Prefecture. At large farms, the infection risk may be higher when the number of sows is larger, leading to a larger number of employees engaged in the management of breeding and feeding, as well as the larger number of people entering and leaving the farm for purposes such as transporting feed and shipping fattening pigs. These conditions have not been investigated at non-affected farms, therefore, case-control studies would be necessary for further analysis. On the CSF outbreaks in the Netherlands, a case-control study (25) and a survival analysis (24) reported that the risk of CSF infection was higher on farms with more than 500 animals.

In the CSF outbreak reported in this paper, the median number of days from diagnosis to complete stamping-out was 2 days, including farms with ∼10,000 animals. Minimizing the period between infection and stamping-out is important in order to prevent the spread of diseases. In 2010, the spread of FMD in Japan was worsened by delays in stamping-out (22), and due to this fact, the guidelines for specific animal diseases including CSF was revised to include the time limit for the containment

TABLE 5 | Preventive measures implemented at the 39 farms affected by classical swine fever.

	No. of farms	Proportion (*N* = 39)
1. Measures at the boundaries of the farms		
Installation of fences without electricity[a]	26	67%
- part of fences were left open and/or gaps or damages present	12	31%
- without any defects	14	36%
Installation of electric fences[a]	15	38%
- parts of fences were left open and/or gaps or damages present	3	8%
- without any defects	12	31%
Covering ground with hydrated lime at entry points	15	38%
Disinfection of vehicles at entry points	28	72%
- by power sprayer [b]	25	64%
- by disinfection baths [b]	6	15%
- by portable sprayer [b]	2	5%
- by disinfection mats [b]	2	5%
Changing footwear of persons entering the farms	29	74%
Changing clothes of persons entering the farms	23	59%
2. Measures to prevent intrusion into pig houses		
Covering windows of pig houses with bird-proof nets	25	64%
- gaps or damage present	10	26%
- without any defects	15	38%
Changing boots at the entrances of each pig house	22	56%
Changing gloves and clothes at the entrances of each pig house	6	15%

[a] *Fourteen farms had some electric fences and some fences without electricity; 12 farms had fences without electricity only; one farm had electric fences only; and 12 farms did not have any fences.*

[b] *Farms were applying one or more of the ways to disinfect vehicles at entry points.*

measures. The Guideline stipulates that stamping-out should be completed within 24 h, for farms with 1,000 to 2,000 heads of fattening pigs, and that carcasses should be buried or burned within 72 h after confirmation of infection. On the CSF outbreaks in the Netherlands between 1997 and 1998, the median size of the affected farms was 1296.5 animals (25–75th percentile: 800–1,800 animals), and it was reported that 70% of the affected animals were killed within 1 day (26, 27). The relatively longer days required in the affected farms in Japan would reflect relatively large number of animals (median size was 1,271 and 25 and 75th percentile: 625–3,622) reared at infected farms and caused shortage in the available human resources. To complement the shortage of human resources, MAFF coordinated mobilization of official veterinarians of MAFF and surrounding prefectural governments to the affected farms and for several large farms, and the Self-Defense Forces were also deployed to assist activities related to the containment measures. For example, in Gifu Prefecture, a median number of 1,670 (25–75th percentile: 1,108–4,321) people engaged in control activities at an affected farm and the Self-Defense Forces were deployed at 6 out of 20 outbreaks, in which 1,662 to 9,858 animals were subject to stamping-out.

Clinical Manifestations and Transmission of Virus Within Farms

In the recent outbreaks of CSF in Japan, fever and leukopenia were observed in many cases. In the infection experiment of Japanese isolates, fever over 40°C and leukopenia (<10,000 cells/μl) were observed before the fever had started (19). Fever and leukopenia have been reported as common symptoms of CSF infection in the experiments of other strains (1, 2, 26, 28). Non-specific symptoms such as fever and loss of appetite are common for many diseases and frequently observed at farms, hence they are unlikely to lead to notification. Previous reports have also pointed out that there are cases where notification is delayed due to these symptoms being misdiagnosed as other diseases with clinical symptoms not detected until secondary infection occurs (1, 2, 15, 26).

The results of laboratory tests on the affected farms indicate that the infection in fattening pigs tended to take time to develop clinical manifestations, leading to late notification. Fattening pigs are kept in groups with continuous feeding, therefore, it might be difficult to recognize abnormalities when their appetite is low in the early stages of infection. Notifications may only be made after the appearance of dead pigs, after the number of infected pigs has increased. In addition, there were significantly more cases of respiratory symptoms leading to notifications among fattening pigs than among sows and piglets. It is also possible that CSF infections worsen the clinical symptoms of fattening pigs which have already been infected with other respiratory diseases.

The piglet deaths at the time of notifications were observed in the weaning pigs of each farm. In CSF, piglets suffering from vertical infection are known to be persistently infected and it is possible that these piglets only developed clinical symptoms after weaning. For the piglets showing PCR(+)/ELISA(+), it is thought that viruses are not eliminated despite antibody production. The same condition has been reported in infection

experiments with low to moderately pathogenic strains of CSF (29, 30).

In most cases, increases in the number of dead animals to more than 0.5% of the total number was not observed. This result was concordant with the result of the study of experimental infection using the virus strain isolated from the 2018 Japan outbreak. In that study, no infected animals died during the study period up to 28 days post-infection (19). These data suggest that detection of CSF infections by death is difficult in sub-acute CSF infections because the proportion of animals dying does not change significantly.

About 80% of the farms (23/29 farms) had infection in pigs at multiple stages. Infections confined to a single stage were observed mainly in the farms housing only fattening pigs. In addition, this study showed the association between the infection in sows and the occurrence of infection in multiple pig houses. It is also suggested by a previous study using a simulation model that the on-farm infection started in sows could only be noticed clinically when transmitted to weaning or fattening pig groups (31). These studies suggest that infection in sows would cause infection in piglets. These piglets move to other pig houses and become a source of infection to other pig houses and finally detected when they show clinical signs. It is important to note that transmission through the movement of infected pigs cannot be prevented by strengthening biosecurity measures such as disinfection.

Source of Infection to the Farms

The geographical distribution of outbreaks and infected wild boars in Gifu Prefecture suggested that infected wild boars around the farm might have been the source of infection. The fact that many of the outbreak farms were within 5 km of the proximate infected wild boar detection sites suggested that infected wild boars were the source of infection to the farms. A study on the geographic analysis of the risk of infection from infected boars for the recent Japanese CSF outbreak indicated that the risk was dependent on the distance to the infected boar and that the risk of infection extended to farms within 5 km (20). However, among the farms located within 5 km of the proximate infected wild boar, the intrusion of wild boars into farms was not confirmed by witnessing any signs or footprints of food exploring on livestock, except for one outbreak at the Gifu Prefectural Park. This could indicate that the virus carried by wild boars in the area surrounding the farms might have been secondarily carried into the farms by other wild animals or persons. It is also possible that small wild animals, such as rats and wild birds including crows, could also carry the virus into the farms, although it has not been proven that these animals can transmit the virus to date, and further verification is needed (25, 32, 33).

To enhance the biosecurity measures conducted at pig farms, MAFF has provided several rounds of guidance since the first outbreak in September 2018 on how to comply with the biosecurity standards at farms, including countermeasures against the entry of wild animals. Although vaccination for pigs began in prefectures with infected wild boars in October 2019, biosecurity measures at farms will continue to be important.

As for the outbreaks at the southern peninsula of Aichi Prefecture, infected wild boars were not found near the affected farms. Therefore, the involvement of infected wild boars on those outbreaks is unclear. Genetic analysis has shown that the there is a relationship between strains isolated from infected farms in an area with infected wild boars in Gifu Prefecture and strains isolated from some of the outbreak farms in the southern peninsula area of Aichi Prefecture. Since there was no epidemiological relationship found between those farms, it is considered that the long-distance transmission may have occurred indirectly through vehicles or other fomites traveling between these areas (34).

Considering the role of wild boars in the spread of the disease, the surveillance in wild boars is an important issue. At present in Japan, there are difficulties in conducting and continuing the surveillance of wild boars to monitor CSF infection mainly because of the lack of specific legal and organizational system for disease surveillance in wild animals. Future control plans on CSF in Japan should be discussed with more detailed investigation on the interaction between the infection in pigs and that in wild boars.

If the distance between farms is <1 km, there is a possibility of occurrence of local transmission (35). As for the affected farms, the farms possibly affected by local transmission were located in two areas in Aichi Prefecture, that is, one in the southern peninsula area and one in the northern area adjacent to Gifu Prefecture. Full genome analysis showed that the virus strains isolated from two of the three farms in the northern area were closely related to each other, but the remaining one was different. The virus from this one farm and the virus isolated from five farms in the southern peninsula area were closely related (34). This suggests that in some cases, transmission of the virus was considered to be occurring between neighboring farms. In contrast, more farms were located more than 1 km apart from each other, suggesting that in many farms, outbreaks were caused by factors other than local transmission such as transmission through people, fomites, or wildlife that had some contact with infected wild boars.

For the previous CSF outbreaks, feedstuffs such as kitchen residues were considered to be one of the major sources of infection on farms (15). It is unlikely that swill feeding was the cause of the outbreaks during the 2018 Japan outbreak as only one of the six farms using non-commercial feeds was feeding kitchen waste residues. The other four farms using non-commercial feeds mainly used food plant residues which did not include meat. Therefore, there was no possibility of contamination by the meat. For the two farms that also fed vegetable scraps and weeds, the possibility that these feeds were the source of infection cannot be ruled out, as infected wild boars have been found in the areas surrounding the vegetable scrap and weed collections.

This study mainly focuses on the features of the affected farms and the comparison between affected farms and non-affected farms have not been conducted. To elucidate the factors influencing the risk of CSF infection, comparison between infected and non-infected farms will be necessary. Whether there is a difference in the risk of occurrence due to the structure

of pig houses or other status of biosecurity measures will need to be verified through case-control studies and other studies in the future.

CONCLUSION

It appears that the current CSF outbreaks in Japan were caused by a virus originating from neighboring countries that spread to pig farms, but the specific route of entry into Japan is unknown. Since most of the infections have occurred in the areas where infected wild boars have been detected and the areas with infected farms are expanding with the expansion of the range of infected wild boars, it is likely that infected wild boars are the main source of infection. Clinical symptoms are non-specific and are difficult to detect during the early stages of the infection. In areas at high risk of infection, daily clinical observation and early testing for pigs showing loss of appetite and listlessness are required. Areas with infected wild boars are still expanding more than a year after the first outbreak. It is necessary to continue to strengthen biosecurity measures at farms located in areas with infected wild boars and to monitor the distribution of infected wild boars.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

YH conceived the study. YS and TY analyzed the data and wrote the main manuscript text. YH, YM, KS, and EY contributed to the interpretation of the results and helped draft the manuscript. All authors reviewed the manuscript.

FUNDING

This study was conducted under the research project on Regulatory research projects for food safety, animal health and plant protection (JPJ008617.18065101 and JPJ008617.20319390) funded by the Ministry of Agriculture, Forestry and Fisheries of Japan (FY2018-2022 and FY2020-2024).

ACKNOWLEDGMENTS

We appreciate the kind cooperation of the Ministry of Agriculture, Forestry and Fisheries, Government of Japan and the local governments for providing epidemiological information on the CSF outbreaks in Japan.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.573480/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 22 September 2020
doi: 10.3389/fvets.2020.566446

A NanoLuc Luciferase Reporter Pseudorabies Virus for Live Imaging and Quantification of Viral Infection

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Pseudorabies (PR), also known as Aujeszky's disease, is an acute infectious disease of pigs, resulting in significant economic losses to the pig industry in many countries. Since 2011, PR outbreaks have occurred in many Bartha-K61-vaccinated pig farms in China. The emerging pseudorabies virus (PRV) variants possess higher pathogenicity in pigs and mice than the strains isolated before. Here, a recombinant PRV (rPRVTJ-NLuc) stably expressing the NanoLuc (NLuc) luciferase fusion with the red fluorescent protein (DsRed) was constructed to trace viral replication and spread in mice. Moreover, both DsRed and NLuc luciferases were stably expressed in the infected cells, and there was no significant difference between wild-type and recombinant viruses in both growth kinetics and pathogenicity. Seven-week-old BALB/c mice were infected with 10^3 50% tissue culture infective dose rPRVTJ-NLuc and subjected to daily imaging. The mice infected with rPRVTJ-NLuc displayed robust bioluminescence that started 4 days postinfection (dpi), bioluminescence signal increased over time, peaked at 5 dpi, remained detectable for at least 6 dpi, and disappeared at 7 dpi, meanwhile, the increased flux accompanied by the spread of the virus from the injection site to the superior respiratory tract. However, the signal was also observed in the spinal cord, trigeminal ganglion, and partial region of the brain from separated tissues, not in living mice. Our results depicted a new approach to rapidly access the replication and pathogenicity of emerging PRVs in mice.

Keywords: pseudorabies virus, NanoLuc luciferase, *in vivo*, image, mouse

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *28 May 2020*
Accepted: *13 August 2020*
Published: *22 September 2020*

Citation:
Wang Y, Wu H, Wang B, Qi H, Jin Z, Qiu H-J and Sun Y (2020) A NanoLuc Luciferase Reporter Pseudorabies Virus for Live Imaging and Quantification of Viral Infection. Front. Vet. Sci. 7:566446. doi: 10.3389/fvets.2020.566446

INTRODUCTION

Pseudorabies (PR), a devastating disease in the pig industry worldwide, is characterized by neurological signs, respiratory signs, high morbidity, and mortality of piglets, whereas older pigs mostly exhibit respiratory and reproductive diseases (1). Pseudorabies virus (PRV) is the causative agent of the acute infectious PR in swine. Due to control efforts and strict implementation of national eradication programs, PR has been eradicated from domestic pigs in North America and several European countries. However, the disease is sporadic in many other countries, including China (2). In late 2011, there was a PR outbreak on many large pig farms where piglets were vaccinated with Bartha-K61vaccine, and quickly the disease occurred in six provinces in China. The mortality rate of infected piglets was from 10 to 50%, which caused great economic loss (3). Moreover, about 57.8% of 5,033 serum samples isolated during the year 2013 to 2016 were positive for PRV gE antibody, so PRV variant strains were still prevalent in China (4). The PRV variants

shared 97.1–99.9% nucleotide (nt) and 96.6–99.5% amino acid (aa) homology with PRV reference strains, and they belonged to different clades (3). Mutation of glycoproteins C and D of PRV variants led to the escape from Bartha-K61 vaccine-induced immunity (5), so gE/gI/TK-deleted PRV variant strains as a substitute for Bartha-K61 vaccine were used in China to control PR at present. Except for the immunogenicity change, the pathogenicity of PRV variant strains enhanced compared with the classic strain in both pigs and mice; however, it is not clear about the mechanism of enhanced pathogenicity to its hosts (6).

Fluorescent proteins or luciferase-tagged viruses have been widely used in the researches of viral infection and replication mechanisms (7–9). Reporters that have luminescence properties possess greater advantages in some areas of virological research due to higher signal–noise ratio and sensitivity compared with fluorescent proteins (10). Many studies have demonstrated several significant advantages of investigating viral pathogenesis with imaging. Bioluminescence imaging (BLI) is a powerful alternative, enabling rapid measurements of viral load and tissue distribution (11–14). Spatial and temporal progression of infection can be quantified, and viral replication and dissemination in the animals can be identified (15). The traditional approaches for viral pathogenicity studies require the killing of animals at diverse time points for the determination of viral titers in excised organs and tissues, whereas BLI does not (16). In addition, BLI can identify unexpected sites or patterns of viral infection that could be missed if organs are not collected or if entire organs are not analyzed for viral titers (14). This approach has been exploited in multiple viruses, including dengue virus, herpes simplex virus type 1, Sindbis virus, influenza virus, and Sendai virus (12, 14, 15, 17, 18).

Here, we generated a recombinant PRV (rPRVTJ-NLuc) stably expressing the engineered luciferase variant NanoLuc and red fluorescent protein DsRed. NLuc is a 19-kDa luciferase engineered from the deep-sea shrimp that possesses ∼150-fold greater specific activity than firefly luciferase (19, 20). The reporter gene of NLuc that fused with DsRed was inserted immediately downstream of the *US9* gene. The replication dynamics of the recombinant PRV is similar to PRVTJ. Moreover, rPRVTJ-NLuc possesses pathogenicity and lethality indistinguishable from those of PRVTJ in mice. These results demonstrated that the recombinant PRV was not attenuated both *in vitro* and *in vivo*. Furthermore, we reported the visualization of PRV infection in mice. These data suggest that imaging of the recombinant PRV can be used to rapidly assess the replication and pathogenicity characteristics of emerging PRVs; these will provide a reference for control PR caused by PRV variants.

MATERIALS AND METHODS

Cells, Viruses, and Plasmids

The PRVTJ strain (GenBank accession number: KJ789182.1) was isolated from a pig farm outbreak in Tianjin of China, propagated in PK-15 cells, and stored at −70°C. PK-15 and Vero cells were maintained at 37°C with 5% carbon dioxide (CO_2) in Dulbecco's modified Eagle medium (DMEM, Thermo-Fisher Scientific, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, United States). Both cell lines were obtained from the China Center for Type Culture Collection (Wuhan, China).

The left and right homologous arms (flanking the PRV *US9* gene, named as L and R) of transfer vector were amplified by using primer pairs P1S/P1R and P2S/P2R. The NLuc gene was amplified with primers P4S/P4R from pNL2.1 vector (Promega, Madison, WI) and inserted into the DsRed expressing vector pDsRed2-C1 (Clontech, USA) through *Eco*RI site firstly, and then, the inserted fragment together with CMV promoter and polyA terminator was amplified by PCR with primers P3S/P3R. The resulting L arm, R arm, and exogenous gene were amplified; 100–300 ng of DNA template was used per reaction for overlap PCR; the long DNA segment was cloned into blunt T-vector and sequenced to make sure to get the target sequence. Then, the large DNA segment was amplified, purified, and ligated into the pOK12 vector (Novagene, USA) between the *Kpn*I and *Xho*I DNA restriction enzyme sites to get the recombinant vector pOK-NLuc-DsRed using T4 DNA ligase (Thermo Scientific, USA).

Transfection, Virus Rescue, and Plaque Purification

The genomic DNA of PRVTJ was extracted using the phenol–chloroform extraction method. Vero cells seeded in six-well culture plates were co-transfected with 1-μg pOK-DsRed-NLuc plasmid and 1-μg genomic DNA of PRVTJ strain using 4 μl of the X-tremeGENE HP DNA transfection reagent (Roche, USA) according to the manufacturer's instructions. The first generation recombinant viruses were collected at 2–3 days after transfection.

For plaque purification, PK-15 cells seeded in six-well cell culture plates were infected with 10-fold serially diluted rPRVTJ-NLuc strain from 10^{-1} to 10^{-5} for 1 h at 37°C in a 5% CO_2 incubator; then, the supernatant was removed; cells were covered with 1% agarose gel and incubated for 2 days till clear cytopathic effect with red fluorescent of DsRed protein formed. Marked plaques were picked by pushing the 200-μl tip through the overlay agarose, and this was diluted in 1-ml DMEM for the next generation of plaque purification. A total of five generations of plaque purification were performed to obtain the purified recombinant viruses.

Virus Titration and One-Step Growth Assay

The viral titer was determined by 50% cell culture infectious dose ($TCID_{50}$). In brief, PK-15 cells seeded in 96-well plates were infected with 10-fold serially diluted viruses (10^{-2} to 10^{-8}) and cultured at 37°C in a 5% CO_2 incubator for 72 h. The number of wells with red fluorescence was counted, and the viral titers were calculated using the Reed & Muench method (21).

One-step growth kinetic of rPRVTJ-NLuc was compared with PRVTJ. The monolayers of PK-15 cells in the 24-well cell culture plates were infected with rPRVTJ-NLuc or PRVTJ at a multiplicity of infection (MOI) of 10 for 1 h at 37°C. Extracellular viruses were inactivated by low-PH treatment (22); supernatant and cells were harvested at different time points (12-h interval) till 60-h post-infection; three repeat samples were harvested at each time point and stored at −80°C. After the sample

returned to room temperate, the cellular debris was removed by centrifugation, and the $TCID_{50}$ of supernatant was detected on PK-15 cells. Average values and standard deviations of the three independent experiments were calculated.

Polymerase Chain Reaction

Genomic DNA was extracted from PK-15 cells infected with rPRVTJ-NLuc or PRVTJ at an MOI of 1 for 12 h using the Tissue DNA Kit (Omega). The primer pairs P5S/P5R, P6S/P6R, and P7S/P7R that were complementary to the glycoprotein B (gB), glyco-protein I (gI), NLuc luciferase, and DsRed fluorescent protein genes are listed in **Table 1**. The amplification was conducted in a total volume of 50 μl containing 25 μl of 2× PrimeSTAR buffer (TaKaRa, Japan), 3 μl of DNA, 9.5 μl of sterilized water, and 1.0 mM for each primer. The reaction was heated at 95°C for 5 min, followed by 35 cycles of 98°C for 10 s, 58°C for 30 s, and 72°C for 2 min, with a final elongation step of 72°C for 10 min. The PCR product was analyzed using 1.0% agarose gel electrophoresis.

Western Blot Assay

The monolayers of PK-15 cells in the 6-well cell culture plates were infected with rPRVTJ-NLuc, rPRVTJ-DsRed, or PRVTJ A at an MOI of 1 at 37°C for 24 h. The total protein of cells was collected after adding an NP40 lysis buffer containing 1% phenylmethylsulfonyl fluoride (Solarbio, Beijing, China) prot, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk for 2 h at 37°C and incubated at room temperature for 2 h with specific mouse anti-gD, an anti-gB monoclonal antibody (a gift of Jing Zhao, State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China) and anti-DsRed polyclonal antibody (Solarbio, Beijing, China). The membranes were washed with phosphate-buffered saline (PBS) with Tween buffer for three times and incubated with DyLight 800 goat anti-mouse IgG (1:8,000) (Thermo Fisher Scientific) at 37°C for 45 min; the membranes were washed for another three times, then visualized and analyzed with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Luciferase Assay

PK-15 cells seeded in 96-well cell culture plates were infected with 10-fold serially diluted rPRVTJ-NLuc strain from 10^0 to 10^{-5} at 37°C in a 5% CO_2 incubator for 12 h. The supernatants were removed, and cells were washed once with PBS before cell culture lysis buffer (Promega) was added. Cell lysates were assayed for luminescence activity with the Nano-Glo Assay System (Promega), and luminescence was detected with TD-20/20 luminometer (Turner Designs).

Infection of Mice With rPRVTJ-NLuc or PRVTJ and Tissue Collection

All the mice were handled according to the Guide for the Care and Use of Laboratory Animals of Harbin Veterinary Research Institute (HVRI), Chinese Academy of Agricultural Sciences, China. Two mice experiments were made in this work: one for the pathogen detection and another one for the live imaging test. For the pathogen detection, 35 6-week-old specific-pathogen-free (SPF) female BALB/c mice were used in this study. The mice were randomly allocated into seven groups, five mice in one box. Mice of groups 1, 2, and 3 were injected intramuscularly (i.m.) with 10^4, 10^3, or 10^2 $TCID_{50}$ of PRV TJ strain in 100-μl DMEM, respectively. Groups 4, 5, and 6 were injected i.m. with 10^4, 10^3, or 10^2 $TCID_{50}$ rPRVTJ-NLuc in 100 μl of DMEM, respectively. Group 7 was the mock-inoculations (medium only) in parallel. Mice were scored daily for symptoms of PRV infection using the following three-point system adapted from the protocol previously described (23). All the mice in the infected and control groups were anesthetized by CO_2 before euthanasia, using the broken-neck method at 7 dpi. Fresh tissues from the heart, liver, spleen, lungs, kidneys, brain, spinal cord, and trigeminal ganglion of mice were collected, one part of the samples was fixed in buffered formalin for hematoxylin and eosin assay, and 100 mg of another part of the samples was stored at −80°C for DNA extraction. Total DNA was extracted using Tissue DNA Kit (Omega) according to the manufacturer's instructions and stored at −20°C for quantitative PCR (qPCR) analysis.

TABLE 1 | Sequences of oligonucleotides used in PCR.

Fragments	Primers	Sequences of primers (5′-3′)	Length (bp)
L arm	P1S	GGTGCCTGCTGTACTACGTGTACGA GCCCTGCATC	1274
	P1R	CTACACGTGCCTGGCGACGATGCC	
R arm	P2S	CGAGCGAGCGAGCGAACGGGAG	1023
	P2R	CTAGGAGATGGTACATCGCGGGGCGC GCTCGCG	
CMV-DsRed -polyA	P3S	TAGATAACTGATCATAATCAGCCATACCA	1486
	P3R	CGCCGTTTAAACGCAGTGAAAAAAATGCTTTA	
NLuc	P4S	GTCTTCACACTCGAAGATTTC	513
	P4R	TTACGCCAGAATGCGTTCGCAC	
gB	P5S	GGGGTTGGACAGGAAGGACACCA	198
	P5R	AACCAGCTGCACGCGCTCAA	
gI	P6S	TGGCTCTGCGTGCTGTGCTC	343
	P6R	CATTCGTCACTTCCGGTTTC	
DsRed	P7S	ATGGCCTCCTCCGAGAACG	747
	P7R	TTATCTAGATCCGGTGGAACCCG	

Real-Time PCR (Quantitative PCR)

Real-time PCR (qPCR) was used to quantitatively analyze the viral loading in the brain, heart, liver, spleen, lung, kidney, spinal cord, and trigeminal ganglion using the reported method of Meng (24). Briefly, viral genomic DNA was amplified using the *gI* gene-specific primer PRV-F1 (5′-GCC GAG TAC CTC TGC C-3′), PRV-R1 (5′-CGA GAC GAA CAG CCG-3′), and TaqMan probes HEX-PRV-Var (HEX-5′-CCG CGT GCA CCA CGA AGC CT-3′-BHQ1); each sample was done in triplicate, deionized water as the negative control.

Pathology and Histopathology

The samples were fixed in buffered formalin and embedded in paraffin wax. Tissue sections were prepared and stained with hematoxylin and eosin assay for histopathological examinations.

In vivo Imaging

For the live imaging test, a total of 30 7-week-old SPF female BALB/c mice were separated into six groups for PRV infection, five mice in each group, and another five mice were injected with DMEM as control. Mice were infected with 10^3 $TCID_{50}$ rPRVTJ-NLuc or PRVTJ by three different inoculation routes: inoculated intraperitoneally (i.p.) (in the lower abdominal region), i.m. (in the right hind leg muscles), or subcutaneously (s.c.) (in the back of the neck). *In vivo* imaging was performed from 3 to 7 dpi with a 24-h interval using the BLI system of the LB 983 NightOWL II (Berthold, Germany) equipped with a cooled slow-scan CCD camera and driven by the IndiGo^{TM} software (version 2.0.5.0, Berthold). At each time point, five mice in each group per day were anesthetized with isoflurane (Burbank, CA 91502, USA) and injected with 100 μl of Nano-Glo reagent (Promega) (diluted 1:20 in PBS) *via* the tail vein. Flux measurements were acquired from regions of interest automatically gated to the signal contours, keeping the mice at 37°C during the whole progress. All the mice in the infected and control groups were anesthetized by CO_2 before euthanasia, using the broken-neck method after imaging the experiment. All data in composite images utilized the same scale.

Statistics

Data represent means ± standard deviations ($n \geq 3$). The comparison between groups was performed by a Student's *t*-test with a two-tailed analysis. Data are considered significant when $P < 0.05$.

RESULTS

Generation and Characterization of the Recombinant Pseudorabies Virus Expressing NanoLuc

PRV genome is characterized by two unique regions (U_L and U_S), and the U_S region flanked by internal and terminal repeat sequences. As reported, the noncoding interval sequence between U_S9 open reading frame and U_S2 open reading frame in the U_S regions is long enough to tolerate large exogenous genes (25). A single cassette of NLuc fused with the DsRed was designed immediately downstream of the *US9* gene of the PRV TJ genome (**Figure 1A**). The transfer vector pOK-DsRed-NLuc with two 1.5-kb homologous arms and PRVTJ genomic DNA were co-transfected into Vero cells, and the first generation of recombinant virus was collected at 2–3 days after transfection when the cytopathic effect formed on the Vero cells. We obtained the purified virus through five rounds of plaque purification. The plaque morphology of rPRVTJ-NLuc had no significant difference from its parental virus (**Figure 1B**). We confirmed that the chimeric NLuc cassette was inserted immediately into the downstream of the *US9* gene of PRVTJ using corresponding specific primers by PCR and sequencing. The stability of the reporter genes, NLuc, and DsRed, was also tested by following amplification and serial passages in PK-15 cells (P1 to P20) (**Figure 1D**). These results indicated there were *gB* and *gI* genes in the genome backbone of the recombinant PRV, which was the same as its parental strain. Also, the reporter genes could exist in the backbone simultaneously and stably, which were detected in the following passages. The expression of DsRed and viral protein was checked by Western blot using antibody target DsRed, gD, and gB proteins. The same bands of gB and gD presented in the samples of rPRVTJ-NLuc, rPRVTJ-DsRed, and PRVTJ infected groups; meanwhile, the band of DsRed protein of rPRVTJ-NLuc was larger than rPRVTJ-DsRed due to NLuc fused with it (**Figure 1E**).

We compared the growth kinetics of rPRVTJ-NLuc with the parental strain PRVTJ in PK-15 cells. The titers of rPRVTJ-NLuc in the culture supernatant had no significant difference with those of PRVTJ during 60-h post-infection (hpi), suggesting that the growth properties of rPRVTJ-NLuc were similar to those of PRVTJ (**Figure 1C**). To examine the relationship between NLuc expression level and rPRVTJ-NLuc infection, the correlation between the input of rPRVTJ-NLuc and NLuc luciferase activities was evaluated. PK-15 cells were infected with rPRVTJ-NLuc with different amounts of input virus: ranging from 10^0 to 10^5 $TCID_{50}$/ml. NLuc luciferase activities were measured at 12 hpi. The activities of luciferase in supernatants increased over time, and the yield of luminescent flux was directly correlated with viral titers ($R^2 = 0.96$) (**Figures 1F,G**). The result demonstrated that the luminescent flux could represent the titer of the recombinant PRV in the infected cell.

rPRVTJ-NLuc Possesses Similar Pathogenicity to PRVTJ in Mice

To monitor symptoms and organ lesions preferably, mice were infected with 10^4, 10^3, or 10^2 $TCID_{50}$ PRVTJ or rPRVTJ-NLuc *via* the i.m. route. Clinical signs, including pruritus, anxiety, rolling, and scratching of the injection site, were recorded every day throughout the experiment. The mice infected with 10^4 $TCID_{50}$ viruses (groups 1 and 4) began showing clinical signs around 72 hpi. The mice infected with 10^3 $TCID_{50}$ viruses (groups 2 and 5) showed similar symptoms but delayed. The mice infected with 10^2 $TCID_{50}$ doses of viruses (groups 3 and 6) showed barely clinical symptoms. No difference was observed in the clinical performance of mice injected with the same dose of PRVTJ and rPRVTJ-NLuc (**Table 2**). Mice infected with 10^4, 10^3, or 10^2 $TCID_{50}$ of PRVTJ or rPRVTJ-NLuc lost body weight and led to die in a dose-dependent manner (**Figures 2A, B**). The mice infected with the same dose of PRVTJ or rPRVTJ-NLuc exhibited similar weight loss and mortality. These results demonstrated that the clinical symptoms of the recombinant PRV did not change after the insertion of the reporter genes.

Next, we determined whether the fatal clinical outcome and distinct pathology of the mice that were infected could be attributed to viral replication in specific tissues. Various tissues, as listed earlier, were collected after euthanasia for qPCR analysis to determine PRV DNA loads. As shown in **Figure 2C**, the virus could be detected in the brain, kidney, spinal cord, and

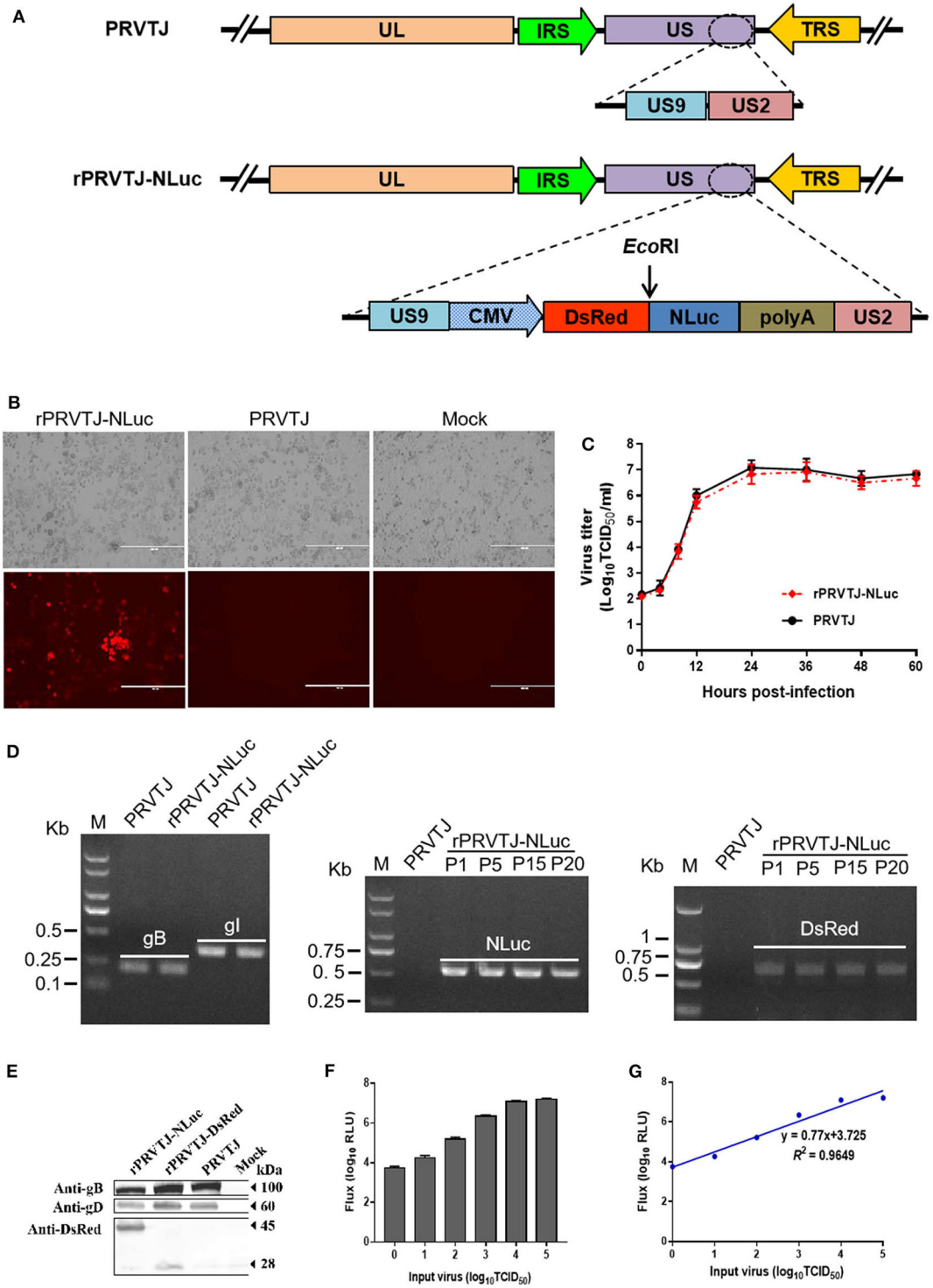

FIGURE 1 | Generation and characterization of the recombinant PRV expressing NLuc luciferase. **(A)** Schematic representation of NLuc and DsRed cassette inserting immediately downstream of the *US9* gene. **(B)** Recombinant PRV can form plaques and express DsRed in the PRV-infected PK-15 cells. Original magnification ×200; bar, 400 μm. **(C)** One-step growth curve of rPRVTJ-NLuc. **(D)** Identify of rPRVTJ-NLuc by PCR. Left panel, verification of the *gB*, *gI* gene in the genome of rPRVTJ-NLuc, and PRVTJ. Middle panel and right panel, identification of the NLuc and DsRed reporter genes in different passages in infected PK-15 cells, respectively. **(E)** Expression of NLuc fused with DsRed in rPRVTJ-NLuc-infected cells was detected by Western blot, the DsRed expression of rPRVTJ-DsRed as control; moreover, viral protein gD and gB of all PRV strains were also detected using anti-gD and anti-gB monoclonal antibodies, normal cells as mock. **(F)** Average luciferase activity of the recombinant PRV in the PK-15 cells at 12 hpi with defined amounts of input virus ($n = 5$). **(G)** Correlation curve between titers of virus and luciferase activities using Pearson's correlation coefficient.

TABLE 2 | Clinical signs score of the mice infected with rPRVTJ-NLuc or PRVTJ.

Virus	Dose ($TCID_{50}$)	Days post-infection						
		1	2	3	4	5	6	7
PRVTJ	10^4	0	0	11 ± 1	-	-	-	-
	10^3	0	0	6.6 ± 0.55	-	-	-	-
	10^2	0	0	4 ± 0.71	7.25 ± 0.5	4 ± 1	2 ± 1	2 ± 0
rPRVTJ-NLuc	10^4	0	0	9.75 ± 0.96	-	-	-	-
	10^3	0	0	4.5 ± 1	10 ± 1.73	5 ± 0	-	-
	10^2	0	0	3 ± 0.71	5.8 ± 0.84	2.6 ± 0.89	2 ± 1	3 ± 0
DMEM	100 μl	0	0	0	0	0	0	0

Extended scoring systems for clinical signs. Clinical signs were recorded every day, incorporating 4 parameters such as:pruritus, anxiety, rolling and scratching of the injection site, each parameter was scored from 0 = no clinical symptom; 1 = mild symptoms, such as subtle neurological symptoms, untidy hair and minor depression; 2 = common symptoms, such as pruritus, rolling and scratching the injection site; 3 = severe symptoms, such as severe pruritus, self-mutilate even acute death. The results are expressed as mean value ± s.d. (n = 5) of total value of 4 parameters. -, death.

trigeminal ganglion of peracute death mice infected with 10^4 or 10^3 $TCID_{50}$ PRVs groups (groups 1, 2, 4, and 5). No virus was detected in the 10^2 $TCID_{50}$ groups (groups 3 and 6), as well as the control group (group 7). PRV replication was detected principally in kidneys, brain, spinal cord, and trigeminal ganglion tissues after i.m. injection, but there was no significant difference in PRV DNA loads between rPRVTJ-NLuc and PRVTJ infected groups, which was consistent with the clinical signs score and pathological analysis, indicating that the replication and spread properties of rPRVTJ-NLuc were similar to those of the parental strain.

Histopathological examination of several tissues (brain, lungs, and kidneys) was performed to check the difference between the mice infected with PRVTJ and rPRVTJ-NLuc (**Figure 2D**), and tissue samples were taken from three mice in each group at the humane endpoint. Firstly, there were local hemorrhage, degeneration, necrosis of partial neuron, Purkinje cell, and glial cell proliferation in the brain of both PRV infected groups. The lungs of the PRV-infected groups showed congestion, the proliferation of alveolar epithelial cells, and a few lymphocytes infiltration. There were degeneration and necrosis of renal tubular epithelial cells in the kidneys of the PRV infected groups. Secondly, it is noteworthy that low-dose PRV infection induces significant histopathological changes in the central nervous system (CNS) of mice as well. The histopathological changes of these mice organs infected with PRVTJ or rPRVTJ-NLuc did not differ on every dosage. Finally, the heart, liver, and spleen of all the PRV-infected mice and all the mice infected with 10^2 $TCID_{50}$ (groups 3 and 6) PRV had no histopathological changes. Collectively, these data indicated that the insertion of NLuc into the PRV genome did not influence histopathological changes of PRV-infected mice.

In summary, there is no difference in clinical symptoms, replication, spread, and histopathology between the mice infected with rPRVTJ-NLuc and its parental virus. So, the insertion of NLuc into the PRV genome was stable, and the recombinant rPRVTJ-NLuc could be used as a tool to study the pathogenicity of PRVTJ.

In vivo Imaging of rPRVTJ-NLuc Showed the Viral Replication and Dissemination in Mice

BLI has been exploited in multiple viruses, including dengue virus, herpes simplex virus type 1, Sindbis virus, and Sendai virus (15, 17, 18). Here, we used rPRVTJ-NLuc to visualize PRV replication and spread in mice. Seven-week-old BALB/c mice were infected with 10^3 $TCID_{50}$ rPRVTJ-NLuc and subjected to daily imaging (**Figure 3A**). Bioluminescence was detected as early as 4 dpi at the site of injection. Luminescent flux increased throughout infection and peaked on day 6, then waning. The enhanced signal could be detected when rPRVTJ-NLuc spread from the injection site to the superior respiratory tract.

To get better *in vivo* images, mice were infected with rPRVTJ-NLuc and PRVTJ by three different inoculation routes. Mice were infected with 10^3 $TCID_{50}$ viruses *via* inoculated i.p. (in the lower abdominal region), i.m. (in the right hind leg muscles), or s.c. (in the back of the neck). As expected, mice infected with rPRVTJ-NLuc displayed robust bioluminescence, which started 4 dpi, increased over time, peaked at 5 dpi, remained detectable for at least 6 dpi (**Figure 3B**), and disappeared at 7 dpi when the virus was cleared after a sublethal infection. All mice inoculated viruses *via* i.m. route showed severe clinical symptoms at 4 dpi, pruritus, rolling, and scratching the injection site, and all died at 5 dpi. This result illustrated that the i.m. route may more easily cause peracute death. Real-time imaging of the same mouse over time from each group revealed viral load and dissemination from the injection site to the spinal cord and CNS.

To confirm the local tissues scattering bioluminescence, selected mice were killed, and their organs were immediately removed. Isolated organs were placed in a furimazine bath, and BLI was undertaken. Signals of the spinal cord, trigeminal ganglion, and a partial region of the brain were observed (**Figure 3C**). There were robust bioluminescence signals in the spinal cord, brain, and trigeminal ganglion based on the flux of each excised organs (**Figure 3C**). These results were consistent with the viral distribution in diverse organs of mice infected with rPRVTJ-NLuc, which possessed the ability to visualize a pathogenic infection in mice.

DISCUSSION

Since late 2011, there were PR-like outbreaks in a large number of vaccinated pig farms in China (26). It is found that the PRV variant strain and the classical strain belong to different phylogenetic branches. In our previous study, the epidemic strain exhibited enhanced pathogenicity in mice and pigs (6). The reemergence of the PRV outbreak resulted in huge economic losses to the pig industry in China, so the ability that can quickly assess the pathogenicity of the epidemic strains and its susceptibility to antiviral interventions was urgently required.

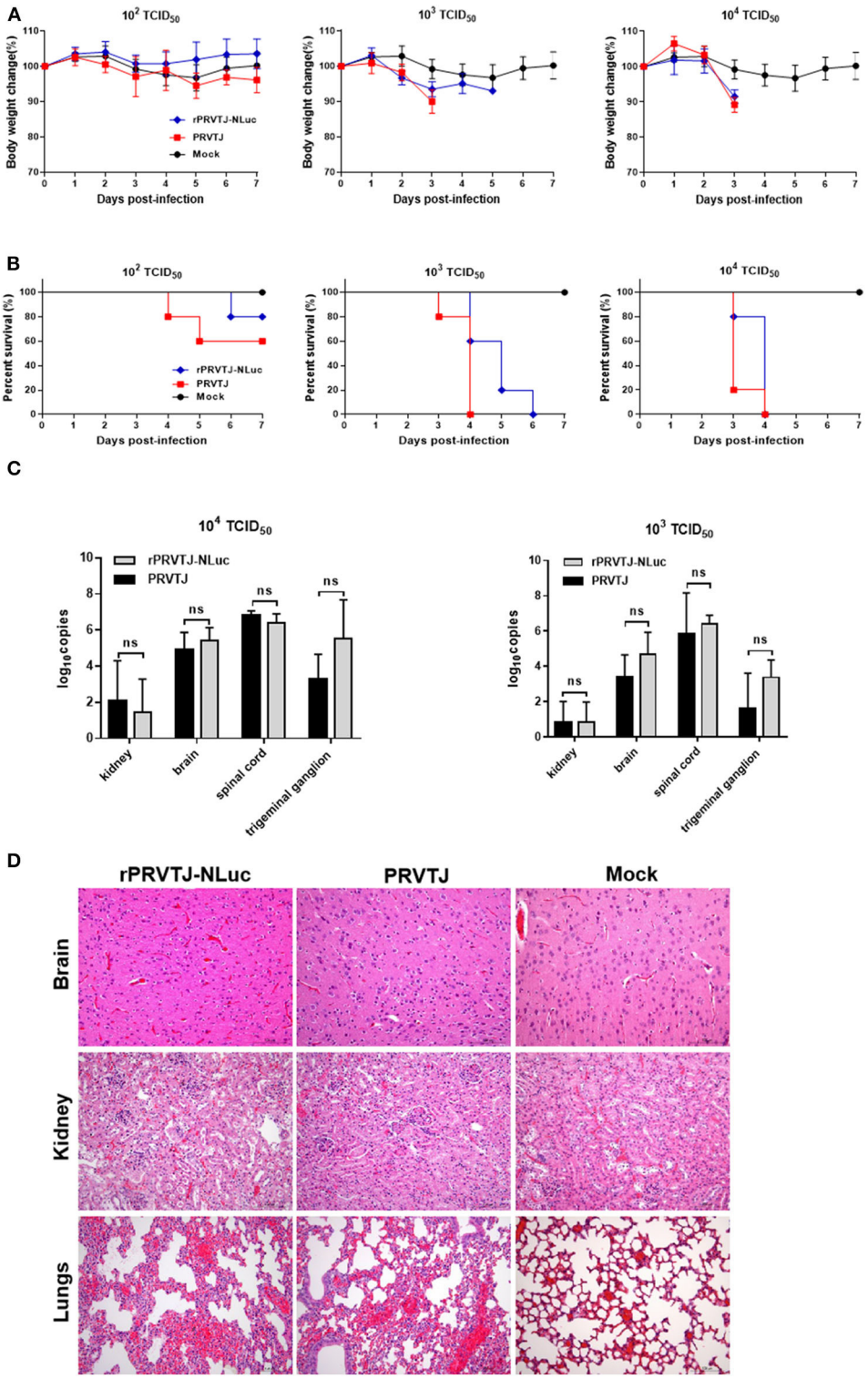

FIGURE 2 | Pathogenicity of recombinant PRV compared with PRVTJ in mice. Six-week-old SPF BALB/c mice were injected intramuscularly with 10^4, 10^3, or 10^2 $TCID_{50}$ of rPRVTJ-NLuc or PRVTJ. **(A)** Average body weights of the infected mice ($n = 5$) at 7 dpi. **(B)** Survival rates of mice infected indicated a dose of viruses. **(C)** Viral DNA copies in different tissue. All the mock-infected and PRV-infected mice were euthanized and subjected to dissection at a moribund stage or 7 dpi. Specific tissues were collected from the mice infected with 10^4 or 10^3 $TCID_{50}$ rPRVTJ-NLuc or PRVTJ and tested by qPCR. **(D)** Histopathological changes in diverse organs of mice infected with rPRVTJ-NLuc or PRVTJ; bar = 100 μm.

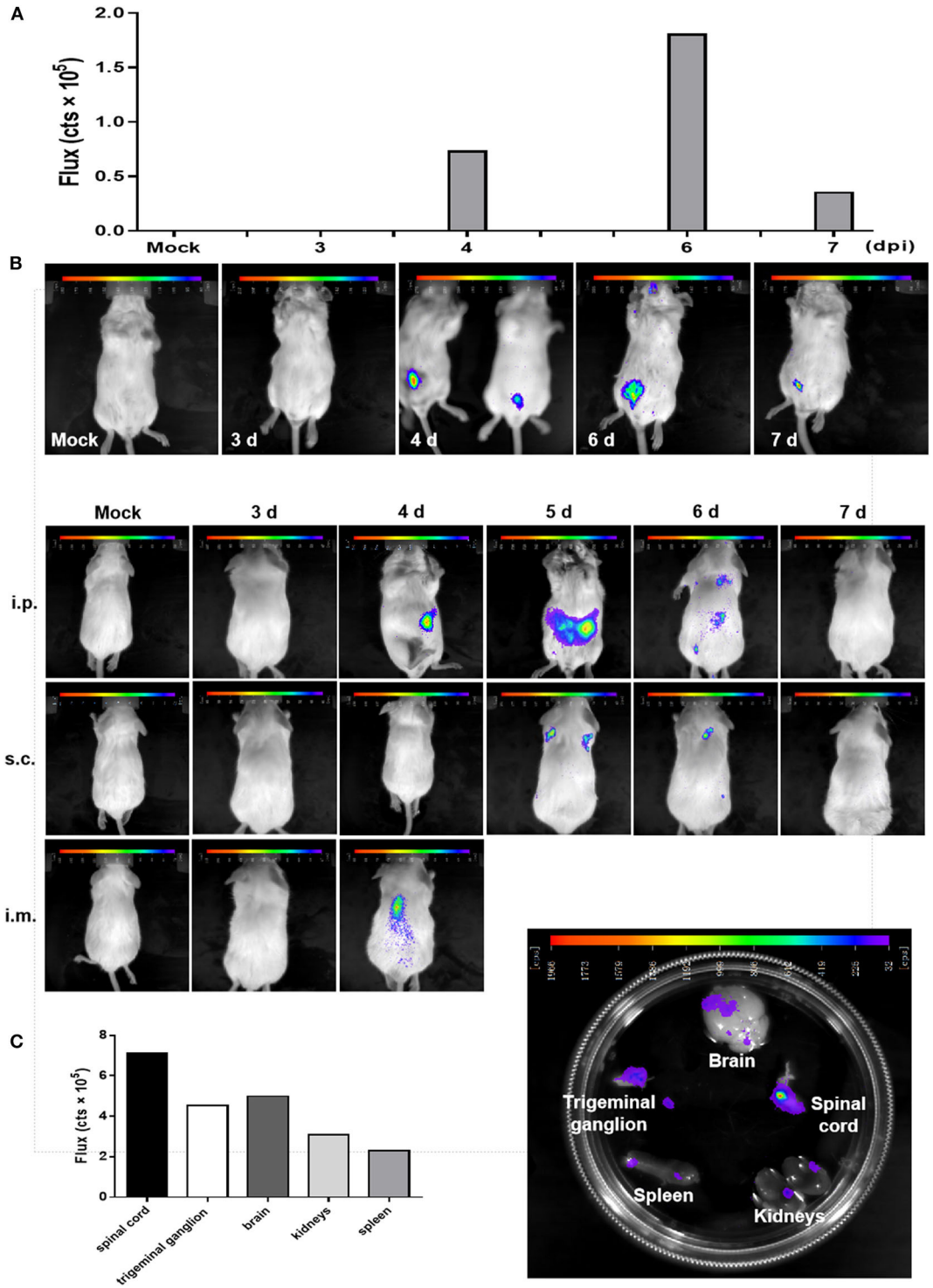

FIGURE 3 | *In vivo* imaging of a PRV reporter in a mouse model. **(A)** Noninvasive imaging detected robust bioluminescence in the mice infected with 10^3 $TCID_{50}$ rPRVTJ-NLuc or PRVTJ and analyzed for bioluminescence at indicated time points. **(B)** Longitudinal *in vivo* imaging of rPRVTJ-NLuc in mice showed viral replication and dissemination. Mice were inoculated intraperitoneally, subcutaneously, or intramuscularly with 10^3 $TCID_{50}$ rPRVTJ-NLuc and PRVTJ. *In vivo* imaging was performed every day from 3 dpi at the indicated time points. **(C)** *In situ* localization of NLuc luciferase activity in the excised organs of mice infected with the recombinant PRV. Seven-week-old SPF BALB/c mice were i.p. inoculated with 10^3 $TCID_{50}$ rPRVTJ-NLuc and killed at 4 dpi. Organs were dissected out and placed separately in a substrate bath. Bioluminescent images were taken 10 min later, and the representative image shows the distribution of luminescent activity in the specimen (right). Quantification of the luminescent activity in the excised organs. After an exposure time of 30 s, a region of interest was manually defined, and the luminescent activity was calculated by the IndiGo™ software (left). All the images of the mice shown in the figure were one representation of five repeats.

In this study, we constructed a recombinant PRV stably expressing the NLuc luciferase fusion with red fluorescent protein DsRed. The double-labeled strategy is convenient for *in vitro* screening of the recombinant virus with DsRed and the *in vivo* imaging with NLuc luciferase. There was no difference in the growth properties of rPRVTJ-NLuc and PRVTJ. Moreover, the recombinant rPRVTJ-NLuc possessed pathogenicity and lethality in mice indistinguishable from those of PRVTJ.

The infection of PRV in rodents routinely shows severe clinical manifestations, including pretty itchy, frantically clawing, and biting of the inoculation site, even self-mutilation and acute death eventually (27). To monitor the symptoms and organ lesions preferably, we infected mice with a lower dosage, 10^4, 10^3, or 10^2 $TCID_{50}$ PRVTJ, or rPRVTJ-NLuc *via* i.m. route. Similar symptoms were found in mice infected with 10^4 or 10^3 $TCID_{50}$ PRVTJ or rPRVTJ-NLuc; the groups of 10^2 $TCID_{50}$ viruses and control were no symptomatic; all these results were the same as Brittle reported. Previous studies demonstrated that mice infected with PRV-Becker develop severe pruritus in the inoculation site, resulting in self-mutilation and peracute death with no detectable behavioral CNS pathology and no obvious pathological changes in the brain. Merely, the peripheral nervous system (dorsal root ganglia and trigeminal ganglia) has significant pathological changes (2, 28). Nevertheless, we detected the replication of the virus and inflammatory pathological changes in CNS tissue and kidneys of mice; maybe, these explained why PRVTJ has higher pathogenicity than the classic PRV strains.

The sensitivity and noninvasive longitudinal measurements enable the monitoring of PRV infection and clearance of a lower-dose infection of rPRVTJ-NLuc in mice (**Figures 3A,B**). Actually, 6-week-old mice were tended to death because of PRV infection. To obtain continuously spatial and temporal progression of PRV infection, the 7-week-old mice were used to perform *in vivo* imaging. In a mouse inoculated i.p. with the dose of 10^3 $TCID_{50}$, the infection began primarily in i.p. injection site. By 6 dpi, the bioluminescence signal decreased and appeared in the position of the spinal cord, indicating the spread of the virus to the CNS. We confirmed the infection of the spinal cord by imaging of excised organs and qPCR (**Figure 3C**), showing stronger bioluminescence and higher virus load in the spinal cord than *in vivo* image. The infection continued to be cleared and was undetectable at 7 dpi. This longitudinal measurement course could detect the dynamics of PRV by *in vivo* imaging and elucidate mechanisms of viral dissemination in a mouse model. It is compelling that a series of reporter virus expressing the NLuc luciferase has the potential to assess the pathogenicity and properties of viral replication and *in vivo* dissemination of diverse PRV stains. Thus, this strategy should be widely applicable to any PRV isolate and will be useful to rapidly access the replication and pathogenicity characteristics of emerging PRV strains.

Although the BLI potentially offers significant advantages over other traditional virological methods, it also has a few disadvantages, e.g., the attenuation of the signal by hair and organ pigmentation, overlapping signals, and the attenuation of signals due to organ depth from the surface. Bioluminescence was not detected in the brain in any of our experiments of whole-body imaging, which is most probably due to the signal attenuation caused by the skull of mice. We confirmed the bioluminescence signal in the brain and trigeminal ganglion by imaging of excised organs. The result is consistent with detectable viral distribution in diverse tissues. Therefore, these data show that although bioluminescence is promising, precise viral tracking and the exact quantification of viral titers require combinations of these methodologies.

In summary, the recombinant PRV stably expressing NLuc luciferase is shown to be a potential tool for studying the pathogenicity mechanism of the PRV variant. We further showed that the recombinant PRV could be applied to study the infection mechanisms of PRVTJ strain by BLI. In further studies, the reporter virus will be used in the research of pathogenicity mechanism diversity of PRV variants and classic strains.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article.

ETHICS STATEMENT

All the animal experiments were approved by the Committee on the Ethics of Animal Experiments of the State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. Five SPF BALB/c mice were breed in one box, mice were euthanized using sodium pentobarbital anesthetic before living-image and all recommended efforts were taken to minimize suffering.

AUTHOR CONTRIBUTIONS

YS and H-JQ designed research. YW and HW performed experiments. BW wrote the manuscript. The authors YW, HW, and BW have the equal contribution for this work and all authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Key Research and Development Program of China (No. 2016YFD0500105), the National Natural Science Foundation of China (Nos. 31870152 and 31702279), and the Heilongjiang Natural Science Foundation (Nos. C2018067 and QC2018029).

ACKNOWLEDGMENTS

We thank HQ, ZJ, and employees of the Research Institute for Biological Safety Problems for their assistance in the present study.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 25 September 2020
doi: 10.3389/fvets.2020.565773

The Distribution and Localization of Collagen Triple Helix Repeat Containing-1 in Naturally and Experimentally Avian Leukosis Virus Subgroup J-Infected Chickens

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 26 May 2020
Accepted: 17 August 2020
Published: 25 September 2020

Citation:
Pang Y, Zhou D, Zhou J, Xue J, Wang Y and Cheng Z (2020) The Distribution and Localization of Collagen Triple Helix Repeat Containing-1 in Naturally and Experimentally Avian Leukosis Virus Subgroup J-Infected Chickens. Front. Vet. Sci. 7:565773. doi: 10.3389/fvets.2020.565773

Collagen triple helix repeat containing-1 (CTHRC1) has recently been identified as avian leukosis virus subgroup J (ALV-J) replication-dependent factor that remarkably facilitates ALV-J replication via interaction with the envelope glycoprotein (SU) of ALV-J. However, the dynamic distribution and localization of CTHRC1 in various tissues upon ALV-J infection are still unknown. In this study, data revealed that the levels of CTHRC1 were significantly increased in various tissues and that the protein was mainly located in the cytoplasm and nucleus of parenchymal cells in tissues of chickens that were infected by ALV-J naturally and experimentally. Interestingly, CTHRC1 was also observed in leukocytes other than erythrocytes in congested veins of ALV-J-infected tissues. Consequently, the positive cells in these veins were confirmed as lymphocytes by laser confocal microscopy. Taken together, these results conclude that the CTHRC1 is an inducible protein and exhibited ubiquitous expression in ALV-J-infected chickens, which may provide basic information for in-depth study of ALV-J infection and replication mechanisms.

Keywords: ALV-J, Cthrc1, IHC, Myelocytomas, Poultry

INTRODUCTION

Avian leukosis virus subgroups (ALVs) belonging to type C *retroviruses* are generally divided into seven subgroups (A–E, J, and K) (1–3). It is noteworthy that since isolation of the first strain (HPRS-103) from meat-type chickens in the United Kingdom in the late 1980s, more attention has been focused on the J subgroup of ALV (ALV-J), which caused high mortality, wide host ranges, and strong tumorigenicity (4, 5). Thus, it is absolutely imperative that the strategies should be implemented to prevent and control ALV-J infection in poultry industry. Attributed to the implementation of ALV-J eradication program (6), there were rarely reports of ALV-J or myelocytomatosis in China after 2013. Nevertheless, there were another intensive reports about ALV-J breakout almost simultaneously in six provinces of China in 2018, which attracted our attention (7). It is rather remarkable that there were extensive medullary-like tumor cells in the bone marrow, liver, and kidney, and all of the neoplasms were myelocytomas (7).

In recent years, a variety of studies have explored that collagen triple helix repeat containing-1 (CTHRC1) highly related to collagen synthesis was remarkably up-regulated in solid tumors (8–10), including hepatocellular carcinoma (11, 12), oral squamous cell carcinoma (13, 14), gastric carcinogenesis (9, 15), ductal carcinoma (16), and colorectal cancer (17–19), and that the expression abundance of CTHRC1 was significant low in normal tissues. CTHRC1 is a highly conserved cancer-secreted glycosylated protein; human CTHRC1 shares 80% sequence identity with the *Gallus* homolog, which was discovered in a screening for differentially expressed genes in a rat model of balloon-injured vasculature (15, 20–22). Thus far, the studies of CTHRC1 focus on the field of oncology; there is rare coverage on the role of CTHRC1 in the field of viral replication. Recently, we have firstly reported that CTHRC1 facilitates ALV-J replication through interaction with the SU protein of ALV-J, which indicates that CTHRC1 plays a crucial role in regulating ALV-J replication (23). These studies may provide new enlightenment to the study of interaction between host protein(s) and ALV-J, and these expand the biological function of CTHRC1 in the field of virus replication (23). However, the distribution and expression of CTHRC1 in chicken tissues upon ALV-J infection are still unknown, which may provide a theoretical basis for the subsequent research of ALV-J and CTHRC1.

In the study, we investigated for the first time the expression and distribution of CTHRC1 in chickens that were infected with ALV-J naturally and experimentally. Interestingly, we found that ALV-J could infect with lymphocytes and activate the expression of CTHRC1 in the lymphocytes.

MATERIALS AND METHODS

Virus, Antibodies, and Experimental Animals

ALV-J strain GM0209 was isolated from a broiler breeder maintained in our laboratory (7). Monoclonal antibody directed against ALV-J SU protein was produced as described previously (23, 24). Rabbit polyclonal antibody against CTHRC1 was purchased from Absin Corporation (Shanghai, China). Specific pathogen free (SPF) chick embryos were acquired from the SPAFAS Corporation (Jinan, China; a joint venture with Charles River Lab, Wilmington, MA, USA). Chick embryos were incubated in an SPF environment at the Laboratory Animal and Resources Facility, Shandong Agricultural University. Chick embryos were inoculated with ALV-J (GM0209 strain) via allantoic cavity at the sixth embryonic age, with the titer of $10^{3.8}$ tissue culture infective dose ($TCID_{50}$)/0.1 ml. Chicks then were euthanized at 15, 22, and 35 days post-infection. Chick heart, liver, kidney, spleen, lung, brain, duodenum, proventriculus, and bone marrow (thymus and bursa of Fabricius will not be discussed in this article) were collected and fixed in 4% formaldehyde solution. Animal experimental protocols were approved by the Shandong Agricultural University Animal Care and Use Committee (permit No. SDAU 19-098; July 8, 2019).

Tissue Information of Natural Avian Leukosis Virus Subgroup J-Infected Chickens

ALV-J infection broke out in Jiangsu, Shandong, Henan, Hebei, Heilongjiang, and Guangdong provinces of China in February 2018 (7). On-site, a total of 19,500 Ross broiler breeder chickens aged 15–20 weeks suffered from depression, paralysis, and weight loss. Tissues samples including the liver, kidney, and bone marrow were collected from broiler breeder flocks in Gaomi and Binzhou. Samples were verified that the case was only ALV-J infection by performing hematoxylin and eosin (HE) staining and PCR (7).

Immunohistochemistry Staining

The tissue microarray glass slides were prepared using the tissues of naturally and experimentally ALV-J-infected chickens and normal chickens. Then, the tissue microarray glass slides were dried at 60°C for 30 min and then deparaffinized gradually through xylene, 50% xylene, and gradient concentrations of ethanol until being immersed in water (25). Chicken tissue sections were blocked for peroxidase activity with 0.3% hydrogen peroxide for 40 min. Antigen retrieval was performed through boiling in 10 mmol/L citrate buffer (pH 6.0) for 25 min. Then the tissues were incubated with anti-CTHRC1 antibody (1:100 dilution) at 4°C for 12 h. The tissues were washed with phosphate-buffered saline (PBS) for three times and incubated with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody (1:500 dilution) for 70 min at 37°C. Immunostaining was performed by using diaminobenzidine substrate chromogen method. Tissues were immersed in hematoxylin for cell nuclear staining. The slides were then dehydrated via gradient concentrations of ethanol, cleared with xylene, and cover slipped with neutral balsam (Shenggong, Shanghai, China) (26).

Immunofluorescence Staining

Lymphocytes and erythrocytes were separated from the peripheral blood of ALV-J-infected and normal chickens. Lymphocytes and erythrocytes were fixed with 4% paraformaldehyde and then permeabilized with methanol for 20 min prior to the addition of primary antibody (anti-mouse ALV-J SU protein and anti-rabbit CTHRC1) and incubation at 37°C for 60 min. These two types of cells then were washed in Tris-buffered saline-Tween 20, followed by incubation with Alexa Fluor 488-conjugated donkey anti-rabbit or 594-conjugated donkey anti-mouse antibodies. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Finally, lymphocytes and erythrocytes were mixed in a 1:1 ratio. Fluorescent images were obtained with a confocal laser scanning microscope (Leica SP8).

Reverse Transcription and Quantitative Real-Time PCR and PCR

Total RNA was extracted from cells according to manufacturer instructions (Qiagen), and RNA concentration was measured using a spectrophotometer (Qiagen). We used 1 μg of total RNA as a template to synthesize cDNA using a

TABLE 1 | Primer used for real-time PCR.

Name	Sequence (5′-3′)	Reference or GenBank
GAPDH	F: GAACATCATCCCAGCGTCCA	(23)
	R: CGGCAGGTCAGGTCAACAAC	
CTHRC1	F: ACGCTG GCTTGGTGGA	(23)
	R: CAGTTCTTCAATGATGATACGG	
ALV-A	F: GATGTTCACTTACTCGAGC	GenBank: MF926337.1
	R: CGTTTACGTCTTATACCTG	
ALV-B	F: ATGTCCACTTACTCGAGCA	GenBank: KC282901.1
	R: TCGTTTGCGTCTTATACCTG	
ALV-J	F: AACAGGTTACATCTGAGCAAGC	GenBank: DQ115805.1
	R: TGTTCCATTGTCATCGCTAACG	
ALV-K	F: CTCGAGCAGCCAGGGAAC	GenBank: KY581580.1
	R: CTTCGTTTACGTCTTATACC	
MDV	F: GCCTTTTATACACAAGAGCCGAG	(27)
	R: TTTATCGCGGTTGTGGGTCATG′	
REV	F: CATACTGGAGCCAATGGTT	(28)
	R: AATGTTGTAGCGAAGTACT	

reverse transcriptase kit (TaKaRa, Shiga, Japan) according to manufacturer instructions. A SYBR Green I kit (TaKaRa) was used for cDNA amplification in a total volume of 20 μl. CTHRC1-specific primers and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a housekeeping gene, are shown in **Table 1**. A LightCycler 96 system (Roche, Basel, Switzerland) was used for qPCR with the following cycling conditions: denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s, and 60°C for 34 s. A melting curve was generated at 95°C for 10 s, 65°C for 60 s, and 97°C for 1 s. CTHRC1 levels were analyzed using the $2^{-\Delta\Delta Ct}$ method. Total RNA was extracted from clinical case, and then RNA is reverse transcribed into cDNA. PCR was performed according to the manufacturer's instructions of PrimeSTAR HS DNA Polymerase with GC Buffer (Takara, Japan) (PCR primer is shown in **Table 1**).

ELISA

Virus isolated from clinical cases was inoculated into DF-1 cells, where are a continuous chicken embryonic fibroblast cell line that exhibits normal fibroblastic morphology and is free of endogenous sequences related to leukosis viruses and avian sarcoma (29). Importantly, DF-1 cells are capable of sustaining ALV-J replication, and the cell supernatant was collected at different time points. ELISA kits (SenBeiJia, Nanjing, China) (http://www.sbjbio.com/Products/P1.html) were used to measure CTHRC1 levels in cell supernatant, according to manufacturer instructions. Each experiment included three biological replicates.

Statistical Analysis

All data are reported as the mean ± standard error of the mean (SEM). Prism 7.0 software (GraphPad Software, San Diego, CA, USA) was used to determine statistically significant differences by performing a two-tailed unpaired Student's *t*-test. Differences between groups were considered statistically significant when the *p*-value was less than 0.05 ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

RESULTS

The Expression of Collagen Triple Helix Repeat Containing-1 Is Increased in Naturally and Experimentally Avian Leukosis Virus Subgroup J-Infected Chickens

Data obtained from previous studies of our lab indicated that the chickens from farms were only infected with ALV-J (7). According to Zhou et al. (7), the virus was isolated from the samples of kidneys of tumor-bearing chickens from farms (29). In order to detect the purity of the virus that we isolated, PCR was performed. The results of PCR indicated that all tumor-bearing chickens forming a clinical case were positive for ALV-J and negative for ALV-A, ALV-B, ALV-K, REV, and MDV (**Supplementary Figures 1A–F**) (7).

The expression of CTHRC1 is low in normal tissues, which can be activated with the stimulation of certain pathogens (30, 31). Then, we detected the expression of CTHRC1 in the liver, kidney, and bone marrow of naturally ALV-J-infected chickens. As shown in **Figure 1A**, the levels of CTHRC1 were remarkably up-regulated in the tissues of naturally ALV-J-infected chickens, and the expression of CTHRC1 was mainly and widely distributed with the parenchymal parts of organs, compared with the normal tissues of the same age of SPF chickens. CTHRC1 is widely expressed in the tissues of clinical cases infected with ALV-J. In order to further clarify that CTHRC1 is an inducible protein upon ALV-J infection, we inoculated the virus of isolated into DF-1 cells and then tested whether ALV-J up-regulated the expression of CTHRC1. The results showed that CTHRC1 was elevated upon ALV-J infection (**Figures 1B,C**).

In order to accurately clarify the dynamic distribution of CTHRC1 in various organs upon ALV-J infection and the expression of CTHRC1 with the extension of infection time, SPF chick embryos were inoculated with ALV-J in the allantoic cavity on the 6th day of incubation, and then chicks were euthanized at 15, 22, and 35 days post-infection. As shown in **Figure 1D**, the levels of CTHRC1 were obviously raised upon ALV-J infection than those of normal SPF chickens. Meanwhile, we found that CTHRC1 was mainly expressed in the parenchymal cells of the tissues than those of the interstitial cells of tissues. It was worth noting that the expression of CTHRC1 was lower in the spleen than those of other organs, such as the heart, liver, kidney, lung, brain, duodenum, the proventriculus; and the expression of CTHRC1 featured high intensity in the Purkinje cells of the brain tissue.

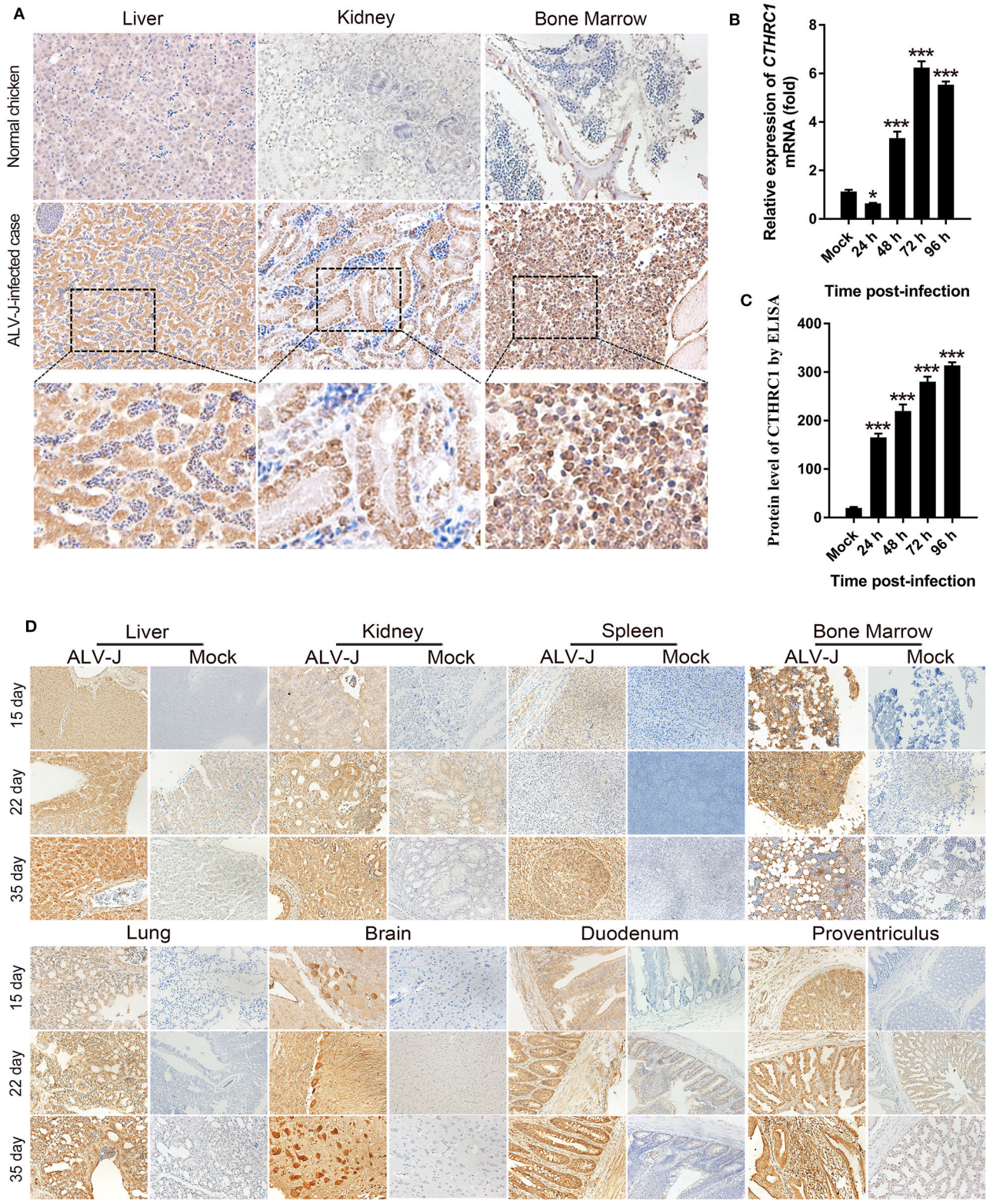

FIGURE 1 | Avian leukosis virus subgroup J (ALV-J) infection facilitates collagen triple helix repeat containing-1 (CTHRC1) expression *in vivo*. Immunohistochemical staining of tissues of naturally **(A)** and experimentally **(D)** ALV-J-infected chickens with the CTHRC1 antibody. The CTHRC1 protein was weakly stained in normal tissues. In contrast, CTHRC1 staining was moderate to strong in ALV-J-infected tissues. Original magnification, 400×. **(B)** Total RNA was extracted from ALV-J-infected and uninfected DF-1 cells. CTHRC1 mRNA was measured by qPCR. **(C)** CTHRC1 protein levels were measured by ELISA in supernatant from ALV-J infected and uninfected DF-1 cells. $^{*}p < 0.05$; $^{***}p < 0.001$.

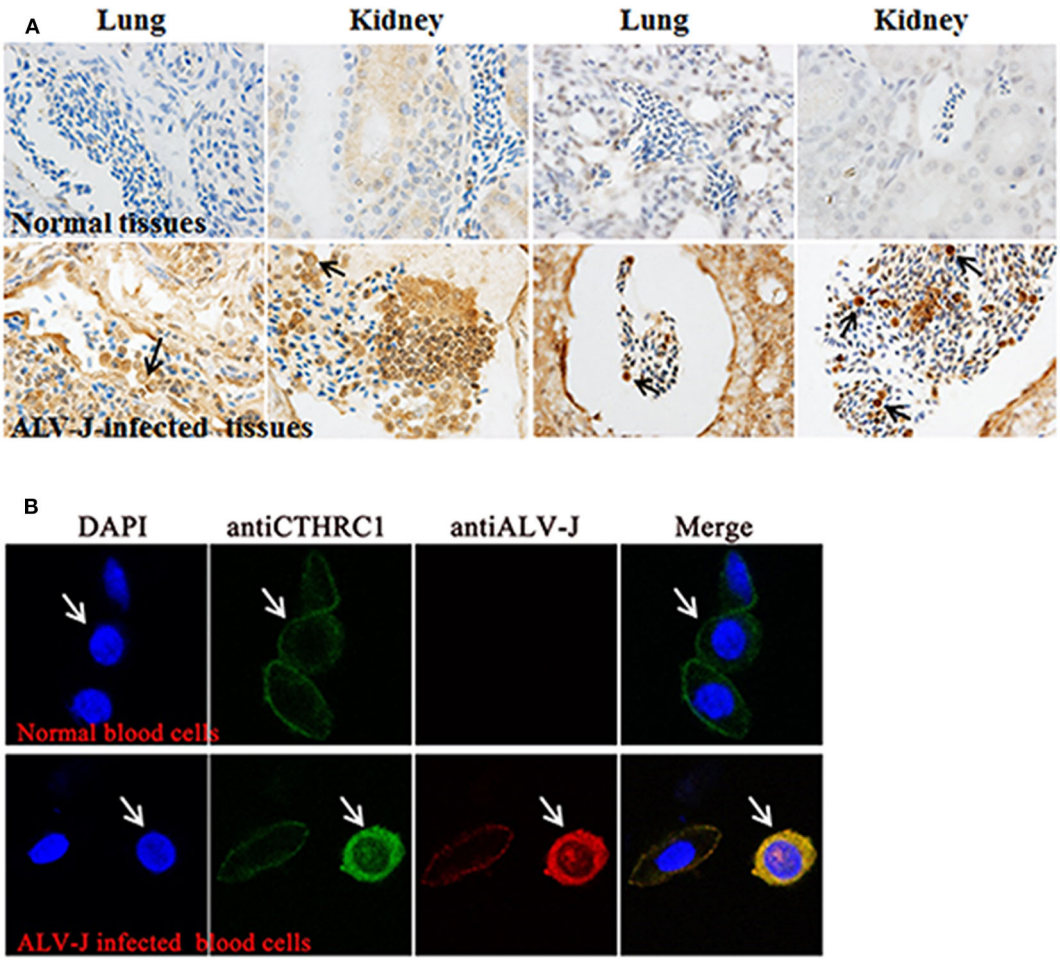

FIGURE 2 | Avian leukosis virus subgroup J (ALV-J) activates the expression of collagen triple helix repeat containing-1 (CTHRC1) in blood lymphocytes **(A)**. The CTHRC1 protein was strongly stained in certain blood cells tested by the immunohistochemistry (IHC) assay. (The lungs and kidneys are derived from different ALV-J-infected chickens.) **(B)** Lymphocytes and erythrocytes isolated from the blood of ALV-J-infected chicken. Cells were fixed for an immunofluorescence assay to detect ALV-J (red) and CTHRC1 (green) with primary antibodies. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (blue). Fluorescent images were acquired with a confocal laser scanning microscope (Leica SP8). White arrows indicate lymphocytes.

Collagen Triple Helix Repeat Containing-1 Is Activated in Blood Lymphocytes of Avian Leukosis Virus Subgroup J-Infected Chickens

Interestingly, during immunohistochemistry (IHC), we found that CTHRC1 was stained in certain blood cells of congested veins and that erythrocytes were not stained. Thus, we speculated that the stained cells were lymphocytes according to cell morphology (**Figure 2A**). To further identify the stained blood cells, lymphocytes, and erythrocytes were isolated from blood of ALV-J-infected chickens and normal chickens to exactly explore the phenomenon by laser confocal assay. As shown in **Figure 2B**, CTHRC1 was mainly located in the cytoplasm and was activated in the blood lymphocytes of ALV-J infected chickens than those of normal chickens. CTHRC1 was interacted with ALV-J SU protein and co-localized in the cytoplasm (23), which was in line with our results obtained in **Figure 2B** that CTHRC1 and ALV-J were co-located in the cytoplasm of lymphocytes. Meanwhile, according to Cui et al. (1999), ALV-J was not infected with erythrocytes of blood, which only transport ALV-J, but it can infect lymphocytes of blood (32). In our study, we found that ALV-J was only located in the cell membrane of erythrocytes (**Figure 2B**), which was coincident with results obtained in previous studies.

DISCUSSION

CTHRC1, as a host-cell protein, plays a significant role in facilitating ALV-J infection (23). Here, we explored the distribution and localization of CTHRC1 in tissues of chickens that were infected with ALV-J naturally and experimentally. Meanwhile, utilizing IHC and laser confocal assay, we found that

CTHRC1 was activated in blood lymphocytes of ALV-J infection, which meant that ALV-J can infect lymphocytes.

In this study, we found that the expression of CTHRC1 gradually elevated with the extensive infection of ALV-J tested by the IHC assay. Furthermore, ALV-J-infected samples with deeper depths of invasion showed higher levels of CTHRC1, illustrating that CTHRC1 may have an irreplaceable role in ALV-J pathogenicity. It was important to note that the result was further substantiated through *in vitro* experiments (23). Therefore, CTHRC1, a candidate tumor marker, may be a potential metastasis-related gene in myelocytomas caused by ALV-J. Moreover, it was a remarkable fact that CTHRC1 staining in ALV-J-infected tissues was strongly intensely located in cytoplasm and nucleus, while the expression of CTHRC1 in normal chicken tissues was of weak intensity (**Figure 1**). CTHRC1 is a secreted-extracellular protein (9). Pyagay et al. (33) found that CTHRC1 may experience proteolytic process, which could acquire the activity of the molecule (31). Thus, it is possible that the secreted form of CTHRC1 overproduced through ALV-J-infection may act on the surrounding microenvironment, including the stromal cells and extracellular matrix (ECM), which elevates tumor invasion and migration. Previous studies have described that the level of CTHRC1 was very low in normal tissues, which was consistent with results obtained in **Figure 1**.

For young animals, the bone marrow in the marrow cavity is mainly red bone marrow, whose main function is to produce hematopoietic stem cells. The red bone marrow in the marrow cavity is gradually replaced with age by yellow bone marrow, whose main component is fat that is not stained by IHC (34). As shown in **Figure 2B**, after 35 days of ALV-J infection, the staining of CTHRC1 was slightly decreased, which may be because the red bone marrow in the marrow cavity is gradually replaced by the yellow bone marrow. Nowadays, effort has mainly been concentrated on the tumorigenic mechanism of ALV-J (7, 35).

As shown in **Figure 1**, compared with other structures of the kidney, the renal tubule is highly colored, which shows that the renal tubule highly expresses CTHRC1 after being infected with ALV-J. Enhanced expression of CTHRC1 could restrict collagen type I and III deposition (33, 36). Meanwhile, the inhibition of the expression of collagen types I and III could alter the host membrane permeability, which could promote viral replication (37). It is well-known that the kidney is the main target organ of ALV-J. It is newly revealed that the high expression of CTHRC1 in renal tubules is caused by ALV-J infection.

ALV-J belonging to an avian C type *retrovirus* can integrate into the host genome to induce tumor and immunological tolerance by impairment in the effect of host lymphocytes, which was consistent with results obtained in **Figure 2B** (32, 38). It can be observed that ALV-J could activate CTHRC1 in blood lymphocytes, but not in erythrocytes (**Figures 2A,B**), which indicated that CTHRC1 might be involved in immunological tolerance induced by ALV-J by impairing lymphocyte function. Here, CTHRC1 was found in the blood lymphocytes of ALV-J-infected SPF chickens, which revealed that it may be a potential key factor influencing ALV-J infection and production.

CTHRC1 was widely up-regulated in multiple human tumors, such as cancers of the liver, pancreas, and gastrointestinal tract and melanoma (39). Meanwhile, the recombinant of CTHRC1 protein could elevate the capacities of invasion and migration of primary gastrointestinal stromal tumors (40). Thus, CTHRC1 plays a vital role in the fields of human tumor (21). ALV-J, MDV, and REV, as the three major neoplastic diseases, cause huge economic losses to the poultry industry every year (35). However, how to prevent and control these three diseases still plague many workers. Our research found that CTHRC1 was activated upon ALV-J (23), MDV, and REV infection through proteomic analysis (data not shown), and we speculated that it may play a crucial role in ALV-J, MDV, and REV replication, tumorigenicity, and pathological mechanism and that it might also become a common target for the prevention and control of ALV-J, MDV, and REV infection. Additionally, CTHRC1 may serve as a common effective biomarker for evaluating the poor clinical pathological characteristics of ALV-J, MDV, and REV infection. In the future, we will investigate the effect of CTHRC1 on ALV-J, MDV, and REV replication, tumorigenicity, and pathological mechanism.

In conclusion, our current study revealed that CTHRC1, as an invasion-driving protein, was widely activated in the parenchymal cells of tissues of ALV-J-infected natural and experimental cases. CTHRC1 was up-regulated in blood lymphocytes, which may be associated with the immunosuppression caused by ALV-J. Thus, combined with previous studies, this research may provide precise information for in-depth study of ALV-J infection and replication mechanisms.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Shandong Animal Care and Use Committee (permit number: SDAU number 19-098, 8 July 2019).

AUTHOR CONTRIBUTIONS

ZC and YP conceived and designed the experiments. YP performed the experiments. YP, DZ, JX, and JZ analyzed the data. ZC, YW, and YP wrote the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from the National Natural Science Foundation of China (31672521), the China-UK Partnership on Global Food Security: Combating Avian Leukosis Virus Subgroup J for Sustainable Poultry Production (31761133002, BB/R012865/1), the Shandong Modern Agricultural Technology and Industry System (SDAIT-11-04).

ACKNOWLEDGMENTS

We are grateful to the teacher of Li Zhang for providing the necessary infrastructure facilities for the study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.565773/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

OPINION
published: 29 September 2020
doi: 10.3389/fvets.2020.581969

Scrapie Control in EU Goat Population: Has the Last Gap Been Overcome?

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Keywords: TSEs: transmissible spongiform encephalopathies, scrapie, PRNP: prion gene name, goat, EU regulation, genetic—disease resistance, biodiversity

INTRODUCTION

Scrapie is a fatal, neurodegenerative disease that affects sheep and goat worldwide, belonging to the group of transmissible spongiform encephalopathies (TSEs).

Since 2002, Member States (MS) of European Union (EU) have implemented active surveillance to control the risk of scrapie. The EU scrapie eradication policy is mainly aimed to eradicate classical scrapie. The choice of population groups and sample sizes have evolved in the years, as well as the eradication measures and control of disease (selective culling, movement restrictions, reinforced surveillance measures, etc.). In this context, over the past two decades, breeding programs to increase the frequency of the resistance-associated ARR allele in sheep populations have been introduced to minimize TSE risk in MS, but there was not a regulatory effort in adoption of analogous measures for goats. However, scientific knowledge related to scrapie resistance associated with goat PRNP gene polymorphisms has considerably expanded in the last 10 years.

Classical scrapie is considered endemic in many MS. Since its publication, the only measures applicable for TSE control in goat contained in Regulation (EC) No 999/2001 obliged farmers to provide a complete culling of whole flock, with great economic loss and serious concerns for the risk of extinction of endangered breeds. However, over the years, additional measures have been introduced such as monitoring of the infected herd without the obligation of total culling and the possibility of reintroducing goats with unknown genotype after biosafety practices. Nevertheless, these measures could allow the goat population to become the main reservoir of scrapie, affecting the disease eradication program in small ruminant population.

Following a request from the European Commission (EC), the European Food Safety Authority (EFSA) was asked to deliver scientific opinions on the scrapie situation in EU to evaluate the introduction of breeding policies in goats. From 2014, EFSA advised to promote selection and introduction of resistant bucks in EU caprine population (1). More recently, in 2017, based on the latest scientific evidence, EFSA concluded that breeding programs for scrapie resistance in goats should be implemented in MS, taking particular attention to potential negative effects of extinction in rare and endangered breeds (2).

With Regulation (EC) No 2020/772 of June 11, 2020, amending Regulation (EC) No 999/2001, EC laid down new approaches as regards eradication measures for TSEs in goats and in endangered breeds. In this context, the authors discuss advantages and critical points related to the different control measures introduced by EU regulations during the last two decades.

OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
Timm Konold, Animal and Plant Health Agency, United Kingdom

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *10 July 2020*
Accepted: *18 August 2020*
Published: *29 September 2020*

Citation:
Migliore S, Puleio R and Loria GR (2020) Scrapie Control in EU Goat Population: Has the Last Gap Been Overcome? Front. Vet. Sci. 7:581969. doi: 10.3389/fvets.2020.581969

STATE OF THE ART

Legislative Basis

Regulation (EC) No 999/2001 establishes rules for the prevention, control, and eradication of certain TSEs, including scrapie in small ruminants. This Regulation dates back to 2001 and, after many subsequent amendments, is still in force today.

In 2003, Regulation (EC) No 260/2003 revised the requirements for eradication measures in case of the detection of TSE in a farm by selective culling of susceptible sheep and by requiring the implementation of measures to increase TSE resistance in the outbreak. Simultaneously, decision 2003/100 (EC) laid down requirements for the establishment of breeding programs for resistance to TSE in sheep, aimed to increase the level of alleles associated with resistance (ARR) and decreasing the frequency of alleles associated with susceptibility (VRQ) in EU sheep population. Commission Regulation (EC) No 1923/20065 and No 727/2007 then integrated the breeding program requirements into Regulation (EC) No 999/2001. In 2006, EFSA confirmed the efficacy of breeding program for TSE resistance in sheep (3).

More recently, on June 11, 2020, Regulation (EC) No 2020/772 amended Annexes I, VII, and VIII to Regulation (EC) No 999/2001 introducing the possibility for the MS to limit slaughtering/culling and destruction to goats which are genetically susceptible to classical scrapie. In addition, the definition of "endangered breed" of Regulation (EU) 2016/1012 replaced the expression of "local breed in danger of being lost to farming" as laid down in Regulation (EU) No 807/2014 (4).

Scrapie in EU Goats

Classical scrapie shows similar epidemiological features in sheep and goats and the involvement of both species in outbreaks is common. Even if the incidence in goats is much lower than in sheep, milk and placenta of infected goats may serve as sources of infection to sheep (5, 6). Scrapie in goat was described for the first time in 1942 (4); since then, clinical cases have been recorded throughout Europe. Animal movements between herds and environmental contamination play relevant roles as risk factors.

In 2019, a total of 325,386 sheep and 138,128 goats were tested in EU. In sheep, 821 cases of classical scrapie were detected in seven MS, whereas 517 cases were reported in goats in seven MS (7). Scrapie in goat is considered endemic in the EU countries with the largest caprine populations with more than 10,500 cases from 2002 to 2017. Between 2002 and 2015, classical scrapie was detected in 10 MS with 2.4 cases out of 10,000 tested heads. In this prevalence study, Cyprus was excluded due to an epidemic over the last 10 years (2).

Genetic Basis

In the last two decades, an extensive review of literature was conducted to identify relevant alleles of goat PRNP to which a breeding program could be based. These studies were conducted within different MS and goat breeds. A considerable dataset has been produced for the following alleles: S127, M142, R143, D145, D146, S146, H154, Q211, and K222. Among them, K222, D146, and S146 alleles confer higher genetic resistance to classical scrapie strains circulating in the EU goat population (2). In 2017, based on a combination of the "weight of evidence" and the "strength of resistance," EFSA provided a ranking of resistance to classical scrapie, as follows: K222 > D146 = S146 > Q211 = H154 = M142 > S127 = H143 > wild type (2).

Goat Breeding in EU

Goat farming plays an important socioeconomic role in several countries, particularly where there are hills and mountains, and remote, marginal, and even semi-arid areas (8, 9). Europe is the continent with the widest caprine biodiversity with 187 goat breeds, which is 33% of the goat breeds acknowledged worldwide (10). In this context, there are breeds with large population sizes, cosmopolitan and often characterized by a high production, and breeds with small population sizes not yet subjected to conservation programs because of their remoteness or because they are less competitive in terms of production than other selected breeds (9). Such different scenarios obviously have required a different scrapie control strategy.

DISCUSSION

In 2017, EFSA, based on prolonged field experience and experimental studies, concluded that the K222, D146, and S146 variants confer genetic resistance to the classical scrapie strain circulating in the EU goat population (2). EFSA highlighted that the protective effect of K222 is greater than D146 and S146 variants and of ARR allele in sheep, when the 2002 Scientific Steering Committee opinion was published (2). In this regard, a substantial difference between sheep and goats in the new Regulation (EC) No 2020/772 still remains. In sheep, the ARR/ARR homozygous genotype in reproductive males is an essential requirement, whereas in goats, heterozygosity for at least one of K222 and D/S146 alleles is sufficient to avoid the stamping out. It should be remembered that heterozygous variants Q222K and N146S/D in goats do not confer full protection against classical scrapie as reported in natural outbreaks in Greece (11) and in Cyprus (12). In addition, the subsequent restocking of outbreak without genotype consideration after biosafety practices is a considerable risk. These are critical points whose efficacy will be assessed in the future.

The EFSA opinion also highlights that a high selective pressure in some breeds with a low frequency of resistant variants would likely have an adverse effect on genetic diversity and that each MS should be able to design its own genetic selection strategy depending on the breed concerned.

Estimating the frequency of candidate alleles is a preliminary step in understanding the feasibility of a breeding program. Several investigations on goat PRNP were performed in MS in recent years, and some breed-related differences emerged (**Table 1**). Higher frequency (>24.5%) of 146D or S variants was described in cosmopolitan Boer goat in Great Britain and Netherlands (13–15) and in native Damascus and related breeds in Cyprus (16.5%) (17). A lower frequency (3%) was also described in local and crossbred in Greece (16). To date,

TABLE 1 | Breeds with S146/D146 and K222 haplotypes reported in literature and their frequencies reported in EU.

Country	Breed	Geographical classification	Local status*	Frequency (%)	Source
146D or 146S					
UK	Boer	Cosmopolitan	At risk	24.5–35.5	(13, 14)
Netherlands	Boer	Cosmopolitan	Unknown	31	(15)
	Nubian	Cosmopolitan	At risk	7.1	
Greece	Local/crossbred	Local	Unknown	3.0	(16)
Cyprus	Damascus and related breeds	Cosmopolitan	Not at risk	16.5	(17)
222K					
UK	Toggenburg	Cosmopolitan	At risk	1.9	(13)
Netherlands	Saanen	Cosmopolitan	Unknown	1.9	(15)
	Toggenburg	Cosmopolitan	At risk	29.5	
Greece	Local/crossbred	Local	Unknown	5.6	(16)
France	Saanen	Cosmopolitan	Not at risk	4.9	(18)
Spain	Saanen	Cosmopolitan	Unknown	1.2	(19)
	Alpine	Cosmopolitan	Unknown	6.4	
	Local breeds	Local	At risk	0–0.03	
Italy (Northern breeds)	Camosciata	Cosmopolitan	Not at risk	2.4	(20)
	Saanen	Cosmopolitan	Not at risk	3.0	
	Roccaverano	Local	Endangered	4.3	
	Valdostana	Local	Critical	1.3	
Italy (Southern breeds)	Garganica	Local	Endangered	17.2	
	Jonica	Local	Critical	7.3	
	Southern crossbred	Local	Unknown	22.5	
	Girgentana	Local	Endangered	18.7	(21)
	Rossa Mediterranea	Local	Critical	12.7	(22)
	Argentata dell'Etna	Local	Endangered	16.3	
	Aspromontana	Local	No at risk	10.3	(23)
	Cilentana	Local	Critical	18.2	

**DAD-IS database—FAO (22).*

this mutation does not seem to be widespread in other MS. In contrast, 222 K variant seems to be more common across the MS. Frequencies between 1.2 and 7.5% were described in cosmopolitan and large population size breeds such as Saanen (1.2–4%) and Alpine (6.4–7.5%) reared in Spain, Netherlands, Italy, France, and Greece (15, 16, 18–20). Very high frequency (29.5%) was described in Dutch Toggenburg in Netherlands (15). Variable frequencies were described in small size of native breeds such as local and crossbred in Greece (0.3–5.6%) (16). In Italy, where a great caprine biodiversity is present, a difference between northern and southern native breeds was described (20), with higher frequencies of 222 K in Southern breed such as Garganica (17.2%), Ionica (7.2%), southern crossbred (22.5%), Girgentana (18.7%), Rossa Mediterranea (12.7%), Argentata dell'Etna (16.3%), Aspromontana (10.3%), and Cilentana (18.2%) (20–23). Many of these breeds are considered in critical or endangered status (24) and for this reason any breeding program should consider the endangered status of each goat population to preserve the genetic variability and the biodiversity together with disease control (21).

Various mutations in the PRNP in different breeds have potentially been positively selected in relation to local circulating scrapie strains originating in specific environmental conditions (25).

A recent study (26) assessed the impact of different breeding strategies in goat using a mathematical model, and it concluded that breeding programs for scrapie resistance could be implemented also in a context of so high biodiversity and also different size of the populations of goats. Nevertheless, the growth rate of resistant goats in some breeds may be slow due to the initial genetic profile not being particularly favorable inside the breed. In cosmopolitan breeds with a large population size, a breeding program in the overall population would be desirable. In contrast, in endangered breeds with a small population, a breeding program should be implemented starting from reproductive nuclei. This scheme is less expansive and protects the endangered breeds even if it takes longer to reach the expected results.

As well as goat breeds, a breeding program for scrapie resistance should consider the particular situation of each MS in terms of the presence of resistant alleles and their relative frequency. For example, in Greece, which has one of the largest goat populations in Europe, a goat-scrapie resistance program targeting the Q211, S146, and K222 alleles was designed (27),

whereas in Italy, pilot projects selected positively a singular variant K222.

Although there is a strong interest in disease control among goat farmers in the Northern MS, breeding for resistance is often compromised by the low frequency of resistant alleles. By contrast, in Southern MS where a satisfying frequency of resistant alleles is present, goat farming is mainly related to pastoralism and in several cases there is a lack of interest in starting genetic programs. For this reason, to be successful, new regulations have to consider engaging farmers' cooperation by appropriate risk communication and involving them in the genetic program as well as providing an adequate financial support for goat genotyping.

Regulation (EC) No 2020/772 laid down an alternative tool for scrapie control in EU goat population. It particularly recognized the genetic resistance to classical scrapie in goats carrying at least one of the most recognized alleles (K222, D146, and S146) and preserving them from culling in the case of outbreak. In addition, the new regulation introduces possible derogation measures for endangered breeds according to Regulation (EU) 2016/1012. This new measure will finally strengthen the control of TSEs in small ruminants in the EU and will also have beneficial effects on farming system and for the conservation of goat breed biodiversity.

AUTHOR CONTRIBUTIONS

SM drafting of the article. RP and GL revised the article. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

CLINICAL TRIAL
published: 07 October 2020
doi: 10.3389/fvets.2020.571697

Evaluation of Antimicrobial or Non-antimicrobial Treatments in Commercial Feedlot Cattle With Mild Bovine Respiratory Disease Based on a Refined Case-Definition

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The study objective was to compare clinical and performance outcomes among feedlot steers treated for bovine respiratory disease (BRD) with tildipirosin (TIL), flunixin transdermal solution (FTS; topical application), or both, based on a refined BRD case-definition. Crossbred steer calves (N = 2,380) were enrolled based on a clinical illness score (CIS) of 1–3; a rectal temperature between >102.5° F and ≤103.9° F; and a Whisper Score (WS) = 1 or ≥2. Within each WS stratum, steers were randomly allocated to Saline, TIL, FTS, or TIL + FTS to reflect a 2 × 2 factorial design. Individual health and performance outcomes were measured on Day 60 and closeout. From Day 0 through Day 60, in both strata, TIL resulted in significantly ($P \leq 0.05$) fewer BRD retreatment events, fewer 3rd BRD treatments, fewer steers that did not finish, and greater average daily gain when compared to steers that were not treated with TIL. From Day 0 through closeout, cattle with a WS ≥ 2, treated with TIL had fewer animals ($P \leq 0.05$) that did not finish compared to steers not treated with TIL. In this study, feedlot steers with clinical signs of BRD and rectal temperatures lower than traditional cutoffs displayed a positive response to antimicrobial therapy. A clear benefit of FTS was not observed in this study. Calves with a WS ≥ 2 were lighter at the time of first BRD treatment compared to calves with a WS = 1. However, standalone TIL therapy was the optimal BRD treatment modality across WS strata in this study.

Keywords: bovine respiratory disease complex, tildipirosin, flunixin transdermal solution, cattle, diagnosis, negative control

OPEN ACCESS

Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

Reviewed by:
Timothy John Mahony, The University of Queensland, Australia
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *11 June 2020*
Accepted: *01 September 2020*
Published: *07 October 2020*

Citation:
Nickell J, Bryant L, Lechtenberg KF and Cull C (2020) Evaluation of Antimicrobial or Non-antimicrobial Treatments in Commercial Feedlot Cattle With Mild Bovine Respiratory Disease Based on a Refined Case-Definition. Front. Vet. Sci. 7:571697. doi: 10.3389/fvets.2020.571697

INTRODUCTION

The traditional case-definition for bovine respiratory disease (BRD) diagnosis in feedlot cattle reflects an animal exhibiting clinical signs of BRD such as anorexia, depression, nasal discharge, cough, respiratory difficulty; and, rectal temperature >104° F (1–6). This temperature threshold is also utilized in the BRD case-definition within the regulatory approval process for antibiotics in the United States (7–11). However, the diagnostic accuracy of that case-definition has previously been shown to be relatively poor (12, 13).

Antimicrobial therapy is indicated for an animal meeting those above criteria for a BRD diagnosis. Tildipirosin (TIL) is an antimicrobial medication indicated for treatment and control of bovine respiratory disease (BRD) associated with *M. haemolytica, P. multocida,* and *H. somni.* This drug has previously shown efficacy when applied to animals meeting the above BRD case-definition (14).

In practice, an animal that exhibits a sufficient clinical illness score (CIS) but fails to meet this specific threshold criterium for rectal temperature may be returned to its home-pen without treatment. Cattle that do not fit the case-definition, by not meeting the threshold for rectal temperature, may be misclassified or mistreated; thereby, jeopardizing animal well-being (not treating an animal that really is sick), misusing medication (wasting medication by treating an animal that won't benefit), subsequently impacting animal performance and profitability. Little data are available regarding "response to treatment" of cattle that have those clinical signs and rectal temperature ≤103.9° F. An additional question that has yet to be answered is if cattle that exhibit a sufficient clinical illness score but fail to meet the rectal temperature criteria of ≥104° F have the potential to respond to non-antimicrobial therapy. Flunixin meglumine Transdermal Solution (FTS) is indicated for control of pyrexia associated with bovine respiratory disease and the control of pain associated with foot rot. Using the conventional BRD case definition, utilizing FTS as an ancillary therapy (i.e., in addition to antimicrobial therapy) has not provided added value compared to the antimicrobial alone (15). However, to date, no data are available (to the authors' knowledge) that has assessed FTS as a standalone therapy among animals that exhibit clinical signs of BRD but fail to meet a traditional rectal temperature cutoff.

Given the perceived lack of accuracy afforded by current BRD diagnostic modalities, additional information (i.e., in addition to CIS and rectal temperature) may be necessary to improve overall accuracy while potentially improving the treatment decision-making process. Whisper® technology offers unique information that estimates the lung health of an individual calf at the time it has been identified with a tentative BRD diagnosis (16–19).

The objective of this study was to use a refined case-definition of BRD based on CIS, rectal temperature (≥102.5° F to ≤103.9° F) and results of computer-assisted lung auscultation (Whisper®) to compare clinical and performance outcomes of cattle treated because of BRD. The null hypothesis was that calves meeting the refined BRD case definition would not respond to antimicrobial, non-antimicrobial, or a combination of both therapies.

MATERIALS AND METHODS

The study protocol was submitted to the MVS Institution for Animal Care and Use Committee (IACUC) where the protocol received approval on 30 January 2018. The assigned IACUC number is AC17100B.

Animals

The same study protocol was followed at each of two study sites (Oakland, NE; Manhattan, KS). Beef or beef-cross feeder steers ($N = 3{,}376$) with moderate to high risk for developing BRD were procured through several livestock auctions in Nebraska, Iowa, South Dakota, and Missouri across 24 shipments from late February to late November of 2018. All steers were weaned at the time of procurement; and, the history of vaccination or treatments was not known. When the steers arrived at the study site, their health was evaluated; they were identified with an individual number; they were vaccinated (modified-live viral, multi-valent clostridial toxoid), dewormed, and implanted with a growth-promotant; and, weighed. No antimicrobials were administered to control BRD (metaphylaxis). Steers that were not healthy were not eligible for enrollment which occurred from March through December of 2018. Steers were housed in open-air, dirt-floor pens. Conditions and management of the pens were according to standard feedlot practices. All steers were fed a ration appropriate for the size, age, and stage of feeding. After a brief step up period (∼1 week), cattle were fed once daily a finisher diet, which included (DM basis): 54.1% high moisture corn, 25.3% wet distillers grain, 12.6% sweat brand 60, 3.4% corn stalks, 2.6% liquid supplement, and 2.0 micromineral mix ingredients [including 400 mg of monensin/animal and 85 mg of tylosin/animal per day (Elanco Animal Health, Greenfield, IN)]. Fresh feed was delivered each morning. Fresh water was available *ad libitum*. Waterers were monitored daily and cleaned when necessary. Any health-related intervention not described in the study protocol was administered at the discretion of the attending veterinarian after consultation with the sponsor. The monitor of the study was notified when such treatments were administered or when any steer was euthanized. The attending veterinarian at each site performed a necropsy on any steer found dead or was euthanized. If BRD was diagnosed at necropsy during the first 60 days of the study (Day 0 to Day 60), real-time polymerase chain reaction (RT-PCR) was performed with samples of lung to identify the following specific pathogens: infectious bovine rhinotracheitis (IBR) virus, bovine viral diarrhea virus (BVDV), bovine parainfluenza virus type 3 (PI3), bovine respiratory syncytial virus (BRSV), bovine influenza virus (BIV), *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*.

Inclusion Criteria

Twenty-four hours after processing at arrival, steers were eligible for enrollment. The case-definition of BRD, refined for this study, was a clinical illness score (CIS) of 1, 2, or 3; rectal temperature >102.5° F and ≤103.9° F; and, a Whisper® score (WS) of 1 or ≥2. The CIS was assigned a number (0–4) based on the description in **Table 1**. Rectal temperatures were measured with digital GLA Agricultural Electronics thermometers that were calibrated prior to the study. The Whisper® Veterinary Stethoscope is a computerized stethoscope that measures and analyzes the sounds of the lungs and heart of individual animals using a machine-learning algorithm that assigns a score of 1 through 5 and estimates lung health at the time of clinical disease identification with increasing severity as scores rise (16–19). A

TABLE 1 | Clinical illness scoring system used for this study.

Score	Description
0 = Healthy	Normal, healthy behavior
1 = Mild	May stand isolated with its head down or ears drooping; but, will quickly respond to minimal stimulation
2 = Moderate	May remain recumbent or stand isolated with head down; may show signs of muscle weakness (standing cross-legged, knuckling, or swaying when walking), depression obvious when stimulated
3 = Severe	May be recumbent and reluctant to rise, or if standing isolated and reluctant to move; when moving, is ataxic, knuckling or swaying evident; head carried low with ears drooping; eyes dull, excess salivation/lacrimation possible, obviously gaunt
4 = Moribund	Unable to stand; approaching death; highly unlikely to respond to any antimicrobial treatment

TABLE 2 | Two strata (WS = 1; or WS > 2) were created based on the Whisper Score (WS) at the time of enrollment.

			Flunixin transdermal solution[b]	
			Yes	**No**
WS = 1	Tildipirosin[a]	Yes	340	340
		No (PSS[c])	340	170
WS > 2	Tildipirosin[a]	Yes	340	340
		No (PSS[c])	340	170

Within the respective stratum, a 2 × 2 factorial (binomial) design was used for assignment to experimental treatment. Two thousand three hundred eighty (2,380) calves were randomly assigned to one of the treatments described below based upon their individual WS at the time of enrollment.

[a]Zuprevo® 18% (180 mg tildipirosin/mL) solution; 4 mg tildipirosin/kg (1 mL/cwt) BW, SC.

[b]Banamine Transdermal® (50 mg flunixin meglumine/mL); 3.33 flunixin meglumine/kg (1 mL/15 kg) BW, applied to dorsal midline of back between withers and tailhead.

[c]PSS, Physiologic Saline Solution; 0.9% NaCl; 1 mL/cwt, SC.

TABLE 3 | Protocol for 2nd and 3rd treatment of BRD if needed.

BRD event	Antimicrobial	Dose (mL/cwt)	PTI (days)
2	Nuflor®	6 [mg/kg]	3
3	Baytril® 100	5.5 [mg/kg]	Considered chronic BRD

The 1st treatment was that assigned when the steer was enrolled in the study. No more than 3 treatments were allowed.

lung health estimate of 1 indicates that the lung tissue of the respective calf is relatively healthy. Conversely, a lung health estimate of 5 reflects severely compromised lung tissue. Scores 2–4 reflect intermediary changes in lung health as the scale increases. The bell of the stethoscope was placed approximately two inches caudal and dorsal to the point of the right elbow of the calf. The area was cleaned if needed and sounds were recorded for 8 s. If the recording was not acceptable ("flagged" by the computer; <M60% of the entire recording present on the computer screen; or, the operator had reason to consider the recording to be inadequate), another recording was obtained.

Enrollment Procedure

A steer with CIS of 1, 2, or 3 was brought to a processing chute where its rectal temperature was measured. If that temperature met the criterion for the case-definition, a WS was obtained. Two levels of stratification were used based on the WS. Stratum 1 was comprised of steers with WS = 1; stratum 2 was comprised of steers with WS ≥ 2. These stratums were determined based upon the prevalence of each score category observed in field settings (data not shown). Within each stratum, steers were randomly assigned to one of 4 treatment groups in a 2 × 2 factorial design (see **Table 2**). On a given day (i.e., block), only full treatment blocks, per strata, were enrolled and commingled within pen. Steers that met one of the BRD case definitions but failed to fill all 4 treatments were no longer eligible for enrollment. When a steer met the inclusion criteria and was enrolled, it was weighed and treated as assigned (**Table 2**). Steers assigned to be negative controls (no treatment administered) were treated with sterile saline (1 mL 0.9% NaCl/cwt, SC). A steer was not eligible for enrollment in the study if it had an unacceptable health condition; had a CIS, a rectal temperature, or a WS outside the criteria stated; or if a full treatment block was not filled.

Post-enrollment Procedures

Daily throughout the study general health of the steers was observed by trained personnel who were blinded to treatments. All adverse events were reported by the same personnel. After enrollment a 2-day post-treatment interval (PTI) was imposed on all treatment groups (including negative controls). Steers were then eligible for additional diagnostic procedures and for retreatment (**Table 3**). The BRD retreatment case definition reflected a CIS of 1 or 2 and a rectal temperature ≥104° F, or a CIS = 3 regardless of rectal temperature across all treatment groups within both WS strata. Steers with a CIS = 4 were eligible for removal and/or euthanasia at the discretion of the Study Investigator. After the third treatment, a steer was retained in the pen of origin unless its well-being was in jeopardy, it required additional treatment, or it died in the pen. If any of those situations occurred, the steer was removed from the pen, weighed, and removed from the study. If a steer was found dead or was euthanized, a necropsy was performed by authorized personnel. Data pertaining to the individual, to the day of removal, were retained for analysis. Values for the response variables were recorded to Day 60. On Day 61, steers enrolled in the study were moved to larger pens where they were commingled with calves that were not in the study. At closeout (average: Day 274; range: Day 255–281) all steers enrolled in the study were transported to one of three facilities where they were commercially harvested and processed. No final body weights were captured; however, an "adjusted final live weight" was estimated using a carcass yield of 63%.

Design and Analysis

A stratified, randomized, 2 × 2 factorial design was used. Two (2) strata were determined *a priori* and were based

on WS. Stratum 1 was comprised of steers with a WS = 1; and, Stratum 2 was comprised of steers with a WS ≥ 2 (**Table 2**). Within each stratum, a 2 × 2 factorial design was applied with two (2) levels of "tildipirosin; TIL" and two (2) levels of "Flunixin transdermal solution; FTS" comprising those factorials. Descriptive statistics were generated (SAS, version 9.4; Cary, NC) for CIS, rectal temperature, and incoming body weight. Inferential statistics were generated (SAS, version 9.4; Cary, NC) for the dependent/outcome variables which included the following: BRD retreatment risk, days on feed at BRD retreatment, BRD 3rd treatment risk, BRD case-fatality risk, removal risk, did not finish risk (DNF; a combination of both calves that died or were removed due to BRD), and average daily gain (ADG). Steers were randomly assigned to one of those experimental treatments. Statistical analyses were performed using generalized linear mixed models that were fitted using binomial (proportional outcomes; PROC GLIMMIX), multinomial (ordinal carcass grades; PROC GLIMMIX), or normal (continuous outcomes, PROC MIXED) distributions. Degrees of freedom were adjusted via Kenward-Roger estimation. A random intercept was included in all models to account for potential clustering effects within the design structure (lack of independence between the 2 study-sites, among pens within each site, and treatment groups within each pen). Random effects for all carcass metrics included an effect for the plant in which harvest occurred. Treatment group was included as the fixed effect. Enrollment body weight and rectal temperatures were evaluated for differences across treatments between both strata. If associations were observed, the respective independent variable was included in the model statement as a covariate.

TABLE 4 | Descriptive statistics of steers at time of enrollment.

Pool (hd)	3,376
Enrolled by site (hd)	NE = 1,708 KS = 672
Enrolled total (hd)	2,380
CIS 1 (hd)	2,149
CIS 2 (hd)	230
CIS 3 (hd)	1
Average body weight (lb) [range]	658.1 lb [418–938]
Average rectal temperature (°F) [range]	103.0 °F [102.5–103.9]

RESULTS

No adverse events associated with the products used were observed during this study.

Three thousand, three hundred seventy-six (3,376) steers comprised the pool from which 2,380 steers (70.5% of the pool) were enrolled in this study at two sites (NE = 1,708 steers; KS = 672 steers). Descriptive statistics for those steers are presented in **Table 4**. Steers with a WS = 1 at the time of enrollment were heavier ($P < 0.05$) compared to steers possessing a WS ≥ 2 (**Figure 1**). Additionally, although minimal, steers with a WS = 1 at the time of enrollment displayed a reduced rectal temperature ($P < 0.05$; 103.03, 95% confidence interval [95% CI]; 103.01, 103.05) compared to steers possessing a WS ≥ 2 (103.07, 95% CI; 103.05, 103.09). Descriptive statistics for health-related outcomes from Day 0 to Day 60; and, from Day 0 to closeout are presented in **Figure 2**.

Fifteen (15) steers died due to BRD during Day 0 to Day 60. Samples of lungs from 11 of those 15 (73.3%) were submitted for detection of pathogens using rT-PCR. Of those 11 steers, nine

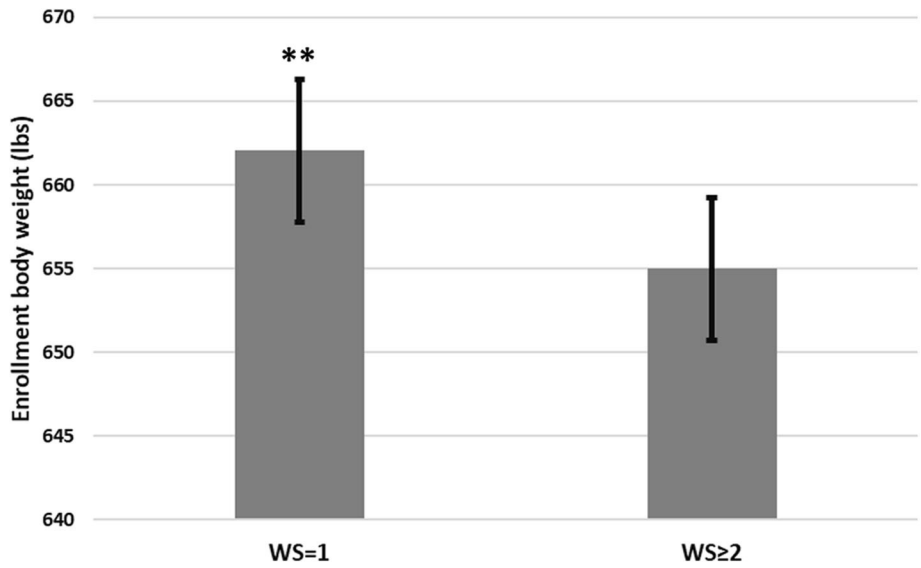

FIGURE 1 | Body weight at enrollment between mixed-breed beef feedlot steers meeting the case definition* of bovine respiratory disease (BRD) with a Whisper Score (WS) of 1 compared to steers with a WS ≥ 2. Error bars denote 95% confidence intervals. *The BRD case definition consisted of an animal displaying a clinical illness score of 1–3 and a rectal temperature of >102.5° F and <103.9° F. A WS was subsequently captured and calves were stratified by WS = 1 or >2. **A statistical difference of $P < 0.05$.

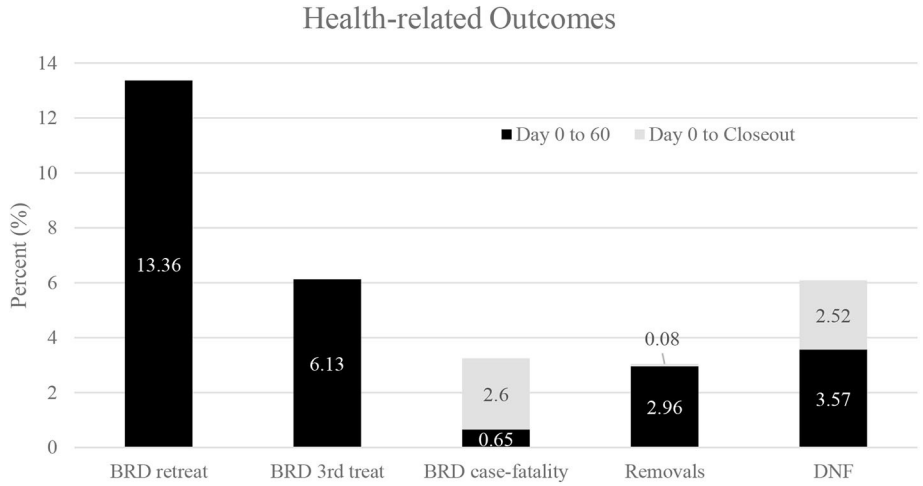

FIGURE 2 | Descriptive statistics for health-related outcomes for all steers from Day 0 to Day 60; and, from Day 0 to closeout. Values for BRD retreatments or for 3rd treatments were not retained after Day 60.

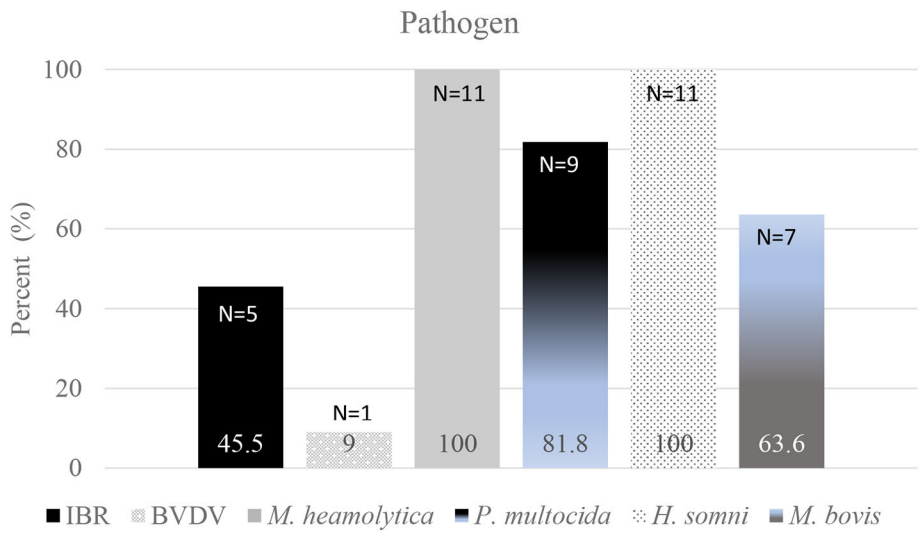

FIGURE 3 | Results of rT-PCR tests to identify pathogens in samples of lung from 11 of 15 steers that died during Day 0 to Day 60. Parainfluenza Type 3 (PI3), BRSV, or BIV were not identified in any sample.

were housed in one pen, and two were housed in one other pen. Results of rT-PCR tests to identify pathogens in samples of lung from those steers are shown in **Figure 3**. Parainfluenza Type 3 (PI3), BRSV, or BIV were not identified in any sample. Non-BRD conditions resulting in deaths or euthanasia were dietary acidosis ($N = 1$), fibrinous peritonitis (hardware; $N = 1$), severe lameness ($N = 2$), and injury ($N = 1$). Between Day 0 and Day 60 the average day of death due to BRD was Day 29; the average day of death from Day 0 to closeout was Day 135.

Model-adjusted estimates for health-related outcomes and for ADG at the end of the study on Day 60 are presented in **Tables 5A**,**B**. Within each stratum (WS = 1 vs. WS $\geq$ 2), tildipirosin resulted in fewer ($P \leq 0.05$) BRD retreatments, longer interval to BRD retreatment, fewer BRD 3rd treatments, fewer BRD removals (steers removed but did not die), fewer steers that did not finish (steers that died or were removed), and greater ADG than did steers that were not treated with tildipirosin.

Model-adjusted estimates for BRD case-fatality, removals, steers that did not finish, and adjusted-ADG from Day 0 to closeout were analyzed and results are presented in **Tables 6A**,**B**. Steers with a WS $\geq$ 2 that were treated with tildipirosin had fewer removals ($P < 0.05$), and fewer steers that did not finish ($P < 0.05$) compared to those that were not treated with tildipirosin. That was not observed for steers in stratum WS = 1. For steers in WS = 1, there was an interaction (tildipirosin x FTS; $P \leq 0.05$) for steers treated concurrently with tildipirosin and FTS; and, fewer

TABLE 5A | Model-adjusted means* (SEM) and corresponding *P*-values for health-related outcomes and ADG from Day 0 to Day 60 by treatment group for steers with a WS = 1.

Parameter	Saline[a]	FTS[a]	TIL[a]	TIL + FTS[a]	*P*-value FTS[α]	*P*-value TIL[β]	*P*-value TIL + FTS[δ]
Incoming body weight (lbs)	663.7 (5.5)	660.0 (4.0)	663.4 (4.0)	660.8 (4.0)	0.48	0.95	0.90
BRD retreatment (%)	15.0 (2.8)	18.5 (2.2)	10.4 (1.7)	8.2 (1.5)	0.95	<0.01	0.17
Day on Feed (DOF) at BRD retreatment (%)	21.6 (3.6)	23.1 (2.7)	31.6 (3.3)	34.1 (3.3)	0.32	<0.01	0.80
BRD 3rd treatment (%)	7.4 (2.1)	9.9 (1.7)	3.4 (1.0)	3.2 (1.0)	0.68	<0.01	0.48
BRD case-fatality (%)	0.57 (0.6)	1.2 (0.6)	0.49 (0.4)	0.85 (0.5)	0.39	0.76	0.91
Removals (%)	2.4 (1.2)	5.0 (1.2)	1.8 (0.7)	1.2 (0.5)	0.67	0.04	0.17
DNF (%)[b]	2.9 (1.3)	5.8 (1.3)	2.3 (0.8)	2.0 (0.8)	0.41	0.07	0.26
ADG (lbs/day)[c]	3.2 (0.1)	3.0 (0.1)	3.5 (0.1)	3.6 (0.1)	0.59	<0.01	0.06

**Mixed models with a random effect to account for lack of independence among treatment groups within pens, and pens within 2 different sites. Mean and SEM listed above reflect the interactive means of the model.*

[a]Saline, negative control; FTS, Flunixin transdermal solution; TIL, tildipirosin; and TIL + FTS, concurrent administration of TIL and FTS.

[b]DNF, did not finish; a combination of both calves that died or were removed due to BRD from Day 0 to 60 of this study.

[c]ADG, Average Daily Gain.

***P-values reflect the overall effect of FTS, TIL, and their interaction, respectively. P-values for each main effect reflect a model-adjusted average between treatments incorporating the product compared to those that do not. For example, the P-value for the FTS main effect reflects the comparison between treatments that implement FTS (i.e., FTS and TIL + FTS) vs. those that do not (i.e., Saline + TIL). Only when the P-value for the interaction is significant (P ≤ 0.05) are direct comparison made between the four treatment groups.*

[α]P-value for main effect of Flunixin transdermal solution.

[β]P-value for main effect of tildipirosin.

[δ]P-value for interaction of tildipirosin and Flunixin transdermal solution.

TABLE 5B | Model-adjusted means* (SEM) and corresponding *P*-values for health-related outcomes and ADG from Day 0 to Day 60 by treatment group for steers with a WS ≥ 2.

Parameter	Saline[a]	FTS[a]	TIL[a]	TIL + FTS[a]	*P*-value FTS[α]	*P*-value TIL[β]	*P*-value TIL + FTS[δ]
Incoming body weight (lbs)	649.5 (5.8)	654.4 (4.2)	653.5 (4.2)	660.0 (4.2)	0.21	0.30	0.86
BRD retreatment (%)	15.9 (3.0)	18.0 (2.2)	10.2 (1.7)	10.0 (1.7)	0.74	<0.01	0.66
Day on Feed (DOF) at BRD retreatment (%)	20.5 (3.5)	20.4 (3.0)	25.1 (3.2)	31.3 (3.1)	0.28	<0.01	0.25
BRD 3rd treatment (%)	6.6 (2.0)	9.8 (1.7)	3.7 (1.0)	3.9 (1.1)	0.39	<0.01	0.48
BRD case-fatality (%)	**Model did not converge[†]**						
Removals (%)	4.0 (1.5)	5.8 (1.3)	1.8 (0.7)	1.8 (0.7)	0.63	<0.01	0.60
DNF (%)[b]	5.2 (1.7)	6.1 (1.3)	2.3 (0.8)	1.8 (0.7)	0.86	<0.01	0.50
ADG (lbs/day)[c]	3.2 (0.1)	3.0 (0.1)	3.5 (0.1)	3.6 (0.1)	0.58	<0.01	0.10

**Mixed models with a random effect to account for lack of independence among treatment groups within pens, and pens within 2 different sites.*

[a]Saline, negative control; FTS, Flunixin transdermal solution; TIL, tildipirosin; and TIL + FTS, concurrent administration of TIL and FTS.

[b]DNF, did not finish; a combination of both calves that died or were removed due to BRD from Day 0 to 60 of this study.

[c]ADG, Average Daily Gain.

***P-values reflect the overall effect of FTS, TIL, and their interaction, respectively. P-values for each main effect reflect a model-adjusted average between treatments incorporating the product compared to those that do not. For example, the P-value for the FTS main effect reflects the comparison between treatments that implement FTS (i.e., FTS and TIL + FTS) vs. those that do not (i.e., Saline + TIL). Only when the P-value for the interaction is significant (P ≤ 0.05) are direct comparison made between the four treatment groups.*

[†] Model did not converge: Lack of enough observations from Day 0 to 60 to generate a model-adjusted estimate.

[α]P-value for main effect of Flunixin transdermal solution.

[β]P-value for main effect of tildipirosin.

[δ]P-value for interaction of tildipirosin and Flunixin transdermal solution.

removals ($P \leq 0.05$) and fewer steers that did not finish ($P \leq 0.05$) than did steers treated with only FTS.

Results of analyses of carcass characteristics are presented in **Tables 7A,B**. For yield and quality grades, data were available for approximately 75% of the steers across all treatment groups within each stratum (data not shown). In stratum WS = 1, tildipirosin resulted in higher marbling scores ($P \leq 0.05$) and thicker backfat ($P = 0.08$) than did steers not treated with tildipirosin. In stratum WS ≥ 2, steers treated with tildipirosin displayed higher ($P = 0.08$) hot carcass weight (HCW) than did steers that were not treated with tildipirosin.

TABLE 6A | Model-adjusted means* (SEM) and corresponding *P*-values for health-related outcomes and ADG from Day 0 to closeout by treatment group for steers with a WS = 1.

Parameter	Saline[1]	FTS[1]	TIL[1]	TIL + FTS[1]	*P*-value FTS$^{\alpha}$	*P*-value TIL$^{\beta}$	*P*-value TIL + FTS$^{\delta}$
BRD case-fatality (%)	3.5 (1.4)	4.2 (1.1)	4.0 (1.1)	2.6 (0.9)	0.73	0.64	0.35
Removals (%)	2.4 (1.2)[a]	5.2 (1.2)[a,b]	3.7 (1.0)[a]	1.2 (0.6)[a,c]	0.66	0.19	0.02**
DNF (%)[2]	5.7 (1.8)[a]	8.7 (1.6)[a,b]	7.4 (1.5) [a]	3.8 (1.0)[a,c]	0.61	0.25	0.03
Adjusted-ADG (lbs/day)[3]	3.3 (0.04)	3.3 (0.03)	3.3 (0.03)	3.3 (0.03)	0.39	0.36	0.51

Mixed models with a random effect to account for lack of independence among treatment groups within pens, and pens within 2 different sites.

***Different superscripts denote significant differences ($P \leq 0.05$) between treatment groups; Pairwise comparisons were only evaluated if the interaction effect (TIL + FTS) was observed to be statistically significant ($P \leq 0.05$). All pairwise comparisons were adjusted for multiple comparisons (Tukey method).*

[1]*Saline, negative control; FTS, Flunixin transdermal solution; TIL, tildipirosin; and TIL + FTS, concurrent administration of TIL and FTS.*

[2]*DNF, did not finish; a combination of both calves that died or were removed due to BRD from Day 0-closeout of this study.*

[3]*Adjusted-ADG: adjusted Average Daily Gain based on a 63% carcass yield.*

***P-values reflect the overall effect of FTS, TIL, and their interaction, respectively. P-values for each main effect reflect a model-adjusted average between treatments incorporating the product compared to those that do not. For example, the P-value for the FTS main effect reflects the comparison between treatments that implement FTS (i.e., FTS and TIL+FTS) vs. those that do not (i.e., Saline + TIL). Only when the P-value for the interaction is significant ($P \leq 0.05$) are direct comparison made between the four treatment groups.*

[α]*P-value for main effect of Flunixin transdermal solution.*

[β]*P-value for main effect of tildipirosin.*

[δ]*P-value for interaction of tildipirosin and Flunixin transdermal solution.*

TABLE 6B | Model-adjusted means* (SEM) and corresponding *P*-values for health-related outcomes and ADG from Day 0 to closeout by treatment group for steers with a WS > 2.

Parameter	Saline[a]	FTS[a]	TIL[a]	TIL + FTS[a]	*P*-value FTS$^{\alpha}$	*P*-value TIL$^{\beta}$	*P*-value TIL + FTS$^{\delta}$
BRD case-fatality (%)	3.1 (1.4)	3.4 (1.0)	3.3 (1.0)	1.5 (0.7)	0.54	0.50	0.44
Removals (%)	5.3 (1.8)	6.3 (1.4)	1.8 (0.7)	2.1 (0.8)	0.66	<0.01	0.95
DNF (%)[b]	8.8 (2.1)	9.4 (1.6)	5.0 (1.2)	3.5 (1.0)	0.68	<0.01	0.31
Adjusted-ADG (lbs/day)[c]	3.3 (0.05)	3.3 (0.03)	3.3 (0.03)	3.3 (0.03)	0.94	0.11	0.79

Mixed models with a random effect to account for lack of independence among treatment groups within pens, and pens within 2 different sites.

[a]*Saline, negative control; FTS, Flunixin transdermal solution; TIL, tildipirosin; and TIL + FTS, concurrent administration of TIL and FTS.*

[b]*DNF, did not finish; a combination of both calves that died or were removed due to BRD from Day 0-closeout of this study.*

[c]*Adjusted-ADG: adjusted Average Daily Gain based on a 63% carcass yield.*

***P-values reflect the overall effect of FTS, TIL, and their interaction, respectively. P-values for each main effect reflect a model-adjusted average between treatments incorporating the product compared to those that do not. For example, the P-value for the FTS main effect reflects the comparison between treatments that implement FTS (i.e., FTS and TIL+FTS) vs. those that do not (i.e., Saline + TIL). Only when the P-value for the interaction is significant ($P \leq 0.05$) are direct comparison made between the four treatment groups.*

[α]*P-value for main effect of Flunixin transdermal solution.*

[β]*P-value for main effect of tildipirosin.*

[δ]*P-value for interaction of tildipirosin and Flunixin transdermal solution.*

DISCUSSION

A traditional diagnosis of BRD is based on the presence of clinical signs of the disease, and a rectal temperature ≥104° F, before treatment is prescribed. Results of this study indicate that steers with clinical signs of BRD and rectal temperature <104° F also respond favorably to antimicrobial treatment. In this study tildipirosin resulted in beneficial outcomes that were measurable from Day 0 to Day 60, and from Day 0 to closeout. There were no statistical benefits (or detriments) for steers treated with FTS alone or concurrently with tildipirosin. In stratum WS = 1, a greater proportion of steers treated with FTS alone did not finish compared to steers treated with FTS + tildipirosin. However, no differences were observed among FTS, Saline, and Tildipirosin across these specific outcomes. Additionally, the use of FTS, alone or in conjunction with tildipirosin, did not augment the outcome among calves in either WS strata.

The normal body temperature of beef cattle ranges from 98 to 102.4° F (20). Therefore, given the diurnal variation in body temperature and the cross-sectional nature of rectal temperature data collection, it is likely that a subpopulation of calves presumptively identified with clinical signs of BRD will possess a rectal temperature <104° F. Response to therapy across both Whisper strata among calves with a rectal temperature <104° F may reflect the reality that current BRD diagnostic modalities involve a one point in time event and do not robustly describe the clinical severity of the individual animal. Theurer et al. observed no direct relationship between rectal temperature at the time of the first BRD treatment and the animal's probability of finishing the feedlot phase of production

TABLE 7A | Model-adjusted* means (SEM) and corresponding *P*-values for carcass characteristics by treatment group for steers with a WS = 1.

Parameter		Saline[1]	FTS[1]	TIL[1]	TIL + FTS[1]	*P*-value FTS$^{\alpha}$	*P*-value TIL$^{\beta}$	*P*-value TIL + FTS$^{\delta}$
HCW[2], lbs		924.4 (6.8)	915.2 (4.9)	926.2 (4.9)	922.7 (4.9)	0.25	0.39	0.61
Ribeye area		15.1 (0.2)	15.3 (0.2)	15.3 (0.2)	15.3 (0.1)	0.45	0.58	0.86
Marbling		462.8 (11.1)	458.2 (8.0)	487.6 (8.0)	482.8 (7.8)	0.59	<0.01	0.99
Backfat		0.57 (0.02)	0.55 (0.01)	0.58 (0.01)	0.60 (0.01)	0.68	0.08	0.26
Calculated Yield Grade		2.9 (0.1)	2.6 (0.1)	3.0 (0.1)	2.9 (0.1)	0.09	0.12	0.29
[a]Yield Grade (*N* = 891)***	1	5.3% (7)	9.9% (25)	10.7% (27)	7.5% (19)		**Model did not converge	
	2	39.8% (53)	39.5% (100)	42.1% (106)	39.5% (100)			
	3	38.3% (51)	36.8% (93)	36.1% (91)	39.9% (101)			
	4	12.8% (17)	12.6% (32)	10.7% (27)	11.5% (29)			
	5	3.8% (5)	1.2% (3)	0.4% (1)	1.6% (4)			
	N	133	253	252	253			
[a]Quality Grade (*N* = 894)	Prime	2.3% (3)	2.4% (6)	1.6% (4)	2.7% (7)	0.73	0.81	0.66
	Choice	61.8% (81)	65.0% (165)	62.9% (158)	59.3% (153)			
	Select	32.8% (43)	30.3% (77)	31.5% (79)	32.2% (83)			
	Other	3.1% (4)	2.4% (6)	4.0% (10)	5.8% (15)			
	N	131	254	251	258			

**Mixed models with a random effect to account for lack of independence among treatment groups within pens, pens within 2 different sites, and sites within 3 different processing plants.*
[a]Each cell within the Yield and Quality grade outcomes reflects the raw proportions and counts for each treatment group.
***Insufficient observations to generate a model-adjusted estimate within the hierarchical structure of the model. Due to non-convergence of the model, the effect of "processing plant" was removed from the random effect and was included as a covariate in the model for "yield grade". However, the model still did not converge within this WS stratum.*
****Total number of steers in each treatment group for Yield and Quality Grade is specified. The proportion of missing data was the same for all treatment groups.*
[1]Saline, negative control; FTS, Flunixin transdermal solution; TIL, tildipirosin; and TIL + FTS, concurrent administration of TIL and FTS.
[2]HCW, Hot Carcass Weight.
***P-values reflect the overall effect of FTS, TIL, and their interaction, respectively. P-values for each main effect reflect a model-adjusted average between treatments incorporating the product compared to those that do not. For example, the P-value for the FTS main effect reflects the comparison between treatments that implement FTS (i.e., FTS and TIL + FTS) vs. those that do not (i.e., Saline + TIL). Only when the P-value for the interaction is significant ($P \leq 0.05$) are direct comparison made between the four treatment groups.*
[α]P-value for main effect of Flunixin transdermal solution.
[β]P-value for main effect of tildipirosin.
[δ]P-value for interaction of tildipirosin and Flunixin transdermal solution.

(21). Rather, this relationship was influenced by additional parameters including time of year, gender, and the days on feed prior to first BRD diagnosis (21). These variables likely contribute to the poor diagnostic performance of current BRD diagnostic methods (12, 13).

In this study, the Whisper technology was used in conjunction with traditional diagnostic modalities (clinical signs and rectal temperature) to delineate severity of BRD and to potentially aid in the BRD treatment decision. Since a WS cannot be randomly assigned to an animal, inferences cannot be made between treatments across WS strata; conversely, those decisions can only be made within the chosen strata analyzed in the present study. Although prior studies have shown an association between a rising Whisper Score and worsening lung health (18, 19) and calves enrolled with a WS $\geq$ 2 were lighter compared to calves with a WS = 1, the data generated in this study indicates that regardless of lung health status, calves in both WS strata require antimicrobial therapy to significantly reduce the risk of BRD relapse. This raises the question as to how to leverage the WS data. As an observation, the positive effects of tildipirosin observed at the 60-day mark within the WS = 1 stratum were not observed at closeout (**Table 6A**). Conversely, a significant tildipirosin effect was observed in the WS $\geq$ 2 strata for both the removal and DNF outcomes from Day 0 to closeout (**Table 6B**). This may suggest that calves identified with clinical signs of BRD, a rectal temperature <104° F and a WS = 1 are more likely to finish the feeding phase of production compared to calves with a WS $\geq$ 2 and a rectal temperature <104° F at the time of first BRD diagnosis. This observation among calves with a WS = 1 raises two potential opportunities to utilize these data: (1) Given that calves in both WS strata responded positively to antimicrobial therapy, perhaps a less potent (and cheaper) antimicrobial may be applicable in this subpopulation of cattle stricken with BRD; and (2) perhaps the value of the WS may be realized in the subsequent management of calves with more severe lung health issues at the time of first BRD diagnosis rather than impacting antimicrobial treatment decisions. More work is necessary to test these theories.

One potential limitation of this study is that the study population was not maintained within their original cohort from the time of enrollment until closeout. Rather, they were placed in a larger general population after 60 days post-BRD diagnosis. This occurrence may have led to an underestimation of overall BRD morbidity due to further reduction in BRD diagnostic accuracy within a larger group

TABLE 7B | Model-adjusted* means (SEM) and corresponding *P*-values for carcass characteristics by treatment group for steers with a WS ≥ 2.

Parameter		Saline[1]	FTS[1]	TIL[1]	TIL + FTS[1]	*P*-value FTS$^{\alpha}$	*P*-value TIL$^{\beta}$	*P*-value TIL + FTS$^{\delta}$
HCW[2], lbs		912.6 (7.2)	914.5 (5.1)	920.4 (5.0)	926.5 (5.0)	0.48	0.08	0.72
Ribeye area		15.2 (0.2)	15.3 (0.1)	15.3 (0.1)	15.4 (0.1)	0.52	0.51	0.94
Marbling		475.7 (10.3)	481.6 (7.4)	470.2 (7.2)	472.3 (7.1)	0.62	0.36	0.82
Backfat		0.57 (0.02)	0.56 (0.01)	0.56 (0.01)	0.59 (0.01)	0.57	0.60	0.40
Calculated Yield Grade		2.9 (0.1)	2.9 (0.1)	2.8 (0.1)	3.0 (0.1)	0.44	0.87	0.26
[a]Yield Grade ($N = 902$)	1	9.4% (12)	9.7% (25)	11.8% (30)	10.3% (27)	0.69	0.83	0.42
	2	32.0% (41)	38.5% (99)	38.0% (97)	34.4% (90)			
	3	49.2% (63)	38.5% (99)	40.8% (104)	43.1% (113)			
	4	8.6% (11)	11.3% (29)	9.0% (23)	9.9% (26)			
	5	0.8% (1)	1.9% (5)	0.4% (1)	2.3% (6)			
	N	128	257	255	262			
[a]Quality** Grade ($N = 902$)	Prime	1.6% (2)	5.8% (15)	1.2% (3)	3.5% (9)	0.19	0.70	0.32
	Choice	64.1% (82)	62.0% (160)	67.2% (172)	65.4% (170)			
	Select	32.8% (42)	29.1% (75)	27.7% (71)	27.3% (71)			
	Other	1.6% (2)	3.1% (8)	3.9% (10)	3.8% (10)			
	N	128	258	256	260			

Mixed models with a random effect to account for lack of independence among treatment groups within pens, pens within 2 different sites, and sites within 3 different processing plants.

[a]*Each cell within the Yield and Quality grade outcomes reflects the raw proportions and counts for each treatment group.*

**Total number of steers in each treatment group for Yield and Quality Grade is specified. The proportion of missing data was the same for all treatment groups.*

[1]*Saline, negative control; FTS, Flunixin transdermal solution; TIL, tildipirosin; and TIL + FTS, concurrent administration of TIL and FTS.*

[2]*HCW, Hot Carcass Weight.*

***P-values reflect the overall effect of FTS, TIL, and their interaction, respectively. P-values for each main effect reflect a model-adjusted average between treatments incorporating the product compared to those that do not. For example, the P-value for the FTS main effect reflects the comparison between treatments that implement FTS (i.e., FTS and TIL + FTS) vs. those that do not (i.e., Saline + TIL). Only when the P-value for the interaction is significant ($P \leq 0.05$) are direct comparison made between the four treatment groups.*

[α]*P-value for main effect of Flunixin transdermal solution.*

[β]*P-value for main effect of tildipirosin.*

[δ]*P-value for interaction of tildipirosin and Flunixin transdermal solution.*

size. Additionally, final body weights were not captured to avoid unnecessary stress on the finished animals which necessitated back calculation and estimation of final weights based on HCW and carcass yields (63%). This likely had an impact on the precision of the ADG estimates at closeout. However, these management decisions were applied universally across the study population and individual identification was accurately maintained through closeout.

In this study, calves exhibiting clinical signs of BRD but with rectal temperatures <104° F (regardless of WS) responded favorably to tildipirosin therapy at the time of first BRD diagnosis. Withholding antimicrobial medication from feedlot animals that have not reached the traditional threshold for rectal temperature may not be prudent for the best interest of the animal or the producer.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the study data are owned by Merck Animal Health. Access to these data would require additional approval beyond that of the authors. Requests to access the datasets should be directed to Dr. John Hutcheson, john.hutcheson@merck.com.

ETHICS STATEMENT

This animal study was reviewed and approved by the study protocol review committee consisted of an independent group compiled by authors CC and KL.

AUTHOR CONTRIBUTIONS

This study was designed and the protocol generated by JN and LB. This study was executed by CC and KL and monitored by JN and LB. Statistical analysis was performed by JN. Manuscript writing reflected a collaboration amongst all authors.

FUNDING

Merck Animal Health fully funded this research.

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Conflict of Interest: This study was fully funded by Merck Animal Health (MAH). Allflex Livestock Intelligence (ALI) is a subsidiary of MAH. JN and LB (employees of ALI and MAH, respectively) were responsible for study design, monitoring, and data analysis. Midwest Veterinary Services (MVS) and Veterinary Biomedical Research Center (VBRC) are contract research organizations wholly owned by KL that provides professional services to support the regulatory research required by FDA, USDA, and EPA for approval of animal health products. MVS's Institution Animal Care and Use Committee (IACUC) is responsible for oversight of the animal program and its components as described in the USDA and PHS guidelines. MVS continues to pass annual to bi-annual audits from government regulatory agencies. The study was executed under the oversight of KL and CC. The study protocol was submitted to the MVS IACUC where the protocol received approval on 30 January 2018. The assigned IACUC number is AC17100B.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 26 October 2020
doi: 10.3389/fvets.2020.542724

Field-Adapted Full Genome Sequencing of Peste-Des-Petits-Ruminants Virus Using Nanopore Sequencing

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Peste-des-petits-ruminants virus (PPRV) is currently the focus of a control and eradication program. Full genome sequencing has the opportunity to become a powerful tool in the eradication program by improving molecular epidemiology and the study of viral evolution. PPRV is prevalent in many resource-constrained areas, with long distances to laboratory facilities, which can lack the correct equipment for high-throughput sequencing. Here we present a protocol for near full or full genome sequencing of PPRV. The use of a portable miniPCR and MinION brings the laboratory to the field and in addition makes the production of a full genome possible within 24 h of sampling. The protocol has been successfully used on virus isolates from cell cultures and field isolates from tissue samples of naturally infected goats.

Keywords: peste-des-petits-ruminants virus, eradication, molecular epidemiology, full genome sequencing, MinION, miniPCR

OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
Eugene Ryabov, United States Department of Agriculture (USDA), United States
Libeau Geneviève, Institut National de la Recherche Agronomique (INRA), France

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *13 March 2020*
Accepted: *30 September 2020*
Published: *26 October 2020*

Citation:
Torsson E, Kgotlele T, Misinzo G, Johansson Wensman J, Berg M and Karlsson Lindsjö O (2020) Field-Adapted Full Genome Sequencing of Peste-Des-Petits-Ruminants Virus Using Nanopore Sequencing. Front. Vet. Sci. 7:542724. doi: 10.3389/fvets.2020.542724

INTRODUCTION

With the development of new and portable sequencing equipment, it is now possible to perform—in very basic laboratories—sequencing that was previously limited to well-equipped laboratories (1–4). With a small thermocycler such as the miniPCR (Amplyus, Cambridge, United States), the hand-held MinION sequencer (Oxford Nanopore Technologies, Oxford, United Kingdom), and portable computational resources, full genome sequencing and advanced molecular epidemiology can be performed in almost any setting (1–4). This is highly advantageous for the diagnosis and control of viral diseases. This approach enables rapid sequencing-based technologies in resource constrained environments, in addition to bringing the laboratory analysis closer to the disease outbreak and reducing the time from diagnosis to full genome and epidemiological investigations.

Peste des petits ruminants (PPR) is a highly contagious and deadly disease in small ruminants (5). The cause is the peste-des-petits-ruminants virus (PPRV), a single-stranded negative-sense RNA virus belonging to the genus *Morbillivirus* (6). Other morbilliviruses include canine distemper virus, measles virus, feline morbillivirus, marine morbilliviruses, and the now eradicated rinderpest virus (RPV) (7).

PPR has a large socioeconomic impact, as small ruminants are mainly kept by poor and rural populations that depend on their animals for income and livelihood. Due to this, the Food and Agriculture Organization of the United Nations (FAO) and the World Animal Health Organization (OIE) have launched a control and eradication program for PPRV to eliminate the disease by 2030 (8). To reach this goal, accurate and well-functioning diagnostic and epidemiological tools need to be in place (9). The Global Strategy for Control and Eradication of PPR (8) highlights that countries in stage 2 in the eradication program (out of four stages), have to strengthen laboratory capacity with molecular methods able to better characterize the collected virus isolates (8). Use of the full genome to characterize isolates, rather than only a partial sequence or genetic marker, ensures detection of important changes within the genome (10).

PPRV is widely distributed in Africa and Asia. In many of these areas, efficient transport of samples, with an unbroken cold chain to a laboratory with the correct equipment, is hard to achieve (9, 11). A broken cold chain during sample transport risks degradation of the sensitive nucleic acid of single-stranded RNA viruses such as PPRV. Analyses performed as close to possible to the sample collection site avoids these long transports (12). More accessible, less expensive, and more timely full genome sequencing will lead to better comprehensive surveillance and detection in the control of a disease such as PPR. The implementation of these mobile methodologies for molecular epidemiology will also increase the chances for successful eradication.

Here we have developed a protocol for a quick, on-site, field-adapted full genome sequencing of veterinary significant virus diseases, with PPRV as an important example. The protocol uses the highly portable miniPCR thermocycler and the MinION sequencer.

MATERIALS AND METHODS

The full wet lab protocol is available at DOI:dx.doi.org/10.17504/protocols.io.pnxdmfn.

Samples

A selection of samples of different origins was used to verify the protocol. These included: (i) viral RNA collected from a cell-culture grown virus (Vero-SLAM cell line), isolate Nigeria 75/1, kindly provided by Dr. Siamak Zohari, National Veterinary Institute (SVA), Uppsala, Sweden; (ii) RNA from field samples representing all currently known lineages of PPRV (cultured on the CV-1-SLAM cell line), kindly provided by Dr. William G. Dundon, International Atomic Energy Agency (IAEA), Vienna, Austria, [KP789375 (13), KR781450, KR781449 (14) and KM463083 (15)]; and, (iii) two field isolates (tissue) collected by Tebogo Kgotlele and Prof. Gerald Misinzo from an outbreak in goats in Dakawa, Morogoro region, Tanzania, in 2013 (16).

Primer Design

Two sets of multiplex full-genome primers were designed using Primal Scheme (http://primal.zibraproject.org) (17). One primer set had an amplicon length of 800 base pairs (bp) and an overlap of 100; the other primer set had an amplicon length of 600 bp and an overlap of 40. Primers were designed using eight full genome sequences representing all known lineages available at the NCBI GenBank (**Table 1**). Primers, for the 600-bp and 800-bp amplicons, are available in the Supplementary Material (**Tables S1, S2**).

TABLE 1 | Complete genomes used to generate the multiplex primers with the primal scheme.

Accession no.	Lineage	Country	Year
EU267273.1	I	Cote d'Ivoire	1989
KR781451.1	II	Cote d'Ivoire	2009
KR828814.1	II	Nigeria	2012
X74443.2	II	Nigeria	1975
KJ867540.1	III	Ethiopia	1994
KJ867543.1*	III	Uganda	2012
KJ867541.1	IV	Ethiopia	2010
KR828813.1	IV	Nigeria	2013

First genome in file.

RNA Extraction, cDNA Synthesis, and PCR Amplification

QIAamp Viral RNA Mini kit (Qiagen) was used according to the manufacturer's instructions to extract RNA from tissue samples from Tanzania (sample type iii). The other samples were shared with us as extracted RNA. cDNA synthesis was performed using Superscript IV First-Strand Synthesis System (Invitrogen) with 11 μl of RNA, according to the manufacturer's instructions. PCR amplification was performed using the Q5 Hot Start High Fidelity Polymerase (New England BioLabs) according to the protocol in (17). The protocol divided the multiplex primers into two pools with an even amount of primer pairs, and was run on the miniPCR thermocycler. The amplicons were then purified using AMPure XP magnetic beads (Beckman Coulter) or HighPrep PCR Clean-up System (MagBio Genomics Inc.) with a 1.8× bead ratio and quantified using Qubit 1.0 Fluorometer dsDNA HS assay (Thermo Fisher Scientific). To verify the amplification, a 1% agarose gel electrophoresis (6–7 V/cm, 50–60 min) was performed, this is however optional in the final protocol.

Nanopore Library Preparation and Sequencing

Sequencing libraries were prepared using the SQK-LSK109 Ligation Sequencing Kit and EXP-NBD104 Native Barcode expansion (Oxford Nanopore Technologies) according to manual and previously suggested modifications (17, 18). The purified PCR amplicons were repaired and A-tailed using the NEBNext Ultra II End Repair/dA-Tailing module (New England BioLabs). Native barcodes and adaptors were ligated to amplicons using Blunt/TA Ligase Master Mix (New England BioLabs). The library was then sequenced on a MinION Flowcell R9.4. for 10 h.

Data Analysis

The docker, as well as guidance for replication of the study is available at (www.github.com/Ackia/Field_Seq). In addition to this, a suggested user protocol is included in the protocol at protocols.io (DOI: dx.doi.org/10.17504/protocols.io.pnxdmfn). The process in short; raw reads were basecalled using GUPPY (version 3.1.5. used for the publication. FASTQ files are available in repository PRJEB35549). Read-set composition and quality were assessed using plots produced by PycoQC (19). Demultiplexed read-sets were checked for purity using Kraken 2, and results were visualized in Pavian (20, 21). The read-sets were aligned to the reference genome (RefSeq assembly accession: GCF_000866445.1) using minimap2 (22). The resulting alignment file was sorted and converted into an index bam-file for further processing with samtools (23). BED files were created, representing the coverage of the sequence reads against the reference genome. BED files were further visualized using R and ggplot (24, 25). Consensus sequence were extracted using samtools and bcftools (23). Whole-genome comparison of sequence identity was performed using sourmash with the sequences of good quality (coverage x50 > 80%) reported from MinION sequencing (26). Based on the sourmash results, representative sequences were selected and whole genome comparison was performed between the consensus sequences produced with the FieldSeq protocol and the reference sequences using Mashtree (27). The tree from Mashtree was visualized using R and ggtree.

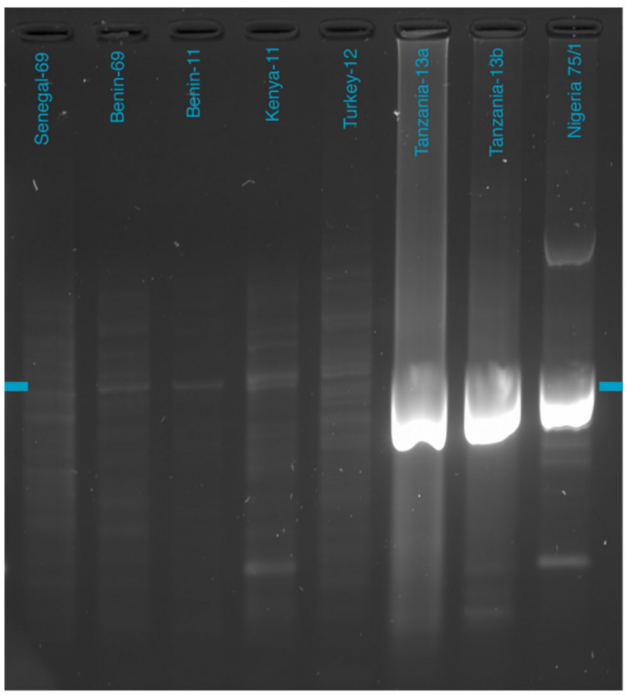

FIGURE 1 | Gel electrophoresis of purified 800-bp PCR amplicons. The blue marker indicates the 800-bp size marker. Full gel image available in **Supplementary Material**.

RESULTS

Gel electrophoresis following PCR amplification of Nigeria 75/1 virus cultured on Vero-SLAM cells showed two bands—one very clear at 800 bp, and a second, weaker band at approximately 2400 bp (**Figure 1**). These longer amplicons are not seen on the gel electrophoresis image for the Tanzanian field samples. However, a strong band is seen at 800 bp. For the samples cultured on CV-1 cells, the gel electrophoresis image shows a narrow band at 800 bp, together with a wide selection of bands of all sizes.

Sequencing of the Nigeria 75/1 isolate produced 741,787 raw reads for the 800-bp primer set and 629,875 raw reads for the 600-bp primer set. The 800-bp primers gave a genome coverage (>50×) of 98.6% and an average coverage of 4,602 reads, whereas the 600-bp primers produced a genome coverage of 99.5%, with an average coverage of 4,586 reads (**Table 2**). Following this first evaluation of the primer sets, we found that the 800-bp primer set

TABLE 2 | Results from sequencing using the Oxford Nanopore MinION sequencer.

Sample (lineage)	Raw reads	Total bp	N50 length (bp)	Reads mapped to PPRV	Average coverage	Genome coverage >50× (%)	Genome coverage >25× (%)	Source
Nigeria 75/1*, 800 bp (II)	741,787	660,217,802	870	672,805	4601	98.6	99.4	Cultured on Vero-SLAM
Nigeria 75/1, 600 bp (II)	629,875	500,972,391	630	597,110	4586	99.5	99.5	Cultured on Vero-SLAM
Senegal-69 (I)	721,283	483,015,988	753	10,196	416	49.6	71.8	Cultured on CV-1**
Benin-69 (II)	945,266	619,883,689	826	35,716	554	78.9	87.5	Cultured on CV-1**
Benin-11 (II)	354,531	221,621,251	779	47,828	460	66.4	79.2	Cultured on CV-1**
Kenya-11 (III)	1,123,782	662,242,080	736	178,526	2311	85.0	88.8	Cultured on CV-1**
Turkey-12 (IV)	776,693	500,690,835	748	11,554	493	67	79.8	Cultured on CV-1**
Tanzania-13a (III)	947,742	707,688,820	782	771,053	4340	91.2	93.0	Field isolate
Tanzania-13b (III)	1,418,713	1,089,046,940	780	1,197,778	4506	93.5	93.5	Field isolate

Mean from duplicate runs.

***Stably transfected with a plasmid expressing the goat SLAM receptor.*

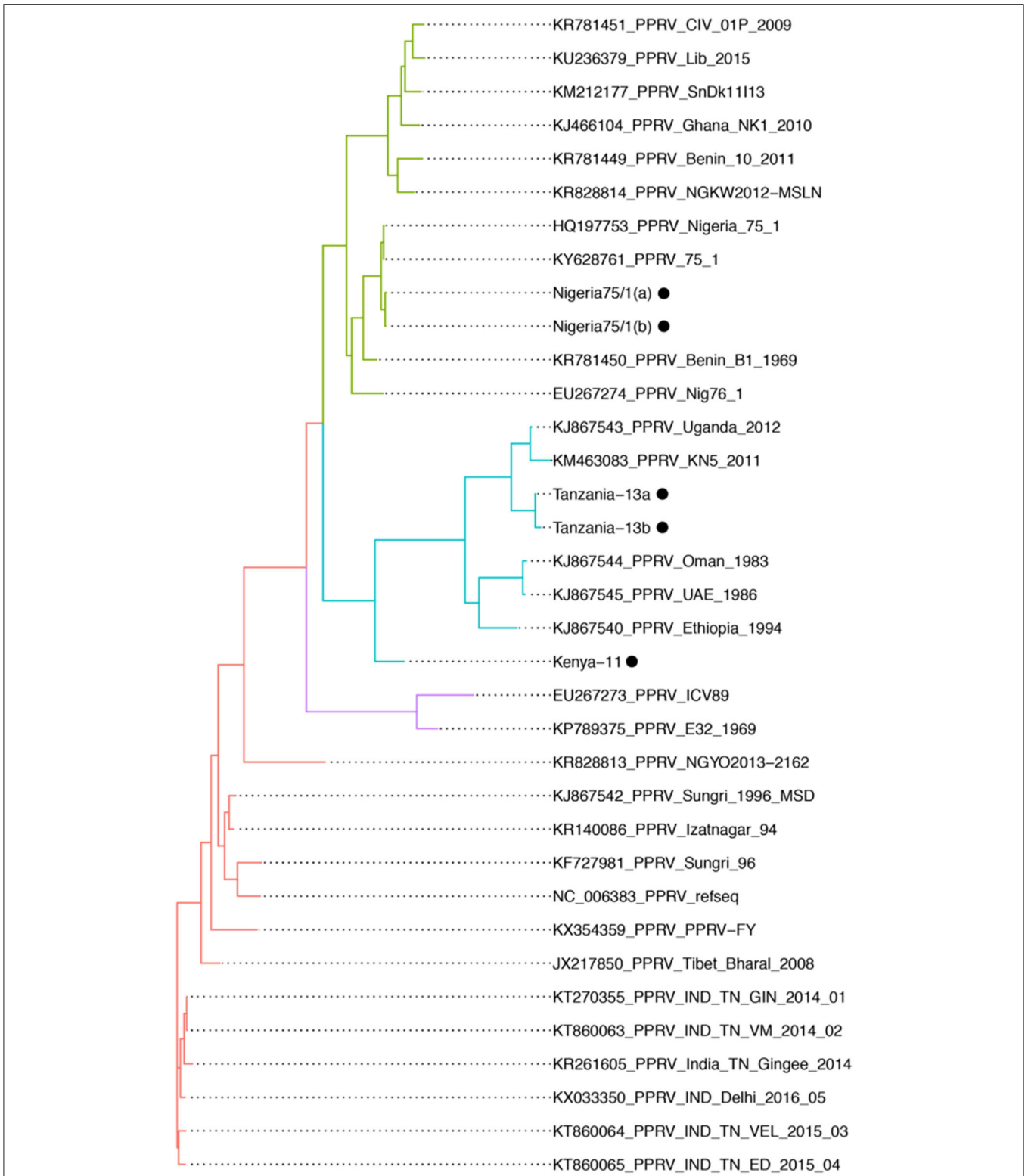

FIGURE 2 | Genomic comparison of whole genome sequences of PPRV from the NCBI GenBank and the isolates with consensus sequences from the minION sequencing that produced quality sequences (>80% of the full genome). All isolates placed in the comparison according to their previously known lineage. Included consensus sequences are indicated by black dots. Isolates with purple branches indicated lineage I, isolates with green branches indicate lineage II, isolates with blue branches indicate lineage III, and isolates with red indicate lineage IV.

gave more even coverage of the PPRV genome, including a higher coverage of the ends of the genome. A possible explanation of this could be the increase overlap of the amplicons for the 800 bp primer set, around 100 bp instead of around 40 bp. On the basis of this result, we decided to continue working with only the 800-bp amplicon primer set for further samples (coverage comparison of both primer sets is available in **Supplementary Material**, **Figure 1**).

The Nigeria 75/1 isolate, the first trial sample, was run in duplicate to evaluate the reproducibility within a single run. The duplicates produced 709,440 and 636,171 reads that mapped against PPRV, with an average coverage of 4,454 and 4,749 reads. This was considered as an equal performance of the duplicates, which were henceforth presented as a mean of the two (**Table 2**). A total of 672,805 reads was mapped to the PPRV genome to give a coverage (above 50×) of 98.4% of the full genome (**Table 2**). For the isolates cultured on CV-1 cells, the protocol was run using the 800-bp multiplex primers. The total number of raw reads varied between 354,531 and 1,123,782; however, most reads did not map against the PPRV reference genome (**Table 2**). Despite this, an average of 69.4% of the genome was covered above 50×. For the two field isolates from Tanzania, the sequencing results were 947,742 and 1,418,713 raw reads, respectively, out of which 771,053 and 1,197,778 reads mapped to the PPRV reference genome (**Table 2**). For these isolates, 91.9% and 93.5% of the genome had coverage above x50. The whole genome sequences with good quality were compared based on nucleic acid similarity and grouped based on distance using mashtree (**Figure 2**). The sequences produced on MinION showed good conformity with previously sequenced genomes based on lineage and previous sequencing.

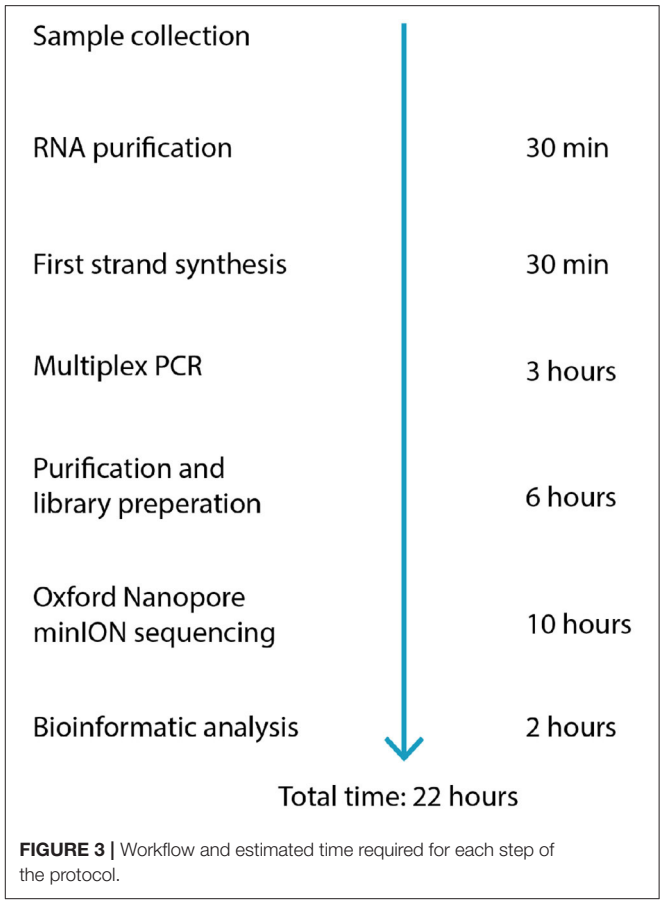

FIGURE 3 | Workflow and estimated time required for each step of the protocol.

DISCUSSION

Here we have presented a protocol for full genome sequencing of the peste-des-petits-ruminants virus (PPRV) using the miniPCR thermocycler and Oxford Nanopore MinION. Both are suitable for use in a minimally equipped laboratory facility or even directly in the field. PPRV is currently the target of a control and eradication program, launched by the FAO and OIE in 2015, with a goal of eradication by 2030 (8). The success of this program depends on vaccination campaigns and the ability to quickly diagnose and trace the source of an outbreak (8). PPRV most often occurs in areas that lack infrastructure and laboratory facilities (11), making it difficult to reach a quick diagnosis or do adequate epidemiological investigations. Moreover, long transports of samples increase the risk of degrading the sensitive viral nucleic acid in the sample, leading to false negative results (5). By bringing the laboratory closer to the outbreak, these risks are minimized and the time from recognizing clinical signs to a molecular epidemiological investigation is significantly reduced.

The proposed protocol does not require an expert laboratory- or sequencing technician, but it does need a basic understanding of contamination avoidance and handling of laboratory equipment. We estimate that, assuming previous training in basic pipetting skills, this protocol can easily be performed following one full run-through auscultation. The loading of reagents to the MinION flow cell requires the most practice, which can be done on used flow cells, or this single step can be performed by more experienced personnel. The time needed to run the full protocol, from the purification of RNA to analyzed sequences, is around 22–24 h (**Figure 3**). The protocol does not include instructions for RNA purification. In a field setting, either a spin column protocol using a small battery-driven centrifuge would be a good option or a magnetic bead-based system (as the latter is also needed in other steps of the protocol). **Table 3** gives a full list of reagents and cost calculation. With our protocol, a full genome is possible to produce for under USD 100 per sample. Washing and reusing the flow cells reduces the cost even further, to around USD 80 per sample.

With good quality virus isolates, this protocol performed well and yielded a full genome with a mean coverage of around 4,500 reads. To standardize the quality assessment of the many new high-throughput sequences being produced, Ladner et al. suggest five standard sequenced viral genomes could be placed in (10). For molecular epidemiology, they suggest the standard "Coding complete," which means 90–99% of the genome is sequenced with no gaps, all open reading frames (ORFs) are complete, and the average coverage is 100×. The sequences produced using our method meet these requirements when the virus isolates are of good quality.

TABLE 3 | Reagents used within the protocol, with cost calculations based on prices stated on suppliers' homepages in September 2019.

Reagent	Product number	Source	Cost/unit	Cost/sample (USD)
RNA extraction			variable	
SuperScript IV first-strand synthesis system	18091050	ThermoFisher Scientific	USD 2978 (200 reactions)	14.89
Multiplex primers		SigmaAldrich	variable, our 800-bp primers cost USD 158 for 100 μM/primer	0.02
Q5 hot start high-fidelity DNA polymerase	M0493L	New England Biolabs	USD 532 (500 reactions)	1.10
dNTPs (10 μM each)	R0192	ThermoFisher Scientific	USD 88 (1 ml)	0.13
HighPrep^{TM} PCR clean-up system	AC-60050	MagBio Genomics	USD 526 (50 ml)	1.40
Qubit dsDNA HS assay kit	Q32854	ThermoFisher Scientific	USD 289 (500 reactions)	1.73
NEBNext Ultra II End Repair/dA-tailing module	E7546L	New England Biolabs	USD 795 (96 reactions)	4.10
Native barcoding expansion 1-12	EXP-PBC001	Oxford Nanopore	USD 288*	4
Blunt/TA ligase master mix	M0367L	New England Biolabs	USD 520 (250 reactions)	20.80
Ligation sequencing kit (incl. FlowCell priming Kit)	SQK-LSK109	Oxford Nanopore	USD 599 (6 reactions)	8.30
MinION flow cell	R9.4.1	Oxford Nanopore	USD 500–900/flow cell, depending on the quantity ordered**	42
Total				USD 98.5

Contains 12 unique barcodes and enough of each to use in 12 different sequencing libraries.

***Possible to wash up to 5 times, then USD 8.4/sample and total USD 81/sample (including the cost of Flow Cell Wash kit).*

For the first run using the cell culture grown Nigeria 75/1 isolate the coverage is over 100× for the entire genome, missing only a piece of the virus poly-A tail (**Figure 4**). There is a slight decrease in coverage in the intergenic region between the matrix (M) and the fusion (F) protein gene (nucleotide position 4,445–5,526), as well as a short region close to the end of the genome. The M and F intergenic region is the longest intergenic region in the PPRV genome and is rich in GC content and secondary structures (28). These properties makes the region difficult for both primer design and amplification. This region have the lowest coverage in all the sequenced isolates, and was problematic for both studied primer sets. In the isolate from Tanzania it is the only region with low coverage (**Figure 5**), however the coverage is above zero and for molecular epidemiology the ORF are of most importance (10).

In the isolates cultured on CV-1 cells, we did not get equally good coverage over the full genome as we did for the Nigeria 75/1 and Tanzanian isolates (**Figure 6**, **Table 2**). The majority of the reads from the CV-1 samples instead mapped against the human genome. We suspect this is due to the low concentration of viral RNA, degradation of the viral genomes in the samples, and that the human sequences were mistakenly interpreted as such but in fact, had originated from the CV-1 cells (African Green monkey kidney cells). Even though this is not a perfect result, it shows how this protocol works with degraded and damaged samples. Despite the reduced coverage of the genome, we were able to extract 49.6–85.0% (with >50× coverage) of the full genomes in these five samples with an average coverage well above 100× for them (**Table 2**). The regions with lowest coverage for these isolates were the same for these as for the isolates of better quality, the M-F intergenic region and a region toward the end of the genome within the large protein, exemplified by the Kenya-11 isolate in **Figure 6**. Coverage plots for all sequenced isolates are available as **Supplementary Material**.

The four samples that produced above 80% of the full genome (Nigeria 75/1, Tanzania-13a/b, and Kenya-11) were used in a genomic comparison together with other available whole genomes (**Figure 2**). The Nigeria 75/1 isolate that performed excellent in the protocol placed together with the Nigeria 75/1 sequence collected from the database. The isolate from Kenya (Kenya-11) was previously sequenced with the accession number KM463083 (15) which is also included in the comparison. These two whole genome sequences is slightly seperated. This is probably due to the sequences produced using the protocol suggested here is not covering 100% of the genome, wheras the published sequence is full and produced by Sanger sequencing. They do, however, place within the same branch, together with other islolates from lineage III of PPRV. Within the same branch, the two samples from Tanzania (-13a and -13b) are also placed closed together, as expected due to the samples being collected from the same outbreak. By comparing, the consensus sequences produced by the described protocol with previously published sequences produced using the other sequencing techniques; we were able to evaluate the performance of the protocol. Other comparisons of the minION sequencing technique to other more traditional, and labor and equipment intensive have equally found that the method produces high quality sequences (29).

A common practice is to use only the genetic marker, the partial nucleoprotein sequence, to study the phylogeny of a PPRV isolate, as these 255 nts is what the lineage is based on. This increases the risk of missing important changes in the genome outside of the marker, but these changes could be important in the transmission routes and the virus evolution (10). Using the full genome also enables the use of advanced phylogenies such as

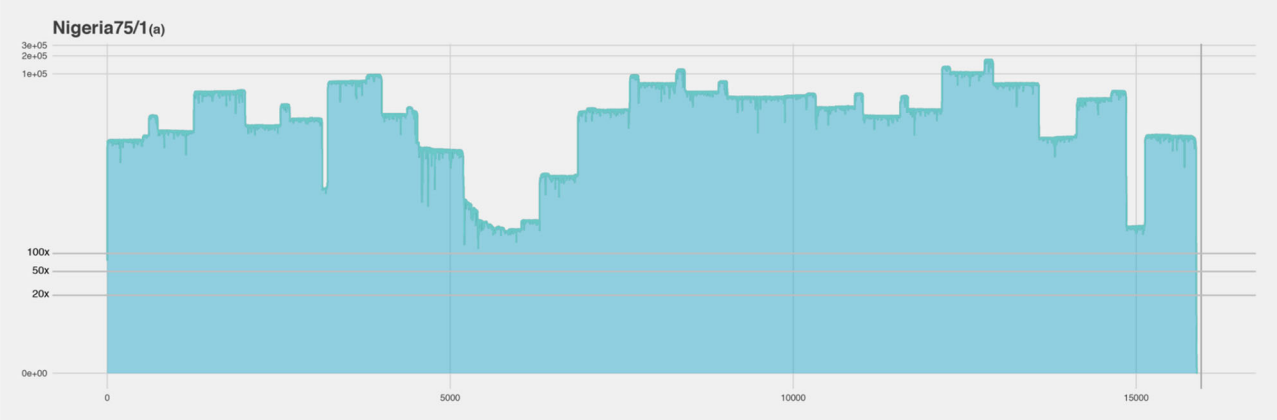

FIGURE 4 | Coverage plot of Nigeria 75/1(a) duplicate. The x-axis represents the length of the genome (15.948 nucleotides). The y-axis represents the sequencing depth on a logarithmic scale. BED files, representing the coverage of the sequence reads against the reference genome, were visualized using R and ggplot.

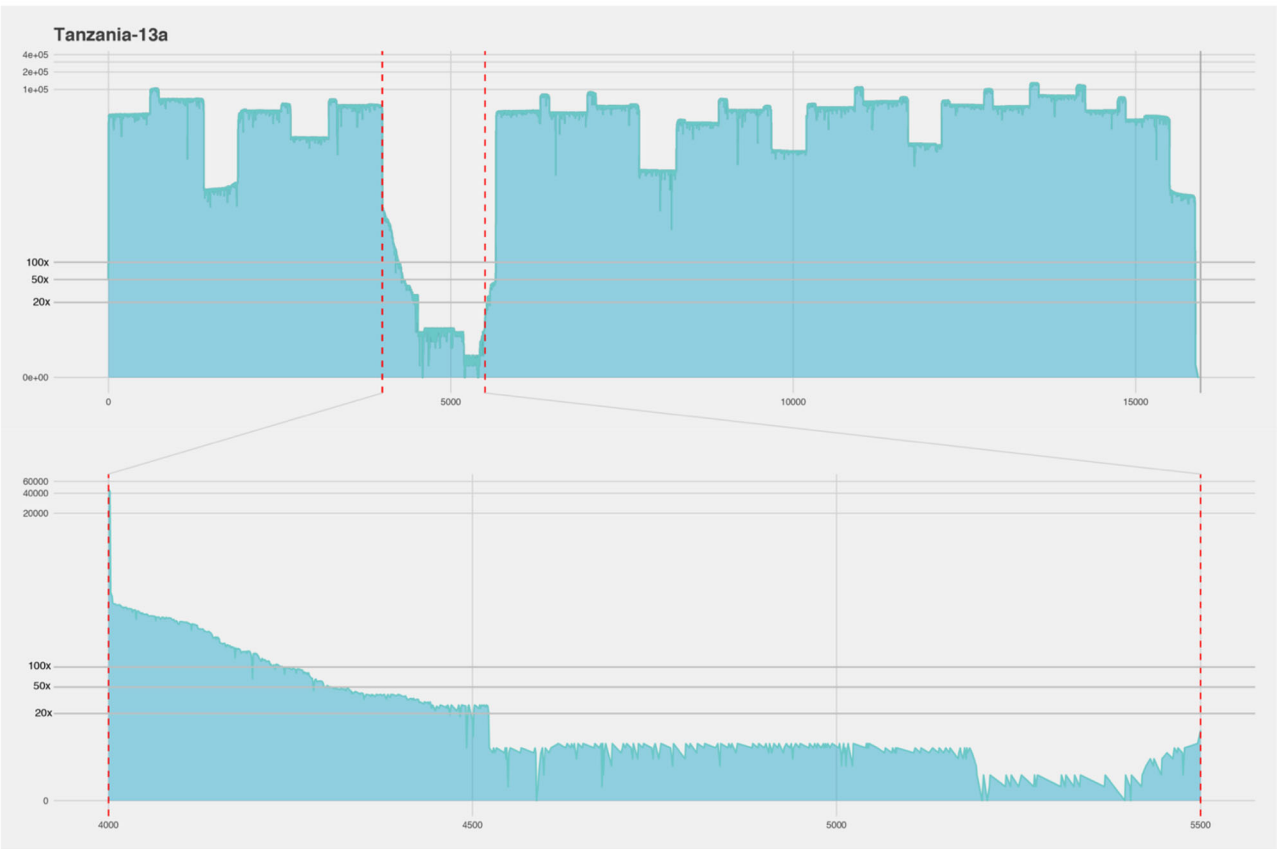

FIGURE 5 | Coverage plot of the Tanzania-13a isolate. The x-axis represents the length of the genome (15.948 nucleotides). The y-axis represents the sequencing depth on a logarithmic scale. BED files, representing the coverage of the sequence reads against the reference genome, were visualized using R and ggplot. A majority of the genome was covered with over 100× sequencing depth, however in the intergenic region between the matrix and the fusion protein genes the sequencing depth falls below ×20 (framed by red dotted lines and showed in detailed in lower half of figure).

those produced by alignments with VIRULIGN (30). The isolates used to verify our protocol are from very different timepoints and geographic regions. If the sequences had belonged to an ongoing outbreak within the same area, this improved resolution of the comparison could help determine the start and transmission route of the outbreak. It would also have made it possible to

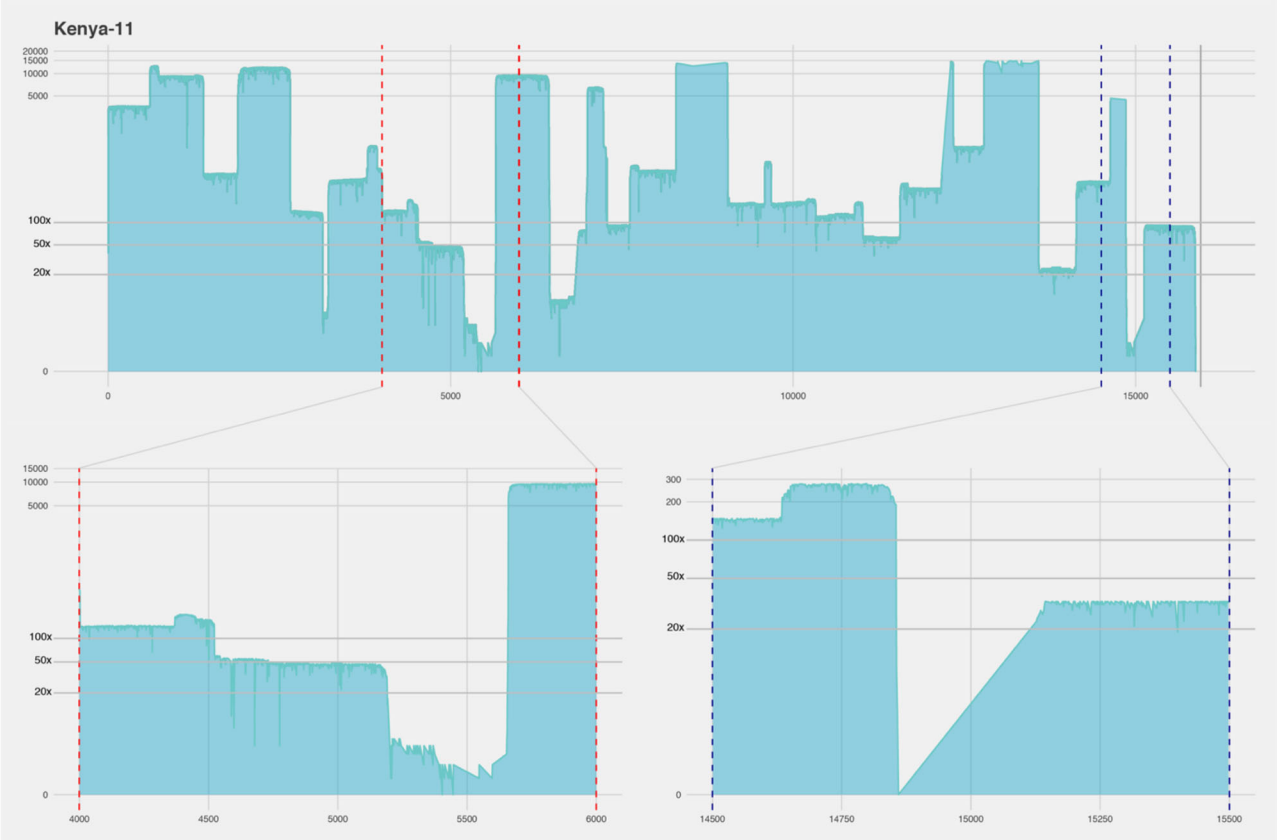

FIGURE 6 | Coverage plot of the Kenya-11 isolate cultured on CV-1 cells. The x-axis represents the length of the genome (15.948 nucleotides). The y-axis represents the sequencing depth on a logarithmic scale. BED files, representing the coverage of the sequence reads against the reference genome, were visualized using R and ggplot. The coverage of this isolate was more uneven, however 85% was covered with ×50 sequencing depth. The lower part of the figure shows a detailed view of two regions with lower coverage, the intergenic region between the matrix and the fusion protein genes (framed by red dotted lines) and a region close to the end of the genome within the large protein gene (framed by blue dotted lines).

track the outbreak in real-time using tools such as Nextstrain (12, 31). For such analyses during outbreaks, the viruses need to be thoroughly sequenced. With our protocol, the production of complete genomes from PPRV field isolates are simplified and will hopefully lead to more full genomes being produced and published.

The use of full genome sequencing for epidemiology and disease surveillance is dependent on the sharing of data and the uploading of the sequences to freely available databases. A genome sequence viewed in isolation can only give limited information (1). Currently, there are 74 complete PPRV genomes available in the NCBI GenBank. Only two are isolated from a wild ruminant: a Dorcas gazelle from a zoological collection in the United Arab Emirates in 1986 (32, 33), and a Capra Ibex in China in 2015 (34). One of the questions in PPR epidemiology is the role of wild ruminants in the spread of the disease. Identified cases in African wildlife are so far considered to be spill-overs from domestic animals, but outbreaks of PPR have occurred several times in Asian wildlife (35). With additional full genome sequences available, this question could possibly be solved.

In conclusion, we have presented a field-adapted, easy to follow, protocol for full genome sequencing of PPRV using the miniPCR thermocycler and the MinION sequencer. With high-quality isolates, the protocol produces a near-complete genome for <USD 100 per sample. We hereby hope to increase the number of complete genomes available for PPRV. More genomes would allow evaluation of the virus evolution and more precise molecular epidemiological investigations. In addition, they would provide a basis for vaccine and drug development (3).

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the European Nucleotide Archive Database under accession number: PRJEB35549.

AUTHOR CONTRIBUTIONS

Conceptualization: ET and OK. Formal analysis: ET, TK, and OK. Writing—original draft preparation: ET. Writing—review and editing: OK, JJ, MB, and ET. Visualization: OK.

Supervision: MB, GM, and JJ. Funding acquisition: ET, JJ, and OK. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by The Royal Swedish Academy of Agriculture and Forestry (Grant No. GFS2018-0165) and Swedish Research Council (Grant Nos. 348-2013-6402, 348-2014-4293, and 2018-03956).

ACKNOWLEDGMENTS

We would like to acknowledge the kind assistance given by Tomas Bergström and Anna Van Der Heiden during this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.542724/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 28 October 2020
doi: 10.3389/fvets.2020.586045

One-Tube Nested Real-Time PCR Assay for Rapid Screening of Porcine Cytomegalovirus in Clinical Samples

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Porcine cytomegalovirus (PCMV) is a pathogen that must be removed from pigs for use as organ donors in xenotransplantation. Recently, it has been found that when donor pigs are infected with PCMV, a pig-to-non-human-primate xenotransplantation lower transplant survival by 2–3 times. Therefore, highly sensitive methods are needed to maintain designated pathogen free (DPF) pig and screen for xenografts. The purpose of this study was to evaluate the performance of commercially available method with one-tube nested real-time PCR assay to quickly detect PCMV infection in clinical samples and compare the results with those of sequence analysis. Molecular diagnostic methods were used to evaluate 127 samples, including tissues and blood samples from pigs suspected of PCMV infection. The detection rate for positive PCMV was 38.6% ($n = 49$), 23.6% ($n = 30$), and 12.6% ($n = 16$) in one-tube nested real-time PCR, nested PCR, and conventional PCR methods, respectively. All PCMV-positive samples in conventional PCR or nested PCR methods were also positive in the one-tube nested real-time PCR assay. All the PCR products in the three methods were checked for amplification of PCMV gene by PCR and subsequent direct sequencing. The results of one-tube nested real-time PCR were found to be consistent with those of sequence analysis for all the samples and showed good agreement ($\kappa = 1$). Our study found that the one-tube nested real-time PCR assay is more sensitive than the other two methods. This assay required approximately 1.5 h for completion. Therefore, we concluded that one-tube nested real-time PCR assay is a fast and reliable method for the characterizing pathogen responsible for PCMV infection.

Keywords: porcine cytomegalovirus, one-tube nested real-time PCR, xenotransplantation, designated pathogen free pig, diagnosis

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *22 July 2020*
Accepted: *30 September 2020*
Published: *28 October 2020*

Citation:
Wang H-y, Song JK, Shin S, Choi KM and Kim H (2020) One-Tube Nested Real-Time PCR Assay for Rapid Screening of Porcine Cytomegalovirus in Clinical Samples. Front. Vet. Sci. 7:586045. doi: 10.3389/fvets.2020.586045

INTRODUCTION

In order to alleviate the shortage of human donor organs available for allograft, xenotransplantation using pig cells, tissues, or organs have been proposed (1–3). This may be related to the transmission of porcine mediated disease, so the maintenance of designated pathogen free pigs is required for xenotransplantation (4–6).

Porcine cytomegalovirus (PCMV), also known as Suid herpesvirus 2 (SuHV2), is an enveloped virus with a double-stranded linear DNA genome. PCMV can cause fever, reduced general condition, loss of appetite, numbness, neurological signs, and respiratory symptoms (e.g., sneezing,

coughing, and dyspnea), acute to subacute disease, and high mortality and morbidity in piglets (3, 7). In a previous studies, the virus have been reported to be immunosuppressive pathogen primarily affecting the immune function of the macrophages and T lymphocytes, which can cause preclinical infection in adult pigs and reproductive failure in pregnant sows (7, 8). PCMV infection is widespread worldwide and has a high prevalence in swine herds. For this reason, many researchers have continued to pay attention to the potential risk to public health in interspecies transmission of PCMV and human xenotransplantation (7). In addition, international xenotransplantation association has a guideline that resource pig for xenotransplantation should be free from PCMV (9). These data indicate that it is essential to quickly detect PCMV infection with high sensitivity and specificity in the early stages of the resource swine herds.

So far, some molecular diagnostic methods, including polymerase chain reaction (PCR) (10–12), enzyme-linked immunosorbent assay (ELISA) (13, 14), loop-mediated isothermal amplification assay (LAMP) (15), and western blot analysis (16) for the detection of PCMV infection has been reported. These assays have low sensitivity, need to agarose gel analysis for amplification products, or have a risk of contamination, which can lead to incorrect results. In addition, companies such as Novateinbio and MyBioSource have ELISA products that detect antibody or antigen of PCMV, there are no commercialized products using molecular diagnostic methods until now. Real-time fluorescent quantitative PCR technology has become a powerful alternative platform for detection and differentiation of pathogenic viruses (3, 17, 18).

In this study, we developed a highly sensitive one-tube nested real-time PCR assay (Opti PCMV-qPCR; Optipharm, Osong, Republic of Korea) that combines nested PCR and real-time PCR to detect PCMV targeting DNA polymerase gene and consists of two sequential reactions in a single tube. The performance of the one-tube nested real-time PCR assay was evaluated using clinical samples suspected of PCMV compared to conventional PCR and nested PCR, and the results were confirmed by sequence analysis.

METHODS

Preparation of DNA Samples

To evaluate the diagnostic performance of the PCR, nested PCR, and one-tube nested real-time PCR methods, a total of 127 field samples, including 37 lung tissues, 30 blood samples, 30 serums, and 30 feces samples, were provided by the Optipharm Animal Disease Diagnostic Center, which was commissioned from January to December 2019. In addition, 10 organs (lung, liver, pancreas, spleen, kidney, brain, heart, small intestine, nasal concha, and tonsil) of six pigs were analyzed to determine the infection rate of PCMV for each organ by one-tube nested real-time PCR assay. According to the manufacturer's recommendation, DNA was extracted from 200 μL of serum or 20 mg of organ tissue homogenate using a commercial automated system (Miracle-AutoXT Automated Nucleic Acid Extraction System, Intronbio, Seongnam, Republic of Korea). To avoid cross-contamination, all samples were individually processed and stored at −20°C. The content and purity of the extracted DNA was analyzed by measuring the absorbance at 260 and 280 nm by a spectrophotometer (Infinite 200 NanoQuant; Tecan, Switzerland).

Conventional PCR and Nested PCR

To evaluate the usefulness of one-tube nested real-time PCR assay, the results of conventional PCR and nested PCR were compared. PCR primers for the conventional PCR and nested PCR used in this study were selected from the nucleotide sequence of the PCMV DNA polymerase gene determined by Hamel (10) and Fryer (11), respectively. PCR was performed using 20 μL of reaction mixture (Genetbio, Daejeon, Korea) containing 2 × master mix, 1 × primer mixture, 3 μL of sample DNA, and ddH_2O added to achieve a final volume of 20 μL. The reaction conditions for conventional PCR and nested PCR using the outer primers (PCMVF1 and PCMVR1) were as follows: pre-denaturation at 94°C for 5 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s; and a final extension at 72°C for 10 min. For nested PCR using the inner primers (PCMVFB and PCMVR2), two microliters of the first-round PCR mixture was transferred to 20 μl of a premixed solution containing the PCR reagents at the same concentrations listed above. The amplification procedure was repeated for 40 cycles with the same time and temperature parameters as described above, except that annealing at 55°C for 30 s was used. The amplified target was visualized as a single band corresponding to a length of 413-bp for conventional PCR and 160-bp for nested PCR using the Chemi Doc system (Vilber Lourmat, Deutschland, Germany).

One-Tube Nested Real-Time PCR Assay

Oligonucleotide primers and probes corresponding to the two strands of the DNA polymerase gene of PCMV (**Figure 1**) were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Primers were prepared as probes corresponding to the complementary strands and used exclusively thereafter. To verify the efficiency of the selected primers and probe, synthesized a positive control DNA sample (Bioneer, Daejeon, Republic of Korea) and amplified with custom PCR primers (forward, 5′-ATGACATTCTTAATCCATATAT-3′ and reverse, 5′-CACTGTCCCTAAAACTACTG-3′) resulting in amplicons of 3,010 bp. The resulting product was mutagenized after subcloning with pBHA vector. Detection of PCMV in clinical samples was performed with Opti PCMV real-time PCR (Optipharm), a quantitative one-tube nested real-time PCR-based assay, using a CFX-96 real-time PCR system (Bio-Rad, Hercules, CA, USA) for thermal cycling and fluorescence detection. Real-time PCR amplification was performed in a total reaction volume of 20 μL containing 10 μL of 2 × Thunderbird probe qPCR mix (Toyobo, Osaka, Japan), 2.5 μL of a mixture of 5 pmol each primers and 5 pmol TaqMan probe that were labeled with fluorophores (FAM-BHQ1), and 3 μL template DNA. The real-time PCR kit consisted of an internal control (IC) DNA and a primer set for IC DNA amplification included in the reaction mixture, which was used to indicate successful nucleic acid extraction, sample quality, and to confirm

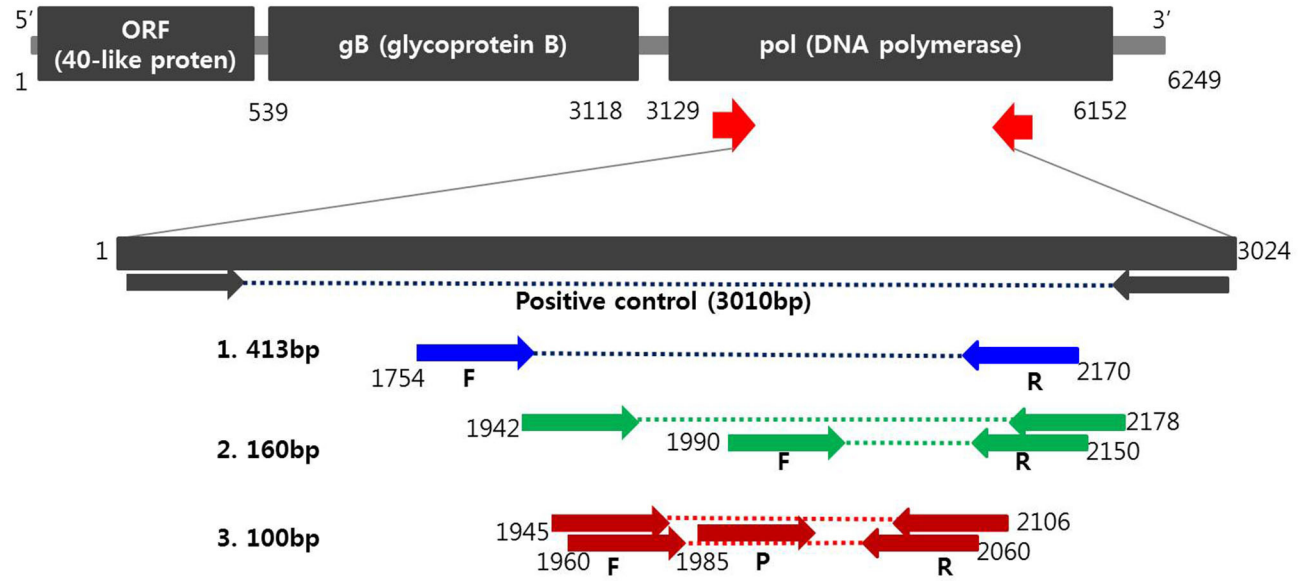

FIGURE 1 | Schematic diagram of the DNA polymerase gene to distinguish PCMV from positions of primers and probes. A positive control was synthesized to verify the efficiency of the developed primers and probes. Primer sequences for conventional PCR and nested PCR were selected from the PCMV DNA polymerase gene determined by Hamel (1) and Fryer (2), respectively. Primers and probe sequences for one-tube nested real-time PCR (3) used in this study were selected at differed locations from the human and mouse cytomegalovirus DNA polymerase gene.

the presence of PCR inhibitors in the reaction. Therefore, it does not compete directly with the amplification of species–specific targets in multiplex real-time PCR. Positive (plasmid PCMV DNA) and negative controls consisting of molecular grade (DNAse/RNAse-free) water (Ultra pure water; Welgene, Gyeongsan, Republic of Korea) without template DNA were included in each assay. The assay was carried out under the following conditions: 95°C for 3 min, then 10 cycles of 3 s at 95°C and 30 s at 60°C, and then by 40 cycles of 3 s at 95°C and 30 s at 55°C. Each sample was tested in duplicate by running the PCR cycle twice and a positive result was obtained when the C_T value was <35.

Interfering Reactions and Reproducibility Analysis

For interfering reactions, we used the following 7 substances by concentration: EDTA and sodium citrate (1, 10, 20, and 50 mM), and heparin (250, 300, 375, and 500 IU) for anticoagulants, phosphate buffered saline (PBS; 1, 5, 10, and 20X) for tissue emulsion, EtOH and xylene (1, 5, 10, 20, and 50%), and blood (1, 5, and 10%). The repeatability and reproducibility of this assay were performed with a total of 240 tests (10 days × 2 runs/day × 4 replicates × 3 lots). The coefficient of variation (CV) was calculated according to the form of the mean C_T values/standard deviation (SD).

Sequence Analysis

To confirm the results of the three molecular diagnostic methods, PCR amplicons of all clinical isolates were sequenced using an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) and the ABI Prism BigDye Terminator (Applied Biosystems) system (CosmoGenetech, Republic of Korea). The primer sets used to amplify the target DNA polymerase gene were 5′-CCTGATCTTAAATGACGAGGACGTGAC-3′ (413F) and 5′-ACCGTCTGAGAGACTGAACTTCTCTGACAC-3′ (413R), 5′-AGGACCCTATGTTGGCAYTGATAC-3′ (1945F) and 5′-TCGTCTGCCTRAGCATGTCC-3′ (2106R), which resulted in a 413-bp and 162-bp PCR product, respectively. The obtained sequence was compared with that of the National Center for Biotechnology Information GenBank database. The primer sequences had been removed from alignment sequences before phylogenetic analysis. Multiple alignments of nucleotide sequences based on the PCR product of the PCMV DNA polymerase gene mentioned above were performed using MUSCLE within the Phylogeny.fr software (19). Then, Gblocks was used as a collection method to align the sequences, assess the phylogenetic relationships by the PhyML using the 49 strains isolated in this study, together with 12 PCMV strains deposited in the GenBank database, and finally visualized the phylogenetic tree by TreeDyn.

RESULTS

Analytical Sensitivity and Specificity of One-Tube Nested Real-Time PCR

The analytical sensitivity of the assay for PCMV detection was determined using a standard curve that 10-fold serially diluted (10^6 copies−1 copy) of plasmid DNA containing cloned PCMV

gene (**Figure 2**). The sensitivity was estimated as the lowest PCMV gene copies yielding a positive result in all 20 replicates, and the corresponding C_T value were selected as the analysis cutoff. A standard curve was generated by plotting the log quantity of PCMV DNA vs. the corresponding C_T value, and the coefficient of determination (R^2) for linear regression was 0.997 with a slope of −4.082. The detection limit of the one-tube nested real-time PCR assay for PCMV was detected at a concentration of 1 copy per reaction. The C_T values of PCMV DNA concentration ranged from 1.2 to 28.5, and mean C_T values were 2.1 ± 2.2 (95% confidence interval [CI], 1.2–3) to 27.1 ± 1.7 (95% CI, 26.4–27.8) and the CV was <3%.

To assess the potential cross-reactivity, analytical specificity was performed with 40 samples with concentrations above 10^4 copies of individual bacterial/viral genes. The one-tube nested real-time PCR assay to detect PCMV-positive showed negative results in all strains except control PCMV. Hence, these primers and probes did not react with any bacterial and viral strains (**Table 1**).

Results of Interfering Reactions and Reproducibility Analysis by One-Tube Nested Real-Time PCR

We performed the interference reaction with 7 substances by concentration. As a result, there was no interference below 50 mM EDTA, 50 mM sodium, 375IU heparin, 10X PBS, 50% EtOH, 50% xylene, and 10% blood (data not shown). For repeatability and reproducibility, the measured number for the 3 concentrations of positive control was 240 (10 days × 2 runs/day × 4 replicates × 3 lots). As shown in **Table 2**, the CV for intra- and inter-assay variability ranged from 0.6 to 2.5% and 1.2 to 2.5%, respectively, which were all <3%. The intra-laboratory reproducibility results at cutoff concentration (about 1 copy) over time were 96.4% (κ = 0.96, 95% CI, 0.926–0.985). Based on the experimental results, we suggest that this assay may have stable results.

Detection of PCMV Using Conventional PCR, Nested PCR, and One-Tube Nested Real-Time PCR Methods in Clinical Samples

To evaluate the performance of one-tube nested real-time PCR assay, a total of 127 clinical samples, including lung tissues (n = 37, 29.1%), whole bloods (n = 30, 23.6%), serums (n = 30, 23.6%), and feces (n = 30, 23.6%), were used. The results were compared with those of conventional PCR (**Figure 3A**) and nested PCR (**Figure 3B**). Of the 127 clinical samples, 49 (38.6%) samples were positive for PCMV, while 78 (61.4%) samples were negative as detected by one-tube nested real-time PCR. On the other hand, 16 (12.6%) and 30 (23.6%) were detected by conventional PCR and nested PCR, respectively (**Table 3**). All clinical samples showed positive IC signals, and the C_T values of the 49 positive and 81 negative samples ranged from 17.6 to 22.5 (mean 20.6, SD ± 1) and 17.7 to 22.5 (mean 20.9, SD ± 0.8), respectively. In addition, the C_T values of PCMV-positive samples ranged from 14 to 28.8 (mean 22.8, SD ± 4.1). In a pilot study, we investigated detection for PCMV infection in 10 organs (lung, liver, pancreas, spleen, kidney, brain, heart, small intestine, nasal concha, and tonsil) of 6 pigs. As a result, the organs with the most prevalent PCMV detected were lung, spleen, and nasal concha (100%), followed by liver, small intestine, and tonsil (83.3%), kidney, heart, and pancreas (66.7%), and brain (50%), respectively (data not shown).

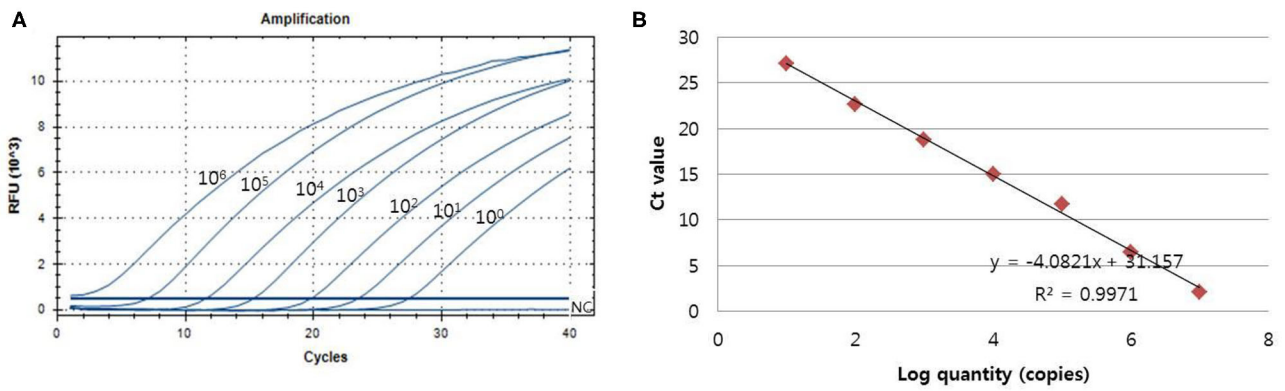

FIGURE 2 | The detection limit of the one-tube nested real-time PCR assay was evaluated using 10-fold serial diluted samples. Serially diluted PCMV control DNA ranging from 10^6 copies to 1 copy per reaction were used to determine the detection limit of the one-tube nested real-time PCR assay. In the one-tube nested real-time PCR assay, the amplification curve of the specific probe **(A)** for detecting PCMV is shown. The overall detection limit of this assay for the PCMV ranged from approximately 1 copy DNA per reaction. C_T was plotted against the input of the quantity of PCMV DNA (repeated 20 times). The linearity **(B)** was generated by plotting the log quantity of PCMV DNA vs. the corresponding C_T value, and the coefficients of determination of the linear regression were 0.997 with a slope of −4.082. The fluorescence intensity is displayed on the Y-axis (R^2 = reporter signal/passive reference signal). RFU, relative fluorescence unit; R^2, fluorescence units.

TABLE 1 | Analytical specificity of the one-tube nested real-time PCR assay to detect PCMV with 40 strains.

No.	Species	Isolate	Sample type	One-tube nested real-time PCR	
				PCMV (Ct)	IC (Ct)
1	*Porcine reproductive and respiratory syndrome virus*	Field isolate	Tissue	N/A	20.58
2	*Porcine reproductive and respiratory syndrome virus*	Field isolate	Tissue	N/A	21.46
3	*Porcine reproductive and respiratory syndrome virus*	Field isolate	Tissue	N/A	20.38
4	*Porcine reproductive and respiratory syndrome virus*	Field isolate	Tissue	N/A	20.54
5	*Swine Influenza virus*	Field isolate	Tissue	N/A	19.72
6	*Hemophilus parasuis*	Field isolate	Tissue	N/A	18.94
7	*Hemophilus parasuis*	Field isolate	Tissue	N/A	20.7
8	*Mycoplasma hyopneumoniae*	Field isolate	Tissue	N/A	22.2
9	*Mycoplasma hyopneumoniae*	Field isolate	Tissue	N/A	20
10	*Mycoplasma hyopneumoniae*	Field isolate	Tissue	N/A	22.28
11	*Porcine rotavirus*	Field isolate	Tissue	N/A	20.66
12	*Porcine rotavirus*	Field isolate	Stool	N/A	20.23
13	*Actinobacillus pleuropneumoniae*	Field isolate	Tissue	N/A	20.31
14	*Porcine epidemic diarrhea virus*	Field isolate	Stool	N/A	21.56
15	*Porcine epidemic diarrhea virus*	Field isolate	Sludge	N/A	20.46
16	*Porcine Parvovirus*	Field isolate	Collagen	N/A	22.21
17	*Porcine Parvovirus*	Field isolate	Collagen	N/A	21.13
18	*Porcine circovirus type 2*	Field isolate	Tissue	N/A	21.07
19	*Porcine circovirus type 2*	Field isolate	Tissue	N/A	21.1
20	*Porcine circovirus type 2*	Field isolate	Tissue	N/A	22.78
21	*Porcine circovirus type 2*	Field isolate	Tissue	N/A	21.19
22	*Porcine circovirus type 3*	Field isolate	Tissue	N/A	22.32
23	*Porcine circovirus type 3*	Field isolate	Tissue	N/A	21.41
24	*Porcine circovirus type 3*	Field isolate	Tissue	N/A	20.11
25	*Porcine circovirus type 3*	Field isolate	Tissue	N/A	20.84
26	*Porcine circovirus type 3*	Field isolate	Tissue	N/A	19.28
27	*Porcine circovirus type 3*	Field isolate	Tissue	N/A	22.45
28	*Escherichia coli*	ATCC 25922	Culture	N/A	21.33
29	*Escherichia coli*	ATCC 35150	Culture	N/A	21.1
30	*Salmonella enteritidis*	ATCC 13076	Culture	N/A	19.36
31	*Salmonella typhi*	ATCC 19430	Culture	N/A	21.13
32	*Salmonella paratyphi*	ATCC BAA-1250	Culture	N/A	20.66
33	*Salmonella Newport*	ATCC 6962	Culture	N/A	20.08
34	*Salmonella typhimurium*	ATCC 14028	Culture	N/A	18.58
35	*Staphylococcus aureus*	ATCC 25923	Culture	N/A	21.18
36	*Staphylococcus aureus*	ATCC 29213	Culture	N/A	21.55
37	*Staphylococcus aureus*	ATCC 6538	Culture	N/A	21.49
38	*Clostridium perfringens*	ATCC 13124	Culture	5.13	21.66
39	*Toxoplasma gondii*	ATCC 50853	Culture	10.74	21.9
40	*Bordetella bronchiseptica*	ATCC 4617	Culture	N/A	20.61
41	*Porcine cytomegalovirus*	PC	Culture0	10.38	20.24
42	*Porcine cytomegalovirus*	PC	Culture	10.74	20.61
43	NC	-	-	N/A	19.8

ATCC, American Type Culture Collection; PCMV, porcine cytomegalovirus.

TABLE 2 | Results of intra- and inter-assay for repeatability and reproducibility analysis.

Copies/$\mu\ell$	*N*	Total			Intra-assay			Inter-assay					
					Within-run			Between-run			Between-day		
		C_T avg	SD	CV(%)	C_T avg	SD	CV(%)	C_T avg	SD	CV(%)	C_T avg	SD	CV(%)
10^5	240	7.7	0.0	0.5	7.7	0.1	0.8	7.7	0.1	1.9	7.7	0.1	1.3
10^3	240	15.6	0.1	0.6	15.6	0.1	0.6	15.7	0.3	1.8	15.6	0.2	1.2
10^1	240	23.8	0.1	0.3	23.9	0.6	2.5	23.8	0.5	2.0	23.6	0.6	2.5

N, test number of inter- and intra- assay; avg, average; SD, standard deviation; CV, coefficients of variation.

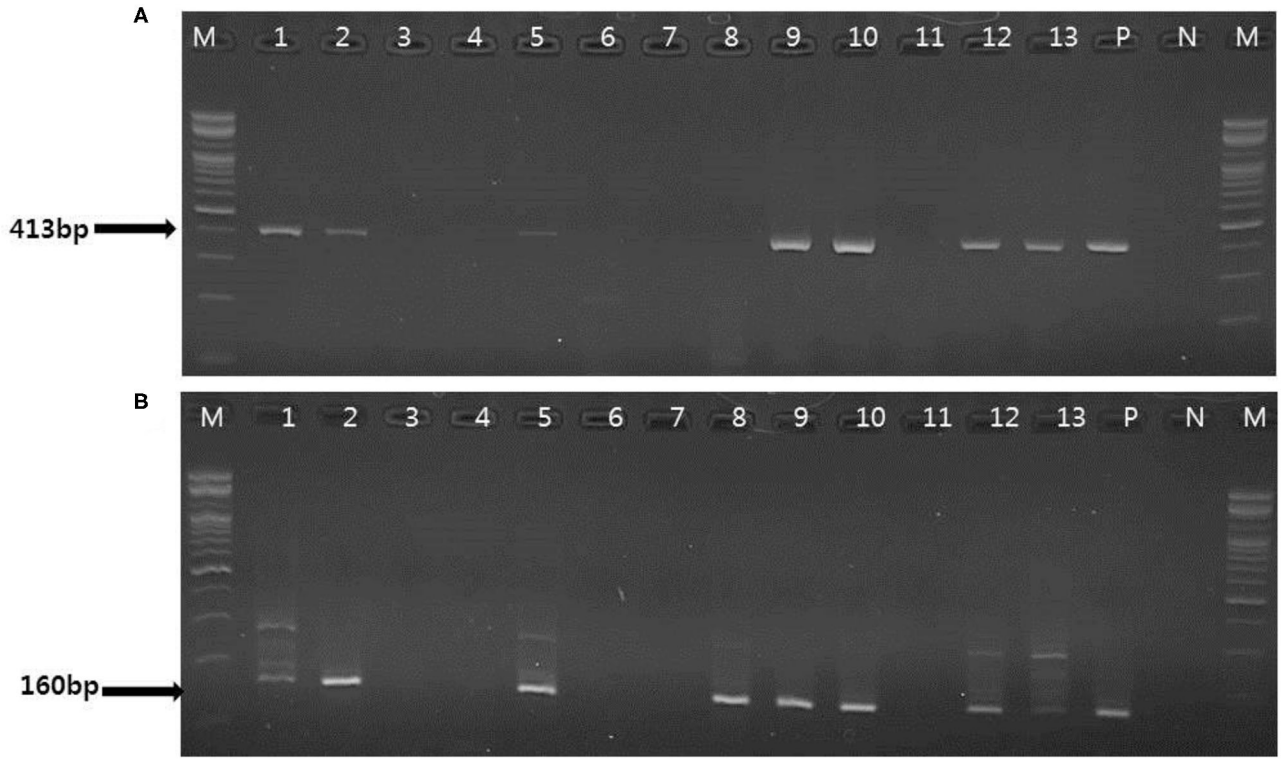

FIGURE 3 | Representative results of the conventional PCR and nested PCR with clinical samples. **(A)** Conventional PCR results. Lanes 1, 2, 5, 9, 10, 12–13: positive; M, 100 bp DNA ladder (Bioneer); P, positive control; N, negative control. **(B)** Nested PCR results. Lane 1, 2 5, 8–10, 12–13: positive; M, 100 bp DNA ladder (Bioneer); P, positive control; N, negative control.

Comparison of the Results Between the One-Tube Nested Real-Time PCR Assay and Sequence Analysis for the Detection of PCMV in Clinical Samples

To confirm the results obtained from the one-tube nested real-time PCR assay, sequence analysis was performed using the same clinical samples. All 49 samples detected as PCMV-positive by one-tube nested PCR assay were consistent with the sequencing results. Our study showed that nested PCR had a higher positive rate than conventional PCR (23.6% vs. 12.6%), but the one-tube nested real-time PCR assay (38.6%) was more sensitive than the other two methods. The agreement rate between one-tube nested real-time PCR assay and conventional PCR or nested PCR was 74% (95% CI 0.654–0.813, $p < 0.001$) and 85% (95% CI 0.776–0.907, $p < 0.001$), respectively (**Table 4**). In addition, the agreement rate of the one-tube nested real-time PCR assay and sequence analysis was 100% (95% CI 0.976–1.000, $p < 0.001$). Using sequence analysis, the sensitivity, specificity, and positive and negative predictive values of PCMV results by one-tube nested real-time PCR assay were 100% ($n = 49$, 95% CI 0.947–1.000, $p < 0.001$), 100% ($n = 78$, 95% CI 0.959–1.000, $p < 0.001$), 100% (95% CI 0.947–1.000, $p < 0.001$), 100% (95% CI 0.959–1.000, $p < 0.001$), respectively. All PCMV-positive samples in conventional PCR or nested PCR methods were also positive in the one-tube nested real-time PCR assay. The

TABLE 3 | Detection of PCMV DNA in 127 clinical samples suspected of PCMV infection using the conventional PCR, nested PCR, and one-tube nested real-time PCR assay.

Sample	Total no. (%) of samples	Detection of PCMV, no. (%) of isolates							
		Conventional PCR		Nested PCR		One-tube nested real-time PCR			
		Positive (%)	Negative (%)	Positive (%)	Negative (%)	Positive (%)	Negative (%)	IC ranged C_T value (mean ± SD)	PCMV ranged C_T value (mean ± SD)
Tissue	37 (29.1)	16 (43.2)	21 (56.8)	25 (67.6)	12 (32.4)	32 (86.5)	5 (13.5)	17.6–22.1 (20.5 ± 0.9)	14–27.3 (21.1 ± 3.9)
Blood	30 (23.6)	0 (0)	30 (100)	1 (3.3)	29 (96.7)	6 (20)	24 (80)	19.3–21.7 (21.1 ± 0.5)	23.1–28.1 (26.6 ± 1.8)
Serum	30 (23.6)	0 (0)	30 (100)	2 (6.7)	28 (93.3)	6 (20)	24 (80)	20.5–22.5 (21.6 ± 0.5)	20–28.5 (21.6 ± 0.5)
Feces	30 (23.6)	0 (0)	30 (100)	2 (6.7)	28 (93.3)	5 (16.7)	25 (83.3)	17.7–21.2 (20.1 ± 0.9)	25.4–28.8 (27.3 ± 1.4)
Total	127 (100)	16 (12.6)	111 (87.4)	30 (23.6)	97 (76.4)	49 (38.6)	78 (61.4)	17.6–22.5 (20.8 ± 0.9)	14–28.8 (22.8 ± 4.1)

PCMV, porcine cytomegalovirus; IC, internal control; SD, standard deviation.

TABLE 4 | Clinical sensitivity and specificity between the one-tube nested real-time PCR assay and conventional PCR, nested PCR, and sequence analysis methods stratified by PCMV suspected samples.

Molecular assays	One-tube nested real-time PCR		Sensitivity, % (*n*) (95% CI)	Specificity, % (*n*) (95% CI)	PPV, % (*n*) (95% CI)	NPV, % (*n*) (95% CI)	Agreement, % (*n*) (95% CI)	κ coefficient (95% CI)
	Positive	Negative						
Conventional PCR								
Positive	16	0	32.7 (16/49) (0.199–0.475)	100 (78/78) (0.959–1.000)	100 (16/16) (0.829–1.000)	70.3 (78/111) (0.608–0.785)	74 (94/127) (0.654–0.813)	0.373 (0.1893–0.5573)
Negative	33	78						
Nested PCR								
Positive	30	0	61.2 (30/49) (0.462–0.748)	100 (78/78) (0.959–1.000)	100 (30/30) (0.905–1.000)	80.4 (78/97) (0.711–0.877)	85 (108/127) (0.776–0.907)	0.659 (0.518–0.800)
Negative	19	78						
Sequence analysis								
Positive	49	0	100 (49/49) (0.947–1.000)	100 (78/78) (0.959–1.000)	100 (49/49) (0.947–1.000)	100 (78/78) (0.959–1.000)	100 (127/127) (0.976–1.000)	1 (0.963–1.000)
Negative	0	78						

PPV, positive predictive value; NPV, negative predictive value; 95% CI, 95% confidence interval.

phylogenetic tree was constructed using Phylogeny.fr software (19) after alignment of the 49 sequenced results. The 49 sequences obtained from direct sequence analysis of clinical samples were found that 69.4% ($n = 34$) in the FJ01 strain (Groups A; accession no. MG696113) groups and 30.6% ($n = 15$) in the B6 strain groups (Groups B; accession no. AF268039) were similar (**Figure 4**).

DISCUSSION

Pigs are frequently infected with PCMV, but infected adult animals do not always show symptoms of disease. Even though the virus remains latent, it can be transmitted the virus to anyone who receives a swine transplantation. Recently, pig-to-non-human-primate xenotransplantation have shown that transplant survival rates are 2–3 time lower when donor pigs were infected with PCMV (4). Therefore, highly sensitive methods are needed to select PCMV-free pigs and to screen for xenografts. The purpose of this study was to evaluate the analytical performance and clinical efficacy of newly developed high-sensitivity one-tube nested real-time PCR assay, taking advantage of conventional PCR and nested PCR for fast and accurate detection based on the DNA polymerase gene of PCMV. One-tube nested real-time PCR is a simple and sensitive method for the detection and identification of PCMV through sequential amplification of the DNA polymerase gene sequence of PCMV in a single tube (20). One-tube nested real-time PCR is about 100 times more sensitive than conventional PCR or nested PCR. Our results are consistent with previous reports indicated that nested PCR improved sensitivity significantly compared to conventional PCR, mostly due to two sequential amplification steps of the target gene (21–23). In addition, one-tube nested real-time

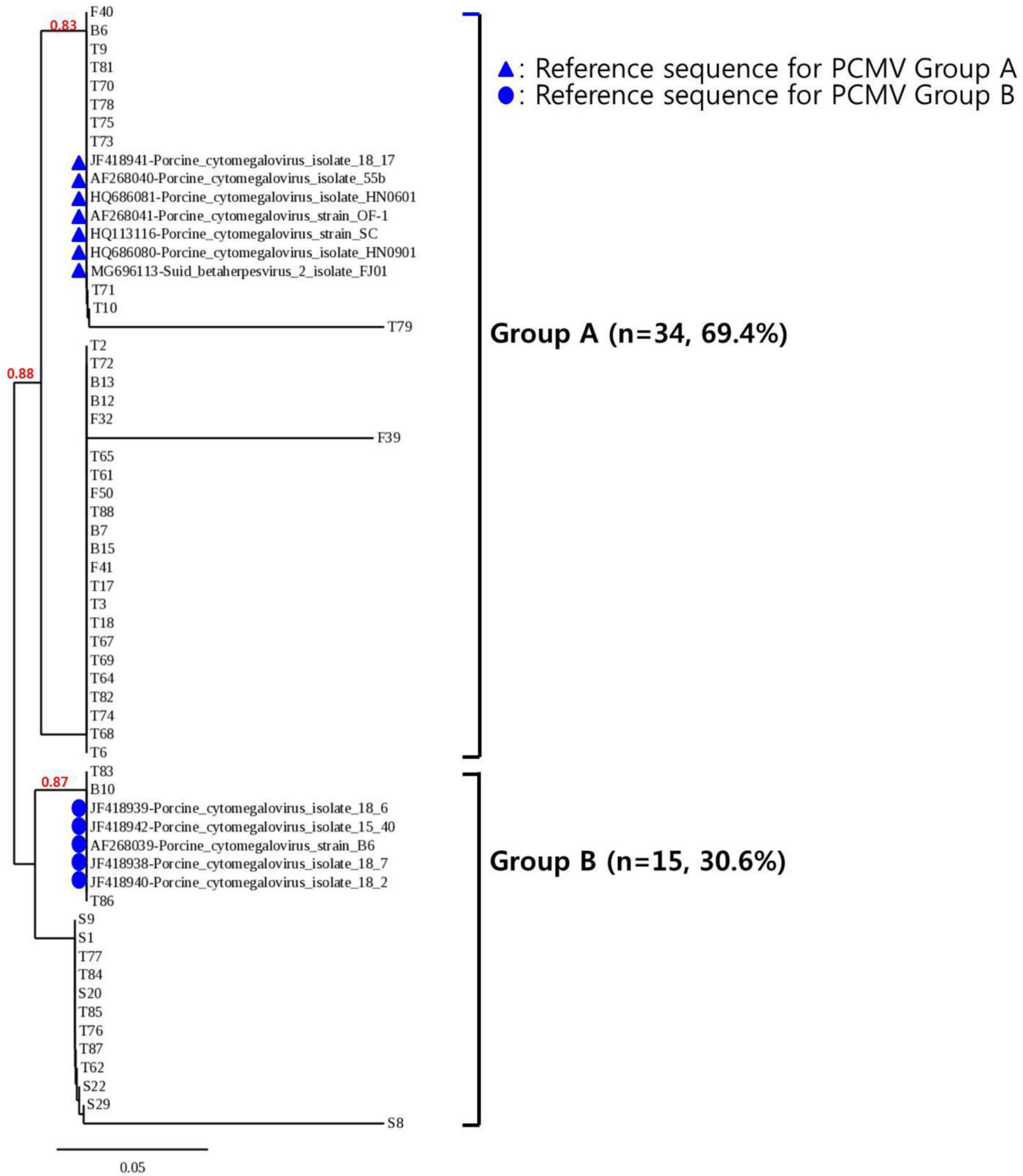

FIGURE 4 | Phylogenetic analysis of 49 PCMV isolates. The phylogenetic tree was constructed using Phylogeny.fr software after the alignment of the 49 sequenced results. The Phylogeny.fr software offers MUSCLE for multiple sequence alignments, Gblocks for alignment curation, PhyML for phylogenetic reconstruction, and TreeDyn for graphical representation of trees. Analysis of phylogenetic tree showed that the sequences obtained from the one-tube nested real-time PCR assay were similar to those of the reference strains FJ01 and B6, with two divided groups (Groups A and B) accounting for 69.4% ($n = 34$) and 30.6% ($n = 15$), respectively.

PCR has the same advantages as conventional real-time PCR, including ease, rapidity (turnaround time of 1.5 h), accuracy, low risk of cross-contamination due to sequential reactions in a single tube, reproducibility, and high-throughput capabilities allows quick screening of multiple samples (24, 25). Moreover, the development of fluorescence-based real-time monitoring allows PCR to be a quantitative method, and PCR amplification or quantification was performed in the same reaction tube to reduce possible errors. Owing to its high sensitivity, one-tube nested real-time PCR has been proposed as an excellent method for determining various diseases (24, 25). In Korea, performance evaluation data such as sensitivity, specificity, reproducibility, and repeatability are required for product approval. To evaluate the performance and accuracy of the one-tube nested real-time PCR assay, the synthesized PCMV plasmid DNA was used as a reference standard, and C_T values were repeatedly measured 20 times. The detection limit of the assay was determined to be 1 copy per reaction. The assay was tested using various strains, no cross-reactivity between strains was observed, and the presence of other samples in PCMV-infected strains was demonstrated to not affect the performance of the assay.

The DNA polymerase gene used in this study was proven to be a highly conserved sequence with no significant variation among several PCMV isolates (3), and showed a difference of 72.7 and 68.9% compared to that of human or mouse DNA polymerase genes, respectively. Nevertheless, the positive rate was high in samples from tissue other than sites. Thus, studies on areas with high PCMV infection in each organ should be conducted to gain further insights into xenotransplantation.

CONCLUSIONS

The one-tube nested real-time PCR assay generally showed high agreement and specificity with sequence analysis. This assay is a fast, accurate, and convenient tool for simultaneously detecting the presence of PCMV infection in many samples. Therefore, using the newly developed molecular diagnostic assay in PCMV screening can help detect the most important disease, while reducing false-positives or false-negatives. It is also likely to be used as a sensitive and specific tool for early detection and diagnosis of PCMV infection.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in the GenBank with accession code AF268039.

AUTHOR CONTRIBUTIONS

H-yW performed evaluation of the experiments, analyzed the data, and drafted the manuscript. JS and SS provided clinical samples and clinical information. KC and HK revised the manuscript. All authors have read and approved the final manuscript.

FUNDING

This research was supported by Revitalization of medical convergence materials industry (Project No: 10065728) from Ministry of Trade, Industry, and Energy and we appreciate this support.

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Conflict of Interest: All authors were employed by Optipharm, Inc.

The authors declare that the research was conducted in the absence of any other commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 28 October 2020
doi: 10.3389/fvets.2020.587885

Serological Cross-Reactivity Between *Bovine alphaherpesvirus 2* and *Bovine alphaherpesvirus 1* in a gB-ELISA: A Case Report in Italy

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 27 July 2020
Accepted: 14 September 2020
Published: 28 October 2020

Citation:
Petrini S, König P, Righi C, Iscaro C, Pierini I, Casciari C, Pellegrini C, Gobbi P, Giammarioli M and De Mia GM (2020) Serological Cross-Reactivity Between Bovine alphaherpesvirus 2 and Bovine alphaherpesvirus 1 in a gB-ELISA: A Case Report in Italy. Front. Vet. Sci. 7:587885. doi: 10.3389/fvets.2020.587885

In this study, we demonstrated for the first time in Italy, the serological cross-reactivity between *Bovine alphaherpesvirus 2* (BoHV-2) and *Bovine alphaherpesvirus 1* (BoHV-1). Five months after arriving at a performance test station in Central Italy, a 6-month-old calf, which was part of a group of 57 animals, tested positive for BoHV-1 in a commercial gB-ELISA test. It was immediately transferred to the quarantine unit and subjected to clinical observation and serological and virological investigations. During this period, the calf showed no clinical signs. The results from laboratory investigations demonstrated the presence of antibodies via competitive glycoprotein B (gB) ELISAs, indirect BoHV-1 ELISAs, and indirect BoHV-2 ELISAs. Furthermore, the plaque reduction assay provided evidence for the presence of antibodies only for BoHV-2, whereas the virus neutralization test showed negative results for both BoHV-1 and BoHV-5. These findings strongly suggest the occurrence of a serological cross-reactivity between BoHV-2 and BoHV-1. Interference of BoHV-2 antibodies in serological BoHV-1 diagnostics should be considered during routine IBR tests, especially when animals are kept in a performance test station.

Keywords: Calf, BoHV-2, BoHV-1, serological cross-reactivity, performance test station

INTRODUCTION

Bovine alphaherpesvirus 2 (BoHV-2) is a member of the family *Herpesviridae* and belongs to the genus *Simplexvirus* (1). The virus was first isolated from a cattle with skin infection on a farm called Allerton in 1957 in South Africa. The aetiological agent is associated with two different clinical forms, a localized skin disease named bovine mammillitis, bovine herpes mammillitis, or bovine ulcerative mammilitis and a generalized disease called Pseudo-Lumpy Skin Disease (PLSD). BoHV-2 infection has been reported in Africa (South Africa, Kenya, Tanzania, Rwanda-Burundi), Europe, the United States, and Australia (2–5). Recently, the virus was isolated from a clinical case of PLSD in northern Italy (6). However, there are very limited data available on the serological evidence of the virus in Italian cattle farms (7). A serological cross-reactivity has been observed

between BoHV-2 and *Bovine alphaherpesvirus 1* (BoHV-1), (5, 8). This phenomenon could lead to severe consequences in BoHV-1 serology, resulting in incorrect diagnosis of BoHV-1, both in areas where there are active control/eradication plans for Infectious Bovine Rhinotracheitis (IBR) and in performance test stations. Moreover, BoHV-2 is similar to BoHV-1 in that it can establish viral latency and be reactivated following an immunosuppressive stimulus, leading to the spread of the virus throughout the herd, causing potential economic losses (9).

In this study, we report, for the first time, the occurrence of serological cross-reactivity between BoHV-2 and BoHV-1 in a calf detained at a performance test station located in Central Italy.

CASE DESCRIPTION

A 6-month-old beef calf (Id. 365/29-04), asymptomatic and seronegative for BoHV-1, was introduced into a performance test station located in Central Italy in October 2018. Following the due protocol for the evaluation of morphological and genetic characteristics, the animal was initially quarantined for 30 days. Two consecutive serum samples were taken 24 days apart. The samples were tested for antibodies against glycoprotein B (gB) of BoHV-1 using a commercial competitive ELISA test (gB-ELISA). They were also tested for neutralizing antibodies against BoHV-1 using virus neutralization (VN) test. The protocol of performance test station does not include investigations against Bovine alphaherpesvirus 2 (BoHV-2). Further, upon testing negative for both the antibodies (gB, VN), the animal was introduced into a group of 56 calves of the same age. These animals were selected from different cattle farms know to be IBR free. Serum and blood samples were taken from all the animals, on a monthly basis, for serological and virological investigations of BoHV-1. The serum samples were tested for the specific antibody via competitive gB-ELISA and VN test. In addition, the EDTA blood samples were used for the detection of BoHV-1 DNA via real-time PCR.

The competitive gB-ELISA test was carried out using the protocol provided by the kit, and the results were expressed according to manufacturer's instructions. VN test and real-time PCR were performed according to the protocols described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (10). All the animals tested negative until February 2019.

In March 2019, the above-mentioned calf (Id. 365/29-04) tested positive in the competitive gB-ELISA test. Although, no clinical IBR symptoms were observed, the animal was immediately placed in quarantine for 30 days. Clinical observations were performed on a daily basis and further serological and virological investigations were carried out. In particular, nasal swabs and EDTA blood samples were collected for virus isolation and real-time PCR, respectively, following the procedures described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (10).

The serum samples were tested for BoHV-1 using different commercial ELISAs: (i) competitive gE-ELISA (A, B, C); (ii) competitive gB-ELISA (D, E), and (iii) indirect-ELISA (F, G, H, I). Additionally, we also performed plaque reduction assay and VN test against BoHV-1.

In order to assess any serological cross-reactivity with other herpesviruses, the serum samples were tested for antibodies against the following aetiological agents: (i) *Bovine alphaherpesvirus 2* (BoHV-2), (ii) *Bovine gammaherpesvirus 4* (BoHV-4), (iii) *Bubaline alphaherpesvirus 1* (BuHV-1), and (iv) *Bovine alphaherpesvirus 5* (BoHV-5). Different indirect ELISA tests were employed to detect BoHV-2 (L), BoHV-4 (M), and BuHV-1 (N). Further, plaque reduction assay and VN test was performed against BoHV-2 and BoHV-5, respectively. The presence of BoHV-2 genome was surveyed via PCR using blood samples.

The ELISA tests were performed following the protocols provided by the kits and the results were expressed according to manufacturer's instructions. Additionally, for the plaque reduction assay BoHV-1 strain Schönböken and BoHV-2 strain RVB 0064 (Biobank, Friedrich-Loeffler-Institut, Insel Riems, Germany) were adjusted to 25–50 plaque forming units (pfu) per 50 μl. Sera were subjected to one freeze-thaw cycle followed by heat inactivation for 30 min at 56°C. Further, 50 μl of 2-fold serially diluted serum was incubated with the test virus for 24 h at 37°C to enable virus neutralization. The serum-virus suspensions were inoculated onto 1-day old Madin-Darby Bovine Kidney cells (1.25×10^5 cells per well). The cells were obtained from the collection of cell lines in veterinary medicine (CCLV, FLI, Insel Riems, Germany), identified by the code MDBK-261. After incubating for 1 h at 37°C, supernatants were removed and replaced with semi-solid overlay medium containing 0.25% methylcellulose (11). Plaque counts were determined 3 days later. Titres were defined as highest dilutions that induced relevant neutralization ($\leq$50% of control values).

The VN test for BoHV-5 was performed on 96-well-tissue culture microtiter plates using the NA67 strain of the virus. Sera were heat-inactivated at 56°C for 30 min. Briefly, 50 μl of each 2-fold serial dilutions were mixed with 50 μl of 100 $TCID_{50}$ of virus in duplicates. The plates were incubated at 37°C and 5% CO_2 for 1 h, and then MDBK cells were seeded at a density of 30,000 cells/well (100 μl). The cells were provided by Biobanking of Veterinary Resources (BVR, Brescia, Italy) and identified by the code BS CL 63. Readings were taken after 72 h, when the cytopathic effect was complete in virus positive control cultures. The titer of each serum was expressed as the highest dilution neutralizing the virus. The BoHV-2 genome was detected using a protocol described by De Giuli et al. (12).

RESULTS

No clinical signs were observed in the calves during the quarantine period. The serological results are shown in **Table 1**. The calf (Id. 365/29-04), tested seropositive in 1 out of 2 competitive gB-ELISAs and in 2 out of 4 indirect-ELISAs. BoHV-2 antibodies were also detected via indirect ELISA. However, no seropositivity was observed in competitive gE-ELISA and indirect BoHV-4 and BuHV-1 ELISAs. Additionally, the plaque reduction assay provided

TABLE 1 | Antibody response obtained from different ELISA tests against BoHV-1, BoHV-2, BoHV-4, and BuHV-1 in the serum sample obtained from a performance station in Central Italy.

ELISA											
BoHV-1									BoHV-2	BoHV-4	BuHV-1
Competitive gE-ELISA			Competitive gB-ELISA		Indirect-ELISA				Indirect-ELISA	Indirect-ELISA	Indirect-ELISA
A	B	C	D	E	F	G	H	I	L	M	N
−	−	−	+	−	+	+	−	−	+	−	−

evidence for a positive result only for BoHV-2, with a mean antibody titer of 1:384, while the VN assay showed no evidence for BoHV-1 and BoHV-5. The virological investigations were consistently negative.

DISCUSSION

In this study, we reported a case of serological cross-reactivity between BoHV-2 and BoHV-1 in a calf detained in a performance test station in Central Italy. BoHV-2 infections have also been described in Africa, Europe, the United States, and Australia (2–4). Several European countries have reported unexplained cases of gB-positive singleton reactors and they were found to be gE-negative (5, 8, 13, 14).

In this report, we have shown that 1 out of 2 commercial competitive gB-ELISAs gave a positive result which was not confirmed by BoHV-1 plaque reduction assay, VN, or competitive gE-ELISA tests. These serological results were inconsistent with immune responses usually developed by a BoHV-1 infected animal (15–17). Antibodies against glycoprotein B of BoHV-1 or neutralizing antibodies appear after 7–14 days post-infection, increase at constant levels, and persist for long periods. In contrast, antibodies against glycoprotein E (gE) appear 30–35 days post-infection and also persist for long periods (18, 19). However, Mars et al., reported that non BoHV-1 related gB-singleton reactors were found to be negative in the gE-ELISA test. Our study showed that, the calf detained at the performance station tested negative for all the three gE-ELISA tests. This was in concordance with the findings of previous studies (5, 8, 13). Increase in gE-reactivity was not detected over a period of 3 months. Seroconversion for gE would be expected in unvaccinated animals within this timespan.

The results obtained in this study could be attributed to non-specific reactivity, as indicated by Beer et al., such as batch variation between ELISA kits, sample quality, or the use of fresh serum (20). However, all of these factors have been taken into consideration in this study. Furthermore, different studies have shown that the seropositivity of some animals in competitive gB-ELISA could be attributed to serological cross-reactivity with other ruminant alphaherpesviruses (5, 8). This antigenic relationship has been demonstrated using different diagnostic tests (5, 21). In particular, the epitopes responsible for the cross-neutralization are located in the major glycoprotein gB, gC, and gD (22). The gB gene is the most conserved among the major herpesvirus glycoproteins (23, 24). In this context, we investigated potential cross-reactivity of BoHV-1 with the following viruses: *Bovine alphaherpesvirus 2* (BoHV-2), *Bovine gammaherpesvirus 4* (BoHV-4), *Bovine alphaherpesvirus 5* (BoHV-5), and *Bubaline alphaherpesvirus 1* (BuHV-1).

Our results demonstrated that indirect-ELISA detected antibodies against BoHV-2 and this was subsequently confirmed via plaque reduction assay and BoHV-2 neutralization assay. The sanitary protocol of the experimental station, does not efficiently control BoHV-2 infection. Thus, the calf was not serologically checked for this viral infection while entering the experimental station.

It is well-known that reactivation is typical of herpesviruses and generally occurs after an immunosuppressive stimulus (25) or after dexamethasone treatment (9). We speculated that the serological cross-reactivity detected 5 months after arriving resulted from the latency state in a calf passing the first infection, rather than a subclinical primary infection. This hypothesis is also supported by the fact that if a primary infection had occurred after the entrance of the calf into the performance station, other animals had to show clinical signs, and then seroconverted against BoHV-2 as well, consequently some more animals might have been identified by gB-ELISA IBR tests. In addition, BoHV-2 spread might not be efficient in this herd (insect control, no role of milking cluster). Additionally, the performance station benefits from a very high biosecurity level, as it is located in an isolated area and is accessed only by personnel dedicated to the activities of the station. Therefore, an accidental entry of wild-type virus is most unlikely.

Furthermore, as the performance station is equipped with traps for biting flies, BoHV-2 transmission by flies may be excluded. This leads us to conclude that a latent BoHV-2 virus might have been reactivated in the calf as a consequence of an immunosuppressive stimulus, possibly when the animal underwent a change of diet or after its introduction into the performance station group. Unfortunately, as required by performance station regulations, the other animals, all asymptomatic and seronegative to BoHV-1 tests, were separated and sold during the study period. Thus, it was not possible to conduct further investigations on the cohabiting calves. However, the seropositivity ascertained in the calf cannot be attributed to vaccination because (i) the health regulations to regarding access a performance station ban the introduction of animals vaccinated against IBR and (ii) there are no commercially available vaccines against BoHV-2. Moreover, according to their regulations, animals entering a performance station are selected from IBR free herds, for which the practice of vaccination is prohibited. The detection of singleton reactors is crucial for the selection of animals in a performance test station, where animals can be introduced only if antibody negative. Additionally, each animal is checked every month to verify that no latent viral infections are reactivated.

Furthermore, in the context of IBR eradication programs, it is important to accurately identify singleton reactors. As an example, in Italy, where an active plan for the eradication

of BoHV-1 in beef cattle breeds is in place (26), 20 gB singleton reactors were evidenced in different regions, during the 2018–2019 campaign (data not shown).

CONCLUSIONS

In conclusion, the present study highlights latent reactivation of BoHV-2 in a calf, which confirmed serological cross-reactivity with different commercial BoHV-1 ELISA tests. This should be carefully taken into consideration, when uncertain interpretation of IBR serology occurs, especially in performance test stations, where accidental contact to vaccine virus or wild type BoHV-1 infection can be reliably ruled out. In fact, animals erroneously considered as positive for BoHV-1, could be eliminated needlessly, which concomitantly means losing an animal of high genetic and economic value. In addition, the cross-serological reactivity may have an economic and social impact on control and eradication programs (trade restrictions, loss of negative status, decline in acceptance).

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/supplementary material.

ETHICS STATEMENT

In this study, all data analyzed were collected as a part of the routine diagnosis, therefore, according to the national legislation, ethics approval, and written informed consent are not required.

AUTHOR CONTRIBUTIONS

Experimental conception and design were done by SP. Collection of samples was done by CR. Immunological analyses were done by CP, CC, PG, and IP. Analysis, interpretation was done by SP, PK, CR, CI, and MG. Paper writing and editing were done by SP, PK, and GD. All authors read and approved the final manuscript.

FUNDING

This study was financially supported by internal funds of the Istituto Zooprofilattico Sperimentale Umbria-Marche.

ACKNOWLEDGMENTS

The authors are grateful to Prof. J. P. Teifke from Friedrich-Loeffler-Institut, Insel Riems, Germany, for providing the NA67 strain of BoHV-5. A special thanks to Prof. Fernando A. Osorio, School of Veterinary Medicine & Biomedical Sciences, University of Nebraska-Lincoln (USA) for providing a critical review of this manuscript and to Prof. Gigliola Canepa, University of Milan (I), for revising the language of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 November 2020
doi: 10.3389/fvets.2020.588173

Establishment and Application of Multiplex PCR for Simultaneously Detecting *Escherichia coli*, *Salmonella*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* in Minks

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 28 July 2020
Accepted: 22 October 2020
Published: 17 November 2020

Citation:
Li P, Zhang D, Li H, Pang J, Guo H and Qiu J (2020) Establishment and Application of Multiplex PCR for Simultaneously Detecting Escherichia coli, Salmonella, Klebsiella pneumoniae, and Staphylococcus aureus in Minks. Front. Vet. Sci. 7:588173. doi: 10.3389/fvets.2020.588173

To establish a multiplex PCR for simultaneous detection of *Escherichia coli* (*E. coli*), *Salmonella*, *Klebsiella pneumoniae* (*K. pneumoniae*), and *Staphylococcus aureus* (*S. aureus*), four pairs of specific primers were designed according to the conservative regions of *phoA* gene for *E. coli*, *invA* gene for *Salmonella*, *khe* gene for *K. pneumoniae*, *nuc* gene for *S. aureus*. The quadruple PCR system was established through optimization of multiplex PCR and detection of specificity, sensitivity, and stability. The results showed that target gene bands of *E. coli* (622 bp), *Salmonella* (801 bp), *K. pneumoniae* (303 bp), and *S. aureus* (464 bp) could be amplified by this method specifically and simultaneously from the same sample containing the four pathogens, with a detection sensitivity of 100 pg/μL. Meanwhile, no bands of common clinical bacteria, including *Clostridium perfringens*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Streptococcus pneumoniae*, *Streptococcus pneumoniae*, *Proteus mirabilis*, *Staphylococcus sciuri*, *Staphylococcus pseudintermedius*, *Acinetobacter baumannii*, *Enterococcus faecalis,* and *Bacillus subtilis* were amplified. In addition, 380 tissue samples were detected by multiplex and single PCR established in current study, respectively. Among the 368 carcass samples, positive detection rates of *E. coli, K. pneumoniae, Salmonella,* and *S. aureus* were 33.7, 12.0, 10.6, and 13.9%. Among the 12 visceral tissue samples, positive detection rates of *E. coli, K. pneumoniae, Salmonella,* and *S. aureus* were 41.7, 25.0, 16.7, and 8.3%, respectively. Positive detection rates of multiplex PCR were consistent with that of single PCR. Compared with single PCR, the multiplex PCR method had the advantages of time-saving, high specificity and high sensitivity. The results showed that the minks in these farms had mixed infection of these four pathogens, and the method established in this study could be applied to the rapid and accurate detection and identification of these four bacteria. In conclusion, the multiplex PCR method has stable detection results, good repeatability, and short

detection time. It is suitable for the rapid and accurate detection of four kinds of bacteria above the carcass of fur animals, which could be suitable in microbial epidemiology investigation. It can provide a reliable technical reference for rapid clinical diagnosis and detection.

Keywords: minks, *E. coli*, *Salmonella*, *K. pneumoniae*, *S. aureus*, multiplex PCR detection

INTRODUCTION

As a valuable economic fur animal, the domestic breeding of minks has been paid more and more attention. The scale of mink breeding in the world in 2014 is 100 million, while that in China is close to 80 million (1). And Shandong province is the largest province for mink breeding and skin production. The mink skins production in Shandong corresponded to 70% of all skins produced in China in 2016 (2). Although in recent years, due to the influence of foreign market demand, the breeding scale has declined, but mink breeding is still an important pillar industry in Shandong Province. However, a range of pathogenic microbes are causing a wide variety of infectious diseases in Shandong (3–8). Some of which may even have the risk of infecting people, and it would be a threat to mink breeding and public health. At the same time, bacterial disease is one of the main causes of mink death.

Escherichia coli (*E. coli*), *Salmonella*, *Klebsiella pneumoniae* (*K. pneumoniae*), and *Staphylococcus aureus* (*S. aureus*) are the most common pathogens or opportunistic pathogens in minks. They can infect not only minks, but also human, and other animals (9–12). In recent years bacterial diseases have been not only frequent, but also in the state of mixed infection or secondary infection in mink farms of Shandong (13, 14). Therefore, it is necessary to give timely and rapid diagnosis and treatment of multiple infection in mink culture.

The traditional method of bacterial pathogens identification mostly uses bacterial isolation and culture combined with biochemical characteristics detection and analysis. Although the traditional methods are reliable, there are still some shortcomings that affect their application, such as strong specialization and high requirements for operators, high risk, time-consuming, and expensive.

Modern molecular biology technology, such as polymerase chain reaction (PCR) combined with gel electrophoresis, is increasingly used in bacteria isolation and identification, which improves the detection efficiency. Compared with single PCR, multiplex PCR has higher detection efficiency, which can not only detect a variety of pathogens at the same time, but also reduce the cost and save the time. It can be seen that multiplex PCR detection is of great significance for the rapid diagnosis and detection of mixed infection (15–17). However, the research on the simultaneous detection of *E. coli*, *Salmonella*, *K. Pneumoniae,* and *S. aureus* in minks by quadruple PCR in Shandong has not been reported, and there is still a lack of relevant research data.

Therefore, the current study was conducted to design primers according to the published gene sequences of these four mink bacteria, and then to establish a multiplex PCR method for simultaneous detection of four bacteria. So as to provide a reference for the rapid identification of these four common or opportunistic pathogenic bacteria.

MATERIALS AND METHODS

Source of Bacterial Strains

Reference strains of *E. coli* (ATCC8739), *Salmonella* (ATCC13076), *K. pneumoniae* (CMCC46117), and *S. aureus* (ATCC6538) used in this study were all purchased from China microbial strain network. *Clostridium perfringens, Pseudomonas aeruginosa, Pasteurella multocida, Streptococcus pneumoniae, Streptococcus pneumoniae*, *Proteus mirabilis*, *Staphylococcus sciuri*, *Staphylococcus pseudintermedius*, *Acinetobacter baumannii*, *Enterococcus faecalis,* and *Bacillus subtilis* positive strains were all pathogenic bacteria isolated from minks, which were isolated, identified and preserved by the laboratory of College of Animal Science and Technology, Shandong Agricultural University.

Sampling and Isolation

During January 2017 to October 2018, 380 samples, including 368 carcass samples (from right hind leg) and 12 visceral tissue samples from diseased minks, were collected from 35 mink farms in the main mink-producing areas of Shandong Province, China, including Zhucheng, Wendeng, Liaocheng, Rizhao, Haiyang, and Dongping. Among the 12 diseased mink tissue samples, seven minks had obvious clinical symptoms of respiratory diseases, and the remaining five minks had clinical symptoms of digestive tract diseases. These samples were used for bacterial isolation by traditional clinical microbilologic methods, and then the established multiplex PCR method and the single PCR method were used to detect these bacterial isolation, and the multiplex PCR results were compared with the single PCR method to detect the specificity of multiplex PCR method.

Primers

The target genes chosen for their specificity were the *phoA* gene in *E. coli*, *invA* gene in *Salmonella, khe* gene in *K. pneumoniae* and *nuc* gene in *S. aureus* (6, 15–21). Four pairs of specific primers were designed by Premier 5.0 and Oligo 6.0 along with NCBI primer-BLAST comparison. All the primers used in current study were synthesized by Shanghai Sangon Biotech Co., Ltd. The primers and the respective amplification lengths are shown in **Table 1**.

DNA Extraction

The reference strains and the positive isolates identified by traditional clinical microbiologic methods were inoculated on 5 mL Tryptic soy broth (TSB), and then cultured by shaking at 37°C for 18∼24 h. Bacterial genomic DNA was extracted from 1 mL bacterial solution with a TIANamp Bacterial DNA Kit (Tiangen, Beijing, China) according to the Kit instructions. The extracted bacterial DNA was detected

TABLE 1 | Primers used in multiplex PCR.

Bacteria	Primer sequences (5′-3′)	Target genes	Gene ID	PCR product size (bp)
K. pneumoniae	F-CGATGCTACTTATCCCGACA R-ACCACCAGCAGACGAACTT	*khe*	KX842080.1	303
S. aureus	F-AGGCATGGCTATCAGTAATGTTTC R-CATCAGCATAAATATACGCTAAGCCAC	*nuc*	DQ507382.1	464
E. coli	F-TACAGGTGACTGCGGGCTTATC R-CTTACCGGGCAATACACTCACTA	*phoA*	FJ546461.1	622
Salmonella	F-AAAAGAAGGGTCGTCGTTAG R-GGAAGGTACTGCCAGAGGTC	*invA*	MK017941.1	801

by nucleic acid analyzer and diluted to 10 ng/μL for subsequent test.

Genomic DNA of carcass samples and diseased mink visceral tissue samples were extracted using TIANamp Blood/Cell/Tissue DNA Kit (Tiangen, Beijing, China) according to the Kit instructions. The extracted DNA was stored at −20°C.

Control, Optimization, and Establishment of Multiplex PCR Conditions

Firstly, the genomic DNA of four standard positive strains was used as a template to screen the optimal annealing temperature by single PCR reaction. Ten annealing temperature gradients of 52, 52.9, 53.8, 54.9, 56, 57, 58.1, 59.2, 60.1, and 61°C were set to determine the optimal annealing temperature. Next, multiplex PCR was performed using the same volume mixture of genomic DNA of four standard strains as template. Thus, the concentration of primers and annealing temperature were optimized to determine the optimal multiplex PCR reaction conditions.

The results of the preliminary test showed the multiplex PCR reaction were carried out in 25 μL reaction mixtures containing 12.5 μL of 2×Es Taq MasterMix, 1 μL for each of the four bacterial DNA templates, 1 μL for each of the four pairs of primers with the best concentration ratio. Among which Taq MasterMix was composed of Es Taq DNA Polymerase (amplification efficiency: 2 kb/min), $MgCl_2$ (3 mM/L), dNTP(400 μM/L), PCR stabilizer, and enhancer. Finally, the volume of the reaction mixtures was filled up to 25 μL with sterilized double distilled water. The amplification conditions consisted of an initial denaturation at 94°C for 7 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 52 61°C for 30 s, extension at 72°C for 30 s, and final extension for 5 min at 72°C. On the basis of four optimum concentration ratio primers, 10 annealing temperature gradients of 52, 52.9, 53.8, 54.9, 56, 57, 58.1, 59.2, 60.1, and 61°C were also set to select the annealing temperature and optimize the reaction conditions.

Sensitivity Test of Multiplex PCR

The DNA template was serially diluted to 10 ng/μL, 1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, 100 fg/μL by 10 fold gradient with sterile double distilled water, then amplified by the optimized single PCR reaction system. Subsequently, the DNA template was serially diluted to 100 ng/μL, 10 ng/μL, 1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL by 10 fold gradient with sterile double distilled water, then amplified by the optimized multiplex PCR reaction system. Finally, the PCR products were electrophoresis to detect the sensitivity of each primer.

Specificity Test of Multiplex PCR

The mixed DNA or single DNA of four standard strains and the DNA of common bacteria samples in clinic, such as *Clostridium perfringens*, *Pasteurella multocida, Pseudomonas aeruginosa, Streptococcus pneumoniae*, *Streptococcus pneumoniae*, *Proteus mirabilis*, *Staphylococcus sciuri*, *Staphylococcus pseudintermedius*, *Acinetobacter baumannii*, *Enterococcus faecalis,* and *Bacillus subtilis* were used as templates in the optimized reaction system for multiplex PCR amplification to detect the specificity of primers.

Stability Test of the Multiplex PCR

In order to evaluate the stability of the multiplex PCR system, templates of the positive and negative samples, respectively, or mixed were added into reaction mixture, and then were amplified by the optimized multiplex PCR system. The positive control was four bacteria DNA (*E. coli, Salmonella*, *K. Pneumoniae,* and *S. aureus*), which were identified as positive by biochemical detection and 16S rRNA sequencing. The negative control was sterilized double distilled water. The stability test was repeated three times. In addition, the detection effect of multiplex PCR system was evaluated by comparing with single PCR.

RESULT

Establishment of Multiplex PCR Conditions

Single PCR test results of the each reference bacteria DNA showed that, the specific gene amplification products of the four bacteria DNA were obtained, which were 622 bp for *E. coli*, 801 bp for *Salmonella*, 303 bp for *K. Pneumoniae,* and 464 bp for *S. aureus,* respectively. The results of multiplex PCR combined with single PCR showed that when the annealing temperature was 56°C, the amplified bands of four target genes of *E. coli, Salmonella*, *K. Pneumoniae,* and *S. aureus* were uniform, concentrated and highly specific (**Figures 1**, **2**). The subsequent tests were carried out at 56°C.

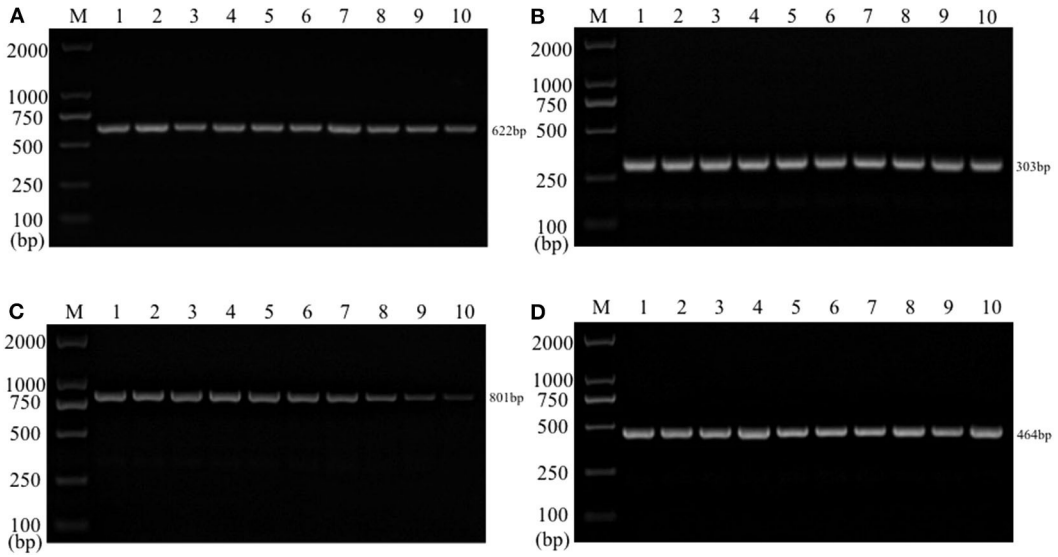

FIGURE 1 | Single PCR amplification at different annealing temperature. Lane M, D2000 DNA marker; Lane 1, 52.0°C; Lane 2, 52.9°C; Lane 3, 53.8°C; Lane 4, 54.9°C; Lane 5, 56.0°C; Lane 6, 57.0°C; Lane 7, 58.1°C; Lane 8, 59.2°C; Lane 9, 60.1°C; Lane 10, 61.0°C. **(A)** *E. coli*; **(B)** *K. pneumoniae*; **(C)** *Salmonella*; **(D)** *S. aureus*.

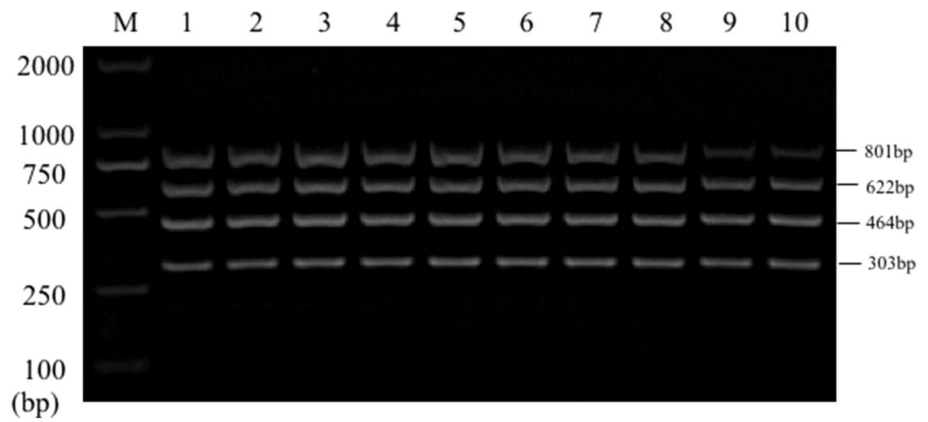

FIGURE 2 | Multiplex PCR amplification at different annealing temperature. Lane M, D2000 DNA marker; Lane 1, 52.0°C; Lane 2, 52.9°C; Lane 3, 53.8°C; Lane 4, 54.9°C; Lane 5, 56.0°C; Lane 6, 57.0°C; Lane 7, 58.1°C; Lane 8, 59.2°C; Lane 9, 60.1°C; Lane 10, 61.0°C.

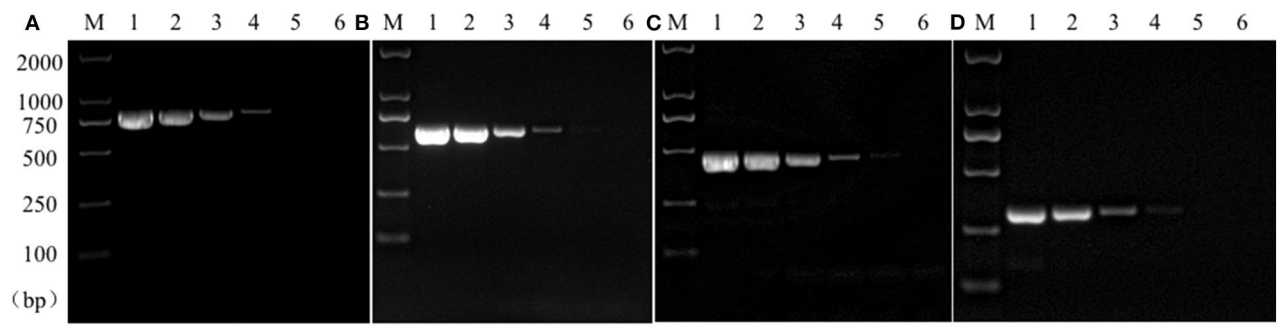

FIGURE 3 | Results of single PCR assay sensitivity experiment. Lane M, D2000 DNA marker; Lane 1∼6: The concentration was 10 ng/μL, 1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL, and 100 fg/μL, respectively; **(A)** *Salmonella*; **(B)** *E. coli*; **(C)** *S. aureus*; **(D)** *K. pneumoniae*.

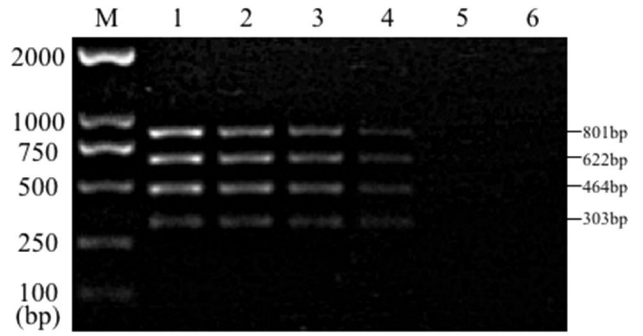

FIGURE 4 | Results of multiplex PCR assay sensitivity experiment. Lane M, D2000 DNA marker; Lane 1, 100 ng of DNA for each of *Salmonella, E. coli, S. aureus,* and *K. pneumoniae*; Lane 2, 10 ng; Lane 3, 1 ng; Lane 4, 100 pg; Lane 5, 10 pg; Lane 6, 1 pg.

Results of Multiplex PCR Sensitivity Experiment

Sensitivity test results of the reference bacteria DNA demonstrated that, the single PCR assay was capable of properly identifying the presence of bacteria at the following lowest concentration, 1.0 pg/μL for *S. aureus*, 10.0 pg/μL for *E. coli*, Salmonella, and *K. pneumoniae* (**Figure 3**). The multiplex PCR assay could properly identify the presence of bacteria at 100 pg/μL of DNA template for *S. aureus*, *E. coli*, *Salmonella,* and *K. pneumoniae* (**Figure 4**).

Results of Multiplex PCR Specificity Experiment

PCR specificity test results of bacteria DNA indicated that, the multiplex PCR assay could be capable to effectively identify the mixed DNA and the single DNA samples. Conversely, no bands of common clinical bacteria, including *Clostridium perfringens*, *P. aeruginosa*, *Pasteurella multocida*, *Streptococcus pneumoniae*, *Streptococcus pneumoniae*, *Proteus mirabilis*, *Staphylococcus sciuri*, *Staphylococcus pseudintermedius*, *Acinetobacter baumannii*, *Enterococcus faecalis,* and *Bacillus subtilis* were amplified by multiplex PCR (**Figure 5**). However, no amplification was achieved from control groups of other bacteria. The results suggested that the established multiplex PCR method showed good specificity.

Results of Multiplex PCR Stability Experiment

The stability test results displayed that the specific target bands were found in all the positive samples, while no amplification products were found in the negative samples (**Figure 6**). The above results were in line with our expectations and the experimental requirements. Together these results suggested that the established multiplex PCR method had good stability and repeatability.

Detection and Identification Results of Clinical Samples

Three hundred eighty samples were detected by multiplex and single PCR method established in current study. And the results were shown at **Tables 2**, **3**. The results of the **Table 2** demonstrated that among the 368 carcass samples, positive detection rates of *E. coli, K. pneumoniae, Salmonella,* and *S. aureus* were 33.7% (124/368), 12.0% (44/368), 10.6% (39/368), and 13.9% (51/368), respectively. Among the 12 visceral tissue samples, positive detection rates of *E. coli, K. pneumoniae, Salmonella,* and *S. aureus* were 41.7% (5/12), 25.0% (3/12), 16.7% (2/12), and 8.3% (1/12), respectively. Positive detection rates of multiplex PCR were consistent with that of single PCR (**Table 2**). At the same time, the results also showed that there were four kinds of bacteria contamination in mink carcasses.

Co-contamination detection results of bacteria in 380 clinical tissue samples by multiplex PCR were shown at **Table 3**. From the results of **Table 3**, it could be seen that single and multiple contamination were present in these samples. Among the 368 carcass samples, positive detection rates of dual contamination for *E. coli, K. pneumoniae*, *E. coli,* and *Salmonella* were 6.0% (22/368) and 4.6% (17/368), respectively. Positive detection rates of dual contamination for *E. coli, S. aureus, Salmonella,* and *S. aureus* were 1.4% (5/368). Positive detection rate of triple contamination for *E. coli*, *K. pneumoniae,* and *Salmonella* was 1.4% (5/368). Positive detection rate of triple contamination for *E. coli*, *Salmonella,* and *S. aureus* was 0.8% (3/368). Positive detection rate of quadruple contamination was 0.8% (3/368). Among the 12 visceral tissue samples, positive detection rates of dual contamination for *E. coli, K. pneumoniae, E. coli,* and *Salmonella* were 8.3% (1/12). Together these results suggest that different degrees of double, triple, or quadruple bacterial infection were present in the minks used for tissue sampling. The multiplex PCR could detect four kinds of bacteria from contaminated mink carcasses.

DISCUSSION

Specific primers were designed for specific target genes of four kinds of bacteria in the current study. The *nuc* gene of *S. aureus* encodes an extracellular thermostable nuclease, which is often used to detect *S. aureus* rapidly and specifically (15, 17, 22, 23). The *invA* gene of *Salmonella* is responsible for encoding the surface protein of the infected epithelial cells, which is common within the genus and unique among the genera, and is closely related to the pathogenicity of *Salmonella* (24, 25). When using *invA* gene as the target gene to design primers for *Salmonella* detection, strong specificity and detection accuracy can be obtained (26). Housekeeper gene *phoA* is used as a specific target gene for detection of *E. coli* (16, 27, 28). *khe* gene encodes the unique hemolysin of *K. pneumoniae* and is widely used in its detection (29, 30). Four pairs of specific primers designed in this study were used for the multiplex PCR, and the size intervals of the expected amplification products were more than 100 bp, so that different target genes could be distinguished after agarose gel electrophoresis.

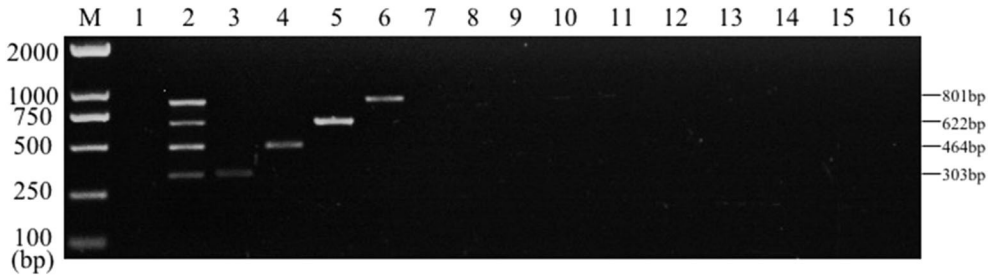

FIGURE 5 | Results of multiplex PCR specificity experiment. Lane M, D2000 DNA marker; Lane 1, Negative control; Lane 2, mixed DNA template of *Salmonella*, *E. coli*, *S. aureus* and *K. pneumoniae*; Lane 3, *K. pneumoniae*; Lane 4, *S. aureus*; Lane 5, *E. coli*; Lane 6, *Salmonella*; Lane 7, *Clostridium perfringens*; Lane 8, *Pasteurella multocida*; Lane 9, *P. aeruginosa*; Lane 10, *Streptococcus pneumoniae*; Lane 11, *Proteus mirabilis*; Lane 12, *Staphylococcus sciuri*; Lane 13, *Staphylococcus pseudintermedius*; Lane 14, *Acinetobacter baumannii*; Lane 15, *Enterococcus faecalis*; Lane 16, *Bacillus subtilis*.

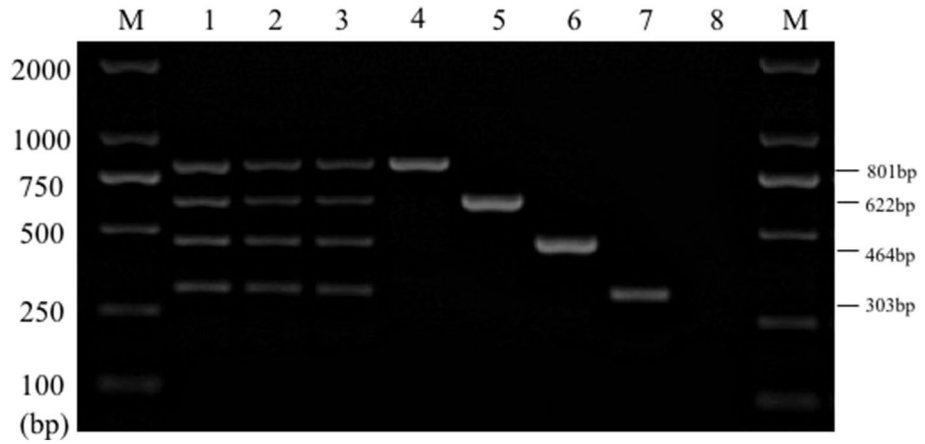

FIGURE 6 | Results of multiplex PCR stability experiment. Lane M, D2000 DNA marker; Lane 1∼3: mixed DNA template; Lane 4, *Salmonella*; Lane 5, *E. coli*; Lane 6, *S. aureus*; Lane 7, *K. pneumoniae*; Lane 8, Negative control.

TABLE 2 | Multiplex and single PCR detection results of clinical samples from Shandong Province, China.

Samples	Pathogens	Proportion of positive samples (%)		Concordance rate (%)
		Multiplex PCR	Single PCR	
Carcass samples	*Salmonella*	39/368 (10.6)	39/368 (10.6)	100
	E.coli	124/368 (33.7)	124/368 (33.7)	100
	K. pneumoniae	44/368 (12.0)	44/368 (12.0)	100
	S. aureus	51/368 (13.9)	51/368 (13.9)	100
Visceral tissue	*Salmonella*	2/12 (16.7)	2/12 (16.7)	100
	E. coli	5/12 (41.7)	5/12 (41.7)	100
	K. pneumoniae	3/12 (25)	3/12 (25)	100
	S. aureus	1/12 (8.3)	1/12 (8.3)	100

The results of single PCR showed that the four pairs designed in this study could amplify the corresponding target genes specifically. Therefore, these primers can be used in multiplex PCR detection system. The optimization of reaction conditions is the key to the construction of multiplex PCR system, the most important of which is the optimization of annealing temperature. Generally, the annealing temperature is determined according to the chain breaking temperature of the upstream and downstream primers, but sometimes the results are not the same as expected (31). Although a single target gene fragment can be amplified specifically at 56–60°C, the annealing temperature of 4–6°C can be reduced in the multiplex PCR reaction, which is conducive to the amplification of all target gene fragments (32). The optimal annealing temperature is

TABLE 3 | Co-contamination detection results of pathogenic bacteria in 380 clinical samples by multiplex PCR.

Pathogens	Proportion of positive samples (%)	
	carcass samples (368)	visceral tissue (12)
E. coli + *K. pneumoniae*	22 (6.0%)	1(8.3%)
E. coli + *Salmonella*	17 (4.6%)	1(8.3%)
E. coli + *S. aureus*	5 (1.4%)	0 (0)
S. aureus + *Salmonella*	5 (1.4%)	0 (0)
E. coli + *K. pneumoniae* + *Salmonella*	5 (1.4%)	0 (0)
E. coli + *Salmonella* + *S. aureus*	3 (0.8%)	0 (0)
E. coli + *K. pneumoniae* + *Salmonella* + *S. aureus*	3 (0.8%)	0 (0)
Total	60 (16.4%)	2 (16.7%)

determined by designing the annealing temperature gradient, and the optimization of primer concentration, primer addition amount and addition proportion is also an important step of the optimization scheme (18). Our study showed that under the same cycle number, the amplification efficiency of specific primers for *E. coli* and *S. aureus* was higher than that for *Salmonella* and *K. pneumoniae*. The amplification efficiency of each pair of primers could be effectively balanced by reducing the concentration of primers with high amplification efficiency and increasing the concentration of primers with low amplification rate (33).

In this study, the sensitivity test results showed that the minimum detection amount of multiplex PCR for four pathogens reached 100 pg. The multiplex PCR sensitivity of *E. coli, Salmonella,* and *S. aureus* in this study is close to or higher than that reported by Xu et al. (34) and Wang et al. (16). The minimum detection concentration of single PCR for bacteria DNA could reach 10.0 pg/μL, even 1.0 pg/μL. The single PCR sensitivity of *E. coli* detection in the current study is the same as that of Xu et al. (34), and it is more convenient and time-saving than that of Guan et al. (35) and Liu et al. (36). In this study, 380 samples were detected by multiplex PCR and single PCR. The results showed that the positive detection rate, accuracy, and sensitivity of multiplex PCR were in agreement with that of single PCR. The multiplex PCR method established in this study can be used to identify and detect bacteria in mink tissue samples. This sensitivity can meet the needs of clinical detection. The specificity test results showed that the multiplex PCR system could not only amplify the mixed samples and single samples, but also could not amplify other kinds of common pathogens or opportunistic pathogens in minks which cause respiratory tract and digestive tract diseases. All of these show that the method is more specific and can be applied to the detection and identification of specific pathogenic bacteria. The research of Guan et al. (37) also showed good stability and repeatability of multiplex PCR, which was consistent with our results.

The detection results of clinical samples showed that the single and co-infection of bacteria in mink visceral tissue samples and carcass samples in Shandong are serious, which suggests that enough attention should be paid to these multiple and single infections. This is not consistent with the research results in pigs (35), which may be due to the different composition of sample pathogens caused by factors such as pre-mortem health status, feeding, and storage conditions.

CONCLUSIONS

In conclusion, the multiplex PCR method is designed to detect and analyze the pathogenic microorganisms in mink carcass and viscera, which provides a rapid, specific and sensitive detection method for the identification of pathogenic bacteria in minks. The establishment of the multiplex PCR is conducive to the harmless treatment, development and utilization of mink carcass resources, and provides technical support for the safe and accurate application of fur animal carcass resources.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations of the Animal Ethics Committee of Shandong Animal Protection and Welfare Institute (Number: SDAUA-2018-47). Moreover, samples collecting treatment and biosafety in this study were performed in accordance with national and local laws and guidelines.

AUTHOR CONTRIBUTIONS

HG conceived and designed the experiments. PL, DZ, and HL are mainly responsible for experimental implementation. JQ and JP are mainly responsible for sample collection and helped to do some experiments. PL and JQ wrote the manuscript. All authors have read and approved the final manuscript.

FUNDING

This work was mainly supported by Shandong Province Modern Agricultural Technology System Innovation Team Program (SDAIT-21-10), the Earmarked Fund for the Protection for the State Forest and Wild Animals (No. 2130211) and Funds of Shandong Double Tops program.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 19 November 2020
doi: 10.3389/fvets.2020.584561

Early Life Inoculation With Adult-Derived Microbiota Accelerates Maturation of Intestinal Microbiota and Enhances NK Cell Activation in Broiler Chickens

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 05 August 2020
Accepted: 26 October 2020
Published: 19 November 2020

Citation:
Meijerink N, Kers JG, Velkers FC, van Haarlem DA, Lamot DM, de Oliveira JE, Smidt H, Stegeman JA, Rutten VPMG and Jansen CA (2020) Early Life Inoculation With Adult-Derived Microbiota Accelerates Maturation of Intestinal Microbiota and Enhances NK Cell Activation in Broiler Chickens. Front. Vet. Sci. 7:584561. doi: 10.3389/fvets.2020.584561

Studies in mammals, including chickens, have shown that the development of the immune system is affected by interactions with intestinal microbiota. Early life microbial colonization may affect the development of innate and adaptive immunity and may contribute to lasting effects on health and resilience of broiler chickens. We inoculated broiler chickens with adult-derived-microbiota (AM) to investigate their effects on intestinal microbiota composition and natural killer (NK) cells, amongst other immune cells. We hypothesized that AM inoculation directly upon hatch (day 0) would induce an alteration in microbiota composition shortly after hatch, and subsequently affect (subsets of) intestinal NK cells and their activation. Microbiota composition of caecal and ileal content of chickens of 1, 3, 7, 14, 21, and 35 days of age was assessed by sequencing of 16S ribosomal RNA gene amplicons. In parallel, subsets and activation of intestinal NK cells were analyzed by flow cytometry. In caecal content of 1- and 3-day-old AM chickens, a higher alpha-diversity (Faith's phylogenetic diversity) was observed compared to control chickens, whereas ileal microbiota were unaffected. Regarding beta-diversity, caecal microbiota profiles could be clustered into three distinct community types. Cluster A represented caecal microbiota of 1-day-old AM chickens and 1- and 3-day-old control chickens. Cluster B included microbiota of seven of eight 3- and 7-day-old AM and 7-day-old control chickens, and cluster C comprised microbiota of all chickens of 14-days and older, independent of inoculation. In 3-day-old AM chickens an increase in the percentages of intestinal IL-2Rα^+NK cells and activated NK cells was observed compared to control chickens of the same age. In addition, an increase in relative numbers of intestinal cytotoxic CD8$\alpha\alpha^+$T cells was observed in 14- and 21-day-old AM chickens. Taken together, these results indicate that early exposure to AM shapes and accelerates the maturation of caecal microbiota, which is paralleled by an

increase in IL-2Rα$^+$NK cells and enhanced NK cell activation. The observed association between early life development of intestinal microbiota and immune system indicates possibilities to apply microbiota-targeted strategies that can accelerate maturation of intestinal microbiota and strengthen the immune system, thereby improving the health and resilience of broiler chickens.

Keywords: poultry, avian immunology, intestinal microbiota, intraepithelial lymphocytes, innate immunity, NK cells

INTRODUCTION

Health and production efficiency of broiler chickens are of major importance, as chicken meat is a key sustainable source of animal protein for the growing human population (1, 2). In poultry production, restrictions of the use of antimicrobials have made other strategies to maintain or improve poultry health, such as enhanced immune responsiveness, increasingly important.

A crucial role in chicken health and production performance is played in many physiological processes by intestinal microbiota, including nutrient digestion and absorption, metabolism, intestinal barrier function, and development of intestinal immunity (3, 4). The maturation of the intestinal microbiota of chickens entails rapid successional changes, developing from a simple, to a more complex and diverse composition due to gradual colonization with microbiota (5–7). Early life exposure to microbiota is an important driver of this development, which can also affect health later in life. This has been shown in human infants (8–10), and other mammals and hatchlings treated with antibiotics early in life or raised under extreme hygienic conditions, e.g., germ-free or SPF environments (11–15). Also, in commercial chickens under normal circumstances, early transiently colonizing bacteria have been shown to have a large effect on intestinal microbiota composition later in life (16–18). However, due to hatching in a hatchery environment, colonization in commercial chickens starts with microbiota from environmental, rather than parental sources. As these environmental microorganisms may include pathogenic bacteria, competitive exclusion products derived from intestinal microbiota of healthy adult chickens have been developed to compete with colonization by pathogenic bacteria and are widely used in poulty production systems to induce a healthy microbiota (19). When supplied *in ovo* or to hatchlings, adult-derived microbiota has been shown to accelerate bacterial colonization (20–22) and to decrease the occurrence of undesirable bacteria such as *Salmonella* and *Escherichia coli* (19, 23, 24).

The intestinal immune system plays an important role in the defense against pathogens that enter a host via the gut. Underneath the mucus layer [the first protective barrier in the intestinal tract (25)], a layer of epithelial cells including immune cells such as the intraepithelial lymphocytes (IEL) is observed. The population of IEL consists of high numbers of γδ T cells, adaptive CD8$^+$ T cells and innate natural killer (NK) cells (26). During embryonic development and early life, when resistance against pathogens relies on innate immune responses since the adaptive immune system is not yet fully developed, NK cells are important players (27, 28). Chicken NK cells have also been reported in multiple organs including the intestine, lung, spleen, and blood (26, 29, 30). Previously, we and others showed that a high percentage of intestinal NK cells in chickens are recognized by the marker 28-4 (26), which was identified as CD25 or IL-2Rα (26). In mammals, the IL-2Rα chain is expressed on NK cells early upon activation (31), and this is followed by enhanced NK cell mediated killing and IFNγ production (31). Another marker found to be expressed on intestinal NK cells was 20E5 (32). It is also expressed on cells that show NK cell activation (29). Furthermore, elsewhere in the body, increased surface expression of CD107 indicative of NK cell activation was observed on primary chicken NK cells in lung, spleen and blood upon infections with avian viruses (30, 33, 34).

In the intestinal tract many interactions occur between the microbiota and immune cells (35, 36). These interactions are important for the development of the immune system, as was shown in mammals (21, 37, 38) and chickens (14, 39). For example, early life transplantation of adult microbiota has resulted in increased natural antibody titers in laying chickens (40) paralleled by long lasting effects on mRNA levels of pro-inflammatory cytokines (41). Disturbing the early life microbiota in 1-day-old broiler chickens by antibiotics resulted in reduced numbers of macrophage-like cells in the jejunum (14), whereas differences in rearing environment, e.g., a reduction in environmental microbial exposure resulted, in two phylogenetically distinct lines of broiler chickens, in lower expression levels of β-defensins (42).

Studies in rodents and humans have shown that specific probiotic microorganisms enhance intestinal NK cell activity and cytokine production (43) either directly via their interaction with receptors expressed on NK cells (44, 45), or indirectly via cytokine production of resident myeloid or epithelial cells (46). Also the adaptive immune system can be modulated via interactions with the microbiota (47–50), or indirectly through innate immune cell activities. As other studies in rodents and humans have shown, the microbiota affects activation of γδ T cells (51, 52) and CD8$^+$ T cells (53). Taken together, this indicates that the composition and activity of the microbiota and its effects on the immune system in early life may have long term consequences on the health of individuals.

In chickens, previous studies addressed the effect of microbiota on innate immune responses in the intestine, spleen and blood by studying mRNA levels of immune related genes (41, 42) by immunohistochemistry (14) and by analysis of natural antibody titers (40). In this study, we used tools that we developed previously for the analysis of the phenotype and the function of

chicken innate immune cells (29, 54) to assess whether and to what extent differences in early life microbial colonization would affect the development of NK cells locally (in the intestine) and systemically (in spleen and blood).

We hypothesized that inoculation with adult-derived microbiota(AM) upon hatch would induce an alteration in microbiota development and affect the presence and activation of intestinal NK cells. To induce early colonization with a rich, complex microbiota to stimulate immune development, we used Aviguard® (MSD Animal Health, the Netherlands), as this product derived from microbiota of healthy adult chickens has been shown to be able to colonize the intestinal tract and induce early maturation of the intestinal microbiota in previous studies with hatchlings (22, 55). In this study, AM inoculation resulted in an accelerated maturation of the intestinal microbiota, an increase of IL-2Rα$^+$ NK cells and enhanced activation of NK cells. The observed association between early life development of intestinal microbiota and the immune system indicates possibilities to apply microbiota-targeted strategies that can accelerate maturation of intestinal microbiota and strengthen the immune system to improve the health and resilience of broiler chickens.

MATERIALS AND METHODS

Birds and Husbandry

Ross 308 broiler 17- and 18-day old embryonated eggs were obtained from the same parent flock of a commercial hatchery (Lagerwey, the Netherlands). ED17 (hatch group A, $n = 52$) and ED18 eggs (hatch group B, $n = 52$) were disinfected with 3% hydrogen peroxide and placed in disinfected egg hatchers. All eggs hatched at ED21. Directly upon hatch, chickens (day 0 in age) were randomly divided into two treatment groups, weighed, labeled and inoculated. Next, the chickens of the two treatment groups were placed in separate floor pens of 2 × 1.5 m (pens 1 and 2), with a solid wall separating the pens. Each pen was divided in two equal parts of 1 × 1.5 m for chickens from hatch group A and B. The pens were lined with wood shavings (2 kg/m^2, sterilized by autoclavation). Non-sterilized standard commercial starter and grower feeds (Research Diet Services, the Netherlands) and water was provided *ad libitum*. No antibiotics, coccidiostatic drugs or commercial vaccines were applied during the experiment. A standard lighting, temperature scheme for Ross broiler chickens was used, and conditions were kept the same for all compartments. The chickens were observed daily for clinical signs, abnormal behavior or mortality and were also evaluated for presence of abnormalities during post-mortem. No signs of disease or impaired health were observed in both groups throughout the experiment. Feed intake and body weight were assessed in both groups at each sampling moment and followed the expectations based on the Ross 308 broiler performance standards in both groups.

The experimental room was equipped with a mechanical negative pressure ventilation system.

The animal experiment was approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee (registration number AVD1080020174425) of Utrecht University (the Netherlands) and all procedures were done in full compliance with all relevant legislation.

Experimental Design

Chickens were inoculated once immediately after hatch to reduce opportunities for prior exposure to microbiota. First, the control group received an oral inoculation with 0.5 ml PBS (Lonza, Basel, Switzerland). The other group, henceforth referred to as the AM group, was inoculated with 0.5 ml of PBS containing 0.05 g/ml of competitive exclusion product Aviguard® (MSD Animal Health, the Netherlands). This is a freeze-dried powder, soluble in water, consisting of fermented, undefined cultures from intestinal microbiota of healthy specific-pathogen-free birds and was used according to manufacturer's instructions. To determine the microbial composition of the AM inoculum and compare this to the microbiota in the chickens, four aliquots of 2 ml were stored at −80°C for DNA extraction. The experimental design of the study is shown in **Supplementary Figure 1**.

Sample Collection

At day 0 (upon hatch), four non-inoculated chickens per hatch group were randomly selected and sacrificed, to collect caecal and ileal content for microbiota analyses, as has been described in (56). Ileal content was collected distal and close to the Meckel's diverticulum. The intestinal content was gently squeezed into a 2 ml sterile cryotube, snap frozen on dry ice and stored at −80°C for DNA extraction. The time between sacrificing and placing the intestinal samples on dry ice was between 3–5 min. To avoid cross contamination, all management and biotechnical procedures were completed first with the control group and for each compartment at the same time. At days 1 (24 h after inoculation), 3, 7, 14, 21, and 35, eight chickens (four from the control and four from the AM group) were randomly selected per hatch group (A/B) and sacrificed to collect caecal and ileal content as described above. At day 0 and day 1, the chickens were too small to collect sufficient cells for immunological analyses. Therefore, ileum tissue, spleen and blood were collected from day 3 onwards from six of these eight chickens ($n = 3$ per hatch group). All chickens were weighed prior to post-mortem analyses.

DNA Extraction

In total, 104 caecal and 104 ileal content samples, consisting of 52 samples per treatment group, and four samples of the AM inoculum were analyzed for microbiota composition. DNA was extracted from 0.25 g content, using 700 µl of Stool Transport and Recovery (STAR) buffer (Roche Diagnostics Nederland BV, the Netherlands). All samples were transferred to sterile screw-capped 2 ml tubes (BIOplastics BV, the Netherlands) containing 0.5 g of zirconium beads (0.1 mm; BioSpec Products, Inc., USA), and 5 glass beads (2.5 mm; BioSpec Products). All samples were treated in a bead beater (Precellys 24, Bertin technologies, France) at a speed of 5.5 m/s for 3 × 1 min, followed by incubation at 95°C with agitation (15 min and 300 rpm). The lysis tube was centrifuged (13,000 g for 5 min at 4°C), and the supernatant was

transferred to a 2 ml microcentrifuge tube. Thereafter, the above-described process was repeated with 300 µl STAR buffer. An aliquot (250 µl) of the combined supernatants from the sample lysis was then transferred into the custom Maxwell® 16 Tissue LEV Total RNA Purification Kit cartridge. The remainder of the extraction protocol was then carried out in the Maxwell® 16 Instrument (Promega, the Netherlands) according to the manufacturer's instructions. DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, DE, USA), and the DNA samples were stored at −20°C until further use.

qPCR, 16S rRNA Gene Amplification, Sequencing, and Data Processing

Extracted DNA was diluted to 20 ng μl^{-1} in nuclease free H_2O. All PCR plastics were UV irradiated for 15 min before use. To validate the AM inoculation, absolute quantification of the bacterial 16S rRNA genes by real-time PCR amplification was performed for the caecal content samples of day-old chickens. For ileal content samples the amount of DNA was too low to reliably determine gene copy numbers. All qPCR assays (CFX384™ real-time PCR detection system, Bio-Rad, Hercules, CA, USA) were performed in triplicate with 25 µl reactions and was described previously (57). For 16S ribosomal RNA (rRNA) gene-based microbial composition profiling, barcoded amplicons covering the variable regions V5–V6 of the bacterial 16S rRNA gene were generated by PCR using the 784F and 1064R primers as described before (58). Each sample was amplified in duplicate using Phusion hot start II high fidelity polymerase (Finnzymes, Finland), checked for correct size and concentration on a 1% agarose gel and subsequently combined and purified using CleanNA magnetic beads (CleanNA, the Netherlands). A detailed description of the PCR conditions is given elsewhere (56). Positive and negative controls were added to the data set to ensure high quality sequencing data. As positive controls we used synthetic mock communities of known composition (58), and as negative controls we used nuclease free water. The resulting libraries were sent to Eurofins Genomics GmbH (Germany) for sequencing on an Illumina Hiseq2500 instrument. The 16S rRNA data was analyzed using NG-tax 2.0 (59). In short, paired-end libraries were filtered to contain only read pairs with a perfect match to the primers and perfectly matching barcodes, to demultiplex reads by sample. Amplicon sequence variants (ASVs) were defined as unique sequences. The ASV picking strategy was based on a *de novo* reference approach. Taxonomy was assigned using the SILVA 128 16S rRNA gene reference database (60). Caecal content samples of day 0 and ileal content samples of day 0 and 1 were excluded from the analysis, because these contained a large number of families associated with the negative control samples, and therefore did not pass our quality control standards. Raw sequence data were deposited into the Sequence Read Archive (SRA) at NCBI under accession number PRJNA670739.

Isolation of Tissues and Cells

Ileum segments (±10 cm distal from Meckel's diverticulum), spleens and blood (5 ml) were collected. Ileum segments were washed with PBS to remove contents and cut in sections of 1 cm^2 and washed again. Subsequently, IELs were collected by incubating three times in EDTA-medium [HBSS 1x (Gibco BRL) supplemented with 10% heat-inactivated FCS (Lonza); 1% 0.5M EDTA (Sigma-Aldrich)] at 200 rpm for 15 min at 37°C. Supernatants were collected and centrifuged 5 min at 1,200 rpm at 20°C. Cells were then resuspended in PBS, lymphocytes were isolated using Ficoll-Paque Plus (GE Healthcare, the Netherlands) density gradient centrifugation for 12 min at 1,700 rpm, washed in PBS using centrifugation for 5 min at 1,300 rpm and resuspended at 4.0×10^6 cells/ml in NK medium [IMDM supplemented with 8% heat-inactivated FCS (Lonza); 2% heat-inactivated chicken serum, 100 U/ml penicillin/ streptomycin, and 2 mM glutamax I; Gibco BRL, United Kingdom]. Spleens were homogenized using a 70 µm cell strainer [Beckton Dickinson (BD) Biosciences, NJ, USA] to obtain a single cell suspension. Next, lymphocytes in spleen and blood were isolated by Ficoll-Paque density gradient centrifugation (20 min at 2,200 rpm), washed in PBS and resuspended at 4.0×10^6 cells/ml in NK medium as described for ileum.

Flow Cytometry

Presence and activation of NK and T cell subsets were determined in IEL, spleen, and blood. Unless described otherwise, all antibodies were obtained from Southern Biotech (AL, USA). Markers known to be expressed on chicken NK cells (hybridomas provided by Göbel), such as mouse-anti-chicken-28-4 (IL-2Rα; IgG3) and−20E5-BIOT (IgG1) were co-stained with mouse-anti-chicken-CD45-FITC (IgM) and -CD3-APC (CT3; IgG1) mAb to exclude T cells. The T cell panel included the following markers: mouse-anti-chicken-CD3-PE (CT3; IgG1), -CD4-APC (CT4; IgG1), -TCRγδ-FITC (TCR-1, IgG1), -CD8α (EP72, IgG2b), and -CD8β-BIOT (EP42, IgG2a). Secondary antibody staining was performed using goat-anti-mouse-IgG3-PE and streptavidin-PercP (BD Biosciences) in the NK cell panel, and goat-anti-mouse-IgG2b-APC/Cy7 and streptavidin-PercP in the T cell panel. To asses CD107 expression on NK cells, lymphocytes were washed in PBA and stained with mouse-anti-chicken-CD3-PE, -TCRγδ-BIOT (TCR-1, IgG1),−28-4, and -CD41/61-FITC (11C3, IgG1, Serotec) to exclude thrombocytes from analysis. Secondary antibody staining was performed using streptavidin-PercP and goat-anti-mouse-IgG3-APC/Cy7. All staining procedures were incubated for 20 min at 4°C in the dark, washed in PBA and subsequently stained with a live/dead marker (Zombie Aqua™ Fixable Viability Kit, Biolegend) for 15 min at RT in the dark to exclude dead cells. Finally, lymphocytes were fixed using 2% paraformaldehyde (Merck, Germany) for 10 min at RT, washed and resuspended in 200 µl PBA. Fluorescence of cells was assessed in 150 µl or 50,000 lymphocytes in the live gate using a FACSCANTO II Flowcytometer (BD Biosciences), and data was analyzed with software program FlowJo (Tree star Inc, OR, USA).

NK Cell Activation Assay

NK cell activation was determined using the CD107-assay, which measures increased surface expression of CD107 as a result of degranulation of perforin and granzymes (29).

Briefly, lymphocytes isolated from IEL, spleen, and blood were resuspended in NK medium, and 1×10^6 lymphocytes per sample were used. Lymphocytes were cultured in presence of 1 μl/ml Golgistop (BD Biosciences) and mouse-anti-chicken-CD107-APC mAb (5G10, IgG1, hybridomas provided by Göbel, T.W., Ludwig Maximilians University, Germany) during 4 h at 37°C, 5% CO_2. Next, cells were washed, stained with monoclonal antibodies and analyzed by flow cytometry.

Data Analysis

Statistical analyses for microbiota and the relation between microbiota and the immune system were performed in R version 3 (R Foundation for Statistical Computing, Austria), using the packages Phyloseq, Microbiome, Vegan, and DirichletMultinomial (61–64). A Kruskal-Wallis test was used to test for difference in 16S rRNA gene counts in caecal content of day-old chickens between treatment groups.

Alpha and beta diversity metrics and multivariate statistical analyses were applied to determine differences in the measured intestinal microbiota between the two treatment groups and with age. The alpha diversity (within sample) data was determined using Faith's phylogenetic diversity. Faith's phylogenetic diversity not only takes the number of different taxa (ASVs) into account, but also the phylogenetic relatedness of these taxa (65). To test for differences in relative abundance of genera between treatment groups, we used a Wilcoxon rank-sum test and corrected for multiple comparisons using the Benjamini-Hochberg (BH) procedure. The beta diversity (between samples) was determined using weighted and unweighted UniFrac metrics (66). Multivariate microbiota data were visualized using principal coordinates analysis (PCoA, multidimensional scaling method), and non-parametric permutational analysis of variance (PERMANOVA) tests were used to analyze group differences within multivariate community data (67).

To assess whether the development of the microbiota proceeded through different stages of maturation in the two treatment groups, Dirichlet Multinomial Mixtures (DMM) modeling was applied, using a probabilistic model, to identify possible clusters (types) of microbial composition 16S rRNA gene sequence data (68) based on the relative abundance of the microbial groups at genus level. Two separate DMM models were used to study clustering of the microbiota data of the caecal content and ileal content separately. Next, to test whether the observed differences in the microbial development between treatments were associated with differences in immune development, Wilcoxon rank-sum test, corrected for multiple comparisons using BH, was used to test for associations between the identified DMM clusters of microbial composition and immunological parameters. As ileal microbiota clustering did not indicate differences in microbial development between treatments, only the clusters identified for the caecal microbiota profiles were used. Associations were tested for a subset of immunological parameters that showed differences between AM and control chickens of the same age. Furthermore, parameters with fewer than four observations per treatment group and day of age were omitted. The final selection of parameters included percentages and absolute numbers of intestinal IL-2Rα$^+$, 20E5$^+$ and CD107$^+$ NK cells, and CD8αα$^+$ T cells.

Statistical analyses for the immunological parameters were done with GraphPad Prism 7.0 software (GraphPad Software Inc., USA), using the Mann-Whitney *U*-test to test differences between treatment groups at a specific day of age. A *p*-value of <0.05 was considered statistically significant.

RESULTS

AM Treatment Influences the Composition and Development of the Intestinal Microbiota in Newly Hatched Chickens

The total bacterial 16S rRNA gene copy numbers 24 h after inoculation were significantly higher in caecal content samples at day 1 in AM inoculated compared to control chickens, indicating the presence of a higher quantity of bacteria after inoculation with AM (**Supplementary Figure 2**).

To investigate the effect of AM inoculation on the microbiota composition at different ages in the broiler chickens, alpha and beta diversities, as well as differences in relative abundance of individual microbial taxa, were assessed. The phylogenetic diversity metric, providing information on the number as well as phylogenetic relatedness of observed microbial taxa at the ASV level, was used as an alpha diversity measure to determine differences between AM and control chickens. The phylogenetic diversity of the caecal content was higher in 1- and 3-day-old AM chickens compared to controls, but not for any of the other ages (**Figure 1A**). In contrast, the phylogenetic diversity of ileal content microbiota did not differ between treatment groups at any age (**Figure 1B**).

Beta diversity, i.e., the similarity in composition between samples, was determined using the weighted and unweighted UniFrac distance metrics to determine the influence of age and treatment on the composition. Two dimensional visualization of the caecal content microbiota profiles in PCoA plots placed 3- and 7-day-old AM inoculated chickens closely together, indicating high similarity in microbiota composition between these age groups (**Figure 2**). PERMANOVA of caecal content microbiota showed that treatment explained 6–9% of the variation in caecal microbiota composition between samples ($p < 5e\text{-}04$; unweighted UniFrac, $p < 2e\text{-}04$, weighted UniFrac), whereas age explained 49–41% of the variation between samples ($p < 5e\text{-}04$; unweighted UniFrac, $p < 2e\text{-}04$, weighted UniFrac). PERMANOVA of ileal content samples showed that treatment explained 4% of the variation in ileal microbiota composition based on unweighted UniFrac, whereas treatment did not significantly contribute to explaining the observed variation using the weighted UniFrac distance metrics ($p = 0.038$; unweighted UniFrac, $p = 0.355$, weighted UniFrac, **Figure 2B**), indicating that differences in microbial profiles of ileal samples between treatment groups concerned mostly the presence/absence of taxa occurring at low relative abundance. Age explained 29–24% of the variation between the ileal content samples ($p < 1e\text{-}04$; unweighted UniFrac, $p < 1e\text{-}04$, weighted UniFrac).

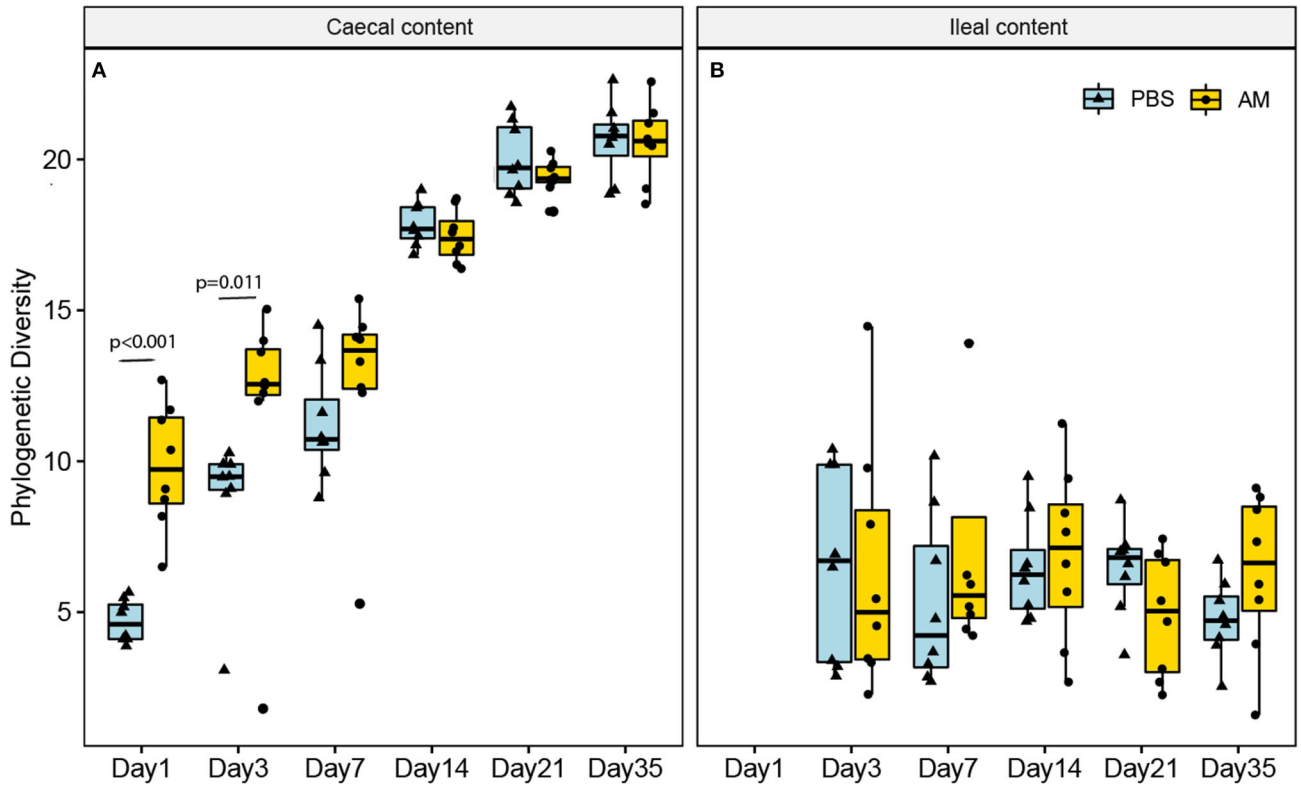

FIGURE 1 | Phylogenetic diversity of the ileal and caecal content microbiota at different ages. **(A)** The phylogenetic diversity (alpha diversity, at ASV level) was only significantly higher in the caecal content of AM chickens compared to controls on day 1 and day 3 ($p < 0.05$). **(B)** In the ileal content microbiota no differences were observed at any of the ages. $n = 8$ chickens per treatment per day of age, whiskers show 95% interval, box 50% interval.

In the AM inoculum 24 different genera were detected, for which the relative abundances in caecal and ileal samples were compared between AM and control chickens. A higher relative abundance in caecal content of AM chickens compared to controls was found for 10 of these 24 genera at day 1, five on day 3, four at day 7, and two at day 14 and 21. At day 35 none of these genera differed in relative abundance between AM and control chickens (**Table 1**). This indicates that AM inoculation had an impact on the relative abundance of genera at an early age, but did not permanently influence the relative abundance of these genera in the caecal content samples. For ileal content, no differences in the relative abundances of the 24 genera of the inoculum were observed at any of the different ages (data not shown).

To assess if AM inoculation affected the development of the microbial composition from hatch toward a mature microbiota, microbial profiles were subjected to DMM clustering of 16S rRNA gene sequencing data based on the relative abundance of microbial taxa at genus level. The DDM method showed the best model fit, based on lowest Laplace approximation, for three clusters in the caecal content profiles (**Figure 3A**). Cluster A contained 26 samples, with all 1-day-old AM and control chickens and all 3-day-old controls. Cluster B consisted of 21 samples, containing seven of the eight 3-day-old AM chickens and 7-day-old AM and control chickens. The remaining 48 samples were in cluster C, which contained all AM and control chickens of 14, 21, and 35 days old. This difference in distribution of AM and control chickens over cluster A and B in the 1st week of life indicates an accelerated maturation of caecal microbiota profiles for AM chickens. In contrast, clustering for the ileal content profiles only showed an effect of age, with cluster D dominated by 3- and 7-day-old chickens of both treatments, and cluster E by chickens of 14, 21, and 35 days old of both treatments (**Figure 3B**). The relative microbial abundance of the clusters observed in the caecal content was analyzed and although PBS and AM chickens varied in their relative abundance of microbial families, PBS and AM chickens can be part of the same cluster based on relative abundance of genera (**Figure 3C**).

AM Treatment Affects Presence of NK Cell Subsets and Their Activation

Possible differences in subsets and activation of intestinal NK cells from AM and control chickens were determined. Local effects of AM inoculation on intestinal NK cells were compared to systemic effects measured in spleen and blood. Within the live lymphocytes, the CD3 negative IL-2Rα$^+$ or 20E5$^+$ NK cells were quantified (**Figure 4A**). In parallel, NK cell activation was determined by analysis of enhanced CD107 surface expression on CD3 negative and CD41/61 negative cells. At day 3, the percentage of intestinal IL-2Rα$^+$ NK cells tended to be higher

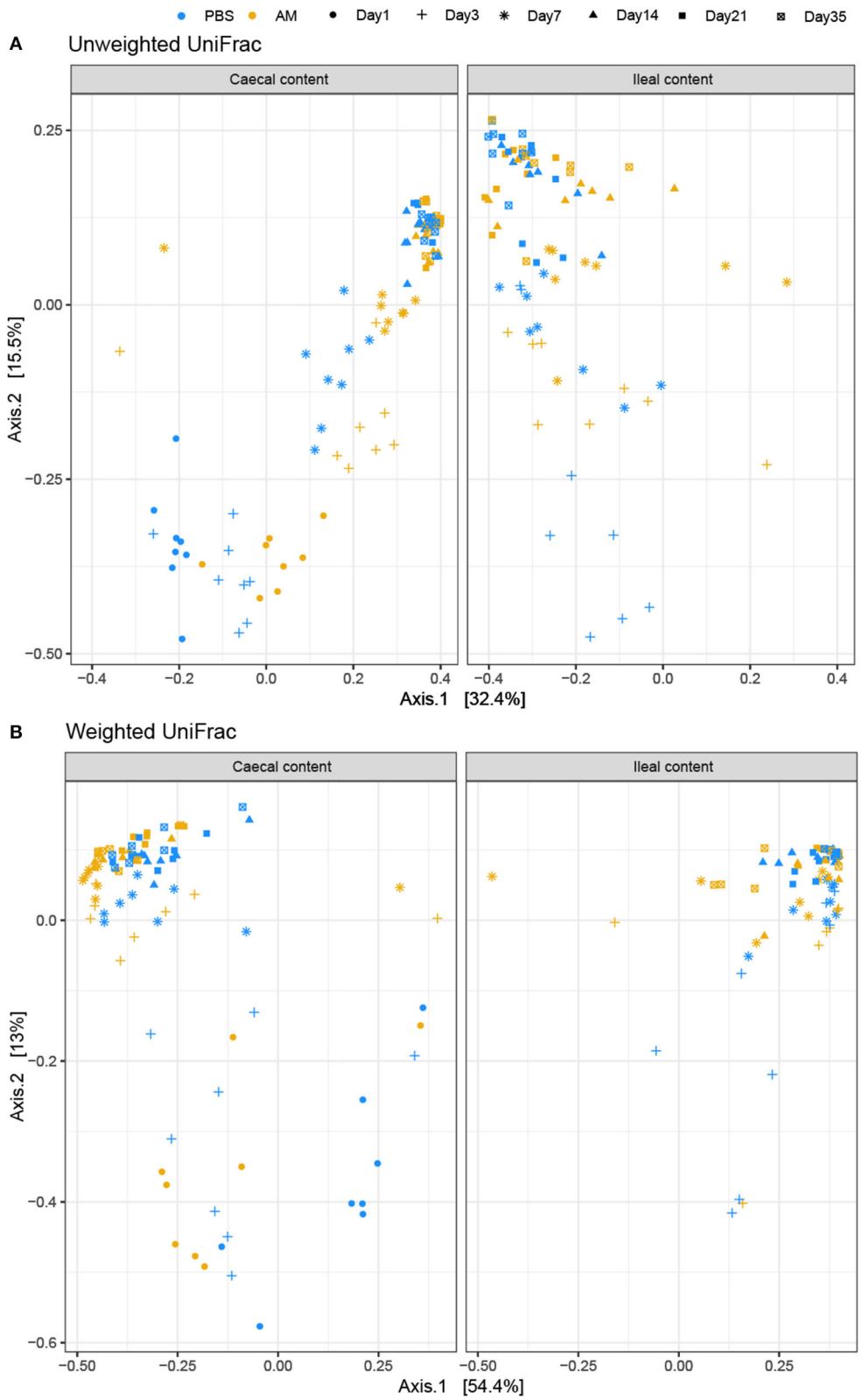

FIGURE 2 | PCoA plot visualizing caecal and ileal content microbial profiles. Unweighted **(A)** and weighted **(B)** UniFrac distance based PCoA on caecal (left) and ileal (right) content samples. **(A)** PERMANOVA of caecal content microbiota showed that treatment explained 6–9% of the variation in caecal microbiota composition

(Continued)

FIGURE 2 | between samples ($p < 5e\text{-}04$; unweighted UniFrac, $p < 2e\text{-}04$, weighted UniFrac), whereas age explained 49–41% of the variation between samples ($p < 5e\text{-}04$; unweighted UniFrac, $p < 2e\text{-}04$, weighted UniFrac). **(B)** PERMANOVA of ileal content samples showed that treatment explained 4% of the variation in ileal microbiota composition based on unweighted UniFrac, whereas treatment did not significantly contribute to explaining the observed variation using the weighted UniFrac distance metrics ($p = 0.038$; unweighted UniFrac, $p = 0.355$, weighted UniFrac). $n = 8$ chickens per treatment per day of age.

in AM chickens (5.61 ± 0.95%) compared to controls (3.25 ± 0.93%, $p = 0.09$, **Figure 4B**). No differences between treatment groups were observed in intestinal 20E5$^+$ NK cells (**Figure 4C**). Increased CD107 expression on intestinal NK cells was observed at day 3 in AM chickens (10.52 ± 0.70%), when compared to controls (8.07 ± 0.47%, $p = 0.06$, **Figure 4D**). At day 35, an increase in activation of intestinal NK cells was observed in AM chickens (14.86 ± 1.27%) compared to the controls (11.71 ± 0.75%, $p = 0.04$, **Figure 4D**). No differences between treatment groups were observed in CD107 expression of intestinal NK cells at other ages (**Figure 4D**).

Relative numbers of IL-2Rα$^+$ and 20E5$^+$ NK cells in spleen and blood were similar in both treatment groups (**Figures 4E,F**, **Supplementary Figures 3A,B**). However, NK cell activation was significantly increased in splenic NK cells in 3-day-old AM chickens (20.74 ± 1.10%) compared to controls (15.35 ± 0.40%, $p = 0.004$, **Figure 4G**). No difference in CD107 surface expression on blood-derived NK cells was found between treatment groups (**Supplementary Figure 3C**). Furthermore, AM inoculation did not affect total lymphocyte numbers in the intestine, spleen and blood (**Supplementary Figures 4A,E,I**). In addition to the percentages of the different NK subsets, absolute numbers were determined. Similar trends were observed in absolute number of IL-2Rα$^+$, 20E5$^+$, and CD107$^+$ NK cells although the differences between treatments were less pronounced (**Supplementary Figure 4**).

AM Treatment Affects Intestinal Cytotoxic CD8αα T Cells in 14- and 21-Day-Old Chickens

In addition to NK cell subsets and NK cell activation, effects of AM inoculation on presence and function of γδ T cells and presence of cytotoxic CD8$^+$ T cells were studied. Within the CD3$^+$ and CD4$^-$ lymphocytes, both TCRγδ$^+$ and TCRγδ$^-$ cell populations were analyzed for CD8αα and CD8αβ expression (**Figure 5A**). In parallel, activation of γδ T cells was determined at day 7, 14, and 21 by analyzing increased surface expression of CD107 on CD3$^+$CD41/61$^-$ TCRγδ$^+$ cells (**Figure 5A**). No differences between AM and control chickens were observed in the percentage of intestinal γδ T cells (**Figure 5B**), CD8$^-$, CD8αα$^+$, and CD8αβ$^+$ gamma delta subsets (data not shown) and activation of γδ T cells (**Figure 5C**). The percentage of intestinal CD8αα$^+$ T cells tended to be higher in 14- (25.3 ± 1.5%) and 21-day-old AM chickens (33.2 ± 4.2%) compared to controls (19.5 ± 1.7%, $p = 0.08$ and 24.0 ± 1.3%, $p = 0.07$, respectively, **Figure 5D**). No differences between groups were found at any age in the percentages of intestinal CD8αβ$^+$ T cells (**Figure 5E**). Furthermore, no differences between AM and control chickens were observed in the percentage of γδ T cells (**Figure 5F**, **Supplementary Figure 3D**), subsets (data not shown), γδ T cell activation (**Figure 5G**, **Supplementary Figure 3E**) and cytotoxic T cells in spleen and blood (**Figures 5H,I**, **Supplementary Figures 3F,G**). Absolute numbers of these parameters were investigated and did not show any differences between AM and control chickens, although an increase in numbers of both treatments was observed with age (**Supplementary Figure 5**).

Association Between Caecal Microbiota Clusters and Immune Cells

Clustering of the caecal content profiles suggests that AM chickens showed an earlier maturation of caecal microbiota profiles compared to controls. Also, differences in IL-2Rα$^+$ NK cells, NK cell activation and CD8αα T cells were observed between AM chickens compared to the controls. To assess a possible relationship between the observed differences in the microbial development between treatments and the detected differences in immune parameters, we used the previously identified DMM clusters to test for correlations between the caecal microbiota profiles (i.e., stages of successive microbiota maturation) and immune parameters.

Clusters A, B, and C were based on relative abundance of genera present in the caecal microbiota of chickens and represent different stages during the early life development of caecal microbiota. Correlations to relative and absolute numbers of IL-2Rα$^+$, 20E5$^+$, CD107$^+$ NK cells, and cytotoxic CD8aa$^+$ T cells in the ileum were investigated. The percentage of intestinal IL-2Rα$^+$ NK cells was higher in cluster B compared to cluster A ($p = 0.026$, **Table 2**), and compared to cluster C ($p = 0.044$, **Table 2**) regardless of treatment (**Figure 6A**). The percentage of IL-2Rα$^+$ NK cells in cluster C tended to be higher compared to cluster A, but this was not significant ($p = 0.068$, **Table 2**, **Figure 6A**). Relative numbers of intestinal 20E5$^+$ NK cells were similar between clusters A and B and highest in cluster C (**Figure 6B**, **Table 2**). Relative numbers of intestinal CD107$^+$ NK cells were highest in cluster C and lowest in cluster A (**Table 2**, **Figure 6C**). Within cluster C, the percentage of CD107$^+$ NK cells tended to be higher in AM chickens (**Figure 6C**). Relative numbers for intestinal cytotoxic CD8αα$^+$ T cells were higher in cluster B and C compared to cluster A and did not differ between cluster B and C (**Figure 6D**, **Table 2**). Similar correlations were observed between clusters and absolute numbers of intestinal 20E5$^+$, CD107$^+$ NK cells and cytotoxic CD8αα$^+$ T cells (**Table 2**).

These results indicate significant associations between caecal microbiota clusters and subsets of intestinal immune cells.

DISCUSSION

In this study, we aimed to induce an alteration in the intestinal microbiota shortly after hatch by administration of adult-derived

TABLE 1 | Relative abundance of genera present in the AM inoculum and differences in relative abundance in caecal content for AM compared to control chickens.

Relative abundance AM inoculum			**Differences in relative abundance AM vs. control chickens**													
			Day 1			**Day 3**			**Day 7**			**Day 14**			**Day 21**		
Genera	**RA%**	**SD%**	**AM%**	**PBS%**	***P***	**AM%**	**PBS%**	***P***	**AM%**	**PBS%**	***P***	**AM%**	**PBS%**	***P***	**AM%**	**PBS%**	***P***
Eubacterium coprostanoligenes group	0.65	0.22	-	-		0.06	-		-	-		0.84	1.12		0.71	0.65	
Bacteroides	0.47	0.06	-	-		3.91	-	**0.045**	2.57	-	**0.018**	2.01	1.12		3.01	3.57	
Blautia	0.30	0.09	9.17	-	**0.006**	5.86	3.92		4.67	16.63		4.35	13.14		6.10	10.68	
Candidatus_Soleaferrea	0.39	0.06	0.56	-	**0.006**	-	-		-	-		-	-		-	-	
Clostridium sensu stricto 1	2.77	0.45	24.32	53.93	**0.033**	1.96	22.30		-	0.19		-	-		0.03	-	
Clostridium sensu stricto 2	0.72	0.12	0.77	-	**0.006**	-	-		-	-		-	-		-	-	
Collinsella	0.53	0.07	0.68	-	**0.034**	4.65	-	**0.018**	3.64	1.04		1.54	4.30		3.60	2.02	
Enterococcus	10.80	1.07	10.12	17.64		16.19	18.55		0.86	0.87		0.32	0.36		0.43	0.48	
Erysipelatoclostridium	2.53	0.09	0.26	0.00		2.56	-	**0.027**	0.05	2.07	**0.018**	0.81	2.01		0.44	1.06	**0.043**
Escherichia-Shigella	0.57	0.02	32.73	3.36	**0.006**	0.72	11.07	**0.044**	0.16	0.73		-	0.03		-	-	
Eubacterium	0.66	0.04	0.30	-	**0.016**	0.46	0.11		0.92	0.28		0.15	0.19		0.07	0.12	
Flavonifractor	1.02	0.14	1.23	-	**0.006**	0.90	0.66		0.13	0.44		0.05	0.43	**0.010**	-	-	
Lachnoclostridium	9.78	0.93	2.30	-	**0.006**	3.28	-	**0.018**	0.77	0.85		0.66	0.72		0.41	0.16	
Lactobacillus	14.96	1.33	8.46	-		6.83	1.12		8.05	3.44		5.34	12.34		13.85	10.14	
Megamonas	1.55	0.56	0.02	-		4.05	-		30.21	-	**0.018**	27.46	-	**0.009**	7.46	-	**0.022**
Megasphaera	3.30	0.74	-	-		-	-		-	-		-	-		-	-	
Negativicoccus	3.62	0.66	-	-		-	-		-	-		-	-		-	-	
Oscillibacter	1.94	0.18	-	-		-	-		-	-		-	-		-	-	
Peptostreptococcus	30.97	4.04	0.19	-	**0.034**	-	-		-	-		-	-		-	-	
Sellimonas	1.31	0.38	-	-		-	-		0.31	0.75		0.60	0.84		0.50	0.88	
Slackia	0.34	0.09	0.02	-		0.03	-		0.43	-	**0.037**	0.01	0.05		0.08	0.10	
Sutterella	1.76	0.21	-	-		-	-		-	-		-	-		-	-	
Uncultured	4.45	3.56	0.00	0.00		1.40	0.27		1.07	2.15		1.37	1.96		1.11	1.48	
unknown	0.08	0.09															

The AM inoculum contained 24 different genera and the genera for which significant differences in relative abundance of AM inoculated chickens (AM) vs. controls (PBS) were found for day 1, 3, 7, 14, and 21 are indicated in bold (n = 8). No differences were observed between treatments on day 35. Results are based on differences of relative abundance tested with Wilcoxon rank-sum test. P = adjusted p-values (<0.05) were corrected for multiple testing with Benjamini-Hochberg (BH). - = not detected. RA = Relative abundance (%) in the AM inoculum. AM/PBS = Relative abundance (%) in AM/control treatment.

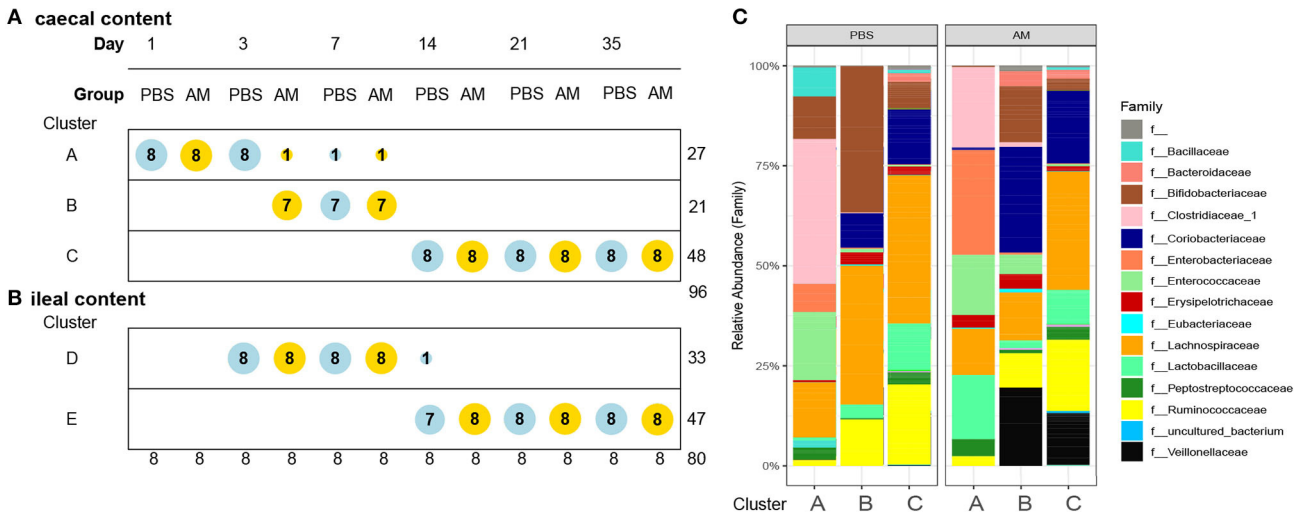

FIGURE 3 | Dirichlet multinomial mixtures (DMM) clustering of 16S rRNA gene sequencing data for caecal and ileal microbial profiles. **(A)** DMM clustering showed the best model fit for three clusters in the caecal content profiles (lowest Laplace approximation, $n = 96$). Cluster A contains 27 samples, Cluster B 21 samples, and the remaining 48 samples are in cluster C. Cluster B contains 3-day old AM chickens, and seven of eight AM and all control chickens of day 7, indicating acceleration of microbiota maturation in the caecal content. Nodes are colored according to intervention (AM or PBS) and ordered according to age. **(B)** In the ileal content samples two distinct clusters were observed, but no evidence for acceleration of the development of the microbiota ($n = 80$). **(C)** Relative microbial abundance of the clusters observed in the caecal content stratified by the intervention at family level.

microbiota, and compared presence and function of NK cells, as representatives of developing innate immunity, to those of non-inoculated controls. We hypothesized that early exposure to adult-derived microbiota would accelerate intestinal microbiota colonization and affect subsets and activation of intestinal NK cells. Our results indicate that the inoculation with the adult-derived microbes mostly affected the early development of the caecal microbiota, and induced an earlier maturation of caecal microbiota compared to control broiler chickens. This development was paralleled by an increase in intestinal IL-2Rα^+ NK cells and enhanced activation of NK cells early in life and CD8$\alpha\alpha^+$ T cells later in life.

The AM inoculation delivered immediately after hatch successfully altered intestinal microbiota composition, especially in the 1st week of life, but did not permanently influence the diversity of caecal microbiota. In addition, with respect to the genera found in the AM product, a higher relative abundance was only found shortly after inoculation. More specifically, a higher relative abundance in AM chickens was found for 10 of the 24 genera in the inoculum on day 1, but this quickly declined to two genera by the end of the 1st week. These findings are in line with previous studies with the same product: inoculation with Aviguard *in ovo* enhanced development of intestinal microbiota of broiler chickens and increased diversity and reduced the abundance of *Enterobacteriaceae* (22). Similar to our study, not all genera present in the inoculum permanently colonized the intestine; they were assumed to have been transient colonizers facilitating the development of a complex microbiota by temporarily altering the microenvironment (22). Similar observations have been reported for 1-day-old laying hens inoculated with Aviguard. Not all bacteria of the product, nor of the mother hen, were effectively transferred to the chickens' gut, but compared to controls, caecal microbiota enriched for the phyla *Bacteroidetes* and *Actinobacteria* was observed within a week in both Aviguard treated chickens and in chickens naturally exposed to a mother hen (55).

Like chickens hatched in commercial hatcheries, the control chickens in our study were gradually exposed to microbiota in the hours and days after hatch from different sources, such as the housing environment, litter, feed, and water. This colonization was delayed compared to the chickens inoculated with AM directly after hatch, as indicated by the clustering of caecal content profiles of 3-day-old controls with 1-day-old AM inoculated chickens, and of 7-day-old controls with 3-day-old AM chickens. This accelerated maturation of caecal microbiota composition has not only been observed in Aviguard studies (22, 55), but also in a study in which topical spray treatment of eggs with adult caecal content significantly altered broiler chicken microbiota immediately after hatch, and accelerated the normal microbiota development (69). As in our study, the effect on the caecal microbiota was highest at 3 days of age, and diminished over time (69). In contrast, swabbing of the egg surface once during incubation with diluted adult caecal content did not lead to significant differences in alpha diversity nor in the pattern of bacterial colonization between treated and control broiler chickens (70). This difference may be a result of the egg inoculation technique, suggesting that perhaps a lower number of spores and vegetative cells was applied to the eggshell in the latter study.

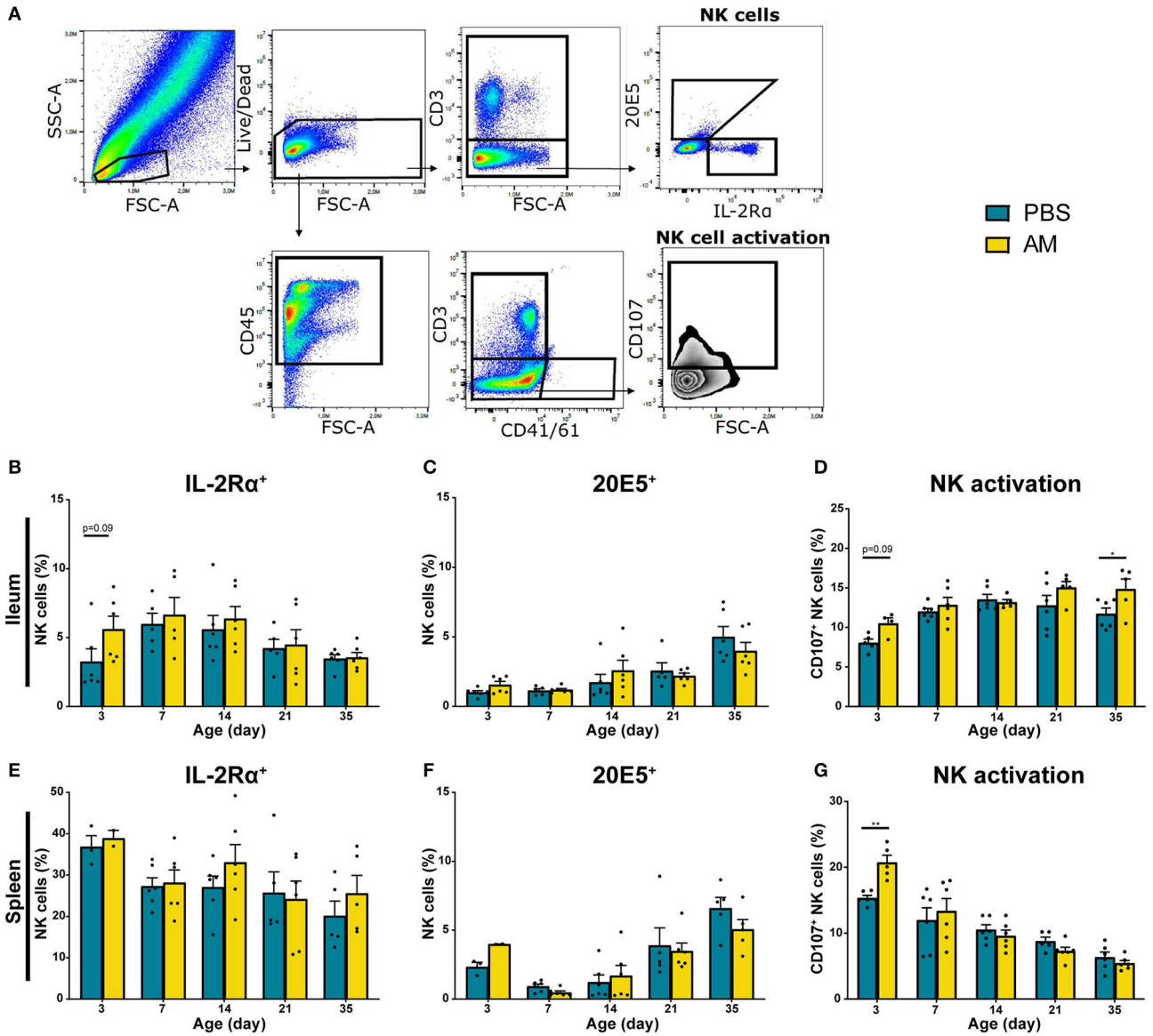

FIGURE 4 | Effect of adult microbiota (AM) on NK cells in broiler chickens. **(A)** Gating strategy after isolation of lymphocytes from IEL to analyze NK cell subsets and activation. **(B,E)** Percentages of NK cell subsets by characterization of surface markers IL-2Rα, **(C,F)** 20E5 during aging in **(B–D)** IEL and **(E–G)** spleen. **(D,G)** Percentages of NK cell activation during aging as assessed by measuring the surface marker CD107. Mean + SEM of chickens is shown ($n = 6$), however, chickens were excluded from analysis when numbers of events acquired in the gate of interest were < 100. Statistical significance is indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

Although many of the available poultry microbiota studies have focused on broiler chickens, its relation with the innate immune system has not previously been elaborately investigated. We observed an increase in IL-2Rα^{+} NK cells and activation of NK cells within the 1st days of life, together with an increase in relative numbers of cytotoxic CD8αα^{+} T cells from day 14 onwards in chickens that were inoculated with AM.

The increased NK cell activation observed in AM chickens may suggest a mildly increased cytotoxic capacity against potential pathogens, as the CD107 expression can increase up to 30% upon viral infections (33), which is more than 2-fold higher than the NK cell activation observed in this study. This result is in line with the observed increase in IL-2Rα^{+} NK cells in this study. Studies in humans have shown that increased IL-2Rα expression is associated with an early stage of NK cell activation (31), and this was also observed in chickens (32, 71, 72). In addition to the local effect on NK cell activation, our observation of increased splenic NK cell activation in 3-day-old AM chickens also indicates there is a systemic effect. No

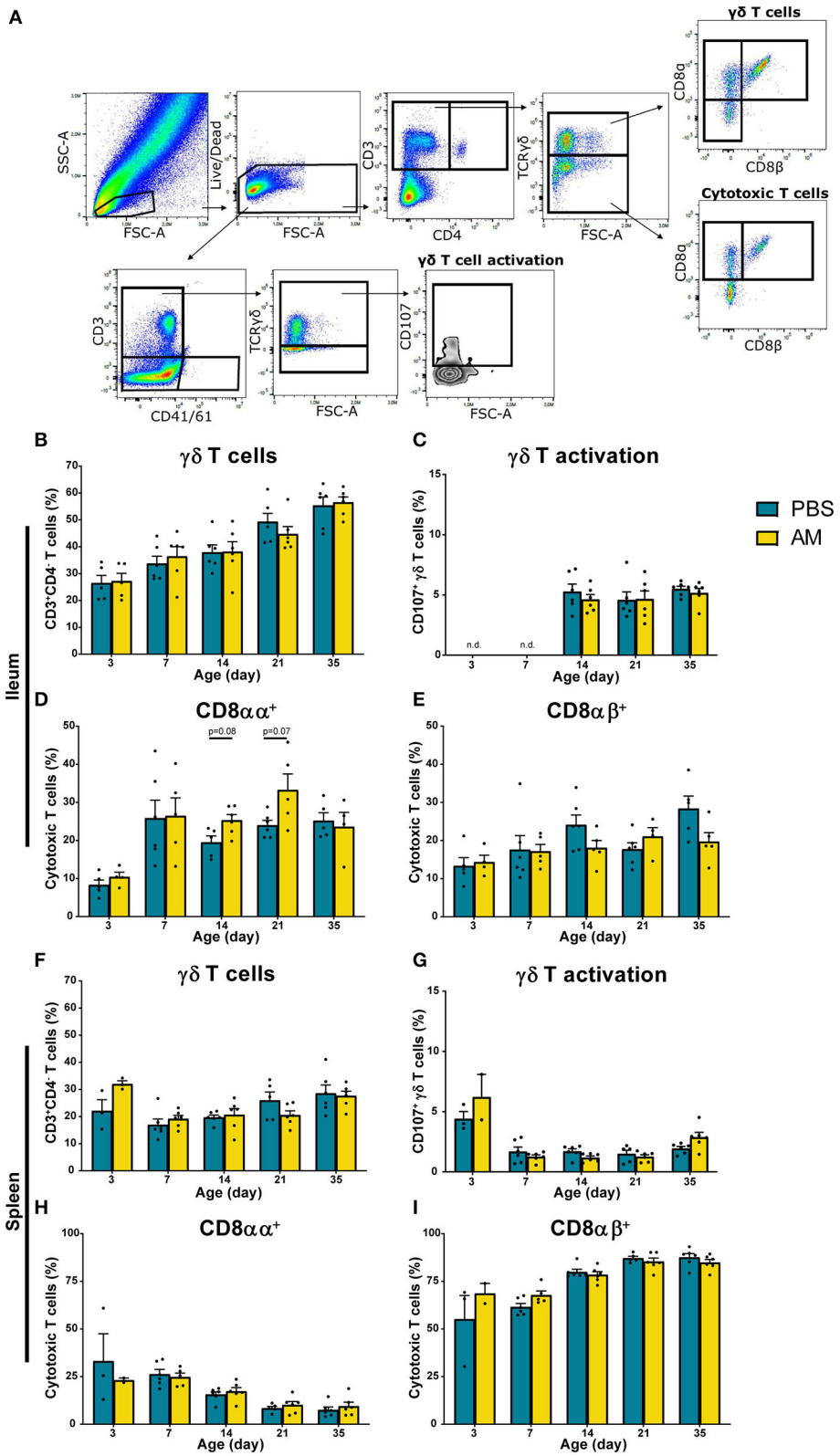

FIGURE 5 | Effect of adult microbiota (AM) on T cells in broiler chickens. **(A)** Gating strategy after isolation of lymphocytes from IEL to analyze T cell subsets and γδ T cell activation. **(B,F)** Percentages of total γδ T cells, subsets (data not shown) and **(C,G)** γδ T cell activation by characterization of surface markers TCRγδ and CD107

(Continued)

FIGURE 5 | during aging in **(B–E)** IEL and **(F–I)** spleen. **(D,H)** Percentages of cytotoxic T cell subsets using the surface markers CD8αα and **(E,I)** CD8αβ during aging. Mean + SEM of chickens is shown ($n = 6$), however, chickens were excluded from analysis when numbers of events acquired in the gate of interest were <100.

TABLE 2 | Statistical differences in relative (%) and absolute (cells/mg) numbers of intestinal immune cells between caecal microbiota clusters.

Immune cells	Cluster A vs. B	Cluster B vs. C	Cluster A vs. C
IL-2Rα$^+$ NK (%)	**0.026**	**0.044**	0.068
20E5$^+$ NK (%)	0.124	**3.0e^{-4}**	**0.001**
CD107$^+$ NK (%)	**0.003**	**0.020**	**0.001**
CD8αα$^+$ T (%)	**0.001**	0.254	**4.1e^{-4}**
IL-2Rα$^+$ NK (cells/mg)	**0.011**	0.051	**2.7e^{-4}**
20E5$^+$ NK (cells/mg)	**0.039**	**5.3e^{-7}**	**2.1e^{-6}**
CD107$^+$ NK (cells/mg)	0.398	**4.0e^{-6}**	**1.1e^{-4}**
CD8αα$^+$ T (cells/mg)	**0.008**	**9.5e^{-6}**	**6.4e^{-4}**

Significant differences are indicated in bold.

effects of AM inoculation on immune cells in the blood were observed.

The observed differences between AM and control chickens with respect to immune parameters suggest an interaction between microbial and immune development. This was further substantiated by the significant associations between IL-2Rα$^+$ NK cells, CD107$^+$ NK, and CD8αα$^+$ T cells and caecal microbiota clusters: cluster A includes chickens with a starting microbiota, cluster B chickens in the middle of the maturation process and cluster C chickens with a more matured successive microbiota composition from day 14 onwards. These clusters follow the successional patterns of microbiota development as previously described for broiler chickens, with bacterial community richness increasing rapidly over time and stabilizing from day 14 onwards (5–7). Our analyses showed that cluster B was associated with an increase in IL-2Rα$^+$ NK cells and an enhanced NK cell activation regardless of treatment. This suggests that the accelerated microbiota colonization due to AM inoculation affected the development of NK cells locally and systemically. Interestingly, the IL-2Rα$^+$ NK cell subset was higher in relative numbers in cluster B compared to the starting microbiota cluster A, but subsequently decreased in relative numbers in the more mature microbiota cluster C. The 20E5$^+$ NK cell subset and NK cells that express CD107 further increased in relative numbers between cluster B and cluster C. This fits with the observation in mammals that an increase in IL-2Rα expression is associated with an early stage of NK cell activation, which is followed by enhanced NK cell mediated killing. Cluster C was associated with an increased relative number of intestinal cytotoxic CD8αα$^+$ T cells. As the caecal microbiota in this cluster shows a matured composition similar in AM and control chickens of the same age, this suggests that early life inoculation with AM also affected the adaptive immune development in the intestine.

Although these results indicate associations between early life microbiota colonization and immune system development, the data from this study cannot elucidate exactly how these processes are related. As has been shown in humans and mice, microbiota can signal to immune cells in various ways either locally or systemically (46, 73). Locally, microorganisms interact directly with NK cells via TLRs and NCRs resulting in cytokine production by NK cells, and indirectly via cytokine production of resident myeloid or epithelial cells that consequently affect NK cell responses (46, 74). Systemically, microbiota can induce instructive signals to non-mucosal antigen-presenting cells and by producing among others IL-15, TNFα and IFN, subsequently prime optimal splenic NK cell responses (73). Since chicken NK cells have been shown to express TLRs (75) and NCRs (76, 77), the interactions between microbiota and NK cells probably follow similar routes to those in humans and mice.

In mammals, specific commensal bacterial strains have been linked to modulation of NK cells. Several reports established that bacteria within the *Lactobacillus* genus can induce IFNγ and cytotoxicity responses in intestinal NK cells as a result of IL-12 production by dendritic cells after TLR engagement with bacteria (43, 78, 79). Furthermore, *Bacteroides fragilis* can stimulate innate and adaptive immune pathways directly through TLR signaling and indirectly by inducing cytokine production (80). Although we did observe significant differences in the relative abundance of genera between AM and control chickens at day 1 and 3, we cannot pinpoint a specific genus responsible for the observed effect on NK cells. Interestingly, the genus *Bacteroides* showed a significantly higher prevalence and relative abundance in 3- and 7-day-old AM chickens and the genus was absent in control chickens of similar age. This could suggest that the observed effects on NK cells in 3-day-old AM chickens may be linked to a higher presence of *Bacteroides* bacteria as shown previously (80). We did not find differences in the prevalence of *Lactobacillus* bacteria due to AM inoculation. Other genera that showed significant differences in their prevalence and/or relative abundance between AM and control chickens at 1 and 3 days of age have not been described as specifically interacting with NK cells.

In addition, microbiota has been shown in mice and humans to interact directly with γδ T cells, and increased frequencies of CD8$^+$ γδ T cells and γδ T cell activation were observed during intestinal inflammation (51, 81). Under non-inflammatory conditions similar to those of our study, application of adult caecal content on eggs altered and accelerated the microbiota of 3-day-old chickens but did not affect γδ T cells in caecal tonsils (69). Furthermore, AM inoculated chickens in our study showed an increased presence of intestinal CD8αα$^+$ T cells at 2 and 3 weeks of age. Although in previous studies with mice no CD8αα and CD8αβ subsets were investigated, microbiota was shown to have direct (53) and indirect (82) effects on cytotoxic T cells, as IFNγ production was induced.

Further research including challenge models is needed to answer the question if chickens with an accelerated maturation of intestinal microbiota and enhanced NK cell responses early in life are indeed more resilient against infections.

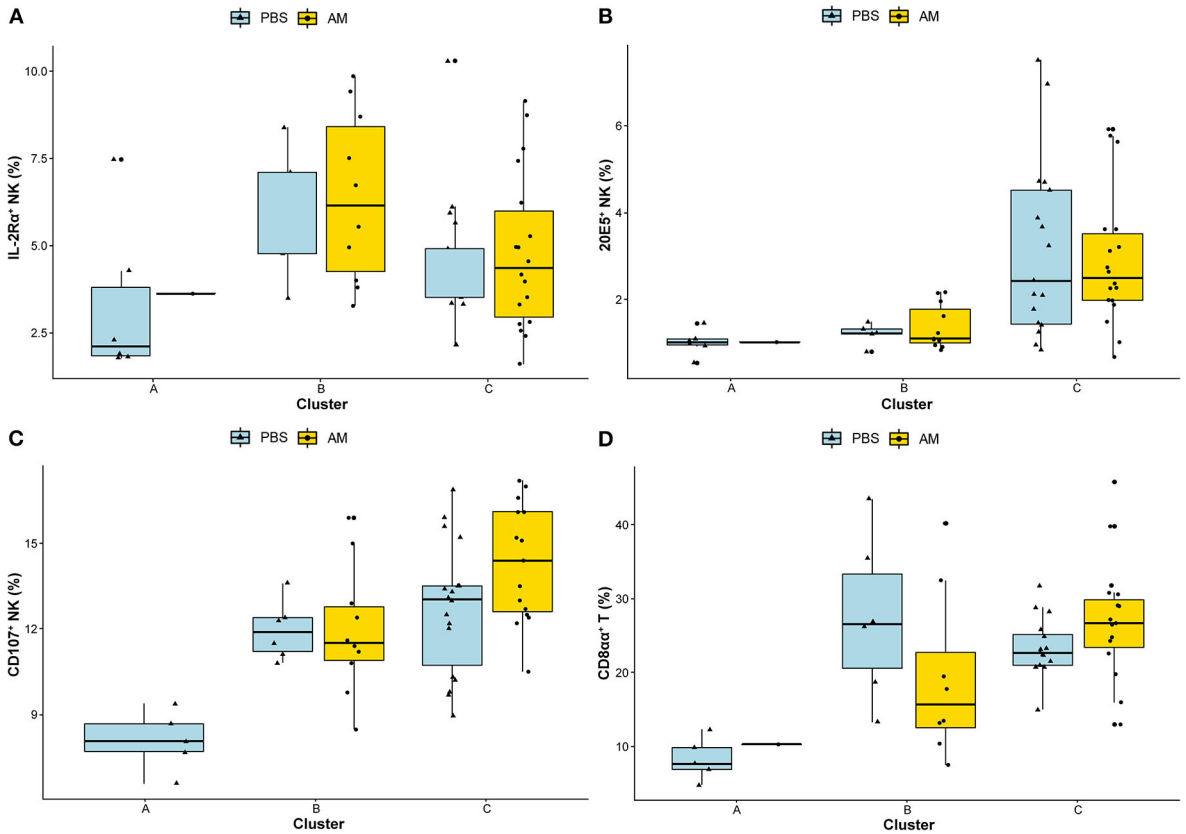

FIGURE 6 | Associations between caecal microbiota clusters and immune cells. Associations between the identified DMM clusters of caecal microbiota composition and relative numbers of intestinal NK cell subsets **(A)** IL-2Rα$^+$, **(B)** 20E5$^+$, **(C)** CD107$^+$, and **(D)** cytotoxic CD8αα$^+$ T cells were analyzed using Wilcoxon rank-sum test. Adjusted *p*-values (<0.05) were corrected for multiple testing with BH.

Interestingly we observed a relation between changes in caecal microbiota and intestinal NK cell responses. It would have been highly interesting to investigate the interaction between immune system and microbiota at caecum level, but unfortunatelythis was not possible since only few NK cells can be obtained from the caecum in young chickens (83, 84). Although we set out to analyze the relation between NK cells and microbiota composition in the ileum, we did not observe differences between treatment groups at any age in the phylogenetic diversity of ileal microbiota nor in the relative abundances of genera. Not being able to show a difference at ileal level, especially considering the relatively small number of chickens at each time point, was not surprising, and exactly the reason why we also collected caecal content. Nevertheless, the shift in microbiota composition as measured in the caeca showed that the AM treatment has successfully affected microbiota development in parts of the intestinal tract. For the AM treatment to be able to alter caecal microbiota composition, the microbiota of the AM product at least must have passed, and to some extent may have colonized upstream parts of the intestinal tract as well, albeit not inducing a measurable shift in microbiota composition in ileum. Therefore, we expect that the observed effects on ileal NK cells are associated with the AM treatment.

In conclusion, our study showed a relation between an accelerated maturation of intestinal microbiota and the enhanced NK cell response early in life. This interaction between microbiota and the developing innate immune system indicates possibilities in developing strategies to improve health and resilience of broiler chickens. One such possibility is through feed interventions or the use of products with adult-derived microbiota directly after hatch, both of which can affect microbiota composition and may accelerate microbiota maturation. Consequently, this can strengthen the innate immune system, conferring direct protective effects early in life as well as influencing adaptive immunity later in life. The combination of a well-developed microbiota and immune system may result in more robust broiler chickens with higher resilience against health challenges, such as disturbances in gut health and invading pathogens. Future research including challenge studies are warranted to test this hypothesis.

DATA AVAILABILITY STATEMENT

Raw sequence data were deposited into the Sequence Read Archive (SRA) at NCBI under accession number PRJNA670739.

ETHICS STATEMENT

The animal study was reviewed and approved by the Dutch Central Authority for Scientific Procedures on Animals and the Animal Experiments Committee.

AUTHOR CONTRIBUTIONS

NM, JK, FV, DL, JO, HS, JS, VR, and CJ contributed to the conception, design of the study, drafting, and critically revising it for important intellectual content. NM, JK, FV, DH, and CJ contributed to acquisition of data. NM and JK performed the analysis of data. FV and CJ supervised the work. All authors approved the final version to be submitted.

FUNDING

This work was part of the research programme of NWO Earth and Life Sciences (ALW) with project number 868.15.020, which is financed by the Dutch Research Council (NWO) and by Cargill Animal Nutrition and Health.

ACKNOWLEDGMENTS

We thanked the animal caretakers of the department Population Health Sciences, division Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, for their help during the animal experiments. We acknowledged R. H. G. A. van den Biggelaar and I. S. Ludwig for their help during the isolation of immune cells. We acknowledged G. J. A. Arkesteijn for maintaining optimal working conditions of the Flow Cytometry and Cell Sorting Facility, Faculty of Veterinary Medicine, Utrecht University. We thanked M. van Gaal and I. Heikamp-de Jong (Laboratory of Microbiology, Wageningen University & Research) for their help with molecular analyses of microbial composition.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.584561/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 02 December 2020
doi: 10.3389/fvets.2020.581342

A Pilot Study to Investigate the Feasibility of a Multiple Locus Variable Number Tandem Repeat Analysis to Understand the Epidemiology of *Dichelobacter nodosus* in Ovine Footrot

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 08 July 2020
Accepted: 09 November 2020
Published: 02 December 2020

Citation:
Giebel K, Green LE and Purdy KJ (2020) A Pilot Study to Investigate the Feasibility of a Multiple Locus Variable Number Tandem Repeat Analysis to Understand the Epidemiology of Dichelobacter nodosus in Ovine Footrot. Front. Vet. Sci. 7:581342. doi: 10.3389/fvets.2020.581342

Dichelobacter nodosus is the essential pathogen in ovine footrot, an important cause of lameness in sheep that reduces productivity and welfare. The aim of this study was to investigate the feasibility of using multiple locus variable number tandem repeat analysis (MLVA) developed to investigate isolates to understand the molecular epidemiology of *Dichelobacter nodosus* in ovine footrot by investigation of communities of strains. MLVA sensitivity was improved by optimizing PCR conditions to 100% specificity for *D. nodosus.* The improved MLVA scheme was used to investigate non-cultured DNA purified from swabs (swab DNA) and cultured DNA from isolates (isolate DNA) from 152 foot and 38 gingival swab samples from 10 sheep sampled on four occasions in a longitudinal study. Isolate DNA was obtained from 6/152 (3.9%) feet and 5/6 yielded complete MLVA profiles, three strains were detected. Two of the three isolate strains were also detected in isolate DNA from 2 gingival crevice cultures. Complete MLVA profiles were obtained from swab DNA from 39 (25.7%) feet. There were 22 *D. nodosus* community types that were comprised of 7 single strain and 15 multi-strain communities. Six community types were detected more than once and three of these were detected on the same four sheep and the same two feet over time. There were a minimum of 17 and a maximum of 25 strain types of *D. nodosus* in the study. The three isolate strain types were also the most frequently detected strain types in swab DNA. We conclude that the MLVA from swab DNA detects the same strains as culture, is much more sensitive and can be used to describe and differentiate communities and strains on sheep, feet and over time. It is therefore a sensitive molecular tool to study *D. nodosus* strains directly from DNA without culture.

Keywords: *Dichelobacter nodosus*, footrot, sheep, MLVA, veterinary epidemiology, bacteriology, PCR

INTRODUCTION

Footrot (FR) is the most common cause of lameness in sheep in the UK and it is a health and welfare concern in sheep flocks globally (1–4). Footrot reduces productivity and sustainability of sheep farming (5, 6), costing the UK industry £20–£80 million per annum (6, 7).

There are two clinical presentations of footrot, interdigital dermatitis (ID), characterized by inflammation of the interdigital skin, and severe footrot (SFR), characterized by separation of the hoof horn from underlying tissues (8). The essential pathogen in footrot is the fastidious gram negative, aerotolerant, anaerobic bacterium *Dichelobacter nodosus* (9, 10) which is key in initiation of ID and in progression to SFR (11–13). *D. nodosus* is present in >90% of UK sheep flocks and causes ∼70 % of lameness (4).

In cross-sectional studies, *D. nodosus* has been detected on healthy and diseased feet (11, 14–18), and in the gingival cavity (19).

Russell et al. (20) developed an MLVA assay as a strain-typing tool for cultured *D. nodosus* isolates based on four polymorphic loci (DNTR02, 09, 10, and 19). The assay was used by Smith et al. (21) to investigate within-flock population dynamics of strains of *D. nodosus*. They reported that *D. nodosus* strains clustered within sheep and were transmitted between ewes over time. *D. nodosus* isolation is challenging and time consuming, because of the organism's fastidious and anaerobic nature and direct PCR from swab DNA is more sensitive than culture (15, 22).

Muzafar et al. (23) used the MLVA developed by Russell et al. (20) to analyse *D. nodosus* directly from DNA extracted from interdigital skin swabs of healthy and footrot affected feet using only DNTR10 and DNTR19. They did not use DNTR09 due to poor amplification nor DNTR02 due to non-specific amplification.

Therefore, the aim of this study was to optimize and validate the full *Dichelobacter nodosus* MLVA for isolate DNA and community DNA and investigate its value in a pilot longitudinal study of persistence of *D. nodosus* in sheep.

MATERIALS AND METHODS

Sample Collection

In 2014, swab samples were collected in a longitudinal study of 10 sheep (5 ewes, 5 lambs) from a UK flock with footrot. All sheep were sampled on four occasions at 2-week intervals from May to June. On each occasion, all four feet and the gingival crevice were swabbed with two swabs each, one for DNA analysis and one for culture. In addition, foot lesions were scored for ID and SFR using two 5-point scales (15). A total of 152 foot and 38 mouth swabs were collected.

Isolation and Detection of *Dichelobacter nodosus*

Swabs for culture were inoculated onto 4% hoof agar (HA) followed by subculture onto a 2% HA (15). Plates were incubated under anaerobic conditions at 30°C for 4–5 days (MACS-MG-1000 anaerobic workstation, Don Whitley Scientific, Shipley, UK, 80% N_2, 10% CO_2, 10% H_2). Isolate DNA was extracted with a DNeasy Blood and Tissue Kit (Qiagen Ltd., Manchester,

TABLE 1 | Input and recovery of MLVA PCR amplicons and peak sizes in *Dichelobacter nodosus* isolates and model communities.

***D.nodosus* isolates**	**Size (bp)**	**Peak size (RFU)***		**Size (bp)**	**Peak size (RFU)**		**Size (bp)**	**Peak size (RFU)**		**Size (bp)**	**Peak size (RFU)**	
		DNTR02			**DNTR09**			**DNTR10**			**DNTR19**	
		Individ	**Comm**		**Individ**	**Comm**		**Individ**	**Comm**		**Individ**	**Comm**
Community 1												
VCS 1703A	545	15,530	209**	985	11,722	2,323**	693	9,795	182**	851	7,855	219**
JIR3918	610	8,091	2,950	768	28,924	16,045	835	22,117	483**	1,019	3,692	1,621
JIR3919	650	13,779	2,681	876	18,892	11,636	646	10,793	101**	1,019	2,078	1,621
JIR3350	560	16,543	4,975	985	23,681	12,323	505	25,910	8,144	932	6,326	1,178
Community 1 (diluted)												
VCS 1703A (1:5)	545	13,092	85**	985	11,812	1,205**	693	5,382	138**	851	8,624	372**
JIR3918	610	8,091	4,034	768	28,924	16,045	835	22,117	1,158	1,019	3,692	3,384
JIR3919	650	37,779	4,501	876	18,892	11,363	646	10,793	3,786	1,019	2,078	3,384
JIR3350	560	16,543	1,597	985	23,681	1,205**	505	25,910	3,030	932	6,326	242**
Community 2												
VCS 1703A	545	15,530	150**	985	11,812	5,194	693	5,382	339**	851	8,627	129**
JIR3918	610	8,091	1,874	786	28,924	10,421	835	22,117	2,322	1,019	3,692	872
4303 LBV	635	9,853	705**	985	19,638	5,194	788	/***	687	1,019	2,714	872
BS8	555	18,255	4,529	985	9,345	5,194	835	3,475	2,322	933	5,191	1,814

**Peak size (RFU), the size of the peak observed in relative fluorescent units; Individ, peak size for a single strain; Comm, Peak size of the strain in a mixed model community. **Recovered products that fall below the established threshold, ***No PCR product detected.*

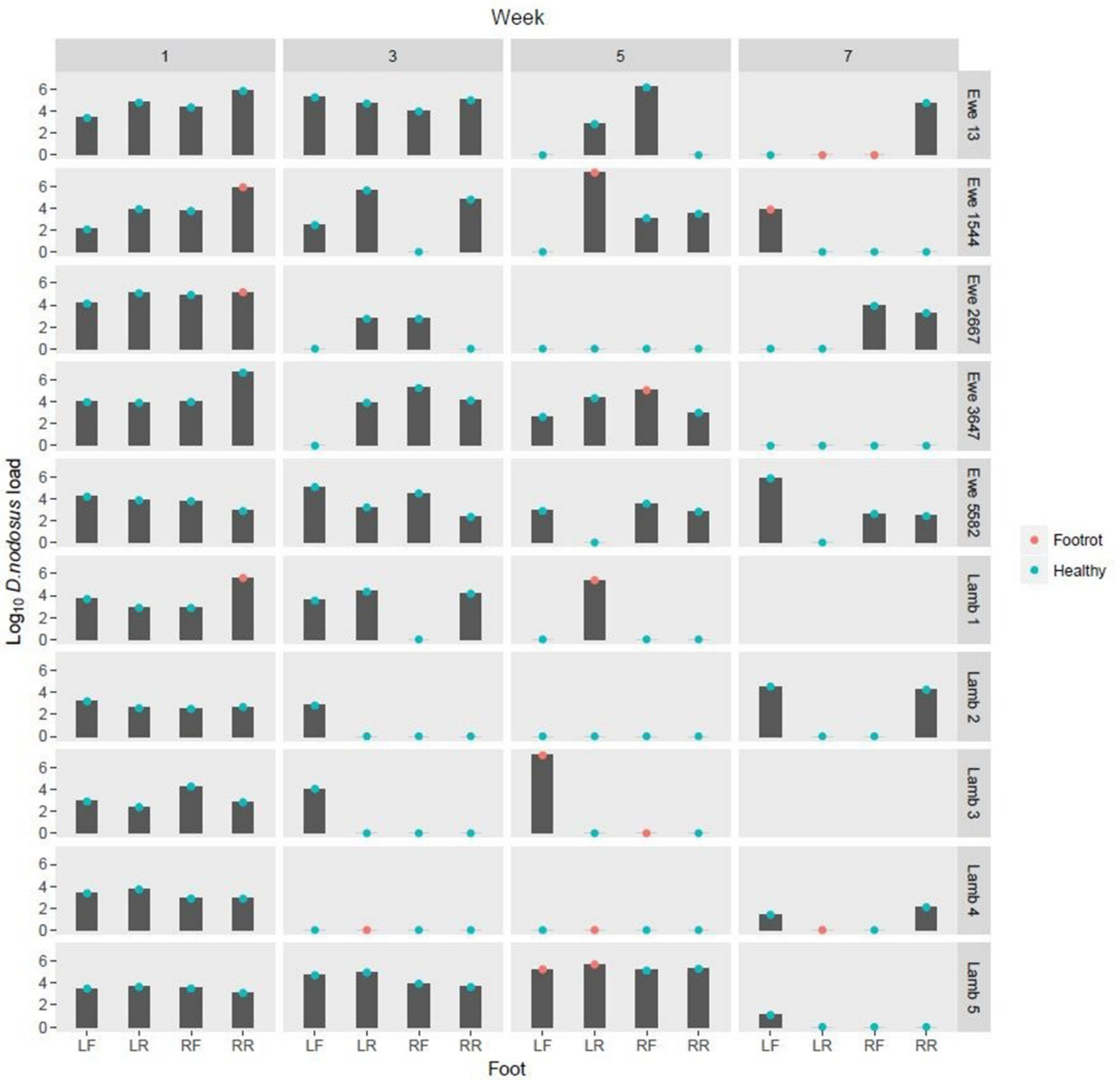

FIGURE 1 | Log_{10} *Dichelobacter nodosus* load on DNA swabs from feet from weeks 1, 3, 5, and 7. Green dots: Healthy foot (Interdigital dermatitis score 0 or 1, footrot score 0) Red dots: Foot classed as diseased with footrot (Interdigital dermatitis score >1 and/or footrot score >0) LF, Left front; LR, left rear; RF, Right front; RR, Right Rear.

United Kingdom) according to the manufacturer's instructions with a lysis time of 1 h. DNA was extracted directly from swabs using the hydroxyapatite spin-column method (24) using only 0.5 ml of the sodium phosphate extraction buffers. *D. nodosus* was detected in DNA extracted from the foot and mouth swabs using a *D. nodosus*-specific *rpoD*-targeted qPCR (25).

MLVA Optimization and Protocol

The sensitivity of the MLVA primers (**Supplementary Table 1**) was determined using DNA from *D. nodosus* isolates and swab samples. Improvements in sensitivity were made by changing the PCR Master mix (From Promega x2 PCR Master Mix to Bioline MyTaq^{TM} Red Mix), increasing primer concentration (from 10 pmoles of each primer in a 50 μl reaction to 10 pmoles of each primer in a 25 μl reaction), DNA template concentration (from 1 μl/50 μl reactions to 1 μl/25 μl reactions) and the number of PCR cycles (from 30 to 40 cycles). The final protocol was, in 25 μl reactions; 12.5 μl MyTaq^{TM} Red Mix (Bioline, London, United Kingdom), 1 μl of each primer (10 μM stock concentration; **Supplementary Table 1**), 1 μl bovine serum

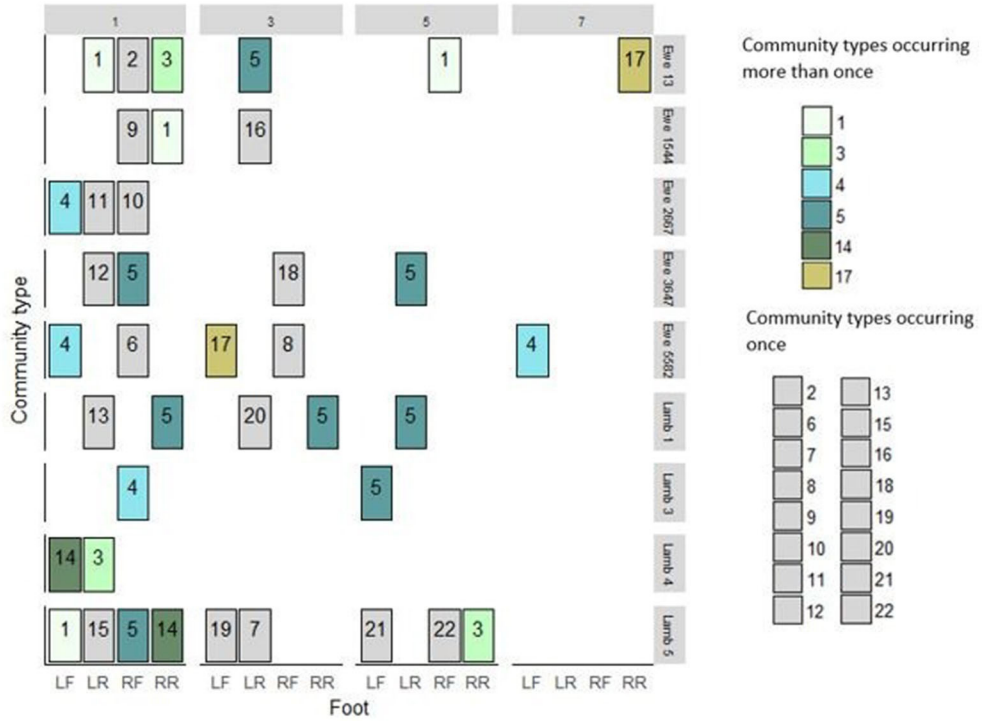

FIGURE 2 | *Dichelobacter nodosus* community profiles from 39 feet of 9 sheep from weeks 1, 3, 5, and 7. Community types in green shades were detected repeatedly. Community types in gray occurred only once in the study. LF, Left front; LR, left rear; RF, Right front; RR, Right Rear.

albumin (20 mg ml^{-1}) (Sigma Aldrich, Dorset, United Kingdom) and 1 μl of DNA template. DNA from *D. nodosus* strain 4303 LBV and nuclease free H_2O were used as positive and negative controls, respectively. All PCR reactions were carried out on an Eppendorf Mastercycler ep gradient machine (Eppendorf, Hamburg, Germany) using the following cycling conditions: One cycle of 95°C for 2 min, 40 cycles of 95°C for 1 min, 59°C for 30 s and 72°C for 1 min with a final extension of 72°C for 2 min. PCR products were visualized by ethidium bromide-stained agarose gel electrophoresis and imaged using a Gene Flash imager (Syngene Bio Imaging, Cambridge, United Kingdom).

Determination of VNTR Amplicon Size Using Fragment Analysis

MLVA amplicon size was determined using fragment analysis. Forward primers for the four loci were labeled with different fluorescent dyes (**Supplementary Table 1**) and amplicons for each locus were submitted separately for fragment analysis (DNA Sequencing and Services, University of Dundee, Scotland). PCR products from *D. nodosus* isolates were diluted 1:100 and products originating from swabs were diluted either 1:20 or 1:100 depending in the PCR band intensity seen on agarose gel. 1200 Liz dye (Applied Biosystems, Warrington, United Kingdom) was used as a size standard and data were analyzed using Peak Scanner? Software (Applied Biosystems, Warrington, United Kingdom). The bin range was set to 4bp (fragment size ± 2 bp) and minimum fragment length cut off values 500, 500, 400, and 550 bp for DNTR02, 09, 10, and 19, respectively, based on the length of each fragment without repeats.

To provide additional information on the accuracy of the assay, the 4 loci were amplified from *D. nodosus* strain 1703A (GenBank Accession number CP000513) and submitted for fragment analysis and Sanger sequencing. The size of the loci from fragment analysis was compared with the published 1703A sequences for the 4 loci (Genebank Accession numbers KC676717, KC676718, KC676719, and KC676720 for DNTR02, 09, 10, and 19, respectively). The sequenced VNTR loci from *D. nodosus* strain 1703A were analyzed using tandem repeat (TR) finding software (26) to determine the numbers of repeats in the sequence.

MLVA analysis of two isolates resulted in a primary peak and a number of small secondary peaks ≤ 20% the height of the primary peak at expected TR intervals (**Supplementary Figure 1**). To investigate secondary peaks, 14 more *D. nodosus* isolates were analyzed (**Supplementary Table 2**). Secondary peaks were observed in some, but not all, isolates. The secondary peaks might have been due to non-axenic *D. nodosus* cultures. However, this is unlikely because multiple peaks were detected at all 4 loci (**Supplementary Figure 1**) which would indicate many isolate strains in each culture. Other explanations for secondary peaks might be rapid, minor, within-strain variation or an artifact in the PCR process. It was not possible to adjust the PCR to prevent the formation of these small secondary peaks, therefore it was

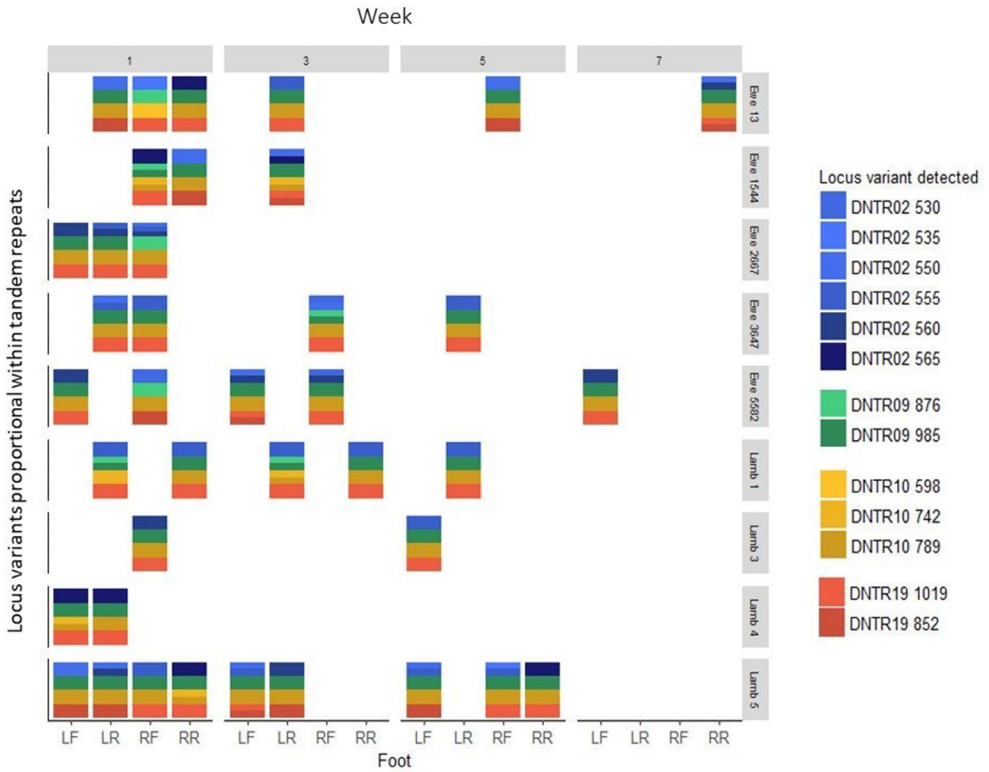

FIGURE 3 | *D. nodosus* MLVA DNTR = *D. nodosus* tandem repeat 02, 09, 10, 19, variants proportional within TR from DNA from swab samples from 39 feet from 9 sheep in weeks 1, 3, 5, and 7. LF, Left front; LR, left rear; RF, Right front; RR, Right rear. Four colors, each of one shade, indicates a single strain.

decided to consider secondary peaks $\leq$ 20% the height of the primary peak as artifacts (**Supplementary Figure 2**).

Validation of the MLVA PCR and Testing on Model Communities

Primer specificity was tested by MLVA analysis of DNA from non-target organisms previously detected on sheep feet or present in soil or feces. These were *Streptococcus uberis, Staphylococcus epidermis, Staphylococcus intermedius, Staphylococcus aureus, Staphylococcus hyicus, Staphylococcus chromogenis, Streptococcus dysgalactidae, Streptococcus agalactidae, Mannheimia spp., Fusobacterium necrophorum, Pseudomonas aeruginosa, Escherichia coli,* and *Mycobacterium tuberculosis.*

To confirm specificity PCR products from 10 *D. nodosus* positive samples from foot swabs and 1 gingival swab were analyzed using MLVA and the resulting VNTR amplicons were submitted for Sanger sequencing (GATC Biotech AG, Cologne, Germany). Sequences were assessed for quality using CodonCode Aligner version 6.0.2. and analyzed using BLAST (27).

MLVA sensitivity was investigated by assessing its limit of detection of *D. nodosus* load. *D. nodosus* strain 4303 LBV was incubated on Eugon Agar (28) for 5 days. After incubation 1 ml of PBS, pH 7.4, was added to the *D. nodosus* plates, creating a cell suspension. Suspended cells were quantified using a Petroff-Hausser counting chamber (Hausser Scientific, PA, USA) and serially diluted. Sterile swabs were spiked with 50 µl of the serially diluted cell suspension resulting in 1.07×10^6 to 1.07 *D. nodosus* cells per swab (equivalent to *rpoD* copies per sample). DNA was extracted from these swabs as above. Samples were then screened for *D. nodosus* using the MLVA assay. To test whether detection could be improved further, all samples were submitted to a second round of the MLVA PCR assay using the same cycling conditions.

The feasibility of using MLVA to analyse multistrain communities was investigated using model communities created from eight *D. nodosus* isolates with different MLVA profiles. Two model communities (1 and 2) were created, each with 4 isolates (**Table 1**). DNA from each isolate was standardized to a concentration of 15 ng/µl and 5 µl of DNA from each isolate mixed in a 1:1:1:1 ratio. A third model community was created with the isolates in community 1, with one isolate (*D. nodosus* 1703A) diluted 5-fold to investigate whether non-dominant isolates could be detected. These model communities were amplified and analyzed using the MLVA protocol above.

Assessment of the MLVA Assay on DNA Extracted From Foot and Gingival Swab Samples

Swab DNA and isolate DNA from feet and gingivae from the longitudinal study that were positive for *D. nodosus* by

TABLE 2 | MLVA *Dichelobacter nodosus* strain types from swab DNA from the feet of sheep by definitely and possibly present.

MLVA strain type	Frequency definitely present	Frequency possibly present
A	4	2
B	1	2
C*	10	12
D*	7	9
E*	8	5
F	2	5
G	2	8
H	5	10
I	1	4
J	1	1
K	0	1
L	0	1
M	1	2
N	1	1
O	1	1
P	0	1
Q	1	2
R	1	1
S	0	1
T	0	1
U	0	1
V	0	2
W	1	2
X	0	1
Y	1	1

*Strain also detected in culture isolates.

rpoD qPCR were analyzed using MLVA and fragment analysis. Secondary peaks $\leq$ 20% the height of the primary peak were excluded (**Supplementary Figure 2**). Distinct community fingerprints were obtained that could be compared visually (**Figure 1**). In addition, strain types within a community could be defined as "definitely present" when a community had only one variant at each locus or when more than 1 variant was detected at one locus only and one variant at the other three loci. When there were 2 or more variants at 2 or more loci strains were defined as "possibly present." The minimum and maximum number of strain types in a community were calculated using the formulae:

Minimum number of strains
= number of variants at the most variable locus
Maximum number of strains
= product of the number of variants at each locus

RESULTS

Validation and Optimisation of the MLVA Scheme

The number of TRs at the four loci for strain 1703A was the same as reported by Russell et al. (20). The MLVA assay was specific for *D. nodosus* with no amplification in any of the 4 loci in non-target species (**Supplementary Figure 3**). DNA sequencing of the amplified products from foot swabs were 99–100% similar to their target sequences confirming specificity of the MLVA PCR. The mouth swab DNA yielded products for three MLVA loci, DNTR02, 09 and 10, which were 96–97% similar to their target sequence. The improved detection limit of the MLVA protocol after a single round of PCR was 10^2,10^3, 10^2, and 10^3 copies μl^{-1} DNA template for DNTR02, DNTR09, DNTR10, and DNTR19, respectively. A second round of MLVA PCR resulted in non-specific amplification and diluting samples did not improve specificity or sensitivity (data not shown).

VNTR Amplicons From *Dichelobacter nodosus* Model Communities

All *D. nodosus* strains in the model communities were detected, including community 3 where one strain was at 5 fold dilution; 17/48 secondary peaks were $\leq$ 20% the height of the primary peak and excluded (**Table 1**); 11 of these 17 were from the VCS strain, which was only detected correctly once, suggesting that this laboratory strain is particularly difficult to detect using MLVA.

Longitudinal Study of Persistence of *D. nodosus* in the Epidemiology of Footrot

FR was detected at least once on the feet of 8/10 sheep (**Figure 1**). *D. nodosus* was detected by qPCR in 97/152 (63.8%) foot swab DNA samples. It was detected on both healthy and diseased feet and on all sheep, but not all weeks (**Figure 1**).

Out of the 97 *D. nodosus* positive foot swab DNA samples, 53 (54.6%) amplified all 4 MLVA loci and in 39/53 (73.6%) samples complete MLVA profiles were obtained (**Figure 2**). The 39 complete profiles had a total of 156 loci with 75/156 (48.1%) with secondary peaks. The total number of peaks in these 156 loci was 220. After application of the $\leq$ 20% threshold (**Supplementary Figure 2**) 106/220 (48.2%) peaks were excluded from further analysis. DNTR02 was the most variable locus with 6 TRs and DNTRs 09 and 10 were the least variable with only 2 TRs (**Figure 3**).

D. nodosus was detected by qPCR in 8/38 (21.1%) gingival swab DNA samples, however, no complete MLVA profiles were obtained. Three loci were amplified in one sample and these matched loci in community types 3 and 4.

Dichelobacter nodosus Community Profiles From Foot Swab DNA

There were more MLVA positive feet in week 1, and detections declined over the 4 visits (**Figure 3**). There were 22 *D. nodosus* community types in total; seven were single strains and fifteen were multistrain; six community types were detected more than once, three of these were detected on the same four sheep and the same two feet over time (**Figure 2**). Community type 5, a single strain community, was most frequently detected (8/39 feet) (**Figure 2**). The results indicate that the MLVA differentiated *D. nodosus* communities spatially between feet, sheep, and over time.

TABLE 3 | MLVA profile of *Dichelobacter nodosus. nodosus* from isolate DNA compared with swab DNA.

Sample origin	Sheep ID	Week of study	Locus fragment size/number of repeats				Isolate DNA strain type	Swab DNA strain types
Foot			DNTR02	DNTR09	DNTR10	DNTR19		
RR	13	1	565/10	985/5	789/8	1,019/5	C	C
LR	3,647	1	555/8	985/5	789/8	1,019/5	C	E, C**
RF	3,647	1	555/8	985/5	789/8	1,019/5	E	E
LF	5,582	1	560/9	985/5	789/8	1,019/5	D	D
RR	13	3	565/10	985/5	789/8	1,019/5	C	NS
LR	13	3	555/8	985/5	789/8	/	E*	E
Mouth	13	3	560,565/9–10	985/5	789/8	1,019/5	C, D	NS
Mouth	3	3	560,565/9–10	985/5	789/8	1,019/5	C, D	∧

*RR, Right rear; RF, Right front; LR, Left rear; LF, Left front. *DNTR19 did not amplify, but strain type E present in corresponding swab. **E, C = both strain types are definitely present, NS, no swab data for all 4 loci, ∧DNTR19 did not amplify, other VNTR's identical.*

Dichelobacter nodosus Strain Type Analysis From Foot Swab DNA

There were 17 strains definitely present and a further 8 possibly present in the 22 *D. nodosus* community types in foot DNA (**Table 2**). Seven strains were, definitely or possibly, present on more than 4 occasions and the remaining 18 strains were present on 1 to 3 occasions. The three most frequently detected single strains were C, D and E (**Table 2**), these were the single strain community types 3, 4, 5 (**Figure 2**).

Dichelobacter nodosus Strains and Communities From Isolate DNA

*D. nodosu*s was cultured from 6/152 (3.9%) foot swabs from 3 sheep in weeks 1, 3, and 5, with 5 complete and 1 partial MLVA profile obtained from these isolates. The three most frequently detected strains, C, D, and E (**Table 2**), were also detected in swab DNA from the same foot at the same time (**Table 3**). Complete MLVA profiles were obtained from 2 isolates from the gingival crevice and *D. nodosus* strains C and D (Community types 3 and 4) were detected in both samples (**Table 3**).

DISCUSSION

The optimized *D. nodosus* MLVA (20) was developed and used successfully to investigate *D. nodosus* isolates and communities from DNA extracted directly from swab samples. The variability in loci and community types in the longitudinal study indicate that the improved MLVA scheme is more sensitive than previous studies (21, 23) and can be used to improve understanding of the epidemiology of communities of *D. nodosus* on feet over time.

Smith et al. (21) investigated transmission and persistence of *D. nodosus* strains on sheep's feet over a 10 months period using the original assay (20) on isolates of *D. nodosus.* They reported 45 animal-level repeat *D. nodosus* isolation events, 47% of those were isolation events of the same strain from the same foot over time. In addition, they detected and isolated the population dominant strain. In this, albeit shorter study, we obtained similar results with 4 animal-level repeat detection of *D. nodosus* communities and one dominant single strain community (community 5) that was detected on the same foot consecutively.

There was a high level of variability in *D. nodosus* communities in our study, with most variation occurring in week 1. This is possibly attributable to rainfall on and preceding the day of sampling as wet weather facilitates persistence of *D. nodosus* (29). A high level of *D. nodosus* variability was also reported by Smith et al. (21) who isolated 87 MLVA types over 10 weeks, which suggest that a large number of strains are present in footrot affected flocks and suggest that our findings represent true variability.

Our optimized MLVA was used in a subsequent longitudinal study to investigate persistence of *D. nodosus* strains on feet; this demonstrated that *D. nodosus* strains persist on the feet of diseased sheep, but not on the feet of healthy sheep (29).

Only 6 isolates were cultured in this study using published *D. nodosus* isolation techniques, however, the strain types from culture isolates were a subset of the strains detected from the non-culture DNA (**Table 3**) at the same site and time, indicating that MLVA analysis of non-cultured DNA is more sensitive than DNA from culture, as reported by others for other bacterial species (30–32). This indicates that previous studies that have compared relationships between *D. nodosus* using MLVA profiles of isolates (21) are incomplete and analysis would have been improved using MLVA from DNA directly.

The MLVA scheme had a limit of detection of $\sim 10^3$ genome copies μl^{-1} of extracted DNA and so there were a number of samples that were positive for *D. nodosus* by qPCR and negative by MLVA. Therefore, even the MLVA is not 100% sensitive. Despite this, the ability to use MLVA on non-cultured DNA to identify strains as definitely and possibly present and to produce fingerprint profiles is novel and adds to the value of MLVA as a tool to investigate strains of *D. nodosus* on feet over time.

This is the first occasion that a *D. nodosus* strain profile has been obtained from the gingival crevice, although complete strain profiles were detected from isolates and only incomplete strain profiles were obtained directly from swab DNA. Strain types C and D were present in mouths and were also among the strains most frequently detected on feet (**Table 3**).

The presence of regular secondary peaks in fragment analysis when testing isolate DNA has not been reported previously,

although artifactual DNA extension during PCR has been reported but usually only for short sequences (33, 34). Further investigation of the secondary peaks was outside the scope of the current study and so a conservative threshold was applied to decrease the probability of artificially increasing the number of loci in a sample. As a consequence, it is possible that some non-dominant strains that were present at low abundance were classified as artifacts.

CONCLUSIONS

A sensitive and specific *D. nodosus* MLVA assay using four VNTR loci was validated and optimized for use on non-culture DNA. The strain types detected from isolate DNA from the same site were a subsample of those detected from swab DNA, but many more strains were present in swab DNA, indicating that it is more sensitive to analyse *D. nodosus* from DNA directly rather than via culture. Because the MLVA can be used to identify communities of *D. nodosus* on the feet of sheep over time it can be used to investigate persistence and transmission of communities of *D. nodosus* and so improve epidemiological understanding of *D. nodosus*. Other MLVA schemes may be developed for use in the non-culture based study other bacterial species.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Welfare & Ethical Review Body (AWERB), University of Warwick.

AUTHOR CONTRIBUTIONS

The work presented in this article was conducted by the KG. LG and KP supervised the research. All authors contributed to the article and approved the submitted version.

FUNDING

KG was funded by a Natural Environment Research Council studentship (NE/K007491/1). LG and KP were supported by BBSRC (BB/M012980/1).

ACKNOWLEDGMENTS

We thank Dr Ruth Kennan, Monash University for providing isolates of *D. nodosus* and Drs Ed Smith, Emma Monaghan, Rachel Clifton, and Jessica Taylor for their assistance with collection of samples from sheep and laboratory support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.581342/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 03 December 2020
doi: 10.3389/fvets.2020.552517

Emergence and Phylogenetic Analysis of a Getah Virus Isolated in Southern China

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Getah virus (GETV) has caused many outbreaks in animals in recent years. Monitoring of the virus and its related diseases is crucial to control the transmission of the virus. In the summer of 2018, we conducted routine tests on clinical samples from different pig farms in Guangxi province, South China, and isolated and characterized a GETV strain, named GX201808. Cytopathic effects were observed in BHK-21 cells inoculated with GX201808. The expression of E2 protein of GETV could be detected in virus-infected cells by indirect immunofluorescence assays. Electron microscopic analysis showed that the virus particles were spherical and ∼70 nm in diameter with featured surface fibers. The multistep growth curves showed the virus propagated well in the BHK-21 cells. Molecular genetic analysis revealed that GX201808 belongs to Group 3, represented by Kochi-01-2005 isolated in Japan in 2005, and it clustered closely with the recently reported Chinese strains isolated from pigs, cattle, and foxes. A comparison of the identities of nucleotides and amino acids in the coding regions demonstrated that the GX201808 showed the highest amino acid identity (99.6%) with the HuN1 strain, a highly pathogenic isolate resulting in an outbreak of GETV infection in swine herds in Hunan province in 2017. In the present study, GETV was identified and isolated for the first time in Guangxi province of southern China, suggesting that future surveillance of this virus should be strengthened.

Keywords: genetic analysis, phylogenetic analysis, isolation, emergence, Getah virus

OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
Santhamani Ramasamy, Public Health Research Institute (PHRI), United States
Wenjie Gong, Jilin University, China

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *16 April 2020*
Accepted: *04 November 2020*
Published: *03 December 2020*

Citation:
Ren T, Mo Q, Wang Y, Wang H, Nong Z, Wang J, Niu C, Liu C, Chen Y, Ouyang K, Huang W and Wei Z (2020) Emergence and Phylogenetic Analysis of a Getah Virus Isolated in Southern China. Front. Vet. Sci. 7:552517. doi: 10.3389/fvets.2020.552517

INTRODUCTION

Getah virus (GETV) is an enveloped, single-stranded positive-sense RNA virus. GETV is a member of the genus *Alphavirus* in the family *Togaviridae*, which are transmitted mostly by various mosquito species (1). The virus comprises a genome of ∼11.7 kb containing a 5′-untranslated region (UTR), two large open reading frames (ORFs), a 3′-UTR, and a poly-A tail (2). The ORF1 is situated at the 5′-end of the genome and encodes non-structural polyproteins (nsP1 to nsP4). The ORF2 is located at the 3′-end of the genome and encodes structural polyproteins that are transcribed into five structural proteins, namely, C, E3, E2, 6K, and E1, respectively (3, 4).

GETV has been shown to be distributed widely in the Asiatic, Australia, and Eurasian regions since the prototype GETV strain (MM2021) was first isolated from mosquitoes in Malaysia in 1955

(5–10). Sero-epizootiologic investigations showed that the virus is present in pigs, horses, goats, cattle, boars, and other animals including humans (9, 11–14), suggesting that the host range of the virus has expanded broadly. GETV infections can cause fever, rashes, and edema of the hindlegs in horses (15), fetal death and reproductive disorders in pigs (16) as well as fever, anorexia, depression, neurologic symptoms, and death in foxes (17). GETV infections in horses and pigs have been reported several times in Japan since the 1970's (18–21), and the outbreak of GETV infections in horses were reported in 1990 in India (22). In China, GETV is widely distributed in 15 provinces ranging from the southwest to northern areas of China since it was first identified from mosquitos in Hainan province, and it has caused several outbreaks in animals in recent years (2, 7, 17, 23–28). In 2017, an outbreak of GETV infection was reported in swine herds in Hunan province, China, resulting in the death of ∼200 piglets and reproductive disorders of more than 150 pregnant sows (25). Lethal infections in blue foxes caused by GETV were also reported in Shandong province, East China (17). The latest GETV outbreak in racehorses occurred in Guangdong province, South China, in 2018 (27). Recently, serum samples from beef cattle showing sudden onset of fever have occurred in GETV-positive animals (26).

TABLE 1 | Detailed information on the GETV strains in this study.

Strain	GenBank number	Year	Host	Country
MM2021	AF339484	1955	*C. gelidus*	Malaysia
Sagiyama virus	AB032553	1956	Mosquito	Japan
M1	EU015061	1956	*Culex* sp.	China
MI-110-C2	LC079087	1978	*Equus caballus*	Japan
MI-110-C1	LC079086	1978	*Equus caballus*	Japan
LEIV 17741 MPR	EF631999	2000	*Culex* sp.	Mongolia
LEIV 16275 Mag	EF631998	2000	Mongolia	Russia
HB0234	EU015062	2002	*Culex tritaeniorhynchus* Giles	China
South Korea	AY702913	2004	Swine	South Korea
YN0540	EU015063	2005	*Armigeres subalbatus*	China
Kochi/01/2005	AB859822	2005	*Sus scrofa*	Japan
HNJZ-S1	KY363862	2011	Pig	China
YN12031	KY434327	2012	*Armigeres subalbatus*	China
YN12042	KY450683	2012	*Culex tritaeniorhynchus* Giles	China
SC1210	LC107870	2012	*Armigeres subalbatus*	China
12IH26	LC152056	2012	*Culex tritaeniorhynchu*	Japan
14-I-605-C2	LC079089	2014	*Equus caballus*	Japan
14-I-605-C1	LC079088	2014	*Equus caballus*	Japan
HNJZ-S2	KY363863	2015	Pig	China
15-I-752	LC212972	2015	*Equus caballus*	Japan
15-I-1105	LC212973	2015	*Sus scrofa domesticus*	Japan
HNNY-1	MG865966	2016	Pig	China
HNNY-2	MG865967	2016	Pig	China
GETV-V1	KY399029	2016	Pig	China
16-I-676	LC223132	2016	*Equus caballus*	Japan
16-I-674	LC223131	2016	*Equus caballus*	Japan
16-I-599	LC223130	2016	*Equus caballus*	Japan
HNPDS-2	MG865969	2017	Pig	China
HNPDS-1	MG865968	2017	Pig	China
AH9192	MG865965	2017	Pig	China
JL17/08	MG869691	2017	Mosquito	China
JL1707	MH722255	2017	Mosquito	China
HuN1	MF741771	2017	Porcine	China
SD17/09	MH106780	2017	Fox	China
JL1808	MH722256	2018	Cattle	China
SC201807	MK693225	2018	Pig	China
GETV-GDFS2-2018	MT086508	2018	Pig	China

In this study, we conducted routine tests on clinical samples for GETV in samples from different farms in Guangxi province, South China, in 2018. A GETV strain was isolated from the serum of a GETV-positive animal. The virus was genetically closely related to recently isolated strains, HuN1, SD17/09, and JL1808 from different animals in Hunan, Shandong, and Jilin province, respectively, indicating a potential national emergence of this virus.

MATERIALS AND METHODS

Cell Culture and Antibody Production

Baby hamster kidney cells (BHK-21; ATCC CCL10) were cultured in modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS) as described in our previous study (29). To generate the E2 antibody against GETV, the E2 coding region was amplified by RT-PCR using forward primer (5′-CGGGATCCAGTGTGACGGAACACTT-3′) and reverse primer (5′-CCGGAATTCGGCATGCGCTCG TGGCGCGCA-3′). The forward and reverse primers carried the EcoR I and BamH I restriction sites, respectively. Thermal cycling conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 90 s, and a final elongation step at 72°C for 10 min. The E2 PCR produced were double digested with the EcoR I and BamH I and then ligated into similarly digested pET-32a (+) expression vector (Novagen), resulting in plasmid pET32a-E2 and BL21 (DE3). *Escherichia coli* cells were transformed with pET32a-E2 and then induced by 1 mM IPTG for 4 h. A HIS binding kit (Novagen) was then used to purify the recombinant E2 protein. New Zealand white rabbits were injected with the purified recombinant E2 protein to generate a polyclonal antibody against the GETV E2 protein. Affinity chromatography with protein A was used to purify the polyclonal antibody (anti-GETV-E2 PcAb).

Sample Collection, Viral RNA Extraction, and GETV Detection

Three hundred fifty field samples (sera) were collected from clinically diseased pigs in Guangxi province, South China. Viral RNA from 200 μl of each sample was extracted using the

Prep Body Fluid Viral DNA/RNA Mini Prep kit (Axygen AXY) according to the manufacturer's instructions. The extracted RNA was then used for cDNA synthesis using M-MLV reverse transcriptase with random hexamers (Takara Bio, Inc., Dalian, China) according to the manufacturer's instructions. RT-PCR was then performed to detect GETV using primers as described in a previous study (23). Thermal cycling involved initially heating at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s, with a final extension step at 72°C for 10 min.

Virus Isolation

The PBS-diluted GETV-positive sera were filtered through 0.22-μm filters (Millipore, Billerica, MA, USA) and plated onto BHK-21 cell monolayers seeded in a six-well plate. After 1 h of incubation at 37°C, the cells were washed twice with PBS and maintained in MEM supplemented with 2% FBS (Gibco) in a 5% CO_2 incubator. The cells were observed on a daily basis for the presence of cytopathic effects (CPE). The supernatants (200 μl) were subsequently used for serial passages into BHK-21 cells. The third passage (P3) identified the presence of the virus by RT-PCR and indirect immunofluorescence assays (IFAs) using a polyclonal antibody against GETV E2 protein. The isolates were plaque purified three times and then used for complete genome sequencing.

Plaque Assay

Viral plaque assays were performed using BHK-21 cells grown in six-well plates. Viral samples were serially 10-fold diluted in MEM. Samples, 200 μl, of each dilution were inoculated onto monolayers of BHK-21 cells and incubated for 1 h. The cells were then overlaid with a mixture of MEM containing 1% low-melting agarose (Cambrex, Rockland, ME, USA) and 2% FBS and incubated at 37°C for 3 days in 5% CO_2. After careful removal of the medium, the cells were stained with 3–4 ml of staining solution consisting of 0.5% crystal violet and 25% formaldehyde solution for 15 min, and visible plaques were observed.

Growth Curve

Viral growth kinetics were determined using BHK-21 cells as described previously (23). Briefly, BHK-21 cells in six-well plates were inoculated with GETV (P3) at a multiplicity of infection

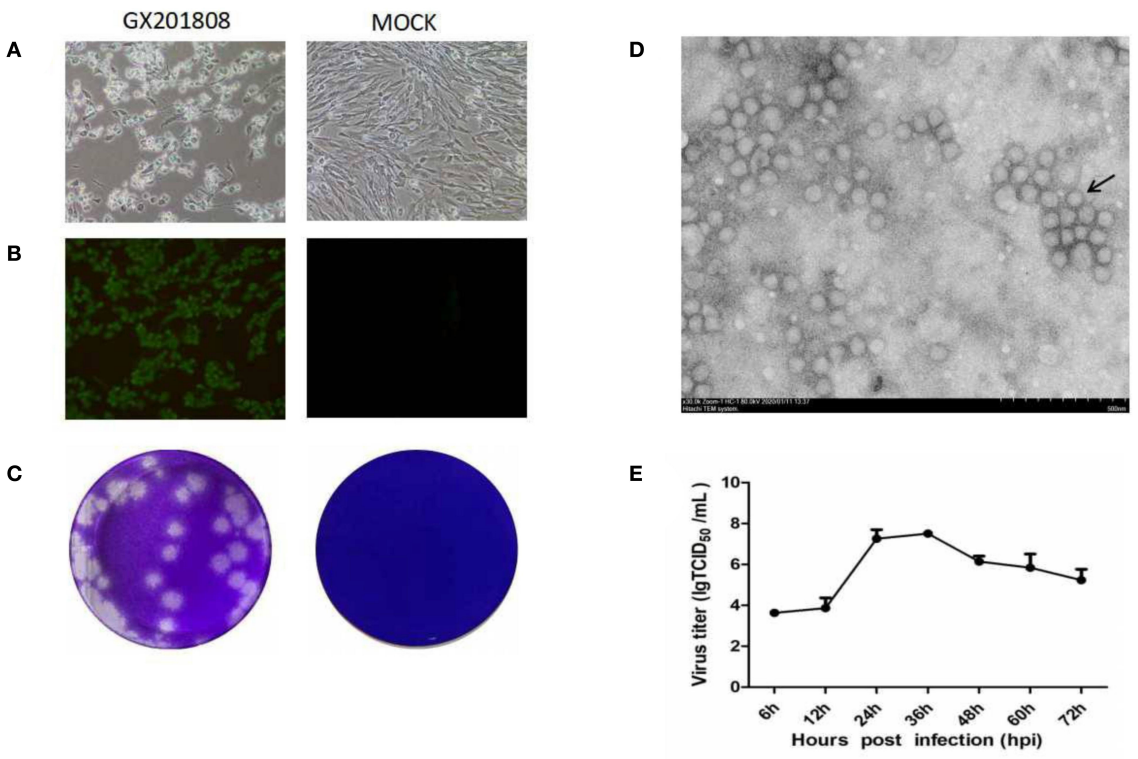

FIGURE 1 | Isolation and identification of the Getah virus (GETV) GX201808 strain grown in baby hamster kidney (BHK-21) cells. **(A)** Cytopathic effects (CPEs) in BHK-21 cells infected with the GETV GX201808 strain. Mock-infected and virus-infected BHK-21 cells were observed at 36 hpi. **(B)** GETV GX201808 strain was identified in infected BHK-21 cells using immunofluorescence assay (IFA) at 24 hpi. The infected BHK-21 cells were fixed and stained using an anti-GETV-E2 PcAb against the GETV E2 protein and goat anti-rabbit H&L IgG. Images were taken using a 20x objective. **(C)** Plaque morphology of GETV GX201808 on BHK-21 cells. Monolayers of BHK-21 cells in six-well plates were infected with GETV GX201808. The cell monolayers were overlaid with 1% agarose and stained with crystal violet at 48 hpi. **(D)** Electron microscopic examination of morphology of GETV particles. BHK-21 cells were infected with GETV GX201808, and the precipitated viruses from the supernatants were processed for electron microscopy. **(E)** The growth of GETV GX201808 at a multiplicity of infection (MOI) of 1 on BHK-21 cells. The viral titers were determined as $TCID_{50}$, and the values obtained were the means of three independent experiments.

(MOI) of 0.1. After 1 h of incubation at 37°C, the BHK-21 cells were washed twice with PBS. Two hundred microliters of BHK-21 cell supernatants were harvested at 6, 12, 24, 36, 48, and 72 hpi and stored at −70°C until use. The virus titers ($TCID_{50}$) for each time point were assessed using BHK-21 cells and calculated according to the Reed–Muench method. The growth curves were determined after measuring the mean titers of three independent measurements at each time point.

Indirect Immunofluorescence Assay

The expression of viral proteins in GETV-infected BHK-21 cell was tested by IFAs. BHK-21 cell monolayers were inoculated with passaged viruses at one MOI. Twenty-four hours post-inoculation, the infected cells were washed twice with PBS, followed by fixation in cold acetone at −4°C for 30 min. The cells were washed five times with PBS and then blocked with 1% BSA (fraction V bovine serum albumin; Roche, Mannheim, Germany), which was diluted in PBS, for 30 min at room temperature. After being washed with PBS, the cells were incubated with primary anti-GETV-E2 PcAb (1:100) for 1 h at room temperature. Then the cells were washed with PBS five times followed by incubation with goat anti-rabbit IgG (H + L; Alexa (Fluor® 488, Abcam) for 1 h at 37°C. The cells were subsequently washed five times with PBS. Finally, images were captured using an inverted fluorescence microscope (Nikon, Tokyo, Japan).

Preparation of Virus Particles and Electron Microscopy

BHK-21 cell monolayers were inoculated with the virus at one MOI. At 24 hpi, 30 ml of supernatant from the infected cells was harvested and filtered through 0.22-μm filters (Millipore, Billerica, MA, USA) and then mixed with 7.5 ml of 50% PEG-8000 to a final 10% concentration. The mixture was gently stirred at 4°C overnight and then centrifuged at 12,000 rpm at 4°C for 2 h. After careful removal of the supernatants, the precipitated viruses were re-suspended in 1 ml of TBS. The virus–TBS mixture was stirred at 4°C for 30 min for being negatively stained and was visualized by transmission electron microscopy (TEM).

Complete Genome Determination

The viral genomic RNA was extracted from BHK-21 cells infected with GETV and then reversed transcribed into cDNA using M-MLV reverse transcriptase (TaKaRa, Dalian, China) according to the manufacturer's instructions. PCR was performed using TaKaRa LA Taq (TaKaRa, Dalian, China) to amplify the complete genomic sequence using the previously published PCR primers (23). The reaction conditions were 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 90 s, and a final elongation step at 72°C for 10 min. The positive amplicons were purified using an E.Z.N.A.TM Gel Extraction kit (OMEGA, USA) and then inserted into a pMD18-T vector (TaKaRa, Dalian, China) for nucleotide sequencing in both directions using universal primers T7 and SP6. The genomic sequence of GETV was assembled using the SeqMan program of DNAstar software, version 7.0 (DNASTAR Inc., Madison, WI, USA).

Sequence Alignments and Phylogenetic Analysis

Differences in sequence of this isolate and all other GETV strains available from GenBank were analyzed using the MegAlign program with DNAstar 7.0 software. The information regarding

TABLE 2 | Nucleotide and amino acid sequences and identity analysis of GX201808 and the other GETV strains.

Strains	GX201808 (%)				
	Complete genome	Non-structural polyprotein		Structural polyprotein	
	nt	nt	aa	nt	aa
MM2021		94.6	97.4		
Sagiyama virus	96.9	96.5	98.0	97.1	99.1
M1	97.6	97.4	98.2	97.6	98.9
Sagiyama virus Original		96.6	98.3		
MI-110-C1	98.1	98.2	99.2	98.0	99.5
MI-110-C2	98.1	98.2	99.3	98.0	99.4
LEIV16275 Mag	97.1	97.0	98.8	97.1	99.3
HB0234	97.5	97.4	98.9	97.4	98.9
Getah virus South Korea	97.8	97.9	99.2	97.7	99.4
Kochi-01-2005	99.0	99.0	99.4	97.4	99.2
YN0540	97.5	97.6	99.2	97.4	99.2
LEIV 17741 MPR	98.1	98.3	99.3	98.0	99.2
HNJZ-S1	97.4	97.5	99.3	97.3	99.0
121H26	97.4	97.5	99.2	97.2	99.1
SC1210	97.4	97.3	99.0	97.4	99.2
YN12031	95.9	95.9	98.0	95.9	98.6
YN12042	97.4	97.4	99.1	97.3	99.1
14-I-605-C1	97.3	97.4	99.2	97.2	99.1
14-I-605-C2	97.3	97.4	99.2	97.2	99.1
15-I-752	97.3	97.4	99.2	97.2	99.1
15-I-1105	97.3	97.4	99.1	97.2	99.0
HNJZ-S2	97.4	97.5	99.1	97.2	99.1
16-I-599	97.3	97.4	99.1	97.2	98.9
16-I-674	97.3	97.4	99.1	97.2	99.0
16-I-676	97.3	97.4	99.1	97.1	98.9
GETV-V1	97.4	97.4	99.0	97.4	99.1
HNNY-1	97.4	97.5	99.3	97.4	99.1
HNNY-2	97.4	97.5	99.3	97.3	99.1
AH9192	97.3	97.1	98.7	97.3	99.0
HNPDS-1	97.4	97.5	99.3	97.4	99.1
HNPDS-2	97.4	97.4	99.3	97.4	99.1
HuN1	99.3	99.4	99.6	99.2	99.4
JL1707	97.4	97.4	98.9	97.3	99.0
JL1708	97.4	97.4	99.1	97.3	99.1
SD17/09	99.4	99.3	99.6	99.4	99.5
JL1808	99.3	99.3	99.7	99.3	99.6
SC201807	97.3	97.4	99.2	97.2	99.2
GZ201808		97.1	98.7	97.2	99.1
GETV-GDFS2-2018	97.2	97.1	98.9	97.3	99.0

the reference GETV strains is listed in **Table 1**. Phylogenetic analyses were carried out based on the complete genome and E2 gene by MEGA version 6.0 using the maximum likelihood (ML) method with p-distances for nucleotide sequences, and the bootstrap test value was calculated using 1,000 replicates.

RESULTS

Virus Detection, Isolation, and Plaque Purification

Routine tests were conducted on clinical samples collected from different pig farms in Guangxi province of southern China. Of the 350 field samples collected from the clinically diseased pigs in Guangxi province, South China, two serum samples from a swine herd were positive for GETV, as determined by specific RT-PCR (data not shown). One GETV-positive sample (GX201807) was collected from 42 weaning piglets of ∼25 days old, which only exhibited fever for 1–2 days in a swine herd in Nanning, Guangxi Province, which has a total piglet population of 307. Another GETV-positive sample (GX201808) was collected from 12 pregnant sows suffering from reproductive disorders in a pig farm located in YuLin, Guangxi Province, which has a total sow population of 196. The GETV-positive sera samples were negative for PRRSV, SVV, PRV, JEV, and CSFV as demonstrated by RT/-PCR (data not shown).

Serum samples, which were GETV positive as determined by PCR, were inoculated into BHK-21 cells for virus isolation. CPE was generated in cells characterized by shrinkage, rounding, and detachment after 48 hpi (**Figure 1A**). The GETV isolates, named as GX201808, were obtained after serial passages and plaque purification in BHK-21 cells. The supernatants of each passage were GETV positive as confirmed by RT-PCR (data not shown). IFA analysis was conducted using an anti-GETV-E2 PcAb to confirm the isolation of the GETV strain. **Figure 1B** shows staining specific for E2, which was evident in GX201808-infected BHK-21 cells. The plaques generated by GETV in BHK-21 cells were regular in shape with distinct edges (**Figure 1C**). No viable particles were isolated in GX201807-inoculated BHK-21 cells, as confirmed by CPE, RT-PCR, and IFA (data no shown).

Electron microscopic examination of precipitated GX201808 strain particles revealed a cluster of typical morphology usually associated with alphaviruses. The virus particles were spherical with an average of 70 nm in diameter and featured surface fibers (**Figure 1D**). The multistep growth curves of the GETV strain was further analyzed using BHK21 cells. As shown in **Figure 1E**, the numbers of GX201808 particles exhibited a gradual increase from 6 hpi and reached a peak titer of $\sim 10^8 TCID_{50}$ at 36 hpi. The titers then decreased slowly, reaching a titer of $\sim 10^5 TCID_{50}$ at 72 hpi. These results revealed that a strain of GETV was successfully isolated when using BHK-21 cells.

Genetic and Phylogenetic Analyses of the Virus

The full-length genome of the GETV strain GX201808 was sequenced and submitted to GenBank under accession no. MT269657. The entire genome of the GETV strain contained 11,691 bp in length excluding the polyA tail and possessed a typical alphavirus genome organization with 2 main ORFs, ORF1, and ORF2, and short UTRs at the 5′- and 3′-termini. All GETV strains available from GenBank were downloaded for sequence comparison and phylogenetic analysis. The results showed that GX201808 shared 94.9–99.4% sequence identity at the nucleotide level with other strains and the highest identity (99.4%) with strain SD17/09, which was recently isolated from foxes in China. The complete genome of GX201808 strain showed 97.3–99.3% identity with strains isolated from pigs in China, and only 97.6% identity with the strain (M1) first isolated in mosquitoes in China. Sequence comparison at the amino acid level of the GX201808 strain with other reported strains showed the sequence identities ranging from 97.4 to 99.7%, and 98.6 to 99.6% in the non-structural and structural polyproteins, respectively (**Table 2**).

Phylogenetic analysis showed that GETVs were divided into four evolutionary groups. Group I only had the oldest GETV strain (MM2021) isolated in 1963. Two GETVs strains isolated in Japan in 1956 formed Group II. Most of the GETV strains isolated from mosquitoes, pigs, horses, cattle, and other animals, including the strain GX201808 in this study were classified as Group III. Two GETV strains, YN12031 and LEIV/16275/Mag, were classified as Group IV (**Figure 2**).

DISCUSSION

GETV has a widespread geographic distribution, and the host range of the virus is expanding gradually (1, 3). Guangxi province of southern China is located in tropical and subtropical areas of the planet. The tropical and subtropical climates of these regions provide a favorable environment for the reproduction of mosquitoes, which play a key role in the spread of GETV among different hosts (30). The existence of GETV-infected animals and mosquitoes in Hunan, Guangdong, and Yunnan provinces, which are the neighboring provinces located in the east and west of Guangxi province, has meant that they are likely to spread, and they have already been reported (23, 25, 27, 28). In this study, we detected and isolated for the first time in Guangxi province, a GETV strain in pig sera using BHK-21 cells, proving the existence of GETV in this region of China. These GETV-positive samples were collected from weaning piglets exhibiting sudden onset fever or pregnant sows suffering from reproductive disorders.

Recently, an outbreak of GETV infection in swine herds resulted in reproductive disorders of pregnant sows and the death of piglets (25), indicating that this virus may pose a potential threat to swine health. Consistent with the findings of the previous study that reported the isolation of a strain of YN12031 (23), the GETV isolate GX201808 could generate CPE in BHK-21 cells characterized by shrinkage, rounding, and detachment. The virus could produce plaques and grow well in BHK-21 cells. Electron microscopic examination revealed that the virus particles were spherical with an average diameter of 70 nm, and

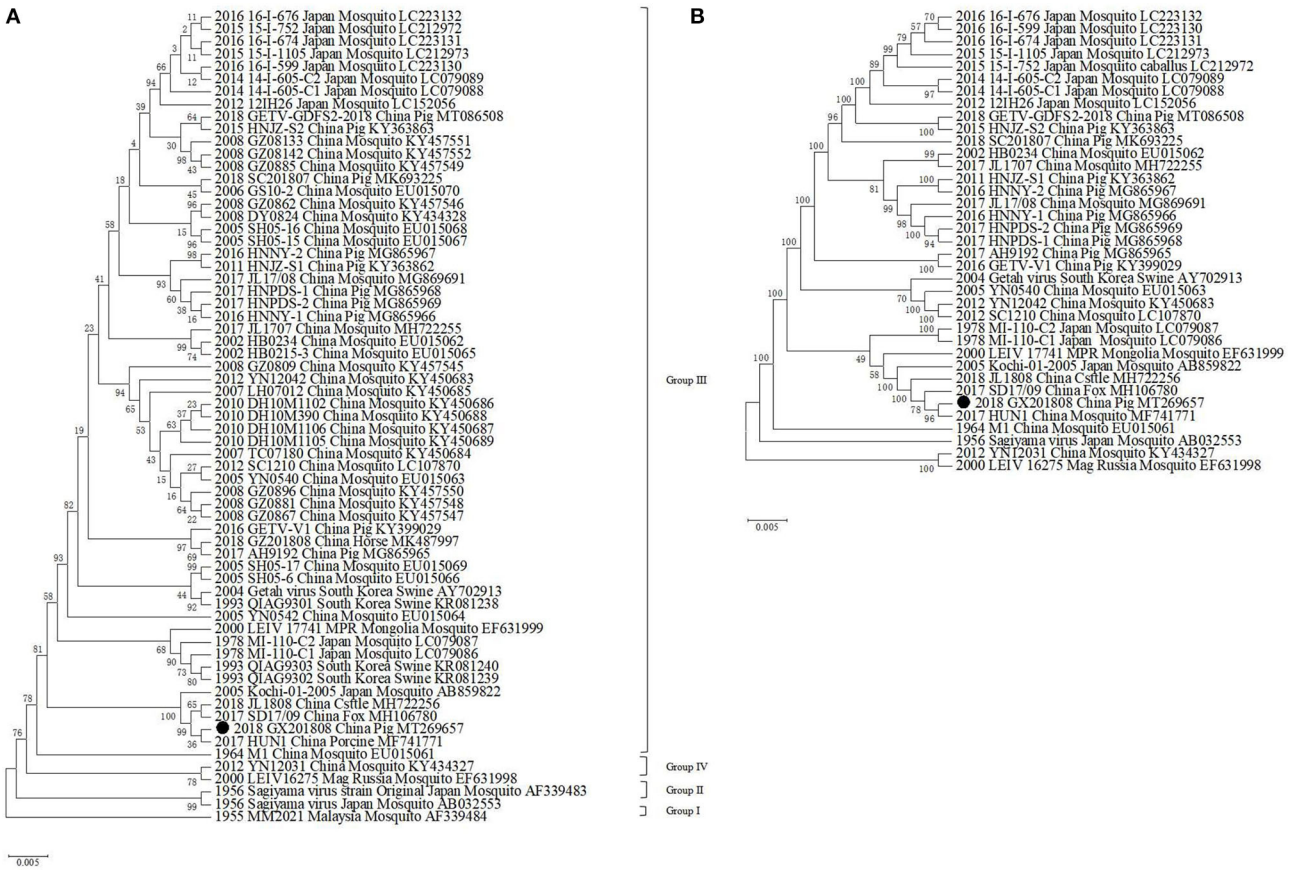

FIGURE 2 | Phylogenetic analysis of GETV GX201808 with the available complete GETV genomes from the GenBank. The phylogenetic trees were generated using the neighbor-joining method implemented in the program MEGA 6.0. Bootstrap values are expressed as a percentage based on 1,000 replications. The strain obtained in this study is indicated by a closed triangle. **(A)** Diagrammatic representation of a tree based on the complete E2 gene nucleotide sequences of GETVs. **(B)** Diagrammatic representation of a tree based on the complete genome sequence of GETV GX201808 and the available complete GETV genomes from the GenBank.

they featured surface fibers, which was consistent with a previous study showing GETV particles display the typical morphology of alphaviruses (23, 31). The expression of E2 in the GX201808-infected cells could be detected by a specific polyclonal antibody raised against the E2 protein of GETV by IFA. This demonstrated that the GETV GX201808 strain was successfully isolated from BHK-21 cells. Viable GETV could not be isolated from BHK-21 cells inoculated with samples of GX201807, and this might be attributed to only a few virus particles in the samples or that the viruses had lost their viability during sample transportation from the pig farms to the laboratory.

It was shown that all the known GETV strains could be grouped into four evolutionary groups (24). The GX201808 strain was clustered in Group III and had the closest relationship with the HuN1 strain, which caused an outbreak of GETV infection in swine herds in Hunan province, China, leading to the death of piglets and reproductive disorders in pregnant pigs in 2017 (25). GX201808 also clustered closely with the recently reported Chinese GETV strains JL1808 and SD17/09 isolated from cattle and foxes, respectively (17, 26). Sequence comparison showed that GX201808 shares high sequence identity at the nucleotide level with the strains HuN1, SD17/09, and JL1808. Group III, represented by the first Chinese strain (M1) that was isolated in 1964 from mosquitoes, contains most GETV strains appearing in mosquitoes, pigs, horses, cattle, foxes, and other animals and has become the dominant viruses circulating among species (17, 23, 26–28).

An increase of high seroprevalence in pigs in the field was detected, and high virus titers were found in pigs infected experimentally with GETV, indicating that this species might be a natural reservoir and amplifiers of GETV (32–34). It has also been shown that GETV strains can circulate among pigs and horses simultaneously within the same region in Japan in 2015, and these were found to be closely related genetically (32). Recent studies also showed that mosquito-borne swine GETVs might play a role in transmitting GETV to blue foxes, cattle, and horses in different provinces of China (26, 27, 29), indicating that this virus has the potential to spread nationally and expand its host range. In response, we recommend that continuous surveillance of GETV infection in animals should be implemented in order to control the circulation of this potentially dangerous virus.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, MT269657.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Guangxi University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

ZW conceptualized the study. TR and QM took part in the data curation. YW and ZN made the formal analysis. KO, YC, and WH were in charge of the investigation. HW, JW, and CN were in charge of the methodology. ZW was in charge of the project administration, wrote, reviewed, and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the Natural Science Foundation of Guangxi Province (No. 2018GXNSFDA281021) and the Foundation of Guangxi University (No. XGZ130959).

ACKNOWLEDGMENTS

The authors would also like to thank Dr. Dev Sooranna of Imperial College London for English language edits of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 07 December 2020
doi: 10.3389/fvets.2020.607662

Molecular Characteristics and Pathogenicity of *Porcine Epidemic Diarrhea Virus* Isolated in Some Areas of China in 2015–2018

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 17 September 2020
Accepted: 16 November 2020
Published: 07 December 2020

Citation:
Yu L, Liu Y, Wang S, Zhang L, Liang P, Wang L, Dong J and Song C (2020) Molecular Characteristics and Pathogenicity of Porcine Epidemic Diarrhea Virus Isolated in Some Areas of China in 2015–2018. Front. Vet. Sci. 7:607662. doi: 10.3389/fvets.2020.607662

Since 2010, *Porcine epidemic diarrhea virus* (PEDV) has caused severe diarrhea disease in piglets in China, resulting in large economic losses. To understand the genetic characteristics of the PEDV strains that circulated in some provinces of China between 2015 and 2018, 375 samples of feces and small intestine were collected from pigs and tested. One hundred seventy-seven samples tested positive and the PEDV-positive rate was 47.20%. A phylogenetic tree analysis based on the entire S gene showed that these strains clustered into four subgroups, GI-a, GI-b, GII-a, and GII-b, and that the GII-b strains have become dominant in recent years. Compared with previous strains, these strains have multiple variations in the SP and S1-NTD domains and in the neutralizing epitopes of the S protein. We also successfully isolated and identified a new virulent GII-b strain, GDgh16, which is well-adapted to Vero cells and caused a high mortality rate in piglets in challenge experiments. Our study clarifies the genetic characteristics of the prevalent PEDV strains in parts of China, and suggests that the development of effective novel vaccines is both necessary and urgent.

Keywords: PEDV, pig, phylogenetic analysis, S gene, pathogenicity

INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) is the etiological agent of porcine epidemic diarrhea (PED), a severe diarrhea disease in piglets that is characterized by severe watery diarrhea, vomiting, dehydration, weight loss, and nearly 100% mortality (1). PED occurred sporadically around the world in 1990–2009, but in 2010, an acute and severe outbreak of PED in piglets occurred in China and spread to other Asian countries, causing large economic losses (2–8). In April 2013, PED suddenly erupted in the United States, causing many piglets to die, and the mortality rate in suckling piglets reached 100% (9, 10). The disease was shown to be caused by a highly pathogenic PEDV variant. The S genes of the classical CV777 strain and the new strain OH851 have the same insertions and deletions (S-INDEL strains), unlike those of the variant PEDV strains (11, 12).

The genome of PEDV is ∼28 kb in length and contains seven open reading frames (ORFs), which encode four structural proteins and three non-structural proteins (13). S is the largest structural protein, and contains neutralizing antibody epitopes and a specific receptor-binding site for viral entry (14). At present, four antigenic epitopes have been characterized in the S protein,

including the CO equivalent (COE) domain (amino acids 499–638), the epitopes SS2 (amino acids 748–755) and SS6 (amino acids 764–771), and epitope 2C10 (1368-GPRLQPY-1374) (15, 16). Because the S protein plays a vital role and the S gene is extensively mutated, it is often used as the target gene in the analysis of viral genetic variation. Based on whether the S gene contains the INDEL sequence or not, PEDV strains can be classified into genogroup II (GII) or genogroup I (GI), respectively. GI is further divided into two subgroups (GI-a, GI-b) according to INDEL sequence differences. At present, most isolates recovered in China belong to GII (17). A new mutation in the S gene of PEDV has recently been reported (18). Different GI-b strains have also been reported in different areas of China (19, 20). Studies have shown that PEDV strains of different genotypes can coexist, in one province in particular. These findings indicate that PEDV has continued to spread widely to most areas of China and has caused serious economic losses in the pig industry, reflecting the complex evolution of the virus. Therefore, extensive research into the evolutionary pathogenic mechanism of these strains in China is essential.

To control the spread of PEDV, a classical-CV777-derived vaccine has been widely used in many areas of China. However, it does not provide adequate protection against PEDV invasion (6, 21). In contrast, the wide-scale use of vaccines has increased the environmental stress upon the virus, causing PEDV to mutate to escape its host's immune defenses. To further and fully understand the prevalence and evolution of PEDV in southern China, diarrhea samples were collected from piglets in this study, and the variation of the S genes of the PEDV-positive samples were analyzed with sequence alignment and a phylogenetic tree.

MATERIALS AND METHODS

Sample Collection

A total of 375 diarrheic samples from the small intestine tissues or feces were collected from suckling piglets on pig farms in eight provinces of China (Fujian, Guangdong, Guangxi, Guizhou, Jiangxi, Shandong, Hubei, Hu'nan, and Hainan) between June 2015 and October 2018. The piglets suffered severe watery diarrhea and dehydration. The diarrheic feces were resuspended in 1 mL of phosphate-buffered saline (PBS) in 1.5 mL Eppendorf tubes. After centrifugation at 10,000 × g for 5 min, 200 μL of each supernatant was transferred to a new tube for RNA extraction and virus isolation.

RNA Extraction and Sequencing

The total RNA from the collected supernatants was extracted with TRIzol Reagent (TaKaRa), according to the manufacturer's instructions. The extracted RNA was subjected to reverse transcription (RT–PCR) with three pairs of newly designed primers to amplify and detect the PEDV S gene (**Table 1**). The three overlapping PCR products were identified with 1.5% agarose gel electrophoresis. The positive PCR products were sequenced by Sangon Biological Engineering Co. Ltd, and the entire sequence of the S gene was determined with the DNAStar software. The complete S gene sequences were submitted to GenBank, under the accession numbers shown in **Table 2**.

TABLE 1 | Primers used for PEDV complete S gene amplification.

Primer name	Nucleotide sequence, 5′-3′	Size(bp)
PEDV S1-F	GGTAAGTTGCTAGTGCGTA	1,630
PEDV S1-R	CACAGAAAGAACTAAACCC	
PEDV S2-F	CTGCCATTCAGCGTATTCTTT	1,768
PEDV S2-R	CTGCGAGTTAACAACCTCTTGA	
PEDV S3-F	GTGCGCAGTATTACTCTGGT	1,559
PEDV S3-R	AAGAAGACGCTTTAAACAGTG	

S Gene Sequence Analysis

The complete genome sequences of reference strains available in GenBank were downloaded and used in a phylogenetic analysis (**Table 3**). A phylogenetic tree was constructed from all the S genes of the representative strains and isolates, using the neighbor joining method with 1,000 bootstrap replicates, with the Molecular Evolutionary Genetics Analysis (MEGA, version 6.0) software (22).

Virus Isolation

Vero cells grown in a 24-well cell culture plate were infected with the previously prepared supernatants and maintained in Dulbecco's modified Eagle's medium (Thermo Scientific) containing 7 μg/mL trypsin without EDTA (Thermo Scientific). The cells were monitored daily for a cytopathic effect (CPE). When the CPE appeared in 70% of the cells, the cells were fixed with anhydrous ethanol. An immunofluorescence assay (IFA) was then performed with an anti-N protein monoclonal antibody (mAb; cat. # PEDV12-F, Alpha Diagnostic International Inc., USA) diluted 1:1,000 and an Alexa-Fluor®-488-conjugated Affinipure goat anti-mouse IgG(H+L) secondary antibody (SA00013-1; Proteintech, USA) diluted 1:400.

Titer Determination for the Viral Proliferation Curve

Vero cells cultured in a 24-well cell culture plate were infected with PEDV at a multiplicity of infection (MOI) of 0.01. The cells and supernatants were collected at 12, 24, 36, 48, 60, 72, and 96 h post-infection (hpi). The cells were then frozen and thawed three times. After centrifugation at 10,000 × g for 5 min at 4°C, the supernatants were collected and the median tissue culture infective dose ($TCID_{50}$) was determined with a microtitration infection assay.

Piglet Challenge Experiment

To determine the virulence of the third-generation isolated strain GDgh16, six healthy 4-day-old colostrum-deprived suckling piglets were artificially fed bovine milk from birth. The colostrum-deprived piglets were randomly divided into two groups, with three piglets in each group. One group was challenged orally with 0.5 mL of PEDV at $10^{5.0}$ $TCID_{50}$/mL. The other group received cell-culture medium. Duplicate samples of small intestine were collected in from all piglets, which had been euthanized at 48 h postchallenge. One of the duplicate samples was crushed in a grinder with 2 mL of PBS. The crushed intestine

TABLE 2 | Information of S genes of 62 PEDV isolates.

No.	Designation	Area	Region	Year	S (bp)	Accession no
1	FJly15	Longyan	Fujian	2015	4161	MN368663
2	FJqz15	Quanzhou	Fujian	2015	4161	MN368664
3	FJzz15	Zhangzhou	Fujian	2015	4161	MN368665
4	GDgz15-1	Guangzhou	Guangdong	2015	4161	MN368666
5	GDgz15-2	Guangzhou	Guangdong	2015	4161	MN368667
6	GDhy15	Heyuan	Guangdong	2015	4161	MN368668
7	GDhz15	Huizhou	Guangdong	2015	4161	MN368669
8	GDjm15	Jiangmen	Guangdong	2015	4161	MN368670
9	GDmm15	Maoming	Guangdong	2015	4161	MN368671
10	GDsg15-1	Shaoguan	Guangdong	2015	4161	MN368672
11	GDsg15-2	Shaoguan	Guangdong	2015	4161	MN368673
12	GDsg15-3	Shaoguan	Guangdong	2015	4161	MN368674
13	GDzq15-1	Zhaoqing	Guangdong	2015	4161	MN368675
14	GDzq15-2	Zhaoqing	Guangdong	2015	4161	MN368676
15	GXnn15	Nanning	Guangxi	2015	4161	MN368678
16	GZgy15	Guiyang	Guizhou	2015	4161	MN368679
17	HBhg15	Huanggang	Hubei	2015	4161	MN368680
18	JXgz15	Ganzhou	Jiangxi	2015	4161	MN368681
19	JXyc15	Yichun	Jiangxi	2015	4161	MN368662
20	FJqz16	Quanzhou	Fujian	2016	4158	MN368683
21	GDfs16	Foshan	Guangdong	2016	4158	MN368684
22	GDhy16	Heyuan	Guangdong	2016	4161	MN368685
23	GDhz16	Huizhou	Guangdong	2016	4161	MN368686
24	GDjm16-1	Jiangmen	Guangdong	2016	4158	MN368687
25	GDjm16-2	Jiangmen	Guangdong	2016	4161	MN368688
26	GDjx16	Jiexi	Guangdong	2016	4158	MN368689
27	GDsg16-1	Shaoguan	Guangdong	2016	4158	MN368690
28	GDsg16-2	Shaoguan	Guangdong	2016	4158	MN368691
29	GDyj16	Ynagjiang	Guangdong	2016	4161	MN368692
30	GDgh16	Guanghui	Guangdong	2016	4158	MG983755
31	GDdg17	Dongguan	Guangdong	2016	4158	MN368693
32	FJfz17-1	Fuzhou	Fujian	2017	4161	MN368695
33	FJfz17-2	Fuzhou	Fujian	2017	4161	MN368696
34	FJqz17-1	Quanzhou	Fujian	2017	4161	MN368697
35	FJqz17-2	Quanzhou	Fujian	2017	4158	MN368698
36	GDhy17	Heyuan	Guangdong	2017	4158	MN368699
37	GDhz17	Huizhou	Guangdong	2017	4158	MN368700
38	GDjm17-1	Jiangmen	Guangdong	2017	4152	MN368701
39	GDjm17-2	Jiangmen	Guangdong	2017	4158	MN368702
40	GDjm17-3	Jiangmen	Guangdong	2017	4161	MN368703
41	GDmm17-1	Maoming	Guangdong	2017	4158	MN368704
42	GDmm17-2	Maoming	Guangdong	2017	4158	MN368705
43	GDsg17	Shaoguan	Guangdong	2017	4161	MN368706
44	HNcz17	Chenzhou	Hunan	2017	4161	MN368707
45	JXnc17	Nanchang	Jiangxi	2017	4158	MN368708
46	FJfz18-1	Fuzhou	Fujian	2018	4161	MN368710
47	FJfz18-2	Fuzhou	Fujian	2018	4158	MN368711
48	FJqz18	Quanzhou	Fujian	2018	4158	MN368712
49	GDhy18-1	Heyuan	Guangdong	2018	4158	MN368713
50	GDhy18-2	Heyuan	Guangdong	2018	4158	MN368714
51	GDhy18-3	Heyuan	Guangdong	2018	4158	MN368715

(Continued)

TABLE 2 | Continued

No.	Designation	Area	Region	Year	S (bp)	Accession no
52	GDhz18	Huizhou	Guangdong	2018	4158	MN368716
53	GDjm18-1	Jiangmen	Guangdong	2018	4158	MN368717
54	GDjm18-2	Jiangmen	Guangdong	2018	4149	MN368718
55	GDmm18-1	Maoming	Guangdong	2018	4158	MN368719
56	GDmm18-2	Maoming	Guangdong	2018	4158	MN368720
57	GDsg18-1	Shaoguan	Guangdong	2018	4158	MN368721
58	GDsg18-2	Shaoguan	Guangdong	2018	4158	MN368722
59	GDst18	Shantou	Guangdong	2018	4158	MN368723
60	GDzj18-1	Zhanjiang	Guangdong	2018	4155	MN368724
61	GDzj18-2	Zhanjiang	Guangdong	2018	4161	MN368725
62	SDbz18	Binzhou	Shandong	2018	4158	MN368709

was then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was collected and their RNA extracted. The PEDV N gene copies in the small intestine were detected with real-time quantitative PCR (qPCR). RT–qPCR was performed with the PowerUp™ SYBR® Green Master Mix (A25742; Thermo Fisher) in a 20 μL reaction containing 1 ng of cDNA as the template, in a CFX96 thermal cycler, under the following cycling conditions: 50°C for 2 min; 95°C for 2 min; and 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 60 s. The other sample was stained with anti-N protein mAb (diluted 1:1,000) for immunohistochemical (IHC) examination.

Statistical Analysis

The numerical data are expressed as means ± standard deviations (SD), and all data were analyzed with the GraphPad Prism software (version 5.02 for Windows; GraphPad Software Inc.).

RESULTS

PEDV Detection and Phylogenetic Analysis Based on the S Gene

As shown in **Table 4**, of the 375 feces and small -intestine samples tested between 2015 and 2018, 177 were positive for PEDV (47.20%). The positivity rates in 2015, 2016, 2017, and 2018 were 48.57% (34 positive samples and 70 test samples), 67.14%% (47 positive samples and 70 test samples), 53.33% (32 positive samples and 60 test samples), and 36.57% (64 positive samples and 175 test samples), respectively. The positive rate was highest in 2016 and lowest in 2018. A sequence alignment showed that these strains shared 92.9–100% nucleotide homology and 91–100% amino acid identity. They also shared 93.1–96.8% nucleotide homology and 91.5–96.8% amino acid identity with reference strain CV777, and 93.8–99% nucleotide homology and 92.5–98.9% amino acid identity with the reference strains isolated from China.

Sixty-two S genes from the test strains and representative strains downloaded from GenBank were analyzed with a phylogenetic tree. As shown in **Figure 1**, the phylogenetic analysis divided these strains into two groups, GI and GII, based on whether the S gene contained the S-INDEL (23). GI included the classical strains (CV777 and SM98) and some isolates from China, the USA, and Japan collected after 2010. Therefore, GI was further divided into three subgroups: GI-a, GI-b. GI-a contained classical S-INDEL strains. GI-b contained a new S-INDEL strain. GII contained non-S-INDEL strains and was also divided into two subgroups, GII-a and GII-b, which consisted of a number of extremely virulent strains from all over the world, isolated since 2010. The strains isolated in the present study belonged to GI-a, GI-b, GII-a, and GII-b. GDjm18-2, was categorized as subtype GI-a, which also included the classical vaccine strains CV777-attenuated and JS2008. GDjm17-1 was categorized in GI-b cluster. The other strains identified in the present study formed eight clusters. Of these strains, 25 isolates from Guangdong, three isolates from Fujian, and one isolate from Jiangxi formed three clusters and belonged to GII-b, with strong similarity to GD-A and CH-GXNN-2012. The other 34 isolates formed five clusters and belonged to GII-a. Among these 34 strains, JXyc15 was closely related to the C4 cluster (North American strains), whereas the other strains showed closer identity to CH-ZMDZ-11, CH-HNAY-2015, and CH-HNCDE-2016L. As shown in **Table 5**, all the strains isolated in 2015 belonged to GII-a (100%). In 2016 and 2017, 46.15% and 43.75% of the isolated strains belonged to GII-a, respectively. Compared with GII-a, the rate slightly increased, and in 2016 and 2017, 53.84 and 50% of the isolated strains belonged to GII-b, respectively. However, in 2018, 72.22% of the isolated strains belonged to GII-b, which was much higher than the proportion that belonged to GII-a in 2017 (22.22%). The comparison result show: Variation of PEDV S gene is continuously occurring and GII-b strains may be the dominant strains in China in the future.

Amino Acid Sequence Analysis of Neutralizing Epitopes in the S Protein

Neutralizing antibodies play an important role in the prevention and control of viral infections. Therefore, it is important to identify and analyze the amino acid sequences of the neutralizing epitopes in viral proteins. To analyze the genetic characteristics of the South China PEDV strains, the deduced amino acid sequences of the S proteins detected in our study were aligned and compared with those of representative

TABLE 3 | Information of the representative strains.

Virus strain	Countries	Year	Accession no.	Virus strain	Countries	Year	Accession no.
CV777	Belgium	2001	AF353511	83P-5	Japan	2013	AB548618
JS-2004-2	China	2004	AY653204	OKN-1-JPN-2013	Japan	2013	LC063836
DX-S	China	2007	EU031893	CH-LXC-2014	China	2014	KT388418
LZC	China	2007	EF185992	PEDV-14	China	2014	KM609207
DR13/virulent	Korea	2007	DQ862099	CH-HNQX-3-14	China	2014	KR095279
JS2008	China	2008	KC109141	CH-HNYF-14	China	2014	KP890336
BJ-2011-1	China	2011	JN825712	CH-GD-22-2014	China	2014	KP870132
CH-JLCC-2011	China	2011	JQ638920	USA-Minnesota271-2014	USA	2014	KR265813
CH-S	China	2011	JN547228	MEX-124-2014	USA	2014	KJ645700
CH-FJND-1-2011	China	2011	JN543367	OH851	USA	2014	KJ399978
SM98	Korea	2011	GU937797	USA-Ohio126-2014	USA	2014	KJ645702
CH-GXNN-2012	China	2012	JX018179	AOM-2-JPN-2014	Japan	2014	LC063837
GD-A	China	2012	JX112709	AOM-3-JPN-2014	Japan	2014	LC063833
GD-B	China	2012	JX088695	KCH-2-JPN-2014	Japan	2014	LC063845
CH-SDDZ-2012	China	2012	KU133240	KPEDV-9	Korea	2014	KF898124
AH2012	China	2012	KC210145	KNU-1310	Korea	2014	KJ451045
JS-HZ2012	China	2012	KC210147	KNU-1401	Korea	2014	KJ451047
CH-ZJCX-1-2012	China	2012	KF840537	KNU-1406-1	Korea	2014	KM403155
CH9-FJ	China	2012	JQ979287	L00721-GER-2014	Germany	2014	LM645057
CV777/attenuated	China	2012	JN599150	FR-001-2014	France	2014	KR011756
CH7	China	2012	JQ239435	PEDV-WS	China	2015	KM609213
CH-HBXX2-11	China	2013	JX501319	CH-XBC-01-2015	China	2015	KR296677
CH-ZMDZY-11	China	2013	KC196276	CH-YGC-01-2015	China	2015	KR296678
CH-SBC-03-2013	China	2013	KC787542	CH-ZWBZa-01-2015	China	2015	KR296680
CH-YNKM-8-2013	China	2013	KF761675	CH-HNAY-2015	China	2015	KR809885
CH-JX-1-2013	China	2013	KF760557	CH-JPYC-02-2015	China	2015	JN547228
CH-HBQX-10	China	2013	JX501318	TW-Pingtung-63	China	2015	KP276250
USA-Indiana-17846-2013	USA	2013	KF452323	CBR2	Thailand	2015	KR610994
USA-Iowa-16465-2013	USA	2013	KF452322	HUA-PED47	Korea	2015	KP455314
USA-Minnesota90-2013	USA	2013	KJ645682	HUA-PED45	Korea	2015	KP455313
MN	USA	2013	KF468752	HUA-PED67	Korea	2015	KP455319
IA1	USA	2013	KF468753	15V010-BEL-2015	Belgium	2015	KR003452
IA2	USA	2013	KF468754	CH-HNCD-2016	China	2016	MF152600
NPL-PEDV-2013	USA	2013	KJ778615	HUA-14PED96	Korea	2016	KT941120
USA-Colorado-2013	USA	2013	KF272920	14JM-226	Japan	2018	KY619763
NK	Japan	2013	AB548623	14JM-126	Japan	2018	KY619740
MK	Japan	2013	AB548624	13JM-291	Japan	2018	KY619768

PEDV strains, including strains from GI-a (CV777 and DR13 virulent), GI-b (OH851 and CH-ZWZBa-01-2015), GII-a (CH-HNQX-3-14, CH-HNAY-2015, CH-ZMDZY-11), and GII-b (CH-GXNN-2012, CD-A). As shown in **Figure 2**, compared with strain CV777, the GI-a strain GDjm18-2 had three amino acid substitutions in the COE domain and one amino acid substitution in epitope SS6. The GII-a strains had amino acid substitutions at 35 positions in the COE domain, at two positions in epitope SS2, and at five positions in epitope SS6. Many new amino acid substitutions were detected in the COE regions of the GII-a strains, at positions 502 (S→ P), 507 (P→ M), 510 (N→ S), 516 (N→ D), 522 (S→ A), 527 (S→ G), 533 (A→ V), 535 (D→ E), 547 (D→ E), 559 (V→ I or A), 562 (S→ D), 567 (S→ A), 568 (K→ T or N), 570 (Q→ H), 571 (D→ N or Y), 575 (P→ L), 580 (S→ A), 588 (S→ G), 594 (T→ R or C), 608 (Y→ H), 613 (S→ I or G), 614 (G→ V), 626 (K→ E or S), and 637 (L→ F or S). Epitope 2C10 was conserved in all GII-a strains. Among the GII-a strains, GDhz16 had four continuous amino acid mutations in epitope SS6, which differed from the epitope sequence in the other strains and the reference strains. Compared with strain CV777, the GII-b strains had amino acid substitutions at 17 positions in the COE domain, and amino acid substitutions at one position in three epitopes (SS2, SS6, and 2C10). As well as the common amino acid mutations that were similar to those in the GII-b reference strains, there were novel amino acid substitutions at

TABLE 4 | The PEDV positive prevalence of different of tested strains in our study.

Year	Province	Positive samples	Total samples	Positive prevalence
2015	Fujian	3	7	42.86%
	Guangdong	26	58	44.83%
	Guangxi	1	1	100%
	Guizhou	1	1	100%
	Hubei	1	1	100%
	Jiangxi	2	2	100%
Total		34	70	48.57%
2016	Fujian	1	1	100%
	Guangdong	45	61	73.77%
	Hunan	1	1	100%
	Guangxi	0	3	0
	Jiangxi	0	4	0
Total		47	70	67.14%
2017	Fujian	4	7	57.14%
	Guangdong	18	34	52.94%
	Hu'nan	3	4	75%
	Guangxi	6	12	50%
	Jiangxi	1	3	33.33%
Total		32	60	53.33%
2018	Fujian	3	3	100%
	Guangdong	52	144	36.11%
	Guangxi	1	10	10%
	Jiangxi	3	10	30%
	Shandong	1	2	50%
	Hu'nan	0	2	0
	Hainan	4	4	100%
Total		64	175	36.57%
All total		177	375	47.20%

TABLE 5 | The PEDV positive prevalence of different groups of tested strains in our study.

Group	2015	2016	2017	2018
GI-a	0	0	0	5.56%
GI-b	0	0	6.25%	0
GII-a	100%	46.15%	43.75%	22.22%
GII-b	0	53.84%	50%	72.22%

eight positions in the COE region: 504 (V→ L), 510 (N→ D), 535 (D→ H), 542 (S→ H), 567 (S→ Y), 614 (G→ V), 626 (K→ T), and 637 (L→ V). These amino acid sequences demonstrate that the neutralizing epitopes of the PEDV S protein are constantly mutating. This phenomenon increases the difficulty of preventing and controlling PEDV infections because existing vaccines cannot effectively protect against PEDV. However, these finding may facilitate the development of effective novel vaccines in the future.

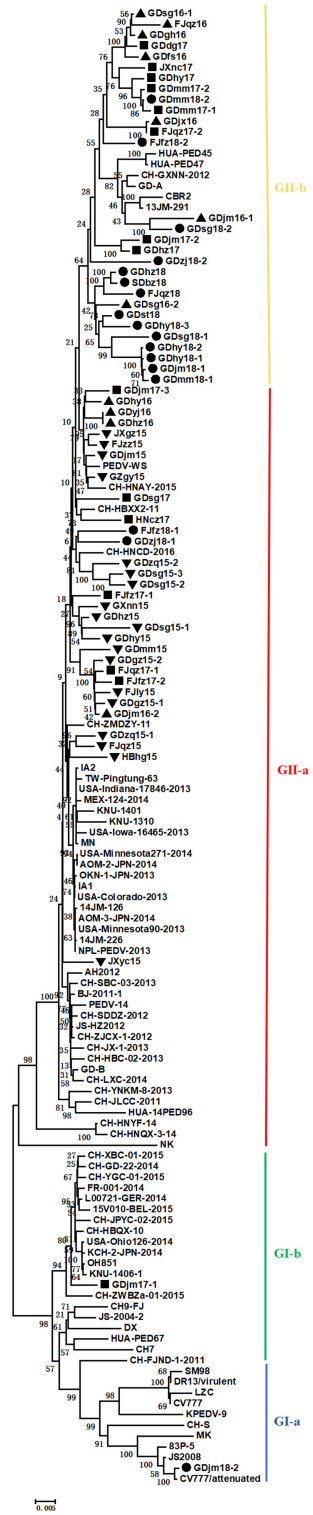

FIGURE 1 | Phylogenetic tree based on the complete S genes of 62 Chinese PEDV strains identified in this study and other global reference strains. The tree was constructed with the neighbor-joining method in the MEGA V.6.0 program. The Chinese PEDV strains isolated in this study are marked with black triangles, black squares, or solid circles.

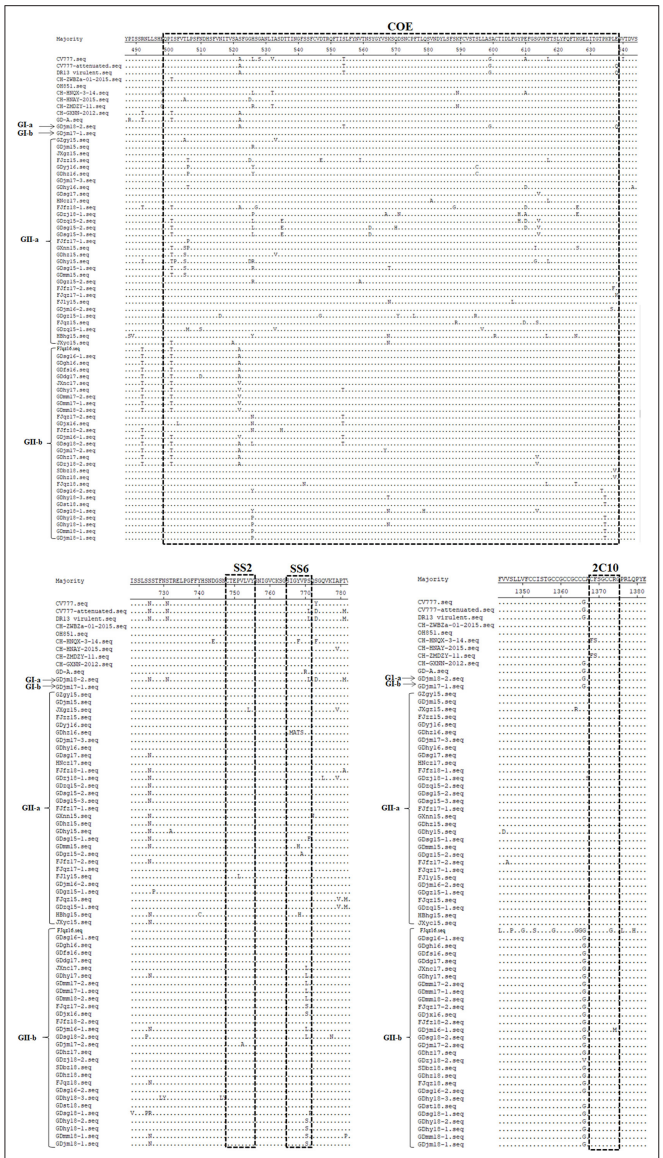

FIGURE 2 | Amino acid sequence analysis of the neutralizing epitopes of the S protein. Amino acid sequence alignment of the neutralizing epitopes of the S proteins of the isolated strains and reference strains, constructed with the Clustal W method.

Numbers of Mutated Amino Acid in Different Domains of the S Protein

To further analyze the amino acid mutations in the different domains of the S protein in these isolates, the different domains of the S protein were aligned with those of CV777, and the average number of amino acid mutations present in each year was calculated. The S protein can be divided into the S1 protein and the S2 protein. The S1 protein contains four domains: SP (amino acids 1–18), S1-NTD (amino acids 19–233), COE and RBD (amino acids 501–629), whereas the S2 protein contains five domains: SS6 (amino acids 764–771), HR1 (amino acids 978–1117), HR2 (amino acids 1274–1313), TM (amino acids 1324–1346), and 2C10 (amino acids 1368–1374). Previous data have indicated that 2C10 is conserved, so we did not analyze the 2C10 domain. As shown in **Figure 3**, in these strains, the S1 sequence had more amino acid mutations than the S2 sequence. From 2015 to 2018, the number of mutated amino acids in S1 remained at a high level, whereas that in S2 decreased. Furthermore, the numbers of mutated amino acids in SP (amino acids 1–18) and S1-NTD (amino acids 19–233) increased slightly, whereas the numbers of mutated amino acids in the COE and RBD domains decreased. SS6, HR1, HR2, and TM in the S2 protein did not change obviously from 2015 to 2018.

Pathogenicity of GDgh16

Because the samples of GDgh16 came from a scale pig farm that had experienced high mortality, and it displayed a high viral titer in Vero cells, we investigated its pathogenicity *in vivo*. As shown in **Figure 4A**, PEDV-infected cells showed a characteristic green color, indicating that PEDV was isolated successfully. The viral proliferation curve indicated that the titer of strain GDgh16 increased to $10^{6.33}$ $TCID_{50}$/mL at 36 hpi but decreased to $10^{4.99}$ $TCID_{50}$/mL at 96 hpi (**Figure 4B**). Six piglets were divided into two groups; one group was challenged orally with GDgh16 and the other group was inoculated with cell culture medium. All the challenged piglets showed classical clinical signs, including vomiting, watery diarrhea, and dehydration, at 16 hpi. The challenged piglets began to die at 24 hpi, and all had died by 48 hpi (**Figure 5A**). The control pigs remained healthy, with no detectable PEDV shedding. The control piglets were euthanized and necropsy was performed on all the piglets. The viral copy numbers in different parts of the intestine were determined with RT–qPCR. The duodenum, jejunum, ileum, cecum, and colon had higher viral copy numbers than the rectum (**Figure 5B**). The duodenums, jejunums, and ileums of the piglets were subjected to an IHC assay. As shown in **Figure 5C**, the tissues from the piglets in the challenged group showed remarkable levels of viral antigens compared to those in the control group. The results of GDgh16 challenge test indicate that the variant strains are a large threat to the pig industry and that the control of PEDV spread has become a critical issue.

DISCUSSION

PEDV has become an important diarrhea virus, causing extensive damage to pig farms worldwide. Because there is no effective vaccine against the emerging prevalent strains in China, the variant PEDV strains occur frequently on many farms in different areas (24). Because there are extensive viral variants and the protection afforded by commercial vaccines is limited, it is necessary to fully understand the genetic variations and epidemiology of PEDV to facilitate the development of next-generation vaccines.

In the present study, the genetic variations in PEDV in parts of China in 2015–2018 were analyzed.

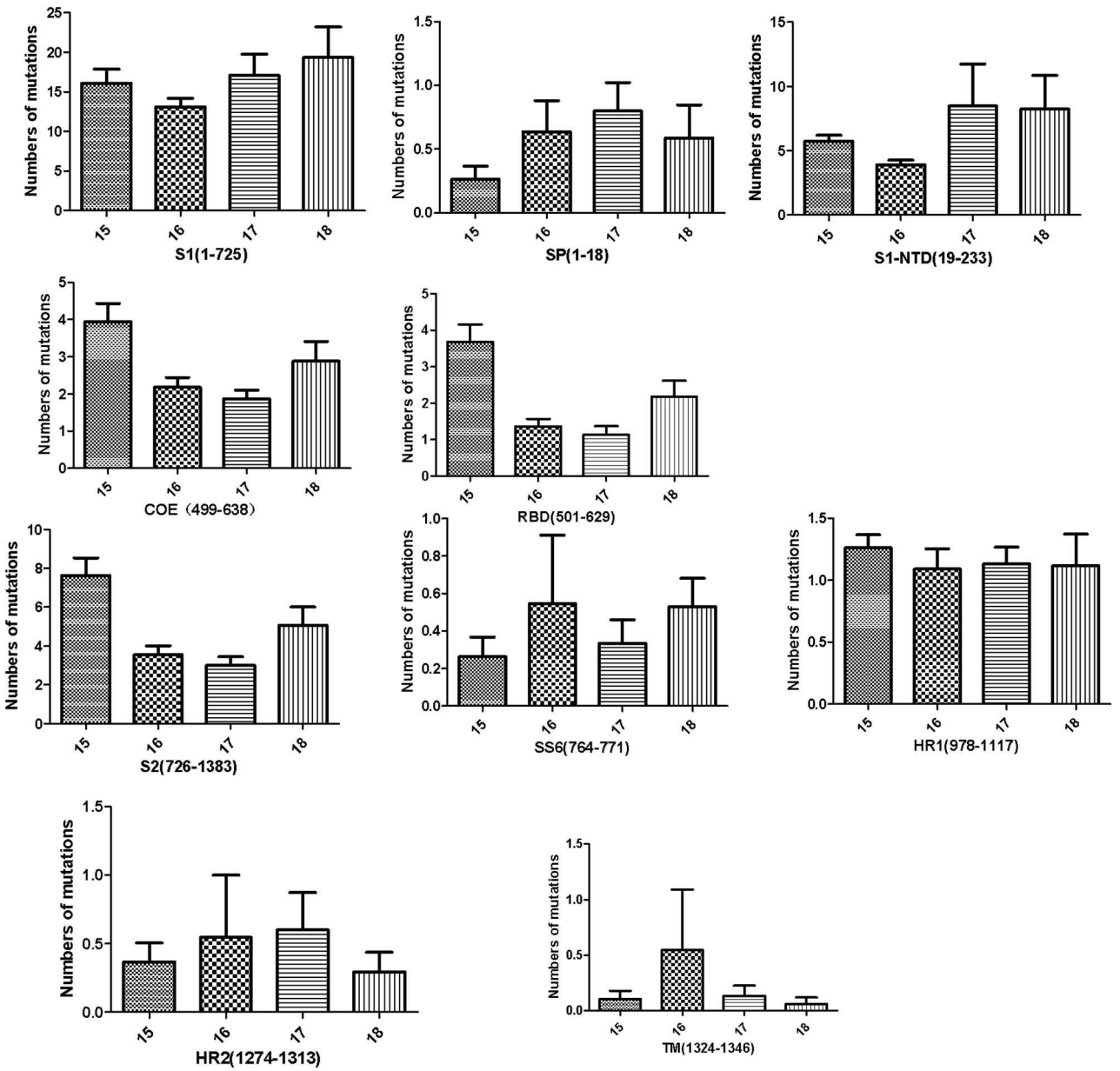

FIGURE 3 | Numbers of amino acid mutations in different domains of the S protein, including the S1 subunit (residues 1–725), S2 subunit (residues 726–1383), signal peptide (SP, residues 1–18), N-terminal domain of S1 (S1-NTD, residues 19–233), the neutralizing epitopes (COE, residues 499–638; SS2, residues 748–755; SS6, residues 764–771; 2C10, residues 1368–1374), two heptad repeat regions (HR1, residues 978–1117, and HR2, residues 1274–1313), and the transmembrane domain (TM, residues 1324–1346).

The S gene encodes the largest structural protein of PEDV and stimulates the host body to produce neutralizing antibodies against the virus. Because its variants are extensive, the S gene is commonly used as the target gene in studies of the genomic characteristics of PEDV (25). A phylogenetic analysis showed that strains from four subgroups of PEDV were present from 2015 to 2018, and that GII-a and GII-b were the two most prevalent subgroups in China at that time. From 2015 to 2018, eight strains belonging to four subgroups (GI-a, GI-b, GII-a, and GII-b) were epidemic in Jiangmen (Guangdong), which suggests that PEDV had mutated widely and the PEDV epidemic was becoming more complex. These results are consistent with those of Wen et al. (26). In 2015, all the isolated strains belonged to GII-a, whereas in 2018, 72.22% of strains belonged to GII-b, and only 22.22% of strains belonged to GII-a. Interestingly, unlike GII-a, which includes strains from other countries, such as America, South Korea, and Japan, the GII-b subgroup only contains Chinese-isolated

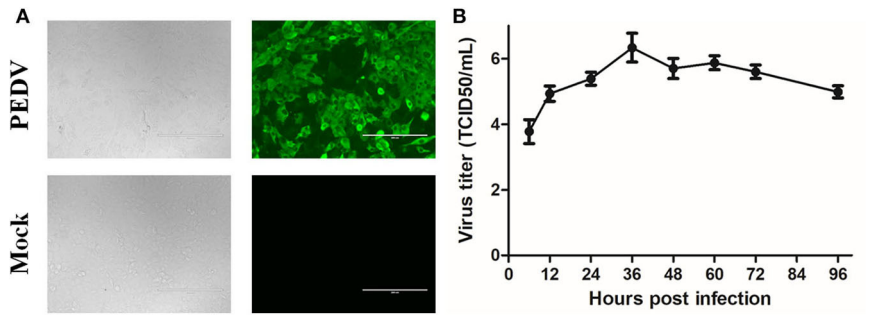

FIGURE 4 | Detection and proliferation curve of PEDV strain GDgh16. **(A)** Identification of GDgh16 in Vero cells. CPE of GDgh16 was observed at 24 hpi under white light and was tested with IFA using a monoclonal antibody directed against the PEDV N protein. **(B)** Proliferation curve of PEDV strain GDgh16. Vero cells were infected with GDgh16 at a multiplicity of infection of 0.01. Cells and culture solution were collected at 6, 12, 24, 36, 48, 60, and 72 hpi, frozen, thawed, and centrifuged. The supernatant was collected and $TCID_{50}$ determined.

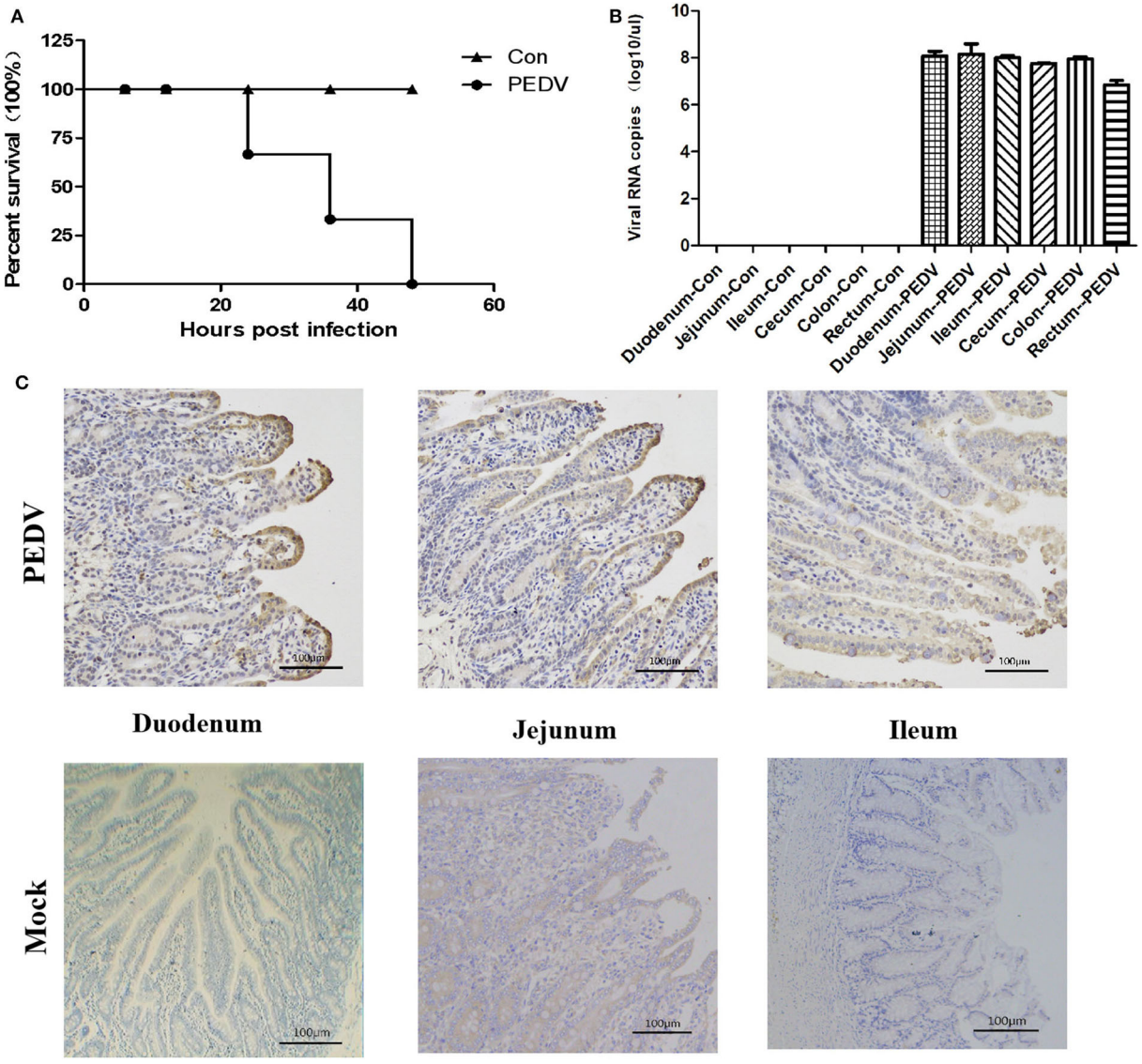

FIGURE 5 | Pathogenicity analysis of GDgh16. **(A)** Survival rate of piglets in each group. **(B)** Quantification of the viral loads in different parts of the intestine. Different parts of the intestine were isolated and the viral load was quantified with TaqMan real-time RT–PCR targeting the PEDV N gene. **(C)** Immunohistochemical analysis of intestines. Duodenum, jejunum, and ileum tissues from each group were stained with monoclonal antibody directed against PEDV N protein (diluted 1:100).

strains. Combined with previous studies, these results suggest that GII-b strains may be the dominant strains in China in the future (27, 28).

The S protein is highly variable, and many studies have shown that amino acid changes in the S protein can affect the virulence and pathogenicity of PEDV. Our study has shown that the numbers of amino acid mutations in the SP1 and S1-NTD domains of PEDV increased in 2017 and 2018. It had been suggested that S1-NTD is a vital domain related to viral virulence (29, 30) and that conformational changes in S1-NTD are related to the high pathogenicity of PEDV strain FJzz1 (18, 27). Increasing numbers of more-virulent PEDV strains have recently emerged (18, 27, 31). Whether the mutations identified in this study alter the major conformation and thus the pathogenicity of these strains will be investigated further in the future. Our data show that the PEDV positivity rate in the provinces tested increased from 2015 to 2016, but decreased from 2016 to 2018, which might be attributable to improvements in disease prevention and control strategies. Many pig farms use the "feed-back" mode to ensure sow immunity to PEDV and to protect piglets against PEDV infection. This is an effective measure to prevent PED, but there is also a risk of virus dispersal, which is responsible for the many GI-b strains reported to date (19, 20, 32–34).

Four neutralizing epitopes of the PEDV S protein have been determined: the COE domain (499–638), epitope SS2 (748–755), epitope SS6 (764–771), and epitope 2C10 (1368–1374) (15, 16). In the present study, we detected amino acid changes at 35 positions in the COE domain. Moreover, one strain, GDhz16, had four continuous amino acid mutations in epitope SS6. Epitopes SS2 and 2C10 also contained amino acid substitutions. The antigenicity, pathogenicity, and neutralization properties of isolated strains are altered by such mutations, especially some insertions and deletions in the S protein (35, 36). Therefore, the vaccine derived from prototype strain CV777 protects against the disease induced by classical strains but not the disease caused by variant strains (24, 37). Whether these amino acid changes affect the antigenicity and neutralization properties of the four neutralizing epitopes warrants investigation in future studies.

Based on previous epidemiological and clinical observations of field strains since 2010, the emerging GII strains are highly pathogenic (38). To investigate the pathogenicity of the isolated variant strains, three piglets were infected orally with GDgh16. The piglets in the infected group began to show clinical signs of diarrhea at 12 h, and developed the typical symptoms of PED at 16 h. Morbidity reached 100%. The piglets began to die at 24 hpi, and all had died by 48 hpi. Moreover, their small intestines contained high viral copies and many viral antigens, indicating that GDgh16 was a highly pathogenic strain. Other researchers have demonstrated that different types of pigs infected with variant PEDV strains shared consistent outcomes (39–42). These results indicate that the variant strains are a large threat to the pig industry, and that the control of PEDV spread has become a critical issue.

In conclusion, the PEDV strains circulating in parts of China between 2015 and 2018 clustered into four subgroups: GI-a, GI-b, GII-a, and GII-b. The GII-b strains became dominant in 2018. Compared with previous strains, these strains displayed multiple variations in the SP and S1-NTD domains and the neutralizing epitopes of the S protein. We successfully isolated and identified a new virulent GII-b strain, GDgh16, which is well-adapted to Vero cells and causes a high mortality rate in piglets. Our study provides insight into the genetic characteristics of the prevalent PEDV strains in parts of China, and suggests that the development of effective novel vaccines is both necessary and urgent.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by The National Engineering Center for Swine Breeding Industry (NECSBI 2015-16).

AUTHOR CONTRIBUTIONS

LY, YL, JD, and CS conceived and designed the experiments. LY, YL, SW, LZ, PL, and LW performed the experiments. LY, JD, and CS analyzed the data and wrote the paper. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Key Research and Development Program of China (2018YFD0501102), the Guangdong Rural Revitalization Strategy Program (201817SY0002), the National Key Technologies R&D Program (2015BAD12B02-5), the Henan Scale Pig Farm Major Disease Purification and Innovative Technology Team, the Henan Science and Technology Project (182102110037), and the Key and Cultivation Discipline of Xinyang Agriculture and Forestry University (ZDXK201702).

ACKNOWLEDGMENTS

This manuscript has been released as a pre-print at Research Square (43).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 16 December 2020
doi: 10.3389/fvets.2020.586056

Evaluation of the Control Options of Bovine Tuberculosis in Ethiopia Using a Multi-Criteria Decision Analysis

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Edited by:
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *22 July 2020*
Accepted: *26 November 2020*
Published: *16 December 2020*

Citation:
Gutema FD, Agga GE, Makita K, Smith RL, Mourits M, Tufa TB, Leta S, Beyene TJ, Asefa Z, Urge B and Ameni G (2020) Evaluation of the Control Options of Bovine Tuberculosis in Ethiopia Using a Multi-Criteria Decision Analysis. Front. Vet. Sci. 7:586056. doi: 10.3389/fvets.2020.586056

Bovine tuberculosis (BTB) is a zoonotic bacterial infection caused by *Mycobacterium bovis* and is characterized by the development of granulomatous lesions in the lymph nodes, lungs and other tissues. It poses serious public health impacts and food security challenges to the agricultural sector in terms of dairy and meat productions. In Ethiopia, BTB has been considered as a priority disease because of its high prevalence in urban and peri-urban dairy farms. However, there has not been any national control program in the country. Thus, in order to initiate BTB control program in the country, information on control options is needed to tailor the best option for the Ethiopian situation. The objective of this study was to identify, evaluate and rank various BTB control options in Ethiopia using a multi-criteria decision analysis based on preference ranking organization method for enrichment evaluations (PROMETHEE) approach while accounting for the stakeholders' preferences. Control options were evaluated under two scenarios: with (scenario 1) and without (scenario 2) bacillus Calmette–Guérin (BCG) vaccination. Nine potential control options were identified that include combinations of three control options (1) test and slaughter with or without government support, (2) test and segregation, and (3) BCG vaccination. Under scenario 1, BCG vaccination, BCG vaccination and test and slaughter with partial compensation by government, and BCG vaccination and test and slaughter with full compensation by government were the top three ranked control options. Under scenario 2, test and slaughter with full compensation by government was the preferred control option, followed by test and segregation supported by test and slaughter with full government compensation, and test and slaughter with half compensation by government. Irrespective of the variability in the weighting by the stakeholders, the sensitivity analysis showed the robustness of the ranking method.

In conclusion, the study demonstrated that BCG vaccination, and test and slaughter with full compensation by government were the two most preferred control options under scenarios 1 and 2, respectively. National level discussions were strongly recommended for further concretization and implementation of these control measures.

Keywords: bovine tuberculosis, multi-criteria decision analysis, stakeholders, control, Ethiopia

INTRODUCTION

BTB is a zoonotic bacterial infection caused by *M. bovis,* a member of the *Mycobacterium tuberculosis* complex (1). It causes a serious public health impact and food security and safety challenges (2). Contaminated dairy products are the main sources of BTB infections in humans, mainly resulting in extra-pulmonary infections such as lymphadenitis (3). According to the World Health Organization, there were 147,000 new cases of zoonotic TB and 12,500 human TB related deaths in 2016 with higher incidence and death rates in Africa than other parts of the world (4). Even though there are no comprehensive studies to estimate the global socio-economic costs of BTB, it causes significant economic losses due to production losses such as reduced milk yield, cost of surveillance and control programs and trade barriers with a major impact on the livelihoods of poor and marginalized communities (5).

In high income countries, public health risk and economic loss associated with *M. bovis* were considerably reduced or eliminated through the implementation of strict test-and-slaughter and meat inspection protocols for cattle, milk pasteurization, financial compensation to farmers and public education (6, 7). However, in most low and middle income countries where BTB is endemic, like in Ethiopia, such measures are hampered by financial constraints particularly for farmer compensation, and by inadequate veterinary services (8). Currently, there are several ongoing efforts to address zoonotic BTB to end the global TB epidemic by 2030 globally (4). However, there are no policies and implementation activities aligning to this global endeavor in the control of BTB in Ethiopia.

The conventional disease prevention and control interventions can have important environmental, social and economic impacts (9). For instance, test and slaughter policy is effective for control of BTB (10). However, it has several impacts such as killing large numbers of test positive animals, raising welfare concerns, and incurring costs for testing and compensation to cattle owners, making it economically difficult to apply particularly in resource limited countries. As a result, decision-making requires systems approach to integrate these multiple aspects of interventions. Multi-criteria decision analysis (MCDA) is an important and effective emerging system approach that can increase the understanding, acceptability and robustness of a decision problem of controlling zoonotic TB considering an integration of epidemiologic, economic and social-ethics value judgments (9, 11).

BTB was reported from 55% of herds and 32.3% of cattle in urban and peri-urban dairy farms in central Ethiopia (12). The national BTB prevalence estimate was 5.8% in individual cattle, with higher prevalence of 21.6% in exotic breeds and their crosses and 16.6% in herds kept under intensive and semi-intensive production systems in urban and peri-urban areas (13). Thus, it is particularly a problem for intensive dairy systems that raise dairy cattle with improved breed. For example, in the years 2005–2011, the maximum production loss due to BTB was estimated at $4.9 million in the urban livestock production systems in Ethiopia (14). Currently, there are no national policies and strategies for the control of BTB although the disease is considered among the top three diseases in dairy producing urban and peri-urban areas of the country in terms of prevalence and household impact (15). Researchers have recommended implementation of control in intensive and semi-intensive dairy farms due to the public health importance of BTB and concerns about spreading the disease through dairy cattle trade from the high prevalence urban system to low prevalence sedentary rural production systems (13, 15). To that end, information for decision making is needed to select and implement control options that are optimally tailored to the country's situations by considering the interests of the dairy farmers and the government. The objective of this study was to identify, evaluate and rank various BTB control options using a multi-criteria decision analysis tool. The outcome of the study would ultimately inform decision makers toward policy formulation and national level discussion to implement BTB control under semi-intensive and intensive dairy farming systems in Ethiopia.

MATERIALS AND METHODS

Assembling Team

The study was conducted between July 2018 and June 2019. A multidisciplinary research team composed of researchers in the fields of veterinary public health, public health, veterinary epidemiology, infectious disease modeling, veterinary animal health economics, biostatistics and multi criteria decision analysis was assembled and involved in the study.

Multi-Criteria Decision Analysis (MCDA)

The comprehensive and stepwise consecutive approaches of MCDA tool for managing zoonotic diseases as developed by Aenishaenslin et al. (9) was used to identify, evaluate and rank various BTB prevention and control options according to stakeholders' preferences to indicate the potential BTB control option under Ethiopian conditions. The approach consists of ten steps that were categorized into seven problem structuring and three decision analysis steps. In the context of the present study, stakeholders refer to key players in the control of BTB

include representatives of governmental organizations, animal health professionals, public health professionals and experts (11).

Problem Structuring

The problem structuring step consisted of the following steps: define the problem, identify the stakeholders, identify key decision issues, define criteria and indicators, identify intervention options, evaluate performance of each intervention option and weight criteria. Before conducting the MCDA, literature review was conducted on the available success stories on BTB controls in other countries to identify different control options. A non-systematic literature review approach was followed to search for focused available information on BTB control options using search engines such as Google scholars and PubMed with key phrases like "control of BTB," "control of TB in cattle" and "control of zoonotic TB." The generated articles were read in-depth for the targeted information and the citations in the articles were further referred when deemed necessary. Moreover, experts working on BTB in the academia and veterinary and medical government offices were consulted through face to face discussions and Skype meeting using check list of the important elements of the problem structuring steps of MCDA. Accordingly, they were consulted to contextualize the key decision issues related to BTB control in Ethiopia in terms of the prevalence of BTB in intensive and semi-intensive dairy farms, the need for BTB control, potential control options and measurements for the evaluation of the control options.

The actual MCDA analysis was performed through an interactive group discussion for which key stakeholders ($n = 15$) from various pertinent organizations in Ethiopia were invited. Out of the 15 stakeholders invited, 10 of them agreed to participate in the MCDA process (**Table 1**). The participating stakeholders conducted thorough interactive discussions to lay out problem structuring phase of the analysis, such as defining the problem and identifying key decision issues, defining the measurement scale on criteria, listing potential BTB control options, and evaluating the control options. The stakeholders identified 10 specific criteria (C1–C10) categorized into six clusters, namely epidemiology (1 criterion), practical applicability (1), economics (3), social ethics (3), public health (1) and animal welfare (1). All criteria were categorical ordinal variables (**Table 2**). Each stakeholder independently weighted each of the identified clusters and criteria and evaluated the performance of each of the control options based on three-point qualitative scale as low, medium or high (9).

Decision Analysis

The decision analysis step included: constructing a matrix based on multi-criteria analysis, sensitivity analysis and interpretation of the results. Since two differing opinions were made during the discussion by the stakeholders regarding inclusion and exclusion of BCG vaccination of calves as a control option under Ethiopian conditions, the comparison and option ranking were performed under two scenarios. Scenario 1 was modeled with the inclusion of BCG vaccination while scenario 2 was modeled by excluding it. The academic version of preference ranking organization method for enrichment evaluations (PROMETHEE) software 1.4 was used to perform pair-wise comparisons of the performance of control options using the preferences of the stakeholders to compute the overall outranking scores (16).

Based on the scores, the identified control options were listed from the most to the least preferred option. For ranking of the control options and visual display of the analysis results, the Geometrical analysis for interactive aid (GAIA) with two dimension (U-V) views and PROMETHEE table were used. The action profiles of the control options were performed for the top ranked control options to evaluate their relative performance on each criterion. The GAIA and sensitivity analysis were performed for scenario 1. GAIA walking weights were run to conduct sensitivity analysis to see the effect of weighing the evaluation criteria by stakeholders on the group ranking when the weights of the criterion were changed and to assess the robustness of the results.

RESULTS

From the 10 stakeholders participate in the MCDA process (S1–S10), nine were from the government organization and one stakeholder represented an association of privately owned dairy farmers (**Table 1**). The stakeholders agreed that BTB is a major problem and that the prevalence is particularly high in dairy herds with exotic cattle breeds and their crosses kept under semi intensive or intensive production system in urban and peri-urban dairy farming. They also emphasized the need for pooling collective efforts toward the control of BTB in the country targeting intensive and semi-intensive dairy farms in urban and peri-urban areas, noting the lack of national BTB control or eradication program in Ethiopia. The research team and stakeholders indicated the occurrence of high prevalence of BTB in semi-intensive and intensive dairy farms and the need for designing and implementing potential control option. The stakeholders identified nine possible control options including combinations of three specific options: test and slaughter with or without financial compensation, test and segregation, and BCG vaccination, with the assumption

TABLE 1 | Composition of the stakeholders participated in the multi-criteria decision analysis for the evaluation of bovine tuberculosis control options in Ethiopia.

Organizations	Number of participants
Ministry of Agriculture	1
Ethiopian Commercial Dairy Producers Association	1
Ethiopian Public Health Institute	1
Adama General Hospital and Medical College	1
Ethiopian Meat and Dairy Industry Development Institute	1
Addis Ababa University, Pathobiology Institute	1
Addis Ababa University, College of Veterinary Medicine and Agriculture	2
Debre Berhan University	1
Ethiopian Institute of Agricultural Research Center	1

that each option can be implemented independently (**Table 3**). For effective implementation of BTB control option in the country, the stakeholders emphasized also the need for stringent prerequisites such as legal framework for implementation, preliminary BTB status testing of each animal and herd, animal identification and animal movement control, biosecurity measures at dairy farms, public education and BTB herd certification as supplementary/complementary measures to the implementation of potential intervention option(s).

All stakeholders generated a specific weighting scheme based on their perceived relative importance of each criterion as defined for the intended decision-making process. For this, each stakeholder was provided 100 points, and was asked to distribute the points to all specified criteria. The weighting of the stakeholders varied among the clusters and within the cluster criteria. Three stakeholders (S2, S3, and S10) gave the highest weight for the economic criteria, while the other seven stakeholders gave the highest weight to the epidemiologic cluster: reduction of BTB prevalence. The social ethics cluster and criteria generally received the least weight by all stakeholders (**Table 4**). Under scenario 1, the ranking of the control options showed that BCG vaccination (OP1), BCG vaccination combined with test and slaughter with cost sharing (OP6), and BCG vaccination combined with test and slaughter with full compensation of the cost by the government (OP7) as the top three potential control options. **Figure 1** shows the relative performance of the top three control options on each criterion. The first ranked option, BCG vaccination, performs well on C2, C6, and C10 while poorly performing on C1 i.e., reduction in the prevalence/incidence of BTB. Conversely, the second and the third control options relatively perform well on C1 while poorly performed significantly on C8 and C10, respectively.

TABLE 2 | Criteria used in the evaluation of bovine tuberculosis control options in Ethiopia.

Criteria cluster	Brief description of criterion
Epidemiology (EPI)	Reduction in BTB incidence or prevalence (C1)
Practical applicability (PA)	Level of difficulty in implementing the control option under Ethiopian condition (C2)
Economics (ECO)	Cost to the farmers -cost of test, loss of milk, replacement cost and other related costs (C3)
	Cost to the government- compensation of slaughtered animals, cost of laboratory test, veterinary costs (cost of farm visit, administrative cost to implement the control option (C4)
	Cost to the industry- inadequate milk supply to processing industries (C5)
Social ethics (SOE)	Acceptability by the Government (C6)
	Acceptability by dairy farmers (C7)
	Social impact - social crisis as a result of the intervention in terms of loss of high performing cow or loss of employment for labor workers (C8)
Public health (PH)	Public health impact - exposure to bovine tuberculosis during implementation of the intervention (C9)
Animal welfare (AW)	Impact on animal - welfare problems as a result of the intervention like slaughtering positive cattle, stress during vaccination and segregation (C10)

TABLE 3 | Descriptions of single and combined control options identified by stakeholders for control of bovine tuberculosis (BTB) in Ethiopia.

BTB control option	Description
BCG vaccination (OP1)	Calf vaccination with BCG vaccine at 6 weeks of age.
Test and segregation (OP2)	Testing and segregating infected animals at early stage of the disease and calf at birth, and switching to test-and-slaughter method in the final stage.
Test and slaughter with cost sharing (OP3)	Testing and slaughtering positive animals with the government and the owner equally sharing the cost of compensation for the slaughtered animals.
Test and slaughter with government support (OP4)	Testing and slaughtering positive animals with the government compensating full cost of the slaughtered animals to the owner.
BCG vaccination and test and segregation (OP5)	Calf vaccination to reduce the prevalence of the disease in the herd, and switch to test and segregation.
BCG vaccination and test and slaughter with cost sharing (OP6)	Calf vaccination and switching to testing and slaughtering of positive animals with the government and the owner equally sharing the cost of compensation for the slaughtered cattle.
BCG vaccination and test and slaughter with government support (OP7)	Calf vaccination, and switch to testing and slaughtering of positive animal with the government compensating full cost of the slaughtered animals to the owner.
Test and segregation and test and slaughter with cost sharing (OP8)	Testing and segregating infected animals, and switch to testing and slaughtering positive animals with the government and the owner equally sharing the cost of compensation for the slaughtered animals.
Test and segregation and test and slaughter with government support (OP9)	Testing and segregating infected animals, and switch to testing and slaughtering positive animals with the government compensating full cost of the slaughtered animals to the owner.

[*] *BTB test refers to application of tuberculin skin test that consists of injecting bovine tuberculin, a purified protein extract derived from M. bovis, intradermally and measuring the skin thickness at the site of injection after 72 h to detect any subsequent swelling at the injection site-sign of delayed hypersensitivity reaction associated with infection.*

TABLE 4 | The relative weight given by stakeholders based on their preference for each cluster and specific criteria for the control of bovine tuberculosis (BTB) in Ethiopia.

Cluster	Criteria	Weights									
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10
Epidemiology	Reduction in BTB incidence (C1)	**25**	**20**	**20**	**30**	**25**	**25**	**25**	**25**	**30**	**20**
Practical applicability	Practical applicability (C2)	**15**	**10**	**10**	**15**	**15**	**15**	**15**	**15**	**20**	**10**
Economics	Cost to the farm owner (C3)	10	15	20	10	15	15	15	15	10	20
	Cost to the government (C4)	10	10	15	9	10	10	10	10	10	20
	Cost to the industry (C5)	10	15	15	6	10	10	10	10	10	10
	Sub total	**30**	**40**	**50**	**25**	**35**	**35**	**35**	**35**	**30**	**50**
Social ethics	Acceptance by government (C6)	6	10	5	5	5	5	5	5	5	5
	Acceptance by owner (C7)	4	5	5	5	5	5	5	5	5	5
	Social impact (C8)	4	5	5	4	5	5	5	5	5	2
	Sub total	**14**	**20**	**25**	**14**	**15**	**15**	**5**	**15**	**15**	**12**
Public health	Public health impact (C9)	**10**	**6**	**5**	**10**	**5**	**5**	**7**	**5**	**3**	**3**
Animal welfare	Impact on animal welfare (C10)	**6**	**4**	**5**	**6**	**5**	**5**	**3**	**5**	**2**	**5**
	Total	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**	**100**

The bold numbers indicate the weight given for each cluster out of 100 points by each stakeholder.

Under scenario 2, test and slaughter with full government compensation (OP4) was the preferred control option followed by test and segregation combined with test and slaughter with full government compensation (OP9), and test and slaughter with half compensation by government (OP3) (**Table 5**).

Figure 2 is the GAIA-scenarios plane visually displaying the positions of the control options and the stakeholders' preferences for scenario 1. As indicated in the GAIA plane, the options OP1, OP7, and OP6 are located on the right positions close to the decision axis representing the preferred control options while OP2 and OP8 are positioned in the left away from the decision axis representing the less preferred options that agreed with the results generated by the PROMETHEE table (**Table 5**). In the plane, most of the stakeholders (8/10) preferences were pointed toward the positive direction of the x-axis, having less variation in their preferences of the control options, while stakeholders 5 and 10 had major deviation from the group. BCG vaccination was not among the top three ranked control options for these two stakeholders. The sensitivity analysis showed that when equal weight was given to each criterion, the ranking of the three top control options remained stable except shifting in the order between the second (OP7) and third option (OP6), indicating the robustness of the study.

DISCUSSION

To the best of our knowledge this is the first study to identify, evaluate and rank different BTB control options using MCDA tool based on the stakeholders' opinions and preferences in Ethiopia. Despite high prevalence of the disease in dairy cattle in the country, currently there is no BTB control and /or eradication program. The participation of stakeholders to achieve the purpose and use of participatory approaches such as MCDA are helpful to identify and evaluate BTB control options. In the present study, we generated the desired data from stakeholders who represented organizations with direct responsibilities or had specific interests in BTB prevention and control in Ethiopia.

In addition to identifying, evaluating and ranking of BTB control options, the stakeholders also identified various pre-requisite measures such as legal framework for the implementation and allied issues as an integral part of a control program prior to its application. Similarly, these measures were identified and their implications on the future prevention and control of BTB in Ethiopia were mentioned as daunting tasks by Dibaba et al. (17). In agreement to these requirements, several BTB eradication programs in many countries succeeded in reducing or eliminating the disease in cattle, by employing such multi-faceted approaches in place (10). Thus, creating enabling conditions, particularly formulation of BTB control policy and implementation guidelines, should be the primary steps in order to implement prevention and control of BTB effectively in the country. This will serve as a springboard toward initiation and implementation of the identified BTB control options in Ethiopia. The role of pertinent stakeholders and researchers would be indispensible in this regard in advising policy and decision makers by creating platforms for national level sensitization and discussion.

In the present study, nine potential BTB control options consisting of three single and six combined options were identified based on the stakeholders' preferences. Under scenario 1, the stakeholders ranked BCG vaccination as the number one control option and complementary measure to the second and third control options in cattle under Ethiopian conditions. BCG vaccination refers to vaccination of calves at 6 weeks of age. Calves are immune-competent at birth and are naturally sensitized to antigens of environmental mycobacteria at a young age. By 6 weeks of age, calves usually show a strong immunological response to such antigens. BCG vaccination of calf at birth induced a high level of immunity. However, it is recommendable to vaccinate calf at 6 weeks of age (18).

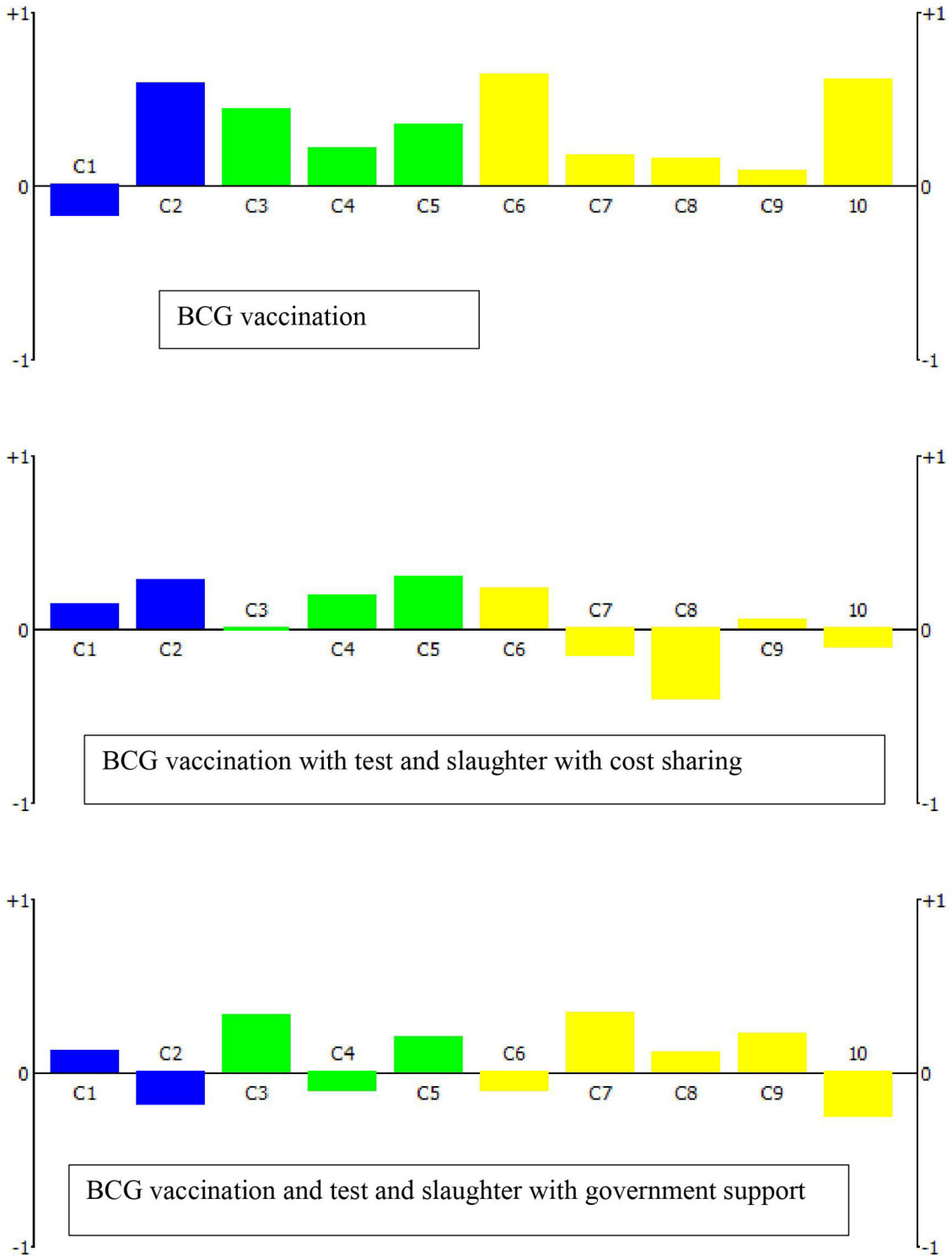

FIGURE 1 | The action profiles of the top three control options under scenario 1 (use of BCG vaccination) indicating the net score per decision criterion (C1–C10; see **Table 2**). A score of −1 means that the evaluated control option has the worst performance for this indicator among all alternatives, while a score of +1 indicates the best performance.

Despite significant knowledge gaps regarding the real impact of BCG vaccination on the incidence of BTB and the inability to distinguish between infected and vaccinated cattle using purified protein derivative (PPD) skin test (19, 20), recent experimental studies have indicated the significance of vaccinating cattle with BCG vaccine in reducing the prevalence, progression and severity

TABLE 5 | Group ranking for bovine tuberculosis control options in Ethiopia.

Alternative control option	Scenario 1		Scenario 2	
	Score	Rank	Score	Rank
BCG vaccination (OP1)	0.250	1	NA	NA
BCG vaccination, and test and slaughter with government support (OP7)	0.111	2	NA	NA
BCG vaccination, and test and slaughter with cost sharing (OP6)	0.071	3	NA	NA
Test and slaughter with government support (OP4)	0.006	4	0.100	1
BCG vaccination, and test and segregation (OP5)	−0.015	5	NA	NA
Test and segregation, and test and slaughter with government support (OP9)	−0.068	6	0.019	2
Test and slaughter with cost sharing (OP3)	−0.074	7	0.018	3
Test and segregation (OP2)	−0.141	8	−0.070	5
Test and segregation, and test and slaughter with cost sharing (OP8)	−0.140	9	−0.067	4

NA, not applicable.

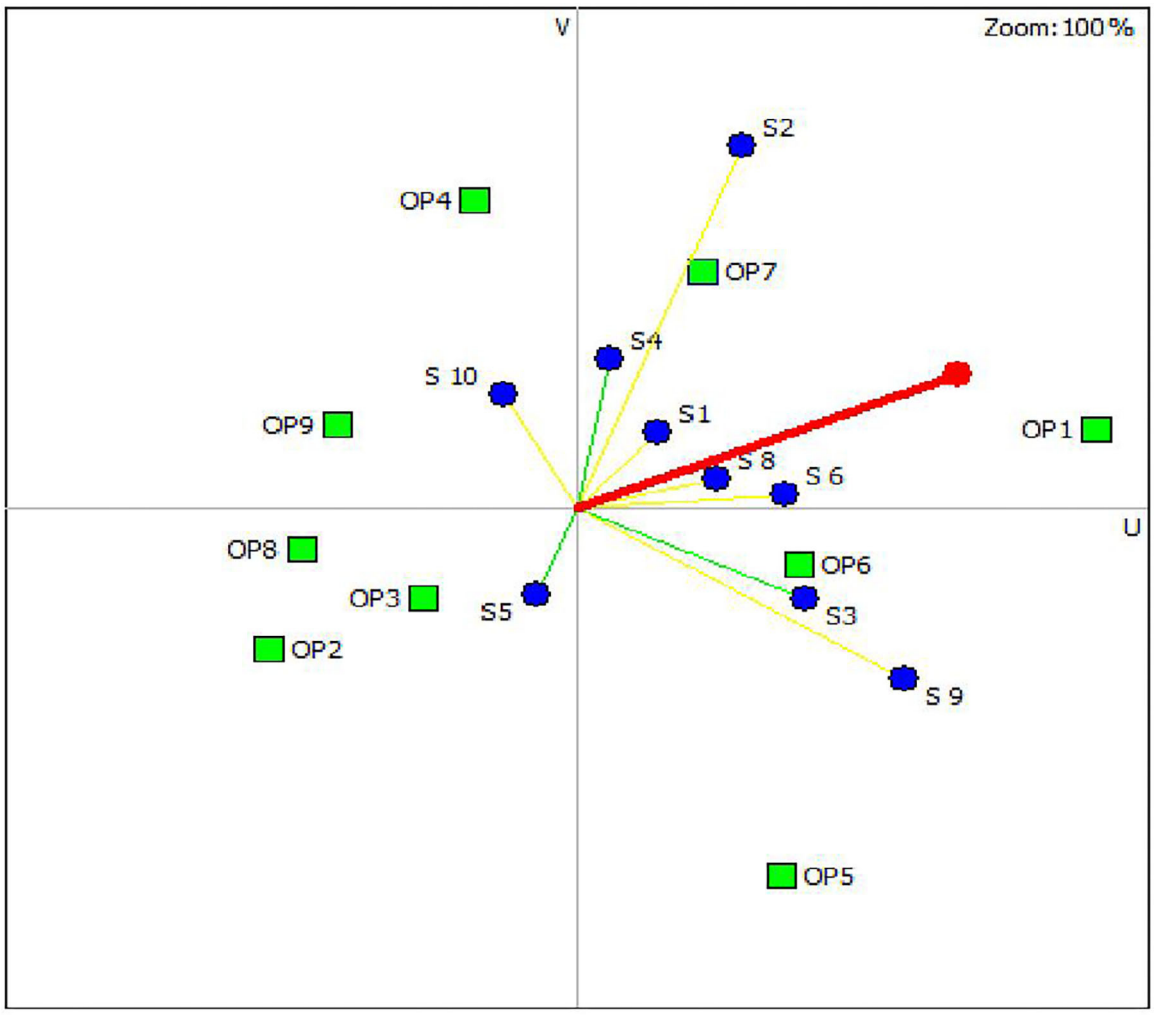

FIGURE 2 | Geometrical analysis for interactive aid decision map for the intervention options under scenario 1 involving BCG vaccination (Delta = 71%, meaning that 71% of the information is conserved in the two-dimensional plane).

of BTB and recommended BCG vaccine as a valuable tool in the control of BTB (21, 22). The use of BCG vaccination has been encouraged because of the development of a new skin test which could differentiate *M. bovis* infected animals from BCG-vaccinated animals (i.e., DIVA role) (23). The experiment conducted in Ethiopia on the evaluation of the efficacy BCG under natural challenge model demonstrated good performance of BCG particularly in reducing the severity and dissemination of the lesion (22). However, approved commercial BCG vaccines for use in cattle are not yet present on the market.

According to the stakeholders, BCG vaccination poorly reduces the prevalence of BTB in cattle. This is likely influenced by the role of BCG in sensitizing cattle to respond to the conventional BTB diagnostic test, combined with the low level of protection of BCG vaccine in cattle compared to its relative effectiveness in humans (22). To overcome the diagnostic limitations of PPD skin test, an alternative skin test that can differentiate infected from vaccinated animals (DIVA) was developed (24). Thus, in the presence of the alternative test with DIVA role BCG vaccination would be the preferred control option for control of BTB, particularly in low and middle income countries like Ethiopia (24).

The second and the third control options involve prior application of BCG vaccination to reduce the number of positive cattle followed by test and slaughter with half and full compensation by the government, respectively. In high income countries, test and slaughter is the most preferred and a widely applied approach to control and eventually eradicate BTB, since the prevalence is low (6, 7, 10). For instance, Australia is among the few countries that eradicated BTB successfully (25) while USA is on the verge of controlling BTB through tracing of infected herds identified through meat inspection, followed by test and slaughter program (26). Many European Union countries also were successful to be recognized as officially TB free countries (27). However, BTB control is not practically feasible in low and middle income countries like Ethiopia through test and slaughter method alone due to lack of resources for rigorous testing, tracing, slaughtering of large number of positive cattle, and compensations to farmers (28).

Alternatively, as revealed by the present study, applying BCG vaccination to reduce the prevalence and progression of BTB and subsequent integration with other control options such as test and slaughter, could be a novel approach for Ethiopia which can also be adopted by other low and middle income countries where BTB is endemic and implementation of test and slaughter policy is practically challenging. To that end, the availability and accessibility of commercial BCG vaccine is critically needed. In addition, mutual understanding, acceptability and cooperation between dairy cattle owners and the government are profoundly needed particularly regarding cost recovery scheme for the implementation of the approach.

Under scenario 2 (which did not consider BCG vaccination as a feasible option), test and slaughter with full compensation by the government ranked the number one preferred control option. Indeed, this could be the most preferred and acceptable option for the cattle owner from the perespective of relatively practical applicability and lower socio-economic impacts. However, this might not be acceptable to the government, as it would be too costly vis-a-vis to other priorities of the government (29). Besides the cost implication, implementation of the test and slaughter approach involves slaughtering and culling of large number of test positive cattle, especially at the beginning of the program, raising concerns of animal welfare and loss of cattle with good milk yield. This might affect the social acceptability of the control option.

Preferably, the second (test and segregation combined with test and slaughter with government support) and third (test and slaughter with cost sharing) options, would be the preferred options in developing countries. There are compelling evidences that test and segregation method significantly reduced the incidence of BTB (30, 31). The method involves segregating test negative animals at early stage of the disease from positive reactors based on whole herd testing. This is particularly important in countries where BTB control is lacking such as in Ethiopia and when BTB control is planned for the first time. For long term surveillance and application of BTB control with this method, testing the herd of all animals >6 weeks of age annually (depending on the incidence rate) and segregating between the positive and negative reactor. For positive reactor pregnant cow and segregating calf at birth is recommended (32). In case of milk from positive reactor cows, pasteurization of milk is effective treatment to avoid public risk and economic loss associated with discarding of milk. *M. bovis* is killed at pasteurization temperature and holding time (33). Use of small scale milk pasteurization and boiling of milk would be an alternative option for smallholder dairy farmers. More specifically, the authors suggested the third control option of scenario 2- test and slaughter with cost sharing as a reliable, feasible and acceptable option from economic point of view in Ethiopia. However, this requires as well advocacy and promotion to create awareness and to convince all stakeholders, particlularly the cattle owners to actively enagage in the implementation of the control options and share the associated costs. However, the social acceptance of slaughtering animals for disease control in this era would be very unlikely since BTB is not a public health emergency and due to the presence of effective public health measures such as meat inspection and milk pasteurization. These measures are not strictly followed in developing countries like Ethiopia and control of BTB in cattle contributed to breaking the cycle of trasmission of zoonotic BTB through meat and milk consumption. Given the high prevalence of BTB in the Ethiopian dairy herds, raising awareness of the public and communities at risk such as dairy farmers about the economic and public health of the diseases by the government would support the initiation of BTB control program.

In this study, the majority (70%, $n = 10$) of the stakeholders gave the highest weight to the epidemiologic criterion (reduction in the prevalence/incidence of BTB) in evaluating the identified control options, while the social ethics criteria generally received the least weight. The epidemiologic criterion is practically important for the control and eradication of BTB. For instance, the application of test and slaughter policy is challenged mainly

due to economic and animal welfare reasons, and this impact is lower when the prevalence of BTB is low (6).

The study has some limitations. The MCDA was based on the stakeholder's opinion and preferences which might result in the difference of the weighing clusters and criteria due to personal bias. In addition, some of the invited stakeholders did not participate. Future nationwide large-scale surveys involving all stakeholders from dairy farmers, academia, veterinary associations, research institutions, NGOs, commodity associations, federal and state agencies would remedy the limitation of small size of stakeholder participation. The other limitation is the absence of commercially licensed BCG vaccine for use in calves at the moment. In this study, BCG vaccine is identified by the stakeholders as a potential control option given its importance in lowering the prevalence and progression of BTB and the development of new skin test, DIVA, which could differentiate *M. bovis* infected animals from BCG-vaccinated animals.

Based on the insights obtained from this MCDA, the following important long-term stepwise approaches were suggested to initiate national level discussions and to create awareness regarding the need for BTB control and eventually work toward controlling/eradicating the disease. First, establishing a national multidisciplinary BTB organizing body/council is needed that is composed of representatives from all stakeholders that would support the government in the formulation of a national level BTB control/eradication program and implementation guidelines. Second, the economic feasibility of the top ranked control options should be assessed, and resources should be mobilized to validate the best control option in a sentinel population (i.e., applying it in selected urban and peri-urban intensive dairy farms where the prevalence of BTB is presumably high). Third, it is necessary to critically evaluate the outcomes of the control program in the selected areas and then extend the best practices to scale up the program to other regions of the country. Finally, conducting persistent surveillance and monitoring of the status of BTB across the country will be needed to develop a national database that would help in periodic evaluation of the effectiveness of the control measures put in place and taking timely corrective actions as needed. An effective and safe BCG vaccine for use in cattle is critical for this approach.

In conclusion, the study used an MCDA tool in identifying and evaluating BTB control options in Ethiopia. According to the stakeholders' preferences, calf vaccination and test and slaughter with full cost compensation by government are the best control options under a scenario that included BCG vaccination and a scenario with no BCG vaccination, respectively. Moreover, the study showed that integrating calf BCG vaccination with other potential control options, in minimizing the number of test positive cattle thereby decreasing the cost of compensation for culled/slaughtered cattle and maintain animal welfare as the most suitable BTB control option in Ethiopia that can also be used by other countries, especially low income countries.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

FG, GEA, KM, TB, MM, and GA designed the study. FG, SL, TT, ZA, BU, and GA collected and summarized the data during multi-criteria decision process. FG analyzed the data. FG wrote the manuscript. FG, GEA, KM, TB, TT, MM, RS, SL, ZA, BU, and GA critically revised and edited the manuscript and approved its submission.

FUNDING

This research was financially supported by Ethiopian Institute of Agricultural Research (Grant Number: 2.2/5961/2011) and Addis Ababa University.

ACKNOWLEDGMENTS

We thank the stakeholders for participating in the study and Tsedale Teshome for technical support. We are also grateful to Cécile Aenishaenslin for providing practical guide on the use of PROMETHEE software.

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frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 10 February 2021
doi: 10.3389/fvets.2021.623786

Concept of an Active Surveillance System for Q Fever in German Small Ruminants—Conflicts Between Best Practices and Feasibility

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OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
David González-Barrio, Complutense University of Madrid, Spain
Monika Szymańska-Czerwińska, National Veterinary Research Institute (NVRI), Poland

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *30 October 2020*
Accepted: *04 January 2021*
Published: *10 February 2021*

Citation:
Winter F, Schoneberg C, Wolf A, Bauer BU, Prüfer TL, Fischer SF, Gerdes U, Runge M, Ganter M and Campe A (2021) Concept of an Active Surveillance System for Q Fever in German Small Ruminants—Conflicts Between Best Practices and Feasibility. Front. Vet. Sci. 8:623786. doi: 10.3389/fvets.2021.623786

Q fever is a zoonotic disease caused by the bacterium *Coxiella burnetii*. Inhalation of contaminated dust particles or aerosols originating from animals (esp. small ruminants) is the main source of human infection. Hence, an active early warning system for Q fever in German small ruminant livestock was conceptualized to prevent human infections. First, we describe the best practice for establishing this system before evaluating its feasibility, as the combination of both evokes conflicts. Vaginal swabs from all husbandry systems with a focus on reproductive females should pooled and investigated by PCR to detect *C. burnetii*-shedding animals. Multistage risk-based sampling shall be carried out at the flock level and within-flock level. At the flock level, all flocks that are at risk to transmit the pathogen to the public must be sampled. At the within-flock level, all primi- and multiparous females after lambing must be tested in order to increase the probability of identifying a positive herd. Sampling should be performed during the main lambing period and before migration in residential areas. Furthermore, individual animals should be tested before migration or exhibition to ensure a negative status. If a flock tests positive in at least one individual sample, then flock-specific preventive measures should be implemented. This approach implies huge financial costs (sample testing, action/control measures). Hence, taking the step to develop more feasible and affordable preventive measures, e.g., vaccinating small ruminant flocks, should replace testing wherever justifiable.

Keywords: surveillance system, *Coxiella burnetii*, small ruminants, one health, early warning

INTRODUCTION

Infectious Agent

Coxiella burnetii is a small, obligate intracellular, pleomorphic Gram-negative bacterium. Because of its high tenacity, *C. burnetii* can be infectious in raw milk for 90–273 days at 4–6°C as well as in raw milk products like butter and soft cheese for 42 days at 20°C. In dust and wool, it can be infectious over 7–24 months depending on the surrounding temperature. *C. burnetii* evokes a zoonotic and mainly airborne disease called Q fever (1, 2).

Affected Species

Humans become infected by inhalation of dust particles or aerosols contaminated with only a few *C. burnetii* organisms (3, 4). Human infections through consumption of raw milk or raw milk products like butter or soft cheese is possible but rare. There is no transmission from man to man except some rare exceptions, e.g., by blood transfusion or during childbirth (5). Infections with *C. burnetii* remain subclinical in nearly 50% of patients. However, unspecific flu-like symptoms, hepatitis, or atypical pneumonia are possible consequences of acute Q fever, which may led to hospitalization (6, 7). In addition, approximately 20% of the patients are at risk of developing chronic fatigue after an infection (Q fever fatigue syndrome, QFS) (8). In <1% of the infected patients, chronic Q fever manifests in the form of endocarditis or hepatitis with a high mortality rate. Furthermore, Q fever is a risk for pregnancy (6, 7).

In animals, many species such as wild or domestic mammals and ticks can be infected by *C. burnetii* and spread this pathogen into the environment (6, 7, 9, 10). In Germany, infected (small) ruminants kept as livestock are the main source of pathogen transmission to humans (1, 11, 12). (Small) ruminants are infected by inhalation of *C. burnetii*, while other transmission routes (e.g., sexual, intrauterine, oral, infestation with ticks) are still being studied (7, 13–16). Therefore, pathogen transmission may take place through reservoir animals, which may be part of a flock or external (e.g., wild mammals but also dogs, cats other ruminant species, or ticks). These reservoir species may have some importance in maintaining *C. burnetii* in livestock as the most important factors here are contamination of the environment by their excretes as well as direct contact with livestock on the pasture. Pathogen transmission from wildlife to humans is rarely documented but is possible and should be considered when the source of infection cannot be identified (6, 7, 10, 11, 15). Concentrating on livestock, *C. burnetii*-infected sheep excrete the bacterium mainly in high concentrations in amniotic fluid, placenta and lochia during physiological birth or abortion. Moreover, excretion in milk, feces, urine, or semen is possible (1, 13, 17, 18). Goats and cattle also excrete the pathogen at the highest concentrations in birth products or with abortion, although the excretion period is longest in milk (17, 19–22). Q fever in sheep is mostly asymptomatic with an abortion rate of approximately 5–20% (23). In contrast, Q fever in goats is connected to abortion in most cases (17, 18, 22). However, abortion in small ruminants can have several causes and is not pathognomonic for an infection with *C. burnetii*. The impact of *C. burnetii* on reproductive disorders in cattle is still under discussion (17, 19, 20).

Pattern of Disease Occurrence

Q fever in humans and animals can be found worldwide except New Zealand and Antarctica. In Europe, an increasing trend was observed between 2012 and 2016, while from 2017 the number of confirmed Q fever cases decreased again. In 2018, 29 EU/EEA countries reported 794 confirmed Q fever cases. The three countries with the most confirmed cases in 2018 were Spain, France and Germany (24). In Germany, outbreaks of Q fever in the human population occur regularly, without nationwide spread, but often in connection with infected small ruminants. The distribution of Q fever was previously described (25–27).

In summary, the number of notified human Q fever cases in Germany has increased over the years and peaked during outbreaks since reporting obligations were regulated in 1962 (25–27). Between 2005 and 2018, the notified cases ranged between 416 and 93 each year as mentioned by the German Robert Koch Institute (RKI), without any detectable trend (24, 28). Due to the concept of passive monitoring, the number of notified cases depends on awareness by the relevant stakeholder groups and may exceed average numbers by far in the case of larger outbreaks as seen in 2003, 2005, 2008, 2010, or 2014, for example (11, 24, 25). In Germany, sporadic cases are reported nationwide, but outbreaks in the human population are reported more frequently in southern federal states and can often be linked to Q fever in small ruminants (11, 25, 26).

In comparison, the average number of notified cases in German (small) ruminant flocks fluctuated between 1970 and 2000 (26). Between 2000 and 2018, a total of 9,920 official individual cases were reported (8,359 cattle; 1,349 sheep; 212 goats) (25). Most of the notified animals were cattle, which can not only be explained by the total numbers of animals, but by the legal requirements for routine diagnostics and the different disease progression in cattle, resulting in more frequent testing of this species instead of small ruminants (26). Again, awareness of the disease is important, as illustrated by the increase of notified cases in cattle, sheep, and goats followed by outbreaks in the human population in Bad Sassendorf/Soest in 2003 and in Jena in 2005 (25, 26, 29).

Supplementary to the official reported cases, only a few surveys were conducted in the past (25). For example, the German within-flock seroprevalence was estimated at 19.3% in cattle, 8.7% in sheep, and 2.5% in goats (11). Moreover, Wolf and colleagues estimated the serological flock prevalence for small ruminants in Germany between 26 and 36.6% [$n = 71$; 14]. In comparison, in a non-representative German study, 7.8% of cattle ($n = 21{,}191$), 1.3% of sheep ($n = 1{,}346$), and 2.5% of goats ($n = 278$) tested positive for *C. burnetii* by PCR (30). Wolf et al. (14) detected that 13.9% of tested sheep and goat flocks ($n = 71$) were positive by PCR. Other studies mainly aimed at sheep farming within German federal states. Therefore, their study results vary widely due to their study design and locations (25). However, it can be concluded that detection of *C. burnetii* antibodies and the pathogen itself differs between animal species, geographic areas, and time of sampling (25).

Zoonotic Potential

Humans are at highest risk of inhaling *C. burnetii* through distant and close contact with animals, especially small ruminants (1, 11, 12, 31). **Distant contact** may occur through geographical proximity to small ruminant flocks as well as by visits to farmers markets where small ruminants are exhibited (11, 29, 31). As Q fever is an airborne disease, the pathogen can be spread over longer distances by wind and may pose a risk for human infection (11, 31). Therefore, the greatest risk of infection is within a radius of 2–4 km from the source of the pathogen. Moreover, in gale force winds, *C. burnetii* may reach distances up to 18 km

(31). Migration of small ruminants through residential areas was identified as a potential risk factor (32). Therefore, it may be a challenge to identify the infection source in human cases that did not have any obvious or known contact with sheep or goats. A higher risk of transmission from small ruminants to humans can be observed in spring/early summer due to the out-of-season lambing of some sheep breeds on pasture (11, 31, 33). Furthermore, **close contact** with contaminated products such as afterbirth or contaminated wool during sheep shearing may also be sources for human infections that can occur sporadically, e.g., animal owners, their family members, or employees (33, 34). Moreover, employees of slaughterhouses or laboratories can become infected at the workplace. Infections by consumption of contaminated raw milk (products) is possible but rare (5). Also possible, but even rarer, is infection through live cell therapy (35).

Rules and Regulations

On an international level, the World Organization for Animal Health (OIE) lists Q fever in the category of multiple species diseases (36). Moreover, OIE recommends protocols for diagnostic testing and vaccination of Q fever in small ruminants to prevent human infections (5). On the level of the European Union, data sampling of zoonoses in humans is regulated under Decision No 211/98/EC and coordinated by the European Centre for Disease Prevention and Control (ECDC). Moreover, Q fever is included in the Community Summary Reports on zoonoses since 2005. To harmonize information about Q fever within the European Union, a scheme for the monitoring and reporting of Q fever in animals was developed in 2010 under Directive 2003/99/EG. This report was prepared by the European Food Safety Authority (EFSA) in collaboration with the ECDC and EFSA's Zoonoses Collaborating Centre (32).

On the national level, Q fever in humans is a notifiable disease with inception of the German Protection against Infection Act (IfSG) in 2001 (25, 37). In accordance with the German National Animal Health Act (TierGesG) and the German Regulation of Notifiable Animal Diseases (TKrMeldpflV), only the direct detection (by culture or PCR) of *C. burnetii* in small ruminants and cattle has to be reported to the local veterinary health authority (25, 38, 39). All notifications are reported in the Animal Disease Reporting System (TSN) by the local veterinary health authorities and contain the date of detection, the species, the flock and its owner as well as the county/city concerned (25). Apart from official rules and regulations, the RKI published recommendations for Q fever after a huge sheep-associated outbreak in 2006 (9, 29). Moreover, an interdisciplinary research association in the German federal state Baden-Wuerttemberg published recommendations for the management of Q fever cases and outbreaks (40). Furthermore, the German Federal Institute for Risk Assessment (BfR) recommends pasteurization of raw milk before consumption and food processing if a *C. burnetii* infection is diagnosed within a ruminant flock (2). A recommendation published by the German Ministry of Food and Agriculture (BMEL) is available that involves hygiene measures in the case of Q fever cases in (small) ruminants (41). However, these recommendations are not mandatory and cannot be imposed by the local veterinary health authorities. In comparison, the local public health authorities may impose regulatory measures for animals based on the IfSG if humans are infected.

Rationale for Using a Monitoring and Surveillance Systems

The German legislation underlines the rationale for using a monitoring and surveillance system (MOSS) for Q fever as it defines this zoonosis as a notifiable disease in both (small) ruminants and humans (37–39). Hence, both MOSS's that are currently in place can be defined as passive monitoring systems, which focus on human health as the predominant rationale instead of health or production effects on animals (25, 42). However, the passive monitoring system of Q fever in small ruminants does not serve as reliable protection for humans against infection. This is especially because infection in small ruminants may proceed with unspecific symptoms or even asymptomatically (17, 18). As a passive reporting system does not detect these kinds of diseases very well, we assume that the occurrence of Q fever in small ruminants is heavily underestimated in Germany (43, 44). To prevent new infections in humans, it is important to gather reliable information about the current Q fever status of small ruminant flocks. With this knowledge, flock-specific action and control measures can be induced, while these measures effectively prevent new cases in the human population, as transmission risk will be reduced (5, 38, 39). It could be discussed that the low number of notified cases in the human population does not directly impose the need for action. However, underreporting of Q fever in humans has to be assumed, as symptoms are unspecific, and zoonotic infections are not high on the list of differential diagnoses (30, 45). Due to the obvious risk for human infection, there is a need to improve passive monitoring in small ruminants toward active surveillance, including measures for the control of Q fever (5, 42, 44). Therefore, the objective of this paper was to conceptualize such a MOSS as an early warning system for Q fever in small ruminant livestock in Germany and to evaluate the concept with regard to feasibility.

METHODS

To comply with international standards, previously developed checklists for the design of a MOSS in the animal health sector were used and modified (42, 43, 46–48). Referring to these international standards, we summarized the main characteristics and the chosen options of our concept in **Tables 1**, **2**. First, we started by defining the **hypothesis and aim** of our MOSS. Afterwards, we discussed the **type of system** in detail. All further options in terms of the described characteristics of this concept are contingent on this aim with regard to international literature about *C. burnetii*, national legislation and regional differences in husbandry of small ruminants. Next, we focused on the characteristics of the **target population** for this MOSS, which is split into the topics ***animal species***, ***sex and age***, ***region,*** and ***husbandry***. After this, we determined the **dependent variables** including the topics ***disease stage***, ***unit of interest,*** and

TABLE 1 | Concept of an active surveillance system for Q fever in German small ruminants—conflicts between best practices and feasibility.

Characteristics (Topics)	Chosen options
Hypothesis	Small ruminants are the main source for Q fever infections/epidemics in the human population in Germany
Aim	Early warning system for Q fever in small ruminant flocks to prevent Q fever infections in the human population
Type of system	(Voluntary) active surveillance system in small ruminants with individually adapted action and control plan
Target population	All female reproductive small ruminants within the flocks regardless of the husbandry systems in Germany
Dependent variable	The flock status of *C. burnetii* shedding detected by PCR of pooled vaginal swabs in female small ruminants in Germany—with the flock-status defined as positive by at least one pool-sample testing positive
Independent variables	Factors influencing the flock status of *C. burnetii* shedding: - Preliminary information (suspicious symptoms, antibody activity, vaccination) - Target population (sex and age)
Sampling technique and sample size	Multistage risked-based sampling
At the flock level	Testing all flocks at risk to transmit the pathogen to public (reproduction, close contact, distant contact)
At the within-flock level	Testing all primi- and multiparous females after lambing in order to increase the probability of identifying a positive herd
Sampling time	During the main lambing period and before migrating in residential areas
Bias	- Selection bias - Information bias
Action and control plan	(Voluntary) individually adapted control plan in positive tested flocks
In the case of positive flock status	- Prevention of close contact between flocks and the public - Prevention of distant contact between flocks and the public - Hygienic measures on the farm - Flock-vaccination - Continuous testing - Examination of the people living and working on the farm
In the case of negative flock status	Continuous testing

TABLE 2 | Implementation and evaluation of an active surveillance system for Q fever in German small ruminants—conflicts between best practices and feasibility.

Characteristics (Topics)	Chosen options
Stakeholders	Small ruminant sector - Animal owners, their employees, family members - Regional/national/breeding associations - Animal traders, abattoirs, dairies, and sheepshearers Veterinary health professionals - Veterinary health authority - Veterinary practitioners/animal health services - Laboratorians Human health professionals - Human health authority - Physicians - Laboratorians Policy makers Animal Disease Funds Public
Economic considerations	Financial costs - MOSS coordination - Sample collection - Sample testing - Costs from modifying usual flock management and housing practices - Vaccination costs - Loss of income Financial benefits - Saved costs for physicians, laboratories, medicine, hospitalization - Saved non-productive time of humans - Quality awareness - Saved cost for action measures Emotional costs - Stigmatism of people in the small ruminants sector - Existential fear of people in the small ruminant sector - Panic in the public Emotional benefits - Knowledge about flock status and necessary consequences - Responsibility of animal owners toward their fellow humans - Gain in confidence of the public in the animal owners - Zoonosis prevention/One Health

diagnosis. Then, the **independent variables and confounder** were discussed. In the next step, we focused on the **sampling technique and sample size** at the ***flock level*** and ***within-flock level*** as well as ***sampling time*** and ***bias***. As Q fever bears a serious risk for the human population, we discussed an **action and control plan *in case of a positive*** or ***negative flock status***. As **implementation and evaluation** of a MOSS are important issues at the time of concept planning, we discussed the involvement of ***stakeholders*** as well as ***economic considerations*** in the context of this concept.

The referred literature was assessed in a non-systematic search strategy using the search database Web of Science (http://apps.webofknowledge.com) performed in English. Investigated search terms were defined and combined with the Boolean Operators AND and OR.

To report about our concept of an active surveillance system for Q fever in small ruminants, the STROBE Statement was used as a guideline (49).

CONCEPT OF THE MOSS

In this paper, we compile a concept of an early warning system for Q fever in small ruminants in Germany. First, we describe the

best practice for this MOSS before taking the next step to address feasibility, as the combination of both evokes conflicts. The basic points of the MOSS are summarized in **Tables 1**, **2**.

Hypothesis and Aim

To conceptualize a MOSS, the hypothesis and the aim are mandatory to define first (42, 43). These generate the basis for every subsequent decision regarding the design of the concept. Q fever has a non-specific aetiopathology as well as little effect on the health status of small ruminants. Nevertheless, there is no active MOSS for Q fever in small ruminants, which is mandatory in all federal states. Based on these facts, we assume that the health status of most small ruminants and thus the potential sources of human Q fever infections in Germany are unknown. As only a few *C. burnetii* organisms can initiate active infection in humans (3), it is imperative to detect a possible source of transmission as soon as possible. Therefore, "early detection in a defined animal population" is the predominant purpose of the MOSS presented here (5, 36, 46).

Type of System

MOSSs are used to gather health data of a defined population (47, 48). However, it should be discussed whether "passive" or "active" data collection is desired and if action should follow a positive finding, i.e., "monitoring" or "surveillance."

In the case of our MOSS, "monitoring" would result in the observation of the Q fever status in small ruminants without any control activities in the case of positive findings (42, 43, 47). However, as we are concerned with a zoonotic agent with considerable human health impact, monitoring without control activities would foil the principles of risk prevention and veterinary public health. Hence, "surveillance" system is preferred here. Those control activities can be either mandatory or voluntary. For mandatory implementation of control activities, rules, and regulations are necessary. In the case of human outbreaks caused by small ruminants or cattle, veterinarians and human local health offices have to work closely together using the IfSG. However, German law does not regulate consistent control activities in the case of Q fever in small ruminants without a link to human Q fever cases (37–39). A competent public health authority can take necessary measures to avert imminent danger only if facts are established, which might lead to the occurrence of Q fever in humans, or if it can be assumed that such facts exist (37). Hence, regulations need modifications. In the meantime, surveillance is contingent upon the voluntary implementation of control activities by animal owners. However, a surveillance system with voluntary control activities calls on the responsibility of animal owners for people in their environment. For the success of this MOSS concept, further considerations presume a 100% participation rate.

Furthermore, it is important to decide whether a "passive" or "active" data collection must take place for this MOSS (42, 43, 47). In the case of a "passive" MOSS, a reporting cascade is necessary to gather health data (43). Such a "passive" method would be an inexpensive way to get information about the Q fever status of small ruminants in Germany because the veterinary health authorities would not need to plan data acquisition, to take diagnostic tests or finance the work of sampling and laboratories. However, this "passive" system contains many sources of error as it is based on the idea that animal owners and practicing veterinarians recognize clinical signs of Q fever very easily and screen the population of interest. Therefore, passive monitoring would overlook small ruminants with positive Q fever status. Furthermore, different knowledge about Q fever and different motivations to report positive flock status negatively influence the quality of health data (42, 43, 50). Animal owners may be less motivated if they distrust the system, especially if consequences after reporting a positive Q fever status are unforeseeable [e.g., stigmatzation; (43)]. Therefore, the reporting cascade would not work reliably for this disease. In contrast, an "active" MOSS collects health data in a systematic way or by regular recording (42). For the active collection method, it is typical to define a specific aim of the MOSS, to use a formal sampling process in a specific population and a specific time of collection as well as standard tests to detect positive findings (42, 43). In general, the local veterinary health authorities are responsible for the coordination of this regular and periodic collection of health data (43). It is important to invest both money and time in structured data acquisition to get valid information about Q fever status in a favorable cost-benefit ratio (46–48). Hence, this leads to an "active" system of data collection in the case of our MOSS. An active MOSS is not restricted to clinical cases with typical symptoms, which is an important advantage in the case of Q fever in small ruminants. Another positive effect for the quality of the data is the control of sampling scheme and information collection by a competent veterinary health authority that guarantees statistically based objectivity as well as increasing comparability of results (42, 43). In conclusion, this MOSS should be implemented as an active surveillance system.

Target Population

The target population is defined as the population that is the focus of a study (42, 51). For example, this can include the whole population at risk that is susceptible to a specifical disease or just a subgroup with special qualities of this population at risk (42). If the target population is not clearly defined, individual interpretation of study results can lead to bias and differential perception of the results (51). To define the target population of this MOSS, we divided their characteristics into the topics ***animal species***, ***sex and age***, ***region,*** and ***husbandry*** (46–48).

Animal Species

Although various domestic and wild mammals can be reservoirs for *C. burnetii*, the focus of this approach is on small ruminant livestock (6, 7, 9, 10). On an international level, (aborting) dairy goats have been the reason for the biggest Q fever outbreaks in humans. However, in Germany most small-scale epidemics have been caused by nondairy sheep (25). Because sheep and goats are often kept together (especially in southern Germany) and because the number of goats is increasing across the country (14, 25), this MOSS has to focus on both species. The German Federal Statistical Office reported 19,556 farms with 1,834,275 sheep and 9,808 farms with 138,810 goats in 2016. Unfortunately, no official

number about these farms keeping sheep and goats together is available (52, 53).

Sex and Age

Possible differences regarding the transmission risk to humans between sex and age groups have to be considered when defining the target population. Both female and male small ruminants can be infected by *C. burnetii* and shed the pathogen via different pathways (13, 14, 18, 21, 22, 54). However, the highest pathogen concentrations are shed in amniotic fluid, placenta, and lochia during physiological birth or abortion and in the milk of small ruminants (18, 21, 22, 54). Hence, female small ruminants after lambing are the subgroup with the highest risk for humans to acquire infection and therefore are focused on in this MOSS. In Germany, approximately 64.42% (n = 1,181,560) of the sheep population and 63.72% (n = 88,451) of the goat population are reproductive females (52).

Region

As the pattern of disease occurrence of Q fever in humans and small ruminants in Germany was previously described (25–27), the reported cases and studies show that Q fever is endemic in Germany (11, 25–27). Therefore, the MOSS should be implemented nationwide. In Germany, rules and regulations for the implementation of a MOSS are split into national, federal, and district tasks. In the districts, the lowest administrative level, veterinary and public health authorities are responsible for general coordination and implementation of disease prevention in animals as well as in humans. These veterinary and public health authorities on the district level can rely on (communication) structures between different stakeholders, which are needed for the implementation of this MOSS (see chapter "Implementation and evaluation"). As the wind and the distance between settlements and small ruminants have an impact on Q fever transmission (11, 31), this MOSS mentions urbanization, density of livestock, and individual weather conditions in different regions of Germany and appeals to the cooperation between neighboring districts in the case of positive findings. In conclusion, this MOSS must be implemented nationwide with the administrative districts as the lowest subregional structure being responsible for implementation of action and control activities.

Husbandry

The current situation of sheep and goat husbandry in Germany was previously described by Bauer et al. (25). In short, the distribution of animals, farms and breeds differs between German federal states (25). Moreover, most German small ruminant farms practice a combination of management systems including grazing enclosure, milk production, and shepherded and migrating flocks, which makes husbandry very inhomogeneous (50). In addition, small ruminant husbandry is mostly associated with **reproduction** for lamb production as well as with landscape conservation and protection. Both are connected with **distant contact** between small ruminants and the public due to lambing and grazing on pasture and the proximity to residential areas. Migrating flocks, which change their location regularly or even cross administrative district borders, are particularly noteworthy here. Furthermore, **close contact** between small ruminants and the public takes place in petting zoos, family farm vacations, and animal-assisted education or therapy. These risk factors for public health will be discussed in the "sampling technique and sample size" section of this manuscript. Finally, this MOSS needs to focus on the whole range of small ruminant husbandry, as there is an overall risk of transmission to humans.

Dependent Variable

Conceptualized as an early warning system, the dependent variable for this MOSS is the status of Q fever in small ruminants.

Disease Stage

To describe a disease status in general, a MOSS can focus on different disease stages such as the infection, the occurrence of symptoms, the presence of antibodies, or the excretion of the pathogen itself. As we have to consider the zoonotic potential of Q fever, pathogen shedding by small ruminants must be identified as early as possible. Since clinical symptoms or the presence of antibodies cannot be connected to the occurrence of pathogen shedding (12, 18, 21, 32, 44), this MOSS has to focus on the detection of pathogen shedding itself. As mentioned in the topic **sex and age,** the perinatal period is the time of highest risk for human infections (18, 21, 22), and this MOSS focuses on the detection of current pathogen shedding early after lambing.

Unit of Interest

"The individual (animal), pen, herd, flock, or farm" or another defined structure may be chosen as an unit of interest (42). Choosing the individual animal as the smallest unit of interest is useful to detect sporadic diseases. Therefore, this strategy is valuable for a disease that is not very contagious. However, Q fever is a contagious disease and can be spread easily within flocks. This means that the occurrence of more than one *C. burnetii* shedding individual within a group of animals is very probable. Choosing the status of pathogen shedding at the flock level, the probability of a positive flock status may change over time with the composition of individuals within the flock (51). As farms may manage more than one flock, and flocks may be housed and handled differently, flocks should be the most appropriate unit of interest here (5, 51, 55). Due to the high concentration and prolonged time of *C. burnetii* shedding, a single *C. burnetii*-infected individual may be a sufficient risk for human infections (3, 29). Therefore, a single animal testing positive is sufficient to designate the flock as positive for *C. burnetii* shedding (14, 44, 55). Conclusively, the unit of interest is the flock status of pathogen shedding defined by at least one individual-sample testing positive.

Diagnosis

Different diagnostic tests are available for direct detection of *C. burnetii*. Only specialized labs perform bacterial cultivation. However, PCR is the most feasible diagnostic test for this MOSS because primer and probe sequences as well as ready-to-use kits are commercially available, and results may be provided with

high speed and accuracy (i.e., high specificity and sensitivity) (1, 5, 25). PCR has been used to detect *C. burnetii*-genome fragments in feces, however further research is necessary to confirm feces as adequate test material to detect acute pathogen shedding in small ruminants (56, 57). As an officially approved diagnostic test, PCR is able to detect *C. burnetii*-genome fragments in milk samples, organs of fetuses, birth products, vaginal swabs, and ticks (25, 58). However, milk production is present on only 2.68% ($n = 524$) of all sheep farms and comprises only 0.98% ($n = 17{,}999$) of the sheep population in Germany in 2016, making this sample material unsuitable for this MOSS (52). In addition to sampling of fetuses and birth products, sampling of vaginal swabs means additional effort and cost, but vaginal swabs provide a high-quality sampling material (5, 25, 59). Furthermore, a vaginal swab can be assigned to an individual animal, so that subsequent measures can be implemented individually (e.g., follow-up testing). In addition, pooled samples of vaginal swabs are applicable to reduce temporal expenditure and financial costs (42). Conclusively, diagnosis must be carried out by conducting pooled vaginal swabs testing by PCR.

Independent Variables and Confounder

Factors influencing the probability of a flock testing positive for pathogen shedding were already discussed before and are only listed here for reasons of clarity:

- Preliminary information: Flocks already known to show **suspicious symptoms such as abortion** at the flock level [the abortion rate ranges between 5 and 90% of pregnant females, while 5–20% is common in sheep flocks, and high abortion rates occur only in some goat flocks (23)] may be an indicator of current and ongoing infection and shedding. Moreover, previously known **antibody titers** in animals may be an indicator of current or ongoing infection and shedding in a flock. This may increase the probability of a flock testing positive for current shedding but there is no guarantee (1, 59). Although previous **vaccination** heavily influences the antibody activity, it also reduces the amount of pathogens shed (5).
- Target population: Female animals with perinatal status are most likely to shed high amounts of the pathogen (see chapter "Sex and age") (18, 21).

Sampling Technique and Sample Size

First, it has to be decided which procedure is applicable for the aim of this MOSS: a census or a sample. The main disadvantage of a census is its great cost in terms of time and money; conversely, an advantage of sampling is its low cost, while leading to reliable estimation of the target population if the sample is selected correctly (42, 51). A solution is to conduct a risk-based sample. This enables lower costs and monitoring of the most relevant samples. Due to the well-known cluster effect of (n) animals in (m) farms, multistage sampling has to be considered, where first the number of flocks and then the number of animals per flock are determined (51).

At the Flock Level

Concerning risked-based sampling, flocks with the following characteristics should be investigated predominantly:

- **Reproduction** in general, but especially when lambing will happen on pasture: Information about the flock management (lambing location i.e., lambing in stable vs. on pasture) as well as the time of parturition (estrus synchronization/artificial insemination, lambing season) must be queried (33).
- **Close contact** in general, but especially when female and pregnant small ruminants are to be exhibited: Information about exhibitions (i.e., conformation shows, farm vacations, open house days). Furthermore, all reproducing female small ruminants kept for petting zoos, animal-assisted education and therapy must be identified and investigated in particular (29).
- **Distant contact** in general, but especially when migration is performed in residential areas: Information about the migration of flocks must be acquired, and the migrating flocks must be tested regularly (31, 33).

As it can be assumed that these characteristics concern most of the German small ruminant flocks, this risked-based sampling may almost be a census.

At the Within-Flock Level

Concerning risked-based sampling, all females after lambing should be investigated predominantly. Assuming that the flock has a positive status given that one individual is shedding *C. burnetii*, we calculated the required sample size (n) to identify freedom from disease considering the absolute number of primi-/multiparous females per flock (N) [see **Supplementary Material**; (5, 36, 51)]. Our calculation shows that the difference between census and sample (N-n) is minimal and therefore this risked-based sampling is almost a census (see **Supplementary Material**).

Sampling Time

Flock level testing should be done during the main lambing period and before migrating into residential areas at least once a year. In addition, an ultrasound examination should be carried out before individual animals are exhibited or transported/slaughtered. Animals in the last trimester of pregnancy should not be exhibited to prevent spontaneous abortions or births near humans and should not be transported/slaughtered to prevent infection of animal traders and abattoirs.

Bias

A sampling process always leads to an uncertainty about the gathered information because a conclusive statement in response to a research question is only available for the sampled individuals (42). To assess the quality of this MOSS, this section discusses possible sources for bias.

Selection bias describes the difference between an estimation and the truth (60). In case of this MOSS, the selection bias may originate from the sampling technique described above. First, it is important to mention that this MOSS currently depends

on voluntary participation of the different stakeholders. Poor participation by these stakeholders could introduce selection bias such that only interested animal owners participate. This could bias the percentage of flocks testing positive toward an overestimation, if these persons already know about their flock having a problem with Q fever. However, it could also bias toward an underestimation, if these persons focus their flock management on a perfect hygiene strategy and therefore participate to prevent further *C. burnetii* infections. A percentage over- or underestimation would not impede the aim of this MOSS, which is to identify as many herds as possible in total that shed *C. burnetii*. Furthermore, if the system becomes obligatory, this bias will be settled. Next, veterinary health officers have to administer the risk-based principle of flock selection and individuals within the flocks. For example, sampling of non-mated female small ruminants, which do not lamb, or sampling of mated female small ruminants during pregnancy and late after lambing also may lead to underestimation. Although this selection process can be supported by the use of a questionnaire, it depends on subjective decisions and may be a source of possible bias. Beyond this, correct assignment of high-risk flocks depends on animal owners' provision of correct information.

Information bias describes the difference between the estimation and the truth that originates from over- or underestimation of results (60). In the case of this MOSS, the greatest information bias is because a few animal owners do not know the time of lambing, as fertile male small ruminants are constantly in the herd. Therefore, the information about time of lambing can only be communicated to the veterinary health officers when it has already taken place. Very good communication between animal owners and veterinarians is therefore a prerequisite for obtaining samples in a timely manner. Further information bias may originate from error classification of pools resulting in false classification of the flock status. This depends on the sensitivity and specificity of the chosen diagnostic test (60), which are very high in the case of PCR testing (1, 5, 25). As *C. burnetii* shedding occurs sporadically, test results can miss a positive individual and therefore the positive status of a flock. By planning to test herds completely and during a time when identification of positive animals is most likely, single false-positive or negative test results can only modify the estimated disease status on the flock level if shedding occurs in very few animals or if the flock is small. Therefore, as the test accuracy is comparably high and pools instead of individual animals are investigated, the test performance does not have to be considered as relevant bias for the determination of disease status.

Action and Control Plan

Recommendations for action and control measures regarding Q fever in small ruminants have been published elsewhere (1, 5, 12, 25, 33, 40, 41). However, German flock management and husbandry vary considerably between each small ruminant (production) system, resulting in a varying risk of pathogen transmission to the human population (5). Therefore, individually adapted action and control plans are preferable over general recommendations and should be developed with the cooperation of veterinary and human health professionals and the animal owners.

In the Case of Positive Flock Status (Pathogen Shedding in a Flock)

If *C. burnetii* shedding is detected, immediate and long-term actions must be defined in a flock-specific action and control plan (41).

Most importantly, prevention of close contact between the positive flock and the public means that animal **exhibitions** as well as **unauthorized persons in the flock** (i.e., farm visits, vacations, open house days) have to cease until the flock is proven negative again. **Authorized persons working in the flock** (animal owners, their employees, animal traders, abattoirs, dairies, sheepshearers) must protect themselves with **personal protection equipment;** wearing FFP3 respirators is most important, and protective work clothing should only be worn within the specific flock (12, 33, 40, 41).

Similarly important is the prevention of distant contact between the flock and the public. Therefore, the animal owners have to organize **lambing inside a stable** and to **store contagious materials** such as afterbirth or aborted material in safe containers until rendering. In addition, **cleaning and disinfection** of lambing areas and stables are necessary to prevent pathogen dissemination. Furthermore, **dung and litter** have to be covered for 9 months before spreading it on farmland (12, 33, 40, 41). Although the alimentary infection pathway is unlikely (2), selling and consumption of **raw milk and raw milk products** must be prohibited (12, 40, 41). Small ruminants must not be used as a source for **live cell therapy** (35). No **migrating** should be allowed, and the flock should be **kept as far as possible from human habitation** until the flock is proven negative again (31). **Shearing and storage of wool** have to take place in a closed room while wearing personal protection equipment. Contaminated wool must be destroyed in a rendering plant (31, 33, 40, 41).

Moreover, an action that concerns the individual animals itself is **vaccination**. However, phase I vaccine should be preferred over phase II vaccine as it is more effective (61). Although phase I vaccination cannot stop the shedding of *C. burnetii*, it can reduce pathogen shedding (1, 17, 33, 61). Oxytetracycline treatment is not recommended in the case of Q fever in small ruminants, as it does not stop pathogen shedding (25, 61).

As Q fever occurs sporadically and may reoccur after some time, a flock with a positive status should be **retested** for at least the next two lambing seasons (22). To prevent further unnoticed pathogen spread, the possible source of infection should be investigated [**tracing on** and **tracing back**, **wildlife as pathogen reservoir**; (10, 12, 33, 34, 45, 54, 61)].

In the Case of Negative Flock Status

If the status of a flock is negative, *C. burnetii* shedding was not detected by PCR of vaginal swabs and transmission to the public is currently unlikely. However, as Q fever occurs sporadically, **annually recurrent testing** is necessary. In cases of **increased risk for transmission** to the public, additional testing should be applied. Furthermore, **animals in their last trimester**

of pregnancy must not be transported, e.g., for exhibition or slaughter, in order to avoid pathogen contamination by spontaneous lambing or slaughter. Accordingly, an ultrasonography examination to determine the pregnancy status of an individual animal has to be carried out prior to transportation.

Implementation and Evaluation

To implement and evaluate this MOSS, it is important to discuss which ***stakeholders*** are potentially concerned and which ***economic considerations*** should be regarded.

Stakeholders

Stakeholders who are affected in some way by this MOSS are different subgroups working in the small ruminant sector as well as veterinary and human health professionals, policy makers, Animal Disease Funds and the public itself (5, 29, 45, 55, 62–64).

The small ruminant sector first includes **sheep and goat owners, their employees, and family members**. Small ruminant owners are organized within **regional and national (breeding) associations**. These associations represent the opinions of their members and are important counterparts to get in contact with small ruminant owners and amplify information regarding MOSS implementation. As long as participation is voluntary, animal owners have to actively agree to take part. Participation will be influenced by the good communication between different stakeholder groups and awareness of the importance of this MOSS for public health. Therefore, we discussed the idea of closely linking voluntary Q fever monitoring to another already well-established (and mandatory) monitoring program for brucellosis with a group of representatives from small ruminant owner associations. It became clear that awareness about the impact of this disease for public health currently is not high enough to trigger willingness to participate. The representatives emphasized that small ruminant owners are already overloaded with regulations and legal documentations and cannot justify further workload and restrictions, which are not predictable in the case of a positive flock status. Hence, it has to be concluded that prior to and in parallel with implementation of this MOSS, great effort must be put into raising awareness, dismantling barriers and fears and increasing knowledge of Q fever and exactly how the MOSS will work. Furthermore, possible economic benefits of a negative test result need to be discussed and emphasized to create an incentive for participation (see chapter "Economic considerations").

Further groups in the small ruminant sector that have to be considered are **animal traders, abattoirs, dairies, and sheepshearers**. These groups need to know the status of flocks in order to adapt their working habits when handling positive flocks with regard to personal protection equipment (i.e., wearing FFP-3 breathing masks) or when processing material derived from small ruminants [i.e., pasteurization of raw milk, separate slaughter, handling and selling of wool; (33, 40, 41)]. Some professionals might even have to postpone or cancel their work in small ruminant flocks that tests positive (e.g., sheepshearers) due to self-protection.

Veterinary health professionals includes the **veterinary health authority officers** who have to organize steps such as selection of most relevant flocks as well as the documentation and analysis of test results. Elaboration of flock-specific action and control plans is another of their tasks. However, these steps must take place in cooperation with the animal owners. At this point, **veterinary practitioners** function as a link between the veterinary health authority and animal owners, as they know the animal owners as their customers and therefore can advise which measures have to be implemented for the affected flock. In addition, veterinary practitioners must perform the vaginal swab sampling on behalf of the veterinary health authority. Since an animal health service exists in most German federal states, these tasks can be transferred to the practicing veterinarians of this organizational unit. **Laboratories** are necessary to do the diagnostic testing of these swabs. To implement necessary safety measures at the laboratory, samples have to be packed safely and accompanied by meaningful preliminary reports.

As Q fever is a zoonosis, human health professionals are additional stakeholders in this MOSS. Therefore, the **public health authority officers** and their colleagues at the veterinary health authority must cooperate. In the case of a positive flock status, an exchange of information must take place automatically, as this provides an early warning to draw attention to possible Q fever cases in the human population. Hence, the public health authority officers should forward information to **physicians** to raise awareness and alertness, without stigmatizing the animal owners and their families. Furthermore, **laboratories** in the human health sector are involved if human cases occur, and they must be informed to be alert about the zoonotic potential as well.

Next, policy makers are an important group of stakeholders in this MOSS. Due to recent German legislation, Q fever is only monitored via a passive MOSS. Hence, implementation of any new surveillance attempts depends on the voluntary participation of animal owners and veterinary health professionals. Therefore, legislation needs to be revised, and an active MOSS has to become mandatory in order to protect the public effectively.

Furthermore, the revision has to include subsidies for animal owners in cases of positive herd status. This leads to the Animal Disease Funds as further stakeholders. These public-law institutions are regulated nationwide by the TierGesG, but within the federal states the reimbursement of costs for monitoring and combating animal diseases varies (38). Therefore, federal legislation is necessary to regulate the subsidization of animal owners by these institutions in the case of positive flock status.

Finally, the public has to be mentioned as a stakeholder group. If there is a risk of a Q fever outbreak in the population, the population should be informed about possible risk factors and preventive behavior. Therefore, it is important that the public relations department of each district cooperates with the local press to inform the public without generating panic or causing stigmatization of small ruminant owners (62, 63).

Economic Considerations

Decisions about this concept are driven by economic considerations that affect all different stakeholder groups.

Financial costs evolve from MOSS coordination, sample collection and testing as well as action and control measures (see **Supplementary Material**). Therefore, the conflict between best practice and feasibility is notable.

For **MOSS coordination**, labor costs for veterinary health authority employees have to be assessed prior to implementation. Here, the effort to collect basic information about the risk-status of the flocks within each governmental district is considered the most time-consuming task. Calculating the financial costs of **sample collection, sample testing,** and vaccination, as an **action and control** measure, we considered the German veterinary fee regulation (GOT), the fee regulation for administration/consumer protection and veterinary health authority (GOVV) in Lower Saxony and the German permanent vaccination commission for veterinary medicine (STIKO Vet) [see **Supplementary Material**; (65–67)]. As **sample collection** has to be carried out by veterinary practitioners on behalf of the veterinary health authority, labor costs, driving costs, materials (vaginal swabs) and sample shipment must be taken into account. Additionally, costs for **sample testing** by the laboratories must be considered, whereby pooling of samples saves costs (see **Supplementary Material**). Concerning **action and control**, all implemented measures have to be supervised by the veterinary authorities, which causes additional personnel costs. For **vaccination**, we assumed four euros per 2 ml dose for a small ruminant individual (25). Further, we assumed that only the gimmers, replacement animals, and purchases of the flock (replacement rate of 20%) need an initial immunization. Here, two doses at intervals of 3 weeks were calculated. If possible, vaccination has to be completed 4 weeks before mating (61), while the other 80% of the flock gets only one booster vaccination per year. **Sample collection, testing, and vaccination** of all reproductive females (n = 1,270,011) in the German small ruminant population include 26,090,430 euros per year in this calculation [see **Supplementary Material**; (52, 53)]. Moreover, animal owners would face (additional) **costs from modifying their usual flock management and housing practices** in order to ensure that pathogen transmission will be prevented. Rendering of contaminated materials may cause additional costs. Furthermore, and most importantly, the **loss of income** for the affected animal owners has to be considered before implementing this MOSS, as well. Financial damage may be substantial depending on the purpose of use and the market value of the flock and its products [i.e., prohibition of trade with wool, raw milk (products), or live animals]. Moreover, **loss of income and threatened jobs** also must be considered. Although Q fever is listed by the OIE, it is not included within "Recommendations applicable to OIE Listed diseases and other diseases of importance to international trade" (5, 36). Since we recommend stopping animal trade from a positive herd, this could result in loss of income for the animal owners. This calculation and summary of financial costs shows that it is not feasible to implement this concept of a best practice MOSS.

Finally, the following steps are necessary to reduce costs in order to make this MOSS feasible. **Preventive measures** should replace testing wherever justifiable. Therefore, the focus on **nationwide vaccination** would be most useful, as this would guarantee lower pathogen shedding by infected small ruminants. Assuming the vaccination costs as stated above for all reproductive females (n = 1,270,011) in the German small ruminant population, a nationwide vaccination of these would include approximately 7,722,000 euros per year. If sample collection and testing are omitted completely, this would result in a cost reduction of 18,368,430 euros per year compared to the best practice concept [see **Supplementary Material**; (52, 53)]. Moreover, current studies are investigating if the **vaccination dose for sheep can be reduced by half** (1 ml per dose) compared to goats (25). Other studies are looking at whether **exclusive vaccination of gimmers** would be sufficient to prevent a positive flock status (68). These approaches would also further reduce costs. Unfortunately, it is not possible to avoid **sample collection and testing in cases of close contact** between small ruminants and the public because vaccination does not prevent pathogen shedding completely (5). On the other hand, vaccination can greatly reduce the risk of Q fever infection in the human population. For further cost reductions, it must also be considered that testing such a large number of animals means that **costs for sample testing and vaccination can be negotiated** between the national veterinary health authority and the industry, resulting in lower costs for each district as in our calculation above. In addition, costs could be saved if the **sample collection** was carried out by independent persons, such as employees of the responsible chamber of agriculture or Animal Disease Founds, who have lower labor costs as veterinarians. However, it must be ensured that employees have the necessary knowledge to guarantee the quality of the samples. Furthermore, it could also be considered whether the animal owners themselves could collect the samples. However, this raises several problems. First, it would have to be ensured that all animal owners have the necessary knowledge to carry out the sampling correctly. Second, the animal owners are directly affected by the results of the samples, which could make them prejudiced in taking the samples.

Regarding MOSS coordination, sample collection and testing as well as vaccination costs, it has to be decided which stakeholder group should participate in the financing. **Animal owners** could be a possible group as it is their responsibility not to compromise public health by animal husbandry (69). On the other hand, animal owners cannot be expected to solely take over these high costs and the responsibility. Therefore, the costs should be shared or assumed. While goat owners may be more willing to accept financial costs, as Q fever leads to losses for their animals (i.e., higher abortion rate), in contrast, sheep owners might be less willing to pay as Q fever usually does not show any health problems in sheep. Given the previous approaches in Germany, it is most likely that financial coverage would be taken over by the **Animal Disease Funds**. In addition, the public health authorities and veterinary health authorities should be considered in regard to joint (cost) management. This is because measures can only be ordered by the public health authorities. This is based on the IfSG. However, veterinary expertise is needed for disease control measures in small ruminant flocks, too. Therefore, it is desirable that an interdisciplinary team coordinates measures. In addition to the legal basis of the IfSG, both public and veterinary

health authorities are responsible for the maintenance of public health as a common good. Although infection with *C. burnetii* poses a greater risk to human health than to animal health, joint financing by animal owners, Animal Disease Founds as well as the public health authorities and the veterinary health authorities is a logical conclusion.

Financial benefits arise from the prevention of human Q fever cases. Therefore, each case that can be prevented by this MOSS saves costs for **physicians, laboratories, medicine, hospitalization, and nonproductive time of humans**. "Expressed in disease adjusted life years (DALYs), Q fever ranked 12th of 32 infectious diseases in the Netherlands over the period 2007–2011, using the methodology developed under the Burden of Communicable Diseases in Europe (BCoDE) project. … The healthcare-associated costs of the Q fever epidemic in the Netherlands was estimated at €18.4–26.5 million and the productivity loss at an additional €1.3–10.3 million" (70). Hence, though not precisely estimable, costs can be high, and outbreaks may affect the health system considerably. Therefore, every preventive measure is financially preferable to ongoing passive monitoring as it is currently in place. The financial benefit to small ruminant owners and the associated incentive to participate is difficult to recognize at the beginning of MOSS implementation. Animal owners are afraid of the financial damage that a positive test will bring. At this point, however, it is very important to emphasize that an active MOSS also leads to **quality awareness**, which in turn brings financial benefits. Once MOSS is implemented nationwide and an awareness of this zoonosis among small ruminant owners, public, veterinary, and human health professionals is available, small ruminant owners can use a negative flock status to prove the quality of their action measures against Q fever. As a financial benefit, animal owners of flocks with a negative test result could sell raw milk (products) for a better price (e.g., by self-marketing or selling to dairies) and escape restrictions such as migrating ban or culling of animals. This MOSS can therefore be the basis for creating a quality mark "Q fever free" for small ruminant livestock in the future. In addition, this active MOSS ensures that Q fever in small ruminant livestock is controlled nationwide and thus also reduces the **risk of pathogen introduction** into Q fever free flocks, e.g., by additional purchases. Therefore, a financial benefit is that Q fever in small ruminant livestock will be less widespread after MOSS implementation and thus fewer costs for **action measures** will be necessary in future.

Emotional costs include **stigmatism** of people in the small ruminant sector, **existential fear** of animal owners and **anxiety of the public about infection**. These costs cannot be enumerated pecuniary, but they have to be considered during the implementation process and when communicating with stakeholders. This MOSS can only act with high efficiency if emotions of animal owners and the public are taken seriously and are addressed properly.

Finally, emotional benefits evolve from protection of the public against Q fever while cooperating with animal owners. Knowing about positive flock status enables animal owners to take safety measures and thus demonstrate their **sense of responsibility** toward their fellow humans. Negative flock status can gain the **confidence** of the public in the animal owners and lead to economic strengthening of their business. Small ruminants are a considerable component of German livestock production especially for landscape conservation and are popular in the private sector. This MOSS does not want to impair the husbandry of small ruminants in Germany. Rather, it intends to support the small ruminant sector with regard to their responsibility in **zoonosis prevention**. Therefore, an emotional benefit is that stakeholders will work together in reaching this aim in a One **Health approach**.

CONCLUSION

In conclusion, this concept of active surveillance of Q fever in small ruminant livestock focuses on an early warning system in order to prevent Q fever infections in the human population. Considering a best practice approach, the aim is to identify flocks currently shedding the pathogen. Flocks should be considered positive if at least one pool of vaginal swabs is positive by PCR. The surveillance approach culminates in flock-specific action and control measures for the affected flocks. If this best practice concept were to be implemented, a huge conflict between economic costs and feasibility would emerge. Therefore, to maintain the aim of this MOSS (prevention of human cases by detecting small ruminant shedders), modifications of the concept are necessary. The system has been developed to serve as a basis for the introduction of a nationwide mandatory surveillance system in the future. Even without the context of subsequent obligation to participate or implement control measures, it is always a challenge to balance necessities and practicability when developing a MOSS.

Currently, successful implementation of this early warning system depends on **voluntary** participation of animal owners. For nationwide and mandatory implementation of this MOSS, a revision of the German law is necessary. Only if the active MOSS is ordered by law can Q fever be prevented safely among the public. In the meantime, and after legal obligation, the most important prerequisite for successful prevention of pathogen transmission is close cooperation between public health authorities and veterinary health authorities at the national and local levels as well as willingness to learn about the possibilities and challenges of the other parties. Furthermore, good and trusting communication with other stakeholders, especially with the animal owners, is mandatory. After successful implementation of this concept focusing on Q fever in small ruminant livestock, a further monitoring and surveillance system for Q fever in other domestic and wild mammals as a target group should be considered. This further development could provide insights into a possible pathogen reservoir in Germany and expand health protection for the population.

In conclusion, this surveillance system is built at the interface of animal and public health, thereby acting as a veterinary public health tool. The responsibility of veterinary and human medicine for public health is already well-recognized, and the One Health concept should be put

into practice with the early warning system for Q fever presented here.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

FUNDING

This work was part of the Q fever—GermAn Interdisciplinary Program for reSearch and was funded by the Federal Ministry of Education and Research (BMBF) under project number 01KI1726B as part of the Research Network on Zoonotic Infectious Diseases.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.623786/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 February 2021
doi: 10.3389/fvets.2021.595511

Epidemiology of Bovine Tuberculosis and Its Zoonotic Implication in Addis Ababa Milkshed, Central Ethiopia

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OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
Eystein Skjerve, Norwegian University of Life Sciences, Norway
Keyvan Tadayon, Razi Vaccine and Serum Research Institute, Iran

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *17 August 2020*
Accepted: *05 January 2021*
Published: *17 February 2021*

Citation:
Tulu B, Zewede A, Belay M, Zeleke M, Girma M, Tegegn M, Ibrahim F, Jolliffe DA, Abebe M, Balcha TT, Gumi B, Martineau HM, Martineau AR and Ameni G (2021) Epidemiology of Bovine Tuberculosis and Its Zoonotic Implication in Addis Ababa Milkshed, Central Ethiopia. Front. Vet. Sci. 8:595511. doi: 10.3389/fvets.2021.595511

Bovine tuberculosis (bTB) continues to be one of the most widely distributed chronic infectious diseases of zoonotic importance, which causes a significant economic loss in animal production. A cross-sectional study was conducted to estimate the prevalence of bTB and its associated risk factors and type the *Mycobacterium bovis* isolated in central Ethiopia. A total of 65 dairy farms and 654 cattle were tested for bTB using a single intradermal comparative cervical tuberculin (SICCT) test. Data on farm management, animal-related characteristics, and the owner's knowledge of the zoonotic importance of bTB were collected using a structured questionnaire. In addition, a total of 16 animals from different farms were identified for postmortem examination. Lowenstein Jensen (LJ) culture was also conducted, and spoligotyping was used to type the *M. bovis* strains isolated. Chi-square test and logistic regression models were used to analyze the herd- and animal-level risk factors. Herd- and animal-level prevalence rates of bTB were 58.5% (95% CI: 46.2%−69.2%) and 39.3% (95% CI: 35.5%−43.5%), respectively. At the herd level, poor farm management was the predictor for bTB positivity ($p < 0.05$). Animal breed, poor BCS, farm type, and poor farm management conditions were significant predictors of bTB positivity ($p < 0.05$) at an individual animal level. All animals identified for postmortem examination were found to have gross TB-like lesions. A total of 14 *M. bovis* strains were identified from 12 animals that were positive for LJ culture. The strain with the largest number of clusters (five isolates) was SB1176, followed by SB0134 (three isolates), SB0192 (two isolates), and SB2233 (two isolates), and two new strains, each consisting of only one isolate. The majority (58.5%) of the respondents did not know the zoonotic importance of bTB. The result of this study showed a high prevalence of bTB in the Addis Ababa milkshed and a low level of consciousness of the owners on its transmission to humans. Therefore, the launching of acceptable control measures of bTB and the creation of public awareness about its zoonotic transmission and prevention measures are required.

Keywords: bovine tuberculosis, Addis Ababa milkshed, zoonotic implication, spoligotyping, farm management

BACKGROUND

Bovine tuberculosis (bTB) is caused by *Mycobacterium bovis*, a member of *Mycobacterium tuberculosis* complex (MTBc). It is a chronic infectious disease of animals characterized by the formation of granulomas primarily in the lungs, lymph nodes, intestine, and kidney. *M. bovis* has the widest host ranges of all MTBc organisms and can readily be transmitted to humans or a variety of domestic and wild animals (1). The most common route of transmission to people is through the consumption of unpasteurized dairy products and inhalation of infectious droplet nuclei (2). In 2019 alone, the World Health Organization (WHO) reported that *M. bovis* was responsible for 143,000 new human TB cases and 12,300 deaths (3). More than 91.0% of the deaths were from the African and Asian countries, where the highest prevalence of bTB has been reported (4).

Many developed nations have reduced or eliminated bTB from their cattle population by implementing effective control strategies that include testing and culling of infected animals, active surveillance, and restrictions of movement in affected areas (5–7). However, in poor and marginalized communities, bTB still continues to cause a significant impact on livestock productivity and on livelihoods of communities (1, 6).

Ethiopia has the largest cattle population in Africa (8) and is also the second most populous country in Africa, with more than 108 million inhabitants (9). The majority of the Ethiopian economy relies on agriculture that depends on traditional farming using cattle force and cattle husbandry (10). Earlier reports showed that in Ethiopia, the prevalence of bTB can reach up to 50% in intensive dairy production systems that are known to serve a large number of people in the urban setup with milk and other dairy products (11, 12).

Therefore, understanding the magnitude of bTB infection and the molecular epidemiology in animal and human populations in the peri-urban area that supplies the major city of Addis Ababa is a key priority. Additionally, information about the knowledge of dairy farm owners or farm workers about the bTB and its zoonotic importance is essential in designing the control strategy and for policy recommendation. To this effect, the objectives of this study were to determine the prevalence of bTB both at the herd and animal level, type the *M. bovis* strains isolated, and assess the knowledge of farm owners or workers about bTB and its zoonotic importance in Addis Ababa milkshed, the capital of Ethiopia.

MATERIALS AND METHODS

Study Setting and Area

The study was conducted between December 2017 and March 2019 in the milkshed of the capital Addis Ababa, central Ethiopia, situated in a range of 80 km toward the North West and North East, namely, Chanco Woreda, Laga-Tafo Laga-Dadi Town, Muka Turi Town, Sandafa-Bake Woreda, and Suluta Town (**Figure 1**). The area has the largest concentration of intensive dairy farms supplying milk to the capital Addis Ababa. In addition to their proximity to the capital Addis Ababa City, where there is a huge demand for dairy products, these localities are known for their conducive climatic conditions for dairy products.

Study Subjects

The study subjects were dairy cattle managed in the selected dairy farms in the study area. The farms were characterized by a mix of small holders at household level and intensive and semi-intensive farms owned by members of private investors (11). Dairy cattle in the selected herds were the study units, and their breed compositions were one of the following: crosses of Holstein Friesian (HF) and Zebu, crosses of Jersey and Zebu, or pure Zebu. The husbandry and farm setting differed somewhat from one study site to the other depending on the level of awareness, educational status of farmers, and access of extension services. The following inclusion and exclusion criteria were used for farms and individual animals.

Farms were included if they had been established for over a year, owned at least five cattle, gave written informed consent for cattle to have SICCT test, and agreed that at least one strong reactor could be slaughtered in return for financial compensation. Individual animals were excluded if they were calves younger than 4 weeks, clinically sick cattle with diseases not suggestive of bTB, or cows in the last 2 months of pregnancy.

Study Design and Sample Strategy

A cross-sectional study was conducted in the farms located in the milkshed of Addis Ababa City, central Ethiopia. Lists of intensive dairy farms with more than five cattle with HF and/or crossbreed were obtained from the local Livestock and Fishery Department offices in the study area. The farms were grouped into three categories; small (<10 animals per farm), medium (10–50 animals per farm), and large (>50 animals per farm). From each study area, the farms and the animals were randomly selected. The farms were approached for their willingness to participate in bTB testing and those farms that agreed were tested. New farms with less than 1 year since establishment were excluded.

A total of 65 farms and 654 individual animals were screened for bTB infection using a single intradermal comparative cervical tuberculin (SICCT) test. After the farms were identified and written informed consent was obtained from the owners for their cattle to undergo SICCT test, study staff administered intradermal injections of purified protein derivatives (PPDs) from *M. bovis* and *Mycobacterium avium* tuberculin to cattle on the day of consent and returned 72 h later to read the tests. A total of 16 highly reactive cows using SICCT test were purchased (one animal per farm), and further laboratory examinations were performed.

Risk factors associated with bTB positivity both at animal and herd levels were recorded before PPD injection. Body condition score (BCS) of the animals was determined as good, medium, or poor according to Nicholson and Butterworth (13). Good BCS was considered for the animals when the fat cover is easily observed in critical areas and the transverse process was not visible or felt. Animals with visible ribs having a little fat cover and barely visible dorsal spines were classified as medium BCS. Poor BCS was considered when there is an extremely lean animal

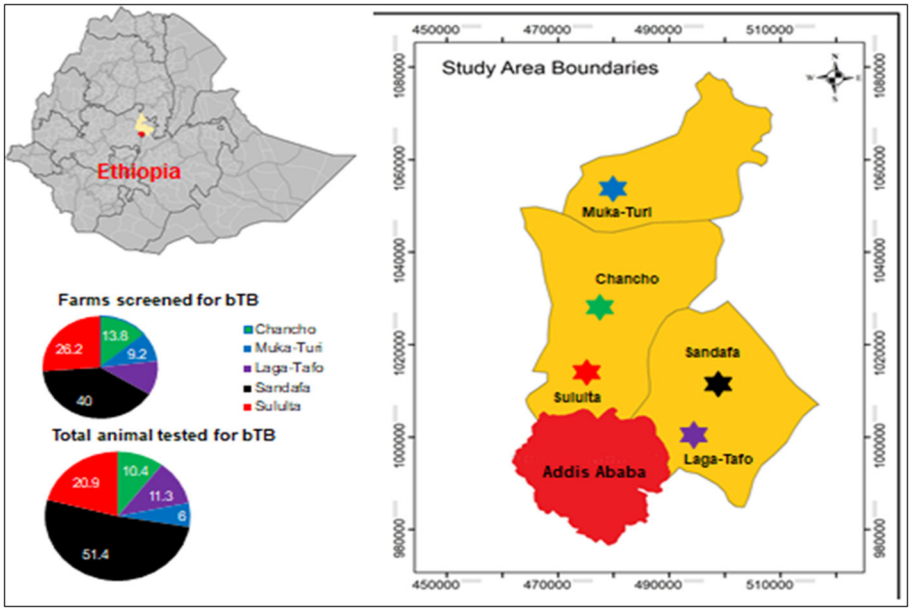

FIGURE 1 | Study area. Source: https://en.wikipedia.org/wiki/Districts_of_Ethiopia.

with projecting dorsal spines pointed to the touch and individual noticeable transverse processes.

The management condition of the farms was categorized based on Ameni et al. (14) as poor, medium (satisfactory), or good. The classification of management condition depends on the housing condition (such as neatness, waste drainage, nature and cleanness of the floor and animals, light source, ventilation, presence of confinement), feeding practice (concentrate and hay), possession of an exercise yard, and contact with other nearby herds and provision with clean water.

Questionnaire Data

Data were collected by study staff using a paper Case Report Format (CRF). Each page of the CRF bears a study ID number unique for each participant farm. Either the owner or the farm manager was interviewed using a predesigned questionnaire about the awareness of bTB transmission, habits of raw milk and meat consumption, and recent TB cases identified from family or workers.

Single Intradermal Comparative Cervical Tuberculin Test

For each cattle selected in this study, SICCT tests were performed using PPDs from *M. bovis* (PPDb) and *M. avium* (PPDa) according to a published protocol (15).

Sample Collection, Processing, and Culturing of Mycobacteria

For all cattle tested by the SICCT test, information about age, sex, type of breeds, and BCS was recorded. Selected positive cattle were purchased and subjected to postmortem examination performed. The criteria used to select the purchased animals were based on the strong PPD response, one animal per farm, and based on the willingness of the farmer to sell the animal. The postmortem examination was done using standard protocols (16).

Seven lymph nodes with suspicious gross lesions were collected per animal, placed in individual 50-ml sterile universal tubes, and transported at 4°C to Aklilu Lemma Institute of Pathobiology (ALIPB) for further processing. At ALIPB laboratory, samples were stored at −22°C, and all samples were processed and cultured for mycobacteria as previously described by the World Organization for Animal Health protocols (17). The tissues were sectioned using sterile blades and were then homogenized with a mortar and pestle. The homogenate was decontaminated by adding an equal volume of 4% NaOH and by centrifugation at 1,865 g for 15 min. The supernatant was discarded, and the sediment was neutralized by 1% (0.1N) HCl using phenol red as an indicator. Neutralization was considered to have been achieved when the color of the solution changed from purple to yellow. Thereafter, 0.1 ml of suspension from each sample was spread onto a slant of Löwenstein–Jensen (LJ) medium. Duplicate slants were used, one enriched with sodium pyruvate and the other enriched with glycerol. Cultures were incubated aerobically at 37°C for at least 8 weeks and with a weekly observation of the growth of colonies.

Identification and Molecular Typing of Mycobacteria

Slants with no growth at week 8 were considered culture negative. Bacterial colonies from culture-positive samples were stained by the Ziehl–Neelsen staining technique to identify acid-fast bacilli (AFB). Spoligotyping was performed following the standard operating procedure that was used by Berg et al. (12) and primarily developed by Kemerbeek et al. (18). The

DNA released by heat killing of the colonies was used as a template to amplify the direct repeat (DR) region of *M. tuberculosis* complex by polymerase chain reaction (PCR) using oligonucleotide biotin-labeled primers derived from the DR sequence, RDa (5′GGTTTTGGGTTTGAACGAC3′) and RDb (5′CCGAGAGGGGACG GAAAC3′) (18).

A total volume of 25 μl and reaction mixtures of 12.5 μl of HotStarTaq Master Mix (Qiagen), a final concentration of 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 2 μl of each primer (20 pmol each), 5 μl suspension of heat-killed cells (approximately 10–50 ng), and 3.5 μl distilled water were used. The mixture was heated for 15 min at 96°C and then subjected to 30 cycles of 1 min denaturation at 96°C, annealing at 55°C for 1 min and extension at 72°C for 30 s. And the final stabilization stage at 72°C for 10 min. Immediately before running spoligotyping, the PCR product was denatured using thermocyler at 96°C for 10 min and then removed from the thermocycler and kept on ice so as to prevent renaturing of the PCR products. Thereafter, the denatured PCR product was loaded onto a membrane covalently bonded with a set of 43 oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus of *M. tuberculosis* complex and then hybridized at 60°C for 1 h. After hybridization, the membrane was washed twice for 10 min in 2× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA (pH 7.7)]-0.5% sodium dodecyl sulfate (SDS) at 60°C and then incubated in 1:4,000 diluted streptavidin peroxidase (Boehringer) for 1 h at 42°C. The membrane was washed twice for 10 min in 2× SSPE-0.5% SDS at 42°C and rinsed with 2× SSPE for 5 min at room temperature. Hybridizing DNA was detected by the enhanced chemiluminescence (ECL) method (Amersham, Biosciences, Amersham, UK) and by exposure to X-ray film (Hyperfilm ECL, Amersham). A mixture of 10 ml of ECL reagent 1 and 10 ml of ECL reagent 2 was prepared and then added onto the membrane, and the membrane was rinsed in the solution for 5 min at room temperature. Then, the membrane was attached onto a film in the dark room and placed in the cassette and incubated for 15 min at room temperature. The film was removed and placed in a developer solution for 2 min, removed from the developer, and rinsed with tap water for 15 s and then placed in a fixer solution for 1 min. Finally, the film was dried and used for interpretation of the result. The presence of the spacer was identified as a black square, while absence of the spacer was identified as a white square on the film. The black squares were converted to 1 while the white squares were converted to 0 and then transferred to the spoligotype international type (SIT)-VNTR international type (VIT) database for the identification of the SITs and the lineages of the isolates.

Statistical Analysis

SICCT test and other questionnaire data were entered and analyzed by SPSS version 21.0. Descriptive statistics like mean, median, and standard deviation were used to summarize data. For both herd and animal levels, prevalence was calculated by dividing the number of reactors to the total number tested. One-sample nonparametric test was used to compute the 95% confidence interval of the prevalence. A chi-square test was used to compare the proportions. Univariable and multivariable logistic regressions were used to rule out the risk factors associated with bTB at the herd and animal levels. Statistical significance was indicated using 95% confidence intervals and p-values <0.05.

Ethical Considerations

The study obtained ethical approval from the Armauer Hansen Research Institute (AHRI) Ethics Review Committee (Ref P018/17); the Ethiopian National Research Ethics Review Committee (Ref 310/253/2017); the Queen Mary University of London Research Ethics Committee, London UK (Ref 16/YH/0410); and the ALIPB, Addis Ababa University (Ref ALIPB/IRB/011/2017/18). Written informed consent was obtained from all the owners of the farms.

RESULTS

Herd- and Animal-Level Prevalence

A total of 65 dairy farms in the milkshed of Addis Ababa City, central Ethiopia, were screened for bTB using SICCT test. The overall prevalence of bTB at herd level was 58.5% (95% CI: 45.6–70.6) at a cutoff value >4.0 mm. When the study sites were considered, 50.0% (19/38) of the positive herds were recorded at Sandafa followed by Sululta (23.7%), Chancho (13.2%), Laga-Tafo (10.5%), and Muka-Turi (2.6%). The overall animal-level prevalence was 39.3% (95% CI: 35.5–43.2) at a cutoff value >4.0 mm. The highest proportion (63.8%) of bTB-positive animals was reported from Sandafa area, followed by Sululta area 19.8% (51/257), Laga-Tafo area 10.5% (27/257), Chancho area 3.1% (8/257), and Muka-Turi area 2.7% (7/257) (**Table 1**).

Herd-Level Risk Factors

Results on the farms' characteristics including the type of farms whether they are traditional or commercial, herd size of the farms, and the farms' management conditions are summarized in **Table 2** below.

In order to identify factors associated with bTB at herd level, binary logistic regression analysis was conducted. The univariable logistic regression analysis showed that the type of farm, management condition, and herd size were factors associated with the presence of bTB in the farms ($p < 0.05$). The multivariable logistic regression analysis showed that farm management conditions remain the predictors for bTB positivity at the herd level ($p < 0.05$) (**Table 2**).

Animal-Level Risk Factors

The mean age of the animals included in this study was 5.1 years (SD = 2.21), the majority of them were in the age group 4.0–9.0 years of age. Eighty-eight percent of the animals were cows, and 93.0% of the animals belong to crossbreed between either HF or Jersey and the local Zebu (**Table 3**).

At the individual animal level, univariable logistic regression showed that factors like the location of animals, being a cow, crossbreed, medium and poor farm management conditions, poor BCS, animals from commercial farms, and animals from medium and large herd size were significantly associated with

TABLE 1 | Herd and animal prevalences of bTB using SICCT test at >4.0mm cut-off.

bTB status	Level	Locations										Total	
		Chancho		Laga-Tafo		Muka-Turi		Sandafa		Sululta			
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	Prevalence (95 % CI)
Negative	Herd	4	44.4	3	42.9	5	83.3	7	26.9	8	47.1	27	41.5 [30.8–53.8]
	Animal	60	88.2	47	63.5	32	82.1	172	51.2	86	62.8	397	60.7 [56.9–64.7]
Positive	Herd	5	55.6	4	57.1	1	16.7	19	73.1	9	52.9	38	58.5 [46.2–96.2]
	Animal	8	11.8	27	36.5	7	17.9	164	48.8	51	37.2	257	39.3 [35.3–43.1]

TABLE 2 | Risk factors associated with bTB in selected dairy farms in the Addis Ababa milkshed, central Ethiopia.

Risk factors		Total (%)	bTB status				
			N (%) positive	Crude OR (95% CI)	*P*-value	Adjusted OR [95% CI]	*P*-value
Locations	Chancho	9 (13.8)	5 (13.2)	1		-	
	Laga-Tafo	7 (10.8)	4 (10.5)	1.1(0.1–7.8)	0.949	-	-
	Muka-Turi	6 (9.2)	1 (2.6)	0.2(0.01–1.9)	0.154	-	-
	Sandafa	26 (40.0)	19 (50.0)	2.2(0.4–10.5)	0.334	-	-
	Sululta	17 (26.2)	9 (23.7)	0.9(0.2–4.5)	0.899	-	-
Farm type	Traditional	44 (67.7)	20 (52.6)	1		-	-
	Commercial	21 (32.3)	18 (47.4)	7.2(1.8–28.0)	0.004	-	-
Management condition	Good	15 (23.1)	6 (15.8)	1		1	
	Medium	17 (26.2)	13 (34.2)	4.8(1.1–22.4)	0.042	9.0(1.1–73.2)	0.040
	Poor	33 (50.8)	19 (50.0)	2.0(0.6–7.1)	0.262	6.7(1.0–43.1)	0.045
Herd size	Small (< 10 animals)	21 (32.3)	8 (21.1)	1		-	-
	Medium (11-50 animals)	29 (44.6)	16 (42.1)	2.0(0.6–6.2)	0.235	-	-
	Large (> 50 animals)	15 (23.1)	14 (36.8)	22.7(2.5–207.7)	0.006	-	-

bTB infection ($p < 0.05$). According to the multivariable logistic regression location of the animals, breed, poor body condition, animals in a commercial farm, and farm management conditions were significant predictors of bTB positivity ($p < 0.05$) (**Table 3**).

Spoligotype Patterns of Cattle Isolates

A total of 16 cows, one cow from each farm were purchased based on their higher SICCT test result. The mean SICCT score was 22.5 mm (SD = 15.59), 8.7 and 71.0 mm being the lowest and the highest score, respectively. Out of the 16 animals that showed gross TB lesions, 12 of them were positive for *M. bovis* culture on LJ medium with a culture positivity rate of 75.0%. All the samples have shown growth on the LJ media supplemented with sodium pyruvate, and only three samples have shown growth on the LJ media supplemented with glycerol. The spoligotype pattern of 14 isolates was shown in **Figure 2**. The isolates from two animals have shown two different spoligotype patterns, which indicate the possibility of double infection. The isolates were grouped into four clusters of *M. bovis* strains of which two were new strains. The genotype with the largest of isolates (five isolates) was SB1176, followed by SB0134 (three isolates), SB0192 (two isolates), and SB2233 (two isolates) and two new strains each with one isolate.

Cattle Owners' Awareness About Bovine Tuberculosis and Its Zoonotic Importance

Farm owners or managers were interviewed to assess their awareness about bTB and its zoonotic transmission. Out of 65 respondents, 31.0% did not know what bTB was, and 58.5% did not know the zoonotic importance of bTB. The participants responded that they had a habit of eating raw meat 83.0% and drinking raw milk 86.2%. Similarly, 87.7% of owners' families or their workers share the same room with their cattle. Finally, 13.8% reported a history of TB in either the owners' family or their workers (**Table 4**).

DISCUSSION

This study reports the epidemiology of bTB and its zoonotic implication in Addis Abba milkshed, central Ethiopia. The country is known to have a high burden of TB in its human (3) and cattle populations (19). In Ethiopia, there are no control and prevention policies of bTB. In addition, human behaviors like drinking raw milk (unpasteurized) and eating raw meat are highly practiced.

In the present study, the herd and animal prevalence rates of bTB using SICCT test were 58.5 and 39.3%, respectively. This is one of the highest reports regarding bTB in the country.

TABLE 3 | Risk factors associated with bTB positive animals from selected dairy farms in the Addis Ababa milkshed, central Ethiopia.

Characteristics		Total (%)	bTB status				
			N (%) positive	Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)	*P*-value
Locations	Chancho	68 (10.4)	9 (3.1)	1		1	
	Laga-Tafo	74 (11.3)	27 (10.5)	4.31(1.79–10.35)	0.002	8.54(2.91–25.04)	<0.001
	Muka-Turi	39 (6.0)	7 (2.7)	1.64(0.54–4.94)	0.378	-	-
	Sandafa	336 (51.4)	164 (63.8)	4.46(1.97–10.05)	<0.001	5.58(2.01–15.45)	<0.001
	Sululta	137 (20.9)	51 (19.8)	7.15(3.32–15.42)	<0.001	12.96(4.94–34.00)	<0.001
Sex	Male	80 (12.2)	16 (6.2)	1		-	-
	Female	574 (87.8)	241 (93.8)	2.89(1.63–5.13)	<0.001	-	-
Breed	Zebu	27 (4.1)	2 (0.8)	1		1	
	Cross breed	613 (93.7)	253 (98.4)	8.78(2.06–37.42)	0.003	6.15(1.26–26.98)	0.025
	HF	14 (2.1)	2 (0.8)	2.08(0.26–16.63)	0.489	-	-
Body condition	Good	93 (14.2)	21 (8.2)	1		1	
	Medium	411 (62.8)	118 (45.9)	1.38(0.81–2.35)	0.234	-	-
	Poor	150 (22.9)	118 (45.9)	12.64(6.77–23.58)	<0.001	12.38(5.96–25.73)	<0.001
Farm type	Traditional	333 (50.9)	64 (24.9)	1		1	
	Commercial	321 (49.1)	193 (75.1)	6.34(4.46–9.02)	<0.001	9.33(4.65-18.68)	<0.001
Management condition	Good	108 (16.5)	64 (24.9)	1		1	
	Medium	268 (41.0)	133 (51.8)	3.29(2.29-4.76)	<0.001	1.86(1.13–3.08)	0.016
	Poor	278 (42.5)	64 (24.9)	4.18(2.61–6.69)	<0.001	-	-
Herd size	<10 animals	95 (14.5)	13 (5.1)	1		-	-
	11–50 animals	273 (41.7)	76 (29.6)	2.43(1.28–4.63)	0.007	-	-
	>50 animals	286 (43.7)	168 (65.4)	8.98(4.78–16.87)	<0.001	-	-

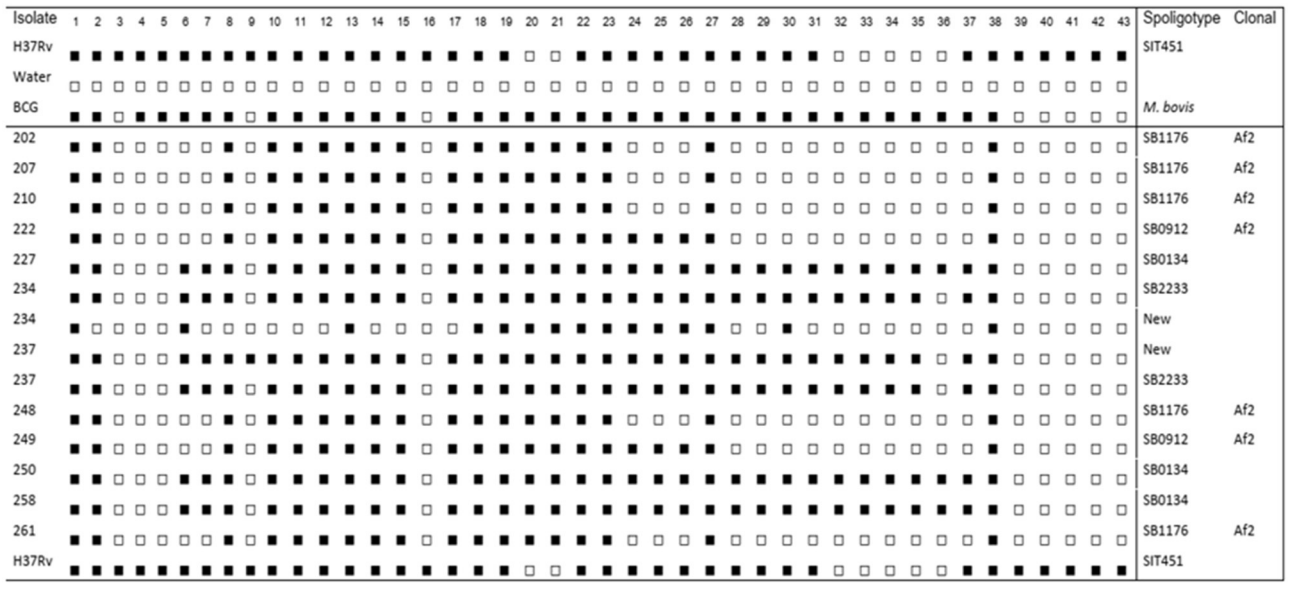

FIGURE 2 | Spoligotype patterns of mycobacterial isolates recovered from tuberculosis lesions in cattle. Four clusters of spoligotype patterns and two new strains of *Mycobacterium bovis* were detected. *Mycobacterium tuberculosis* H37Rv (H37Rv), distilled water (dH_2O), and Bacillus Calmette-Guérin (BCG) are known references.

Previous studies reported lower results compared to the present study (14, 19–23). In a similar area, a previous study by Ameni et al. (11) reported an overall prevalence of 13.5%, with a higher (22.2%) proportion among the Holstein breed. In 2013, Ameni et al. (20) again reported a much lower herd prevalence of 9.4% and individual animal prevalence of 1.8%. On the other hand, a study from the eastern part of Ethiopia reported a prevalence of 51.2% at herd level and 20.3% at individual animal level (23). These significant differences could be attributed to the type of dairy farms and animal breeds included in the study.

TABLE 4 | Owners or farm mangers awareness about bTB and bTB mode of transmission among dairy farm owners.

Questions	Yes (%)	No (%)
Know bTB	45 (69.2)	20 (30.8)
Know bTB is zoonotic	27 (41.5)	38 (58.5)
Eat raw meat	54 (83.1)	11 (16.9)
Drink raw milk	56 (86.2)	9 (13.8)
Sharing rooms with animals	57 (87.7)	8 (12.3)
History of TB in a family or workers	9 (13.8)	56 (86.2)

For example, in the study conducted by Ameni et al. (20), the majority of the farms were smallholder farmers at household level and the animals were the Zebu breeds, which are known to be less susceptible to bTB. In the present study, the majority of the animals from commercial farms were crossbreeds between HF and the local Zebu breeds, which are more susceptible to bTB compared to Zebu cattle (11). Another reason for higher prevalence in this study could be explained by the expansion of intensive dairy farms, which together with an absence of control and prevention policies leads to increased morbidity and transmission of bTB.

Based on multivariable logistic regression analysis of the risk factors in the present study, poor farm management condition was significantly associated with bTB positivity at the herd level. This observation was consistent with the results by Kemal et al. (23) from Eastern Ethiopia. Similarly, Mekonnen et al. (22) reported that farm hygiene is one of the risk factors significantly associated with the bTB at herd level. Hygiene is an essential component in the assessment of farm conditions, and it was assessed in terms of the waste disposal, frequency of waste cleaning, and drainage conditions. Farms with poor management conditions may facilitate the persistence of *M. bovi*s infection, creating a conducive environment for easy proliferation and transmission.

In this study, breed of cattle was one of the predictors of bTB positivity. Previous studies reported by Ameni et al. (11) and Vordermeier et al. (24) indicated that Zebu cattle are more resistant to bTB than either crossbreed or HF breed. Other studies from the United Kingdom (25) and the Republic of Ireland (26) demonstrated that HF cattle have significant heritability to susceptibility to bTB. In the study area, as HFs have a higher milk yield, there is a high tendency to replace the Zebu with HF or crossbreed to increase milk production. This, on the other hand, is a serious challenge in terms of bTB transmission and its impact on the absence of bTB control policies.

In the present study, animals from large commercial farms were more likely to be positive for bTB diseases when compared to traditional farms with smaller herds. This is consistent with the type of breeds that are largely found in such setup. In the study area, commercial farms are largely populated by European breeds and crossbreeds. Studies in Eastern Ethiopia also demonstrated that commercial farms are more likely to be positive for bTB (23). In the commercial dairy system, a large number of cattle are kept in an indoor system with poor ventilation, likely facilitating the transmission of infectious pathogens.

Similar to a previous study by Dejene et al. (27), animals with poor BCS were associated with bTB infection. This study did not define the cause-and-effect relationship between BCS and bTB infection. Either animals with poor BCSs are more susceptible to developing clinical bTB or bTB-positive animals develop a poor BCS as a result of being infected with *M. bovis,* or a combination of both. Clinically, poor body condition is a typical sign that follows *M. bovis* infection in cattle.

Following the SICCT test, selected cows were humanely slaughtered to assess the gross pathology and take samples for mycobacteria isolation and typing. The result showed that all animals slaughtered were positive for gross TB lesions. The severity of the lesion was higher in the lymph nodes of the thoracic region. This observation is related to the route of infection, which is predominantly a respiratory route especially in dairy cattle kept in intensive dairy farms (28). Thus, the thoracic lymph nodes are affected predominantly as they are draining the lungs. On the other hand, in cattle that are kept on pasture, the digestive tract is the predominant site of infection of *M. bovis* and gross pathology (11).

In the present study, the spoligotyping result showed that 58.0% of the known strains (registered on the M.bovis.org) belong to the African 2 (AF2) clonal complex. The AF2 complex is known by the deletion of spacers from 3 to 7 in the spoligotype signatures. These strains of *M. bovis* are known by localized distribution in Eastern African countries including Ethiopia, Uganda, Burundi, and Tanzania (29). The SB1176 strain, which is grouped under AF2 clonal complex, was the dominant *M. bovis* clonal complex in the study area. Consistently, previous studies in a similar study area also reported that SB1176 was the dominant one (30). In the present study, all the known strains of *M. bovis* isolates were clustered. This shows that there is an active *M. bovis* transmission between farms in the study area.

Based on the questionnaire survey about the awareness of the farm owners and/or managers regarding bTB and its zoonotic transmission, a significant number of the respondents did not know the zoonotic importance of bTB. Additionally, the respondents reported that the practice of eating raw meat, drinking raw milk, and their workers sharing the same house with their cattle was very high. Previous studies (14, 23) have also demonstrated a gap in the awareness of the farm owners in this regard. We have observed that all the farms including the commercial and mixed farms sell raw milk/unpasteurized milk to the locals and/or to the milk distributors who collect milk from farms and then transport to Addis Ababa, except one dairy farm that has its own milk and dairy product processing facility. On the other hand, in addition to drinking raw or unpasteurized milk, yogurt, which is prepared from unpasteurized milk, is one of the dairy products being highly consumed in the area. This shows that there is a high zoonotic potential for M. bovis in the study area. Increasing the awareness of the farmers on the zoonotic importance of bTB and its method of transmission is recommended.

The main limitations of the study include not testing of more than 30 animals per farm due to the lack of

willingness of the farmers to allow more animals to get tested. For the same reason, the small numbers of selected animals for postmortem examination because the farmers were not able to sell their animals even if they were told the animals were bTB positive. However, this study covers a wide area of subjects in bTB including the epidemiology, awareness of farmers toward the zoonotic importance of bTB, and the cluster of isolates indicating the active transmission of *M. bovis* in the study area. This information could be used by policymakers working on the control and prevention of bTB.

CONCLUSION

The result of this study showed a high prevalence of bTB in the Addis Ababa milkshed and low level of consciousness of the owners on its transmission to humans. Therefore, launching of control measures of bTB and creation of public awareness on its zoonotic transmission and its prevention measures are required.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The study obtained ethical approved from the Armauer Hansen Research Institute (AHRI) Ethics Review Committee (Ref P018/17), from the Ethiopian National Research Ethics Review Committee (Ref 310/253/2017), the Queen Mary University of London Research Ethics Committee, London UK (Ref 16/YH/0410); and by the Aklilu Lemma Institute of Pathobiology (ALIPB), Addis Ababa University (Ref ALIPB/IRB/011/2017/18). Written informed consent was obtained from all the owners of the farms.

AUTHOR CONTRIBUTIONS

BT and GA conceived the study. BT, AZ, MB, AM, and GA contributed to the study design and development of laboratory assays. BT, AZ, MZ, MG, MT, FI, MB, DJ, HM, MA, TB, BG, AM, and GA contributed to the implementation of the study and data acquisition. BT did statistical analyses, wrote the first draft of the manuscript, and had final responsibility for the decision to submit for publication. All authors reviewed the final draft and agreed with its content and conclusions.

FUNDING

This work was supported by a grant from the United Kingdom Medical Research Council (Reference Number MR/P024548/1 to AM).

ACKNOWLEDGMENTS

We thank all the members of the field and laboratory teams at AHRI and ALIPB; Mr. Getinet Abebe, Mr. Befikadu Assefa, Mr. Mengistu Mulu, Mr. Lemma Terfasa, and Mr. Tadesse Regassa for assistance with fieldwork and postmortem examination; and Mrs. Sofia Yimam, Mr. Selfu Girma, and Dr. Assegedech Sirak for assistance with histopathology and AFB examination. Finally, we would like to acknowledge Mr. Andargachew Abeje for developing the study area map and our drivers Mr. Elias Mulugeta, Mr. Yitbarek Getachew, and Mr. Assefa Mijena.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 17 February 2021
doi: 10.3389/fvets.2020.569901

Isolation and Pathogenic Characterization of Pigeon Paramyxovirus Type 1 via Different Inoculation Routes in Pigeons

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 05 June 2020
Accepted: 28 October 2020
Published: 17 February 2021

Citation:
Chang H, Feng S, Wang Y, Li F, Su Q, Wang B, Du J and He H (2021) Isolation and Pathogenic Characterization of Pigeon Paramyxovirus Type 1 via Different Inoculation Routes in Pigeons. Front. Vet. Sci. 7:569901. doi: 10.3389/fvets.2020.569901

Pigeon paramyxovirus type I (PPMV-1) causes regular outbreaks in pigeons and even poses a pandemic threat among chickens and other birds. The birds infected with PPMV-1 mainly show a pathological damage in the respiratory system, digestive system, and nervous system. However, there were few reports on the efficiency of the virus entering the host *via* routes of different systems. In the present study, a PPMV-1 strain was obtained from a dead wild pigeon in 2016 in Beijing, China. The mean death time (MDT) and the intracerebral pathogenicity (ICPI) of our isolate showed medium virulence. Phylogenetic analysis based on F gene sequence showed that the isolate belonged to subgenotype VIb, class II, which dominated in China in recent years. Then, we evaluated the infection efficiency of different routes. Pigeons were randomly divided into five groups of six as follows: intracephalic (IC), intranasal (IN), and intraoral (IO) infection routes, cohabitation infection (CO), and negative control (N negative). All pigeons were inoculated with 100 μl·10^6 EID_{50} PPMV-1 virus. After infection, pathological lesions, virus shedding, body weight change, survival rate, and tissue tropism were tested to compare the efficiency of the different infected routes. The mortality of groups IC, IN, IO, and CO were 100, 66.7, 50, and 33.3%, respectively. Weight loss in group IC was higher than the other groups, followed by groups IN and IO. The lesions observed in PPMV-1-infected pigeons were severe, especially in the lung and intestine in group IC. Viral shedding was observed from 2 dpi in groups IC and IN, but the shedding rate was higher in group IN than group IC. The longest period was in group CO. Tissue tropism experiment showed that our isolate has a wide range of tissue distribution, and the virus titer in the heart and intestine of group IC and in the brain of group IN was higher. Our data may help us to evaluate the risk of transmission of PPMV-1.

Keywords: pigeon, paramyxovirus, virus, Newcastle disease, pathogenicity, route

INTRODUCTION

Newcastle disease, caused by Newcastle disease virus (NDV), is classified as a notifiable disease by the World Organization for Animal Health (OIE), because of its high morbidity and mortality in avian species. At least 250 avian species, including chickens, ducks, and pigeons were reported to be susceptible to NDV (1–3). NDV is a single-stranded, negative-sense, and non-segment RNA virus, which belongs to the genus Orthoavulavirus, subfamily Avulavirinae, family Paramyxoviridae of the Mononegavirales order (4–7). NDV isolates have been classified into classes I and II. NDV of class I are mostly of low virulence and contain only one genotype (8), and class II virus consists of 18 genotypes (9–13) based on the phylogenetic analysis of nucleotide sequence of the F gene and genomic size (14, 15). Pigeon paramyxovirus type 1 (PPMV-1) found in birds of Columbidae family, mainly turtle dove (*Streptopelia turtur*) and Eurasian collared dove (*Stretopelia decaocto*) is a variant of Newcastle disease virus, and it is almost genotype VI, class II. PPMV-1 first reported in England during the late 1970s, affected racing pigeons with outbreaks in domestic chickens (16), which caused the third worldwide pandemics of NDV (17). In recent years, a high mortality (from 40 to 80%, even to 100% in some cases) caused by PPMV-1 has been observed in pigeons (18–22). Clinical signs of the infected birds involve nervous, respiratory, and digestive system symptoms (23), consisting of moderate to severe depression with neck twisting, ataxia, crouch, paralysis, eyelid edema, diarrhea, and green loose feces. NDV has been documented to remain infectious in feces and carcasses for at least a couple of weeks, several months in feathers, and up to 90 days in soil or water (24). Quite a few times, outbreaks in poultry have been ascribed to PPMV-1 (25–28). The virulence can be enhanced after serial passages in chickens (17, 21, 29, 30), which makes these pigeon-originated viruses a tremendous and continuous threat to the poultry industry (31–33). For these reasons, the potential transmission of the virus and the effective route where the virus will enter the host have been considered points of concern. In the present study, the pathogenicity of a PPMV-1, obtained from a dead wild pigeon in 2016 in Beijing, China, was investigated *via* different inoculation routes. Findings from our study showed intracephalic, intranasal, and intraoral infection routes were effective, but intracephalic was the most.

MATERIALS AND METHODS

Viral Isolation, Amplification, and Full-Length Genome Sequencing

A moribund pigeon with neck twisting, diarrhea, and leg paresis or paralysis was found in Beijing, 5 September 2016. We initially diagnosed that the pigeon was infected with PPMV or avian influenza virus based on clinical symptoms, then, the avian influenza virus was excluded and PPMV-1 infection was confirmed by RT-PCR. The identification of Newcastle disease and the separation of strains are as follows: viral RNA was extracted from the tissues of the pigeon (i.e., heart, liver, spleen, lung, kidney, stomach, brain, trachea, intestine, and pancreas) using Trizol reagent (ambition by Life Technologies, Beijing, China) according to the manufacturer's instructions. Reverse transcription was performed as described (34). The detection gene (a part of fusion gene, 486 bp) was amplified from the cDNA by PCR utilizing Taq DNA Polymerase (CWBIO 2× Taq MasterMix, Cat. CW0682M), and the primers were designed according to the conserved sequence (34) (Primer sequence: F:CAG CTGCGGCCCTAATACA; R:TGGATGCCCAAGAGTTGAG). The program was as follows: 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 25 s; and a final extension at 72°C for 10 min. The PCR products were visualized by 1% agarose gel electrophoresis. Other pathogens (circovirus, avian influenza, and pathogenic bacteria) were negative. The viruses from different organs were plaque-purified three times on primary chicken embryo fibroblasts and inoculated into the allantoic cavity of 9-day-old specific-pathogen-free (SPF) eggs. The virus was isolated and RNA was extracted from the allantoic cavity, detected using PCR as abovementioned. The strain was designated as PPMV-1/pigeon/Beijing/China/01/2016 and abbreviated as PPMV-1/BJ-01/CH. Complete genome of our strain was amplified (primers are shown in **Supplementary Table 1**), sequenced, and submitted to GenBank (GenBank ID: MH807446).

Virulence-Test

There are three virulence evaluation indexes on Newcastle disease virus, and virulence is usually determined by no <2 indexes. The mean death time (MDT) was determined in 9-day-old SPF chicken embryo eggs, and the intracerebral pathogenicity index (ICPI) was determined in 1-day-old chick as previously described (35). The least-fatal dose and egg-infectious dose of the virus were tested in 9-day-old SPF chicken embryo eggs by multiple proportion dilution (from 10^{-3} to 10^{-9}) with five repeats per dilution and calculated using the Reed and Muench method.

Phylogenetic Analysis Based on Complete F Gene

To determine the genetic relationships of the PPMV-1/BJ-01/CH isolate to others, a phylogenetic tree was constructed using the MEGA 7.0 software with the maximum likelihood method *via* the Kimura two-parameter model based on the complete F gene from 21 different subgenotypes of the reference PPMV-1 isolates and 1 APMV-1 as outgroup (8, 12, 29). All complete sequences of the F gene were downloaded from GenBank and aligned utilizing ClustalW. Nucleotide sequences obtained in the present study were aligned with reference sequences available in the GenBank database to determine the subgenotypes.

Animal Experimental Infection

To further determine the pathogenicity of the virus, a total of 31-month-old pigeons, with approximately equal body weight of −5–5%, were used in our study. These pigeons were bought from a hatchery in Miyun District, Beijing, and certified

by hemagglutination inhibition (HI) experiment to have no antibodies of NDV and AIV. These pigeons were randomly divided into five groups, and a marked group was placed in a separate cage in an animal room under biosafety conditions. Adequate food and drinking water were provided. Pigeons were inoculated with the virus by intranasal, intraoral, intracephalic, and cohabitation infections (signed as groups IN, IO, IC, and CO, placing groups CO and IC together), which represented through respiratory system, digestive system, nervous system, and natural infection, respectively. Additionally, the negative control group received phosphate-buffered saline (PBS) solution at pH 7.2. All infected groups were inoculated with a dose of 10^6 median embryo lethal dose (ELD50)/100 μl each, calculated using the Reed and Muench method. Subsequently, all pigeons were observed daily for clinical signs, and clinical symptoms, mortality, and morbidity were recorded. Oropharyngeal and cloacal swabs were taken every day, and body weight was determined every other day until day post-infection (dpi) 14 (0 day post-infection in group CO means the day the pigeons were infected), when there is only one group left to shed virus.

Virus Shedding

All of the oropharyngeal and cloacal swabs were collected, placed in tubes with phosphate-buffered saline solution and 2% fetal bovine serum and stored at −80°C until RNA extraction. RNA was extracted, and reverse transcription and RT-PCR were performed as above to test the virus shedding. In addition, cDNA of the isolate in this study and reagent-grade water were used as positive and negative control, respectively.

Gross Lesion

Lesion formation and lesion size are often used to quantify virulence (36). In our study, organs of all dead pigeons were collected on dpi 5 and 14. The collected tissues (including the heart, brain, lung, intestine, and liver) were pathologically lesion examined. Individuals that died ahead of time point were examined and recorded in advance. Positive rate was tallied by percentage.

Histopathology

Tissues from each group were collected and fixed with 10% neutral formalin. The sections were stained with hematoxylin and eosin (HE), and all HE stained sections were examined for the presence of microscopic lesions.

Tissue Distribution

To understand the distribution and virus load of PPMV-1 in organs from different groups at dpi 5 when clinical symptoms emerged, we randomly choose three from each group, and collected samples from the heart, brain, lung, intestine, and liver. The virus was isolated from tissues of the same weight, and $TCID_{50}$ in DF-1 cells (chicken fibroblast cell line) was used

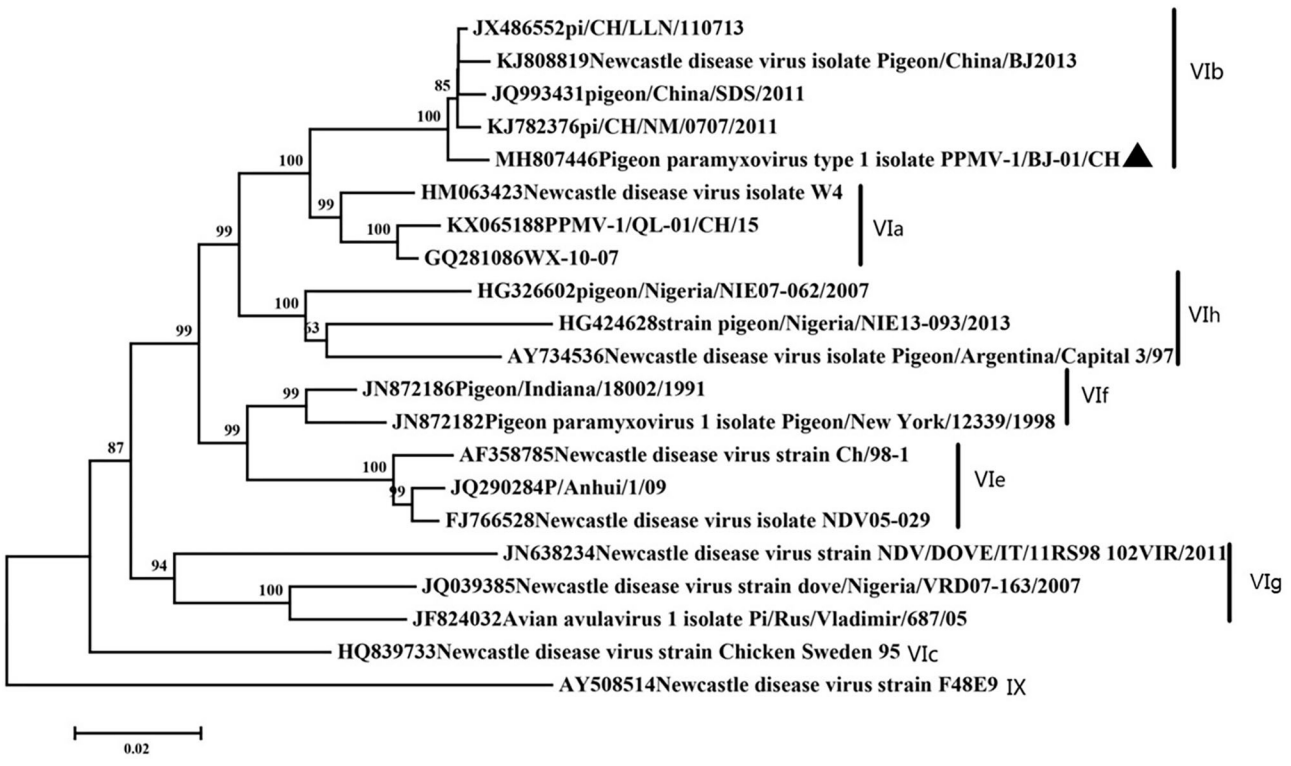

FIGURE 1 | Classification of the virus based on genotype criteria. A maximum likelihood tree of the complete F gene nucleotide sequence. The evolutionary analyses were conducted using the Kimura two-parameter method with 1,000 bootstrap replicates in MEGA7.0. Our isolate was marked with a black triangle.

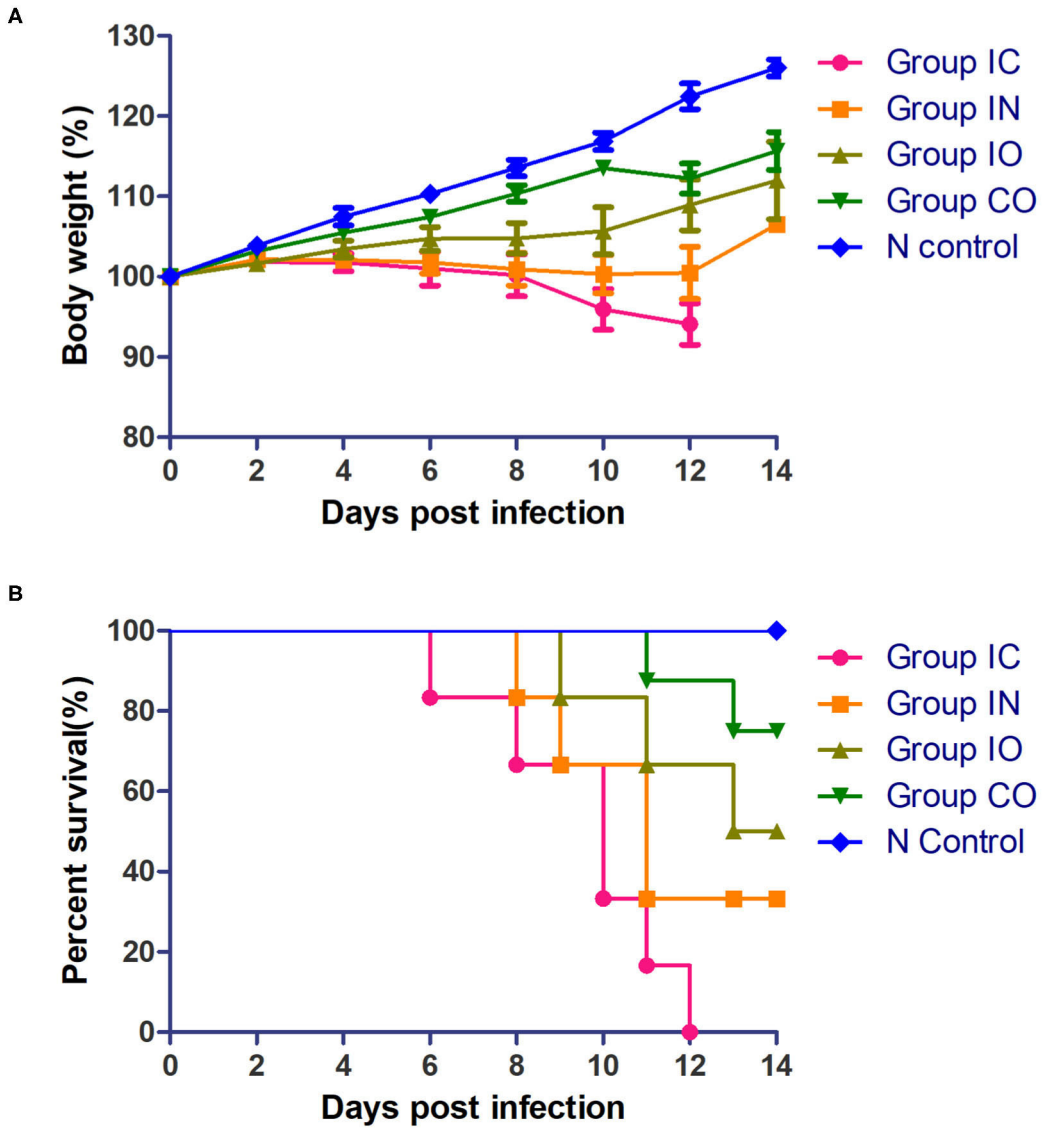

FIGURE 2 | Pathogenicity of the PPMV-1/BJ-01/CH in different routes of infection. **(A)** Body weight change of different groups post-infection. **(B)** Survival curve of different groups post-infection.

to estimate viral loads of the five groups; 3×10^4 DF-1 cells were seeded in 96-well plate with five repetitions 1 day before infection. Twenty-four hours later, the cells were infected with different dilutions of the virus for 1 h at 37°C with shaking every 12 h and confirmed by the hemagglutination assay. $TCID_{50}$ was calculated using the Reed-Muench method. Data were analyzed using Prism (v.5.01) software. Statistical significance was set at a *P*-value of <0.05.

Ethics Statement

These animal studies were performed in strict accordance with the Guidelines for the Care and Use of Animals in Research, which are issued by the Institute of Zoology, Chinese Academy of Sciences (Approval Number IOZ12017).

RESULTS

Phylogenetic Analysis and Genetic Characteristics of PPMV-1/BJ-01/CH Isolate

Phylogenetic analysis showed our isolate belonged to subgenotype VIb (**Figure 1**). Our isolate possessed an ^{112}R-R-Q-K-RF^{117} segment at the cleavage site of F gene. This is characteristic of virulent NDVs. This motif is commonly

found in NDV strains that are highly or moderately virulent in chickens, especially in genotypes VII and IX viruses and some pigeon paramyxovirus strains. Amino acid residues important for receptor recognition of HN is ^{174}R ^{175}I ^{198}D ^{236}K ^{258}E ^{299}Y ^{317}Y ^{401}E ^{416}R ^{498}R ^{516}R ^{526}Y ^{547}E (37), which is the same as most PPMV-1 strains.

Virulence of the PPMV-1/BJ-01/CH Isolate and Sequence Analysis

The MDT and ICPI were 72 h and 0.76, respectively. The results indicated that the PPMV-1 strain was medium according to the criteria (38). Moreover, the minimum lethal dose and 50% egg infection dose of the virus in chick embryo eggs were 10^{-3} and $10^{-5.37}$, respectively.

Pathogenicity of the PPMV-1/BJ-01/CH in Different Routes of the Infection

Clinical signs were observed in all pigeons from 5 dpi. The pigeons lost their weight sharply once clinical symptoms emerged, then soon died. The body weight change (**Figure 2A**) and survival curve (**Figure 2B**) showed the damage of the infected pigeons from group IC were much severer than other groups. A steady increase of body weight was found in negative control group. Post-mortem examination showed the congestion or hemorrhages on the meninx and in the brain. The tracheal

TABLE 1 | Percentage of PPMV-1 positive samples as determined by lesions examination.

Tissues	Groups									
	IC		IO		IN		CO		*N* control	
	D5	D14	D5	D14	D5	D14	D5	D14	D5	D14
Intestine	67	83	0	33	33	33	0	33	0	0
Lung	67	100	33	50	50	83	13	50	0	0
Brain	33	100	17	67	50	67	0	33	0	0
Liver	50	67	0	33	33	33	0	50	0	0
Heart	17	33	0	0	17	50	17	50	0	0

The lesions were shown in ***Supplementary Figure 1****.*

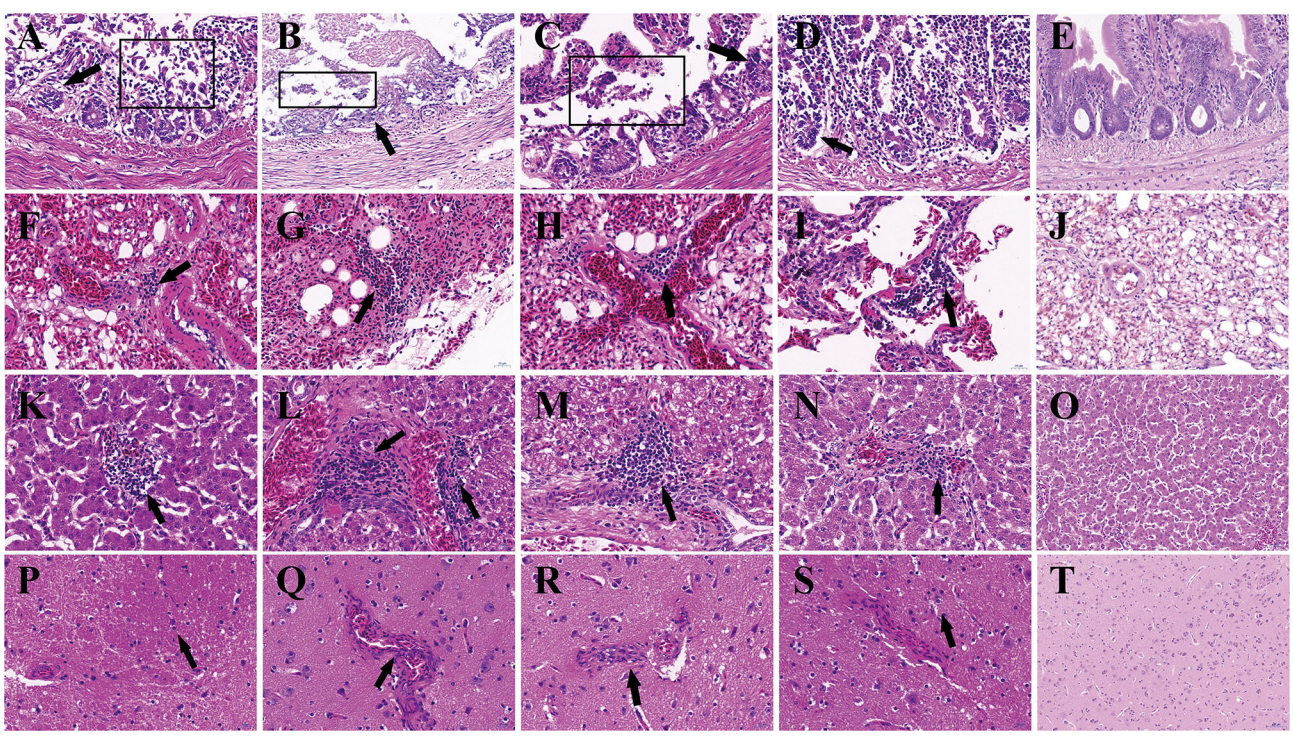

FIGURE 3 | Histopathologic lesions in pigeons of groups. The groups and tissues were marked in the figure. Black arrow indicated the severe histopathologic. **(A,F,K,P)** Histopathologic lesions in group IC. **(B,G,L,Q)** Histopathologic lesions in Group IN. **(C,H,M,R)** Histopathologic lesions in group IO. **(D,I,N,S)** Histopathologic lesions in group CO. **(E,J,O,T)** Histopathologic lesions in group N. **(A–E)** Small intestine. **(F–J)** Lung. **(K–O)** Liver. **(P–T)** Cerebrum. The pictures were magnified ×40.

mucosa was congested or hemorrhagic. Extensive hemorrhages were observed in the mucosa of the small intestine. The spleens were atrophic, friable, and hemorrhagic. Congestion and hemorrhage were found in the pancreas. Our data showed the most serious tissue lesion was discovered in group IC. Groups IO and CO caused less lesions on dpi 5 (**Table 1**).

Histopathological examination showed that the most severe lesions are found in group IC, including the desquamation of small intestine villi, lymphocyte infiltration, and severe epithelial cell loss in the intestine. The alveoli have been destroyed, and a small amount of lymphocyte infiltration and congestion were observed in the lungs. Hepatomegaly and the disappearance of hepatic cord structures were observed in the liver. A small amount of lymphocyte infiltration was also observed in the cerebrum. Milder lesions were observed in other groups (**Figure 3**).

Virus Shedding

The results and mortality of each group are compiled in **Table 2**. Pigeons shed virus from dpi 2 to dpi 14. The virus was detected from oropharyngeal swabs earlier than cloacal swabs in all groups. The earliest start time observed was in groups IC and IN in dpi 2, but the shedding rate was higher in group IN than group IC. The highest shedding rates were observed in group IN from the throat at 2 dpi and cloaca at 7 dpi. Viral shedding ceased at 14 dpi in all groups (**Table 2**).

Tissue Tropism of the PPMV-1/BJ-01/CH in Different Routes of Infection

In this study, we found that the virus can effectively replicate in different tissues, no matter which route of infection, and virus titer in the brain, lung, and intestine were higher than in other tissues. The highest titer of the virus was found in the intestine in group IC and brain in group in (**Figure 4**).

DISCUSSION

In the present study, a PPMV-1 was isolated from a dead pigeon. In order to better understand the genetic characteristics of the strain, the full-length genome was amplified and sequenced. Phylogenetic analysis showed that the isolate belonged to VIb, class II, which was consistent with most of the genotypes found in China (20, 21, 39–41). Amino acid sequence of F gene 112–117 is a typical virulent motif. But the MDT and ICPI showed medium virulence of our isolate. This contradiction often occurs on PPMV-1. Amino acid residues important for receptor recognition of HN are the same as most PPMV-1. All the characteristics of our strain consisted with current epidemic isolates in China (20, 42, 43).

The pathogenicity of the virus may relate to different inoculation routes. For example, the ducks infected intramuscularly with a virulent NDV strain showed the most severe clinical signs, while ducks infected intranasally and intraocularly sometimes also exhibited clinical signs but seldom died (44). To further understand the target system of the virus obtained in this study, several experiments were conducted to evaluate the pathogenicity *via* different system routes. Cohabitation infection was intended to mimic a natural-acquired infection. Intranasal infection was often used by labs as a substitute for aerosol infection of the virus. Oral infection mimic was used to simulate contaminated food and water. Inoculate intracranially was regularly used to evaluate NDV virulence, so it may be the most effective route of infection.

Viral shedding evaluates the effective replication and transmission of the virus in different groups. The results of our study showed infected pigeons of different groups except group CO which shed virus through the larynx from 2 dpi and through cloaca from 4 dpi. Pigeons shed virus through cloaca from 2 dpi after isolating NDV in previous studies (45, 46). The earliest start time observed was in groups IC and IN, and the shedding rate was higher in group IN than group IC; the highest shedding rates were observed in group IN from the throat at 2 dpi and cloaca at 7 dpi. The result indicated that the respiratory tract is the fastest way for PPMV-1 to spread. The shedding rate in group IC remained high, which may confirm that virus can efficiently enter into the hosts through the nervous system. All infection groups shed virus, and the longest persisted until 14 days. Continuous virus shedding may contribute to circulatory infection. Additionally, we used body weight and survival rate to test the pathogenicity. Death occurred in dpi 6, but previous study of a PPMV-1 was inoculated in pigeons through the intranasal route did not result in mortality up to 31 dpi. (47), it may be caused by the differences in virulence of the isolates used (48). The rate of body weight decline of group IC was higher than in the other groups, followed by groups IN and IO. According to the result of $TCID_{50}$ in DF-1 cells, PPMV-1 was able to cause systemic infection in a relatively short time, and virus titer in groups IC and IN was higher than in other groups, which was broadly consistent with gross lesions at dpi 5, resulting in an effective infection process. Groups IO and CO caused less lesions which might be due to lower viral load in the organs at these days. By comparison of the viral load in different tissues, the virus had a wide range of tissue distribution, especially in the lung, brain, and intestine. It can be inferred that the virus could replicate well in these three organs during early infection. At 14 dpi, the gross lesions were more severe in these groups, but the rate of virus shedding was reduced which may be due to higher mortality rates. The results indicated that the virus can effectively infect pigeon *via* different routes, and the most pathogenic was infection through the nervous system and respiratory system, but infection through the nervous showed stronger pathogenicity. Previous studies described neurological lesions in NDV-infected birds, and virulent virus was capable of replication in the brain, but not the avirulent virus (49–51), thus successful replication in the nervous system determines its pathogenicity.

Overall, pigeons play an important role in the epidemiology of PPMV-1. The routes of inoculation greatly influenced pathogenicity of the pathogenic strain isolated in the present study. Considering the growing number of PPMV-1 cases in recent years, it is necessary to develop effective vaccines or other prevention and control methods.

TABLE 2 | Virus shedding of groups.

Groups	No. of positive samples/No. of pigeons tested Days post-infection														
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Group IN	0/6[a]	0/6	6/6	6/6	6/6	6/6	6/6	4/6	3/5	2/4	2/4	1/2	1/2	0/2	0/2
	0/6[b]	0/6	0/6	0/6	0/6	0/6	0/6	3/6	2/5	2/4	2/4	0/2	0/2	0/2	0/2
Group IC	0/6	0/6	4/6	6/6	6/6	6/6	5/5	5/5	3/4	3/4	1/2	1/1			
	0/6	0/6	0/6	0/6	1/6	1/6	0/5	0/5	1/4	1/4	1/2	0/1			
Group IO	0/6	0/6	1/6	5/6	4/6	4/6	3/6	3/6	2/6	1/5	0/5	0/4	0/4	0/3	0/3
	0/6	0/6	0/6	0/6	1/6	1/6	2/6	2/6	3/6	3/5	3/5	3/4	3/4	0/3	0/3
Group CO	0/6	0/6	0/6	2/6	5/6	6/6	5/6	2/6	1/6	1/6	1/6	0/6	0/5	0/5	0/5
	0/6	0/6	0/6	0/6	0/6	2/6	2/6	5/6	5/6	4/6	4/6	4/6	3/5	3/5	0/5
N control	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6

[a] *Positive samples of oropharyngeal swabs.*
[b] *Positive samples of cloacal swabs.*

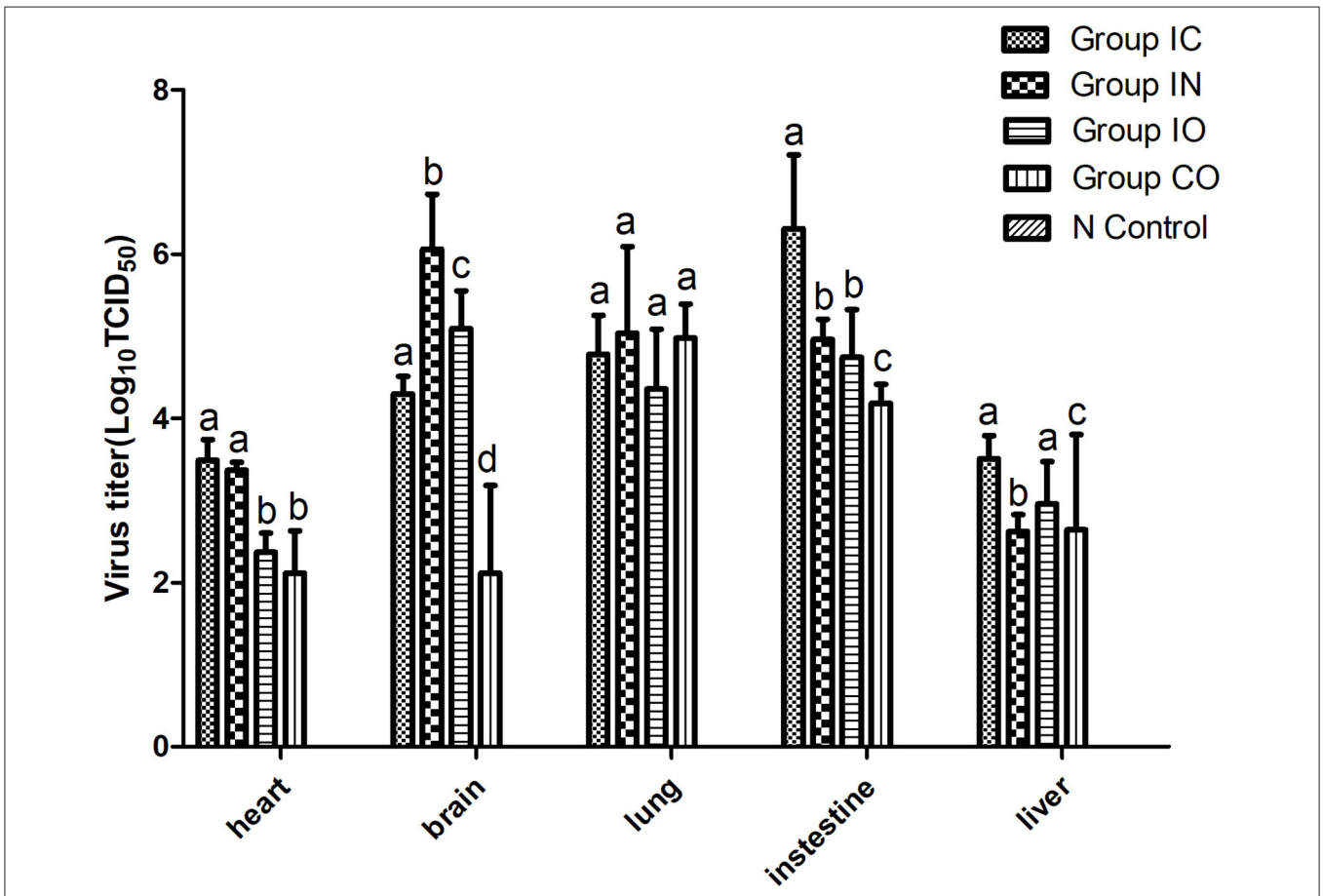

FIGURE 4 | Viral loads in different organs of pigeons post-infection. Virus titer of different tissues from dead pigeons [log_{10} median tissue culture infectious dose/ml (TCID50)] were quantified by serial end-point dilution in 96-well plates using DF-1 cells. The titer was calculated by one-way analysis of variance with standard error bars. Different lowercase letters over the bars denote statistically significant differences ($P < 0.05$) among different tissues after infection calculated by *t*-test. Non-significance ($P > 0.05$) was marked up by the same lowercase letters on different bars. Different lowercase letters marked up on the bars have no relationship among these five organs and represent one organ.

DATA AVAILABILITY STATEMENT

Complete genome of our strain was amplified (primers were showed in **Supplementary Table 1**), sequenced and submitted to GenBank (GenBank ID: MH807446).

ETHICS STATEMENT

The animal study was reviewed and approved by Institute of Zoology, Chinese Academy of Sciences (Approval Number IOZ12017).

AUTHOR CONTRIBUTIONS

HH and HC designed the experiments. HC and SF conducted the experiments and analyzed the data. YW and FL provided the animals. BW performed the RNA extraction and PCR. HC wrote the manuscript. SF, QS, and JD revised the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This work was supported by Beijing Innovation Consortium of Agriculture Research System (BAIC04-2020), Strategic Priority Research Program of the Chinese Academy of Sciences (XDA19050204) and Chinese Academy of Sciences (CZBZX-1).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.569901/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 02 March 2021
doi: 10.3389/fvets.2021.611721

Isolation and Characterization of a Porcine Transmissible Gastroenteritis Coronavirus in Northeast China

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Transmissible gastroenteritis virus (TGEV) is a coronavirus (CoV) that is a major pathogenity of viral enteritis and diarrhea in suckling piglets, causing high morbidity and mortality. In this study, a TGEV strain HQ2016 was isolated from northeast China and characterized its genome sequence and pathogenicity. The phylogenetic analysis indicated that the TGEV HQ2016 strain was more similar to the TGEV Purdue cluster than to the Miller cluster. Both recombination and phylogenetic analysis based on each structural and non-structural gene revealed no recombination event in the HQ2016 strain. Experimental infection study using colostrum-deprived newborn piglets successfully showed that the HQ2016 can cause clinical symptoms including anorexia and yellow-to-whitish watery diarrhea, which are characteristics of TGE, in the inoculated piglets 48 h post-inoculation. These results provide valuable information about the evolution of the porcine CoVs.

Keywords: transmissible gastroenteritis virus, virus isolate, phylogenetic analysis, pathogenicity, coronavirus

OPEN ACCESS

Edited by:
Van Giap Nguyen, Vietnam National University of Agriculture, Vietnam

Reviewed by:
Hye Kwon Kim, Chungbuk National University, South Korea
Ayako Miyazaki, National Agriculture and Food Research Organization, Japan

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *29 September 2020*
Accepted: *05 February 2021*
Published: *02 March 2021*

Citation:
Yuan D, Yan Z, Li M, Wang Y, Su M and Sun D (2021) Isolation and Characterization of a Porcine Transmissible Gastroenteritis Coronavirus in Northeast China. Front. Vet. Sci. 8:611721. doi: 10.3389/fvets.2021.611721

INTRODUCTION

Coronaviruses (CoVs) are the main etiological agents underlying outbreaks of porcine diarrhea, causing substantial economic losses (1). Transmissible gastroenteritis virus (TGEV) is a member of the family *Coronaviridae* that was first reported in 1946 in the USA (2). Since then, the disease always happened in swine-producing areas of the world (1, 3), and reported many times in China in recent years (4–8). Epidemiological investigations have shown that TGEV is often present in the spring and autumn in the northeast of China, sometimes in mixed infections with other diarrhea virus, and caused viral enteritis and severe diarrhea in all ages of pigs, especially with high mortality in suckling piglets (9, 10).

Transmissible gastroenteritis virus is an enveloped virus with a single-stranded, positive-stranded RNA genome of ∼28.5-kb. The genome contains nine open reading frames (ORFs), which encode four structural proteins and five non-structural proteins: the spike glycoprotein (S); envelope protein (E); membrane glycoprotein (M); nucleocapsid protein (N); replicases 1a and 1b; ORF 3a and 3b proteins; and ORF 7 protein. The genes of TGEV are arranged in the order of 5′-rep-S-3a-3b-E-M-N-ORF7-3′ (4–6). The mutation in the spikes protein may be an important indicator for evaluating the tropism and virulence of TGEV. The M protein is the main viral particle membrane protein, which is mainly embedded in the lipid vesicle membrane and is connected to the capsule during assembly of the virus nucleocapsid. The E protein is a transmembrane protein, and the N protein is exists in the viral membrane. The ORF3 is composed of two open frames ORF3a and ORF3b. ORF3a deletion is found in many TGEV strains and PRCV strain. The ORF7

counteracts host-cell defenses and affects the persistence of TGEV, and improves the survival rate of TGEV by negatively regulating the downstream caspase-dependent apoptotic pathways (5, 6, 11, 12).

In this study, we isolated a TGEV from clinical samples collected from farms in northeast China using PK15 cells, characterized its genome based on the whole-genome sequence, and investigated its pathogenicity in colostrum-deprived neonatal pigs in terms of a clinical assessment, viral shedding, virus distribution, histopathological changes, and a mortality analysis. The results suggested that we have isolated porcine enteric coronavirus TGEV HQ2016. The genetic characteristics and pathogenicity of this virus provided valuable information for the evolution of TGEV and will helpful research on the molecular pathogenesis of TGEV.

MATERIALS AND METHODS

Specimen Collection and Screening

In 2016, a total of 50 intestine samples from piglets were collected from eight swine-raising farms in northeast China, in which the piglets showing watery diarrhea and dehydration and as known that all sow without any diarrhea viral vaccine inoculation. The intestinal samples were stored at −80°C. The samples were homogenized and diluted with sterile phosphate-buffered saline (PBS). The suspensions were repeatedly frozen and thawed three times, vortexed and clarified by centrifugation at 12,000 × g for 10 min at 4°C and the supernatants were filtered through 0.22 μm filters (Millipore, Billerica, MA, USA). Semi-nest reverse transcription (RT)-PCR (13) was used to identify the samples positive for TGEV, with two pairs of specific primers (TGEV-N-F: GGTAGTCGTGGTG- CTAATAATGA; TGEV-N-R1: CAGAATGCTAGACACAGATGGAA; TGEV-N-R2: GTT-CTCTTCCAGGTGTGTTTGTT).

Virus Isolation and Plaque Purification

PK15 cells (American Type Culture Collection [ATCC] CCL-33) were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) supplemented with 10% fetal bovine serum (FBS; Bovogen, Australia) at 37°C in a 5% CO_2 incubator. Growth medium was removed from confluent monolayer cells; the cells were washed twice with DMEM and inoculated with a mixture of the supernatants of the positive tissue samples and DMEM containing 20 μg/ml trypsin (GIBCO, 1:250) at a ratio of 1:1. After adsorption for 60 min at 37°C, the cells were washed with DMEM, and maintenance medium consisting of DMEM supplemented with 10 μg/ml trypsin was added. The inoculated cell cultures were observed for CPE for 3–5 days, harvested, and blindly passaged for five times. The viruses in a CPE positive sample was cloned by repeating plaque purify three times and designated as HQ2016.

Virus Titration With a Median Tissue Culture Infective Dose Assay

PK15 cells were seeded on 96-well plates and cultured overnight. The collected TGEV HQ2016 (passaged for 10 times) was 10-fold serially diluted, and used to inoculate cells, with eight replicates per dilution. The cells were then cultured continuously at 37°C under 5% CO_2. The viral CPE was observed for 5–7 days. Tissue culture infective dose ($TCID_{50}$) was determined with the Reed-Muench method (14) and expressed as $TCID_{50}$ per milliliter.

Indirect Immunofluorescence Assay

PK15 cells (1×10^6) were seeded on six-well plates, cultured overnight, and then infected with TGEV HQ2016 (passaged for 10 times) at a multiplicity of infection (MOI) of 1.0. At 24 h after inoculation, the cells were fixed with 4% paraformaldehyde for 15 min and then per-meabilized with 0.2% Triton X-100 for 15 min. The cells were then blocked with 5% skim milk, and incubated overnight at 4°C with a TGEV-specific monoclonal antibody (5E8, supplied by Professor L. Feng, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, Harbin, China) diluted 1:1000. The cells were washed three times with PBS and incubated with a secondary antibody (fluorescein-isothiocyanate-conjugated goat anti-mouse IgG antibody, diluted 1:500) for 1 h at 37°C and then washed three times with PBS. The stained cells were visualized with fluorescence microscopy (Leica DMi8, Germany).

Electron Microscopic Assay

Supernatants from plaque-purified TGEV HQ2016 (passaged for 8 times) infected cell cultures were concentrated by ultracentrifugation method. The supernatants of the cell cultures were centrifuged first at 6,000 × g for 30 min at 4°C, and then at 60,000 × g for 2 h at 4°C. After ultracentrifugation, the samples were negatively stained with 2% ammonium molybdate and adsorbed onto 300-mesh copper net for 2 min. The viral particles were examined with an electron microscope (Hitachi H7500, Tokyo, Japan).

Extraction of Viral RNA and Complete Genome Sequencing

Culture supernatants from plaque-purified TGEV HQ2016 (passaged for 8 times) infected cells were collected and used for preparation of viral RNA. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA samples were sent to testing company (Shanghai Probe Biotechnology Co., Ltd.) to determined complete genomic sequence with the Illumina high-throughput deep sequencing platform (15).

Sequence Analysis

The sequences of TGEV reference strains used in this study were obtained from GenBank, as shown in **Table 1**. The nucleotide and the amino acid sequences of TGEV HQ2016 strain were compared with the corresponding sequences of the TGEV strains in the GenBank database. The sequence was analyzed using the computer program MEGA version 6.0 (16) and DNASTAR (17). Nucleotide and amino acid sequence identities were determined using the Clustal W program. To determine the relationships between representative TGEV isolates and HQ2016 strain, a phylogenetic tree based on the entire genome was

TABLE 1 | Information of the reference TGEV sequences used in this study in the database.

No.	Isolate	Collected year	Country/Origin	GenBank accession no.
1	SHXB	2013	China	KP202848.1
2	Purdue P115	2009	USA	DQ811788.1
3	PUR46-MAD	—	USA	AJ271965.2
4	WH-1	2011	China	HQ462571.1
5	AYU	2009	China	HM776941.1
6	Puedue	—	USA	NC_038861.1
7	HX	2012	China	KC962433.1
8	HE-1	2016	China	KX083668.31
9	SC-Y	2006	China	DQ443743.1
10	Z	2006	USA	KX900393.1
11	HB	1988	USA	KX900394.1
12	Mex-145	2018	USA	KX900402.1
13	Virulent Purdue	1952	USA	DQ811789.2
14	AHHF	2017	China	KX499468.1
15	TS	2016	China	DQ201447.1
16	JS2012	2012	China	KT696544.1
17	Miller M6	2009	USA	DQ811785.1
18	Attenuated H	2009	China	EU074218.2
19	H16	1973	China	FJ755618.2
20	HQ2016	2016	China	MT576083.1

constructed with the MEGA6.0 software through the neighbor-joining method. The reliability of the neighbor-joining tree was estimated by bootstrap analysis with 1,000 replicates.

Recombination Analysis

We used the RDP4 software, including RDP, Bootscan, and SiScan, for a recombination analysis to detect the probable parental isolates and recombination breakpoints of TGEV HQ2016, with the default settings. The criteria used to detect recombination and identify breakpoints were $P < 10^{-6}$ and a recombination score >0.6 (18).

Pathogenicity of TGEV HQ2016 in Newborn Piglets

We used 12 newborn piglets of both sexes without colostrum, who had not been exposed to TGEV before and no anti-TGEV antibodies. The newborn piglets were randomly allocated to the control group ($n = 6$) or the challenged group ($n = 6$). The piglets were fed a mixture of skim milk powder (Inner Mongolia Yi Li Industrial Group Co., Ltd., China) and warm water. The groups were separated by room and ventilation system within the same facility. After acclimation for 1 day, the six piglets in the control group were orally administered 5 ml of DMEM and used as the uninfected controls. The six piglets in the challenged group were orally administered 5 ml of DMEM containing 5×10^6 $TCID_{50}$ of TGEV HQ2016 (passaged for 10 times). All the piglets were observed every 12 h for clinical signs of vomiting, diarrhea, lethargy, and altered temperature or body condition. Rectal swabs were collected from each piglet every 12 h and fecal consistency was scored. The grading standards for the clinical signs and fecal consistency are shown in **Table 2**. Fecal viral RNA shedding was detected with quantitative RT–PCR (19). The sequences of the primers used were: forward, 5′-AAACAACAGCAACGC TCTCG-3′; reverse, 5′-ATTGGCAACGAGGTCAGTGT-3′. The piglets in the two groups were sacrificed at 84 h after challenge. At necropsy, fresh samples of duodenum, jejunum, ileum, cecum, and colon were collected and fixed in 10% formalin solution. The fresh samples were stored at −80°C before a viral RNA distribution analysis with quantitative RT-PCR (19), and formalin-fixed samples were used for histopathological and immunohistochemical analyses. The mortality of the newborn piglets in each group was recorded daily.

Statistical Analysis

The data, including the results of the clinical symptoms, fecal scores and viral load in which inoculated and control piglets, were compared among the different groups by one-way repeated measures ANOVA and the least significance difference (LSD). All data were processed and analyzed using SPSS21.0 Data Editor (SPSS Inc., Chicago, IL, USA). The results for the comparisons among groups were considered different if $^{*}P < 0.05$ or $^{**}P < 0.01$.

RESULTS

Virus Isolation and Identification

A total of 50 intestinal samples were collected from eight pig farms in northeast China. The piglets on these farms suffered vomiting and diarrhea. TGEV was detected in 20% of the samples, and the positive samples were from six farms. The supernatants of the TGEV-positive samples were used to inoculate PK15 cells, 6 of 10 positive samples were tested for virus isolation. Of which, three sample become positive CPE after five passages. No CPE was observed in control

TABLE 2 | The grading standard for clinical symptom and feces of piglets.

Scores	0	1	2	3	4
Clinical symptoms	Normal	Slow movement, normal appetite	Lies, spirit languishes, loss of appetite	Difficult to walk, dehydration	Difficulty standing, dehydrated seriously and weight loss
Fecal consistency	Normal	Soft feces	Liquid with solid feces admixture	Watery feces	Watery diarrhea

PK-15 cells (**Figure 1A**). The CPE was characterized by cell fusion, cell rounding and shrinkage, and the detachment of the cells into the medium (**Figures 1B,C**). TGEV antigen was identified in the cytoplasm of the virus inoculated PK-15 cells but not in mock inoculated cells by IFA using TGEV-specific monoclonal antibody (**Figures 1D,E**). Coronavirus-like particles with a diameter of 100 to 120 nm, similar to the size of TGEV were identified in the culture supernatant of the virus inoculated PK-15 cell by negative staining electron microscopy (**Figure 1F**). The virus isolate was designated as TGEV HQ2016 strain hereafter. And then, the titer of TGEV HQ2016 reached $10^{5.25}$ $TCID_{50}$/0.1 ml at passage 10.

Complete Genomic Sequence of TGEV Strain HQ2016

The genomic sequence of TGEV HQ2016 strain, determined with the illumina sequencing, platform was 28,571 nucleotides (nt) long, and the sequence was submitted to GenBank under accession number MT576083, and exhibited the genomic organization typical of all previously reported TGEV sequences, which are arranged in the order of 5′-rep-S-3a-3b-E-M-N-ORF7-3′ (4–6). The 5′ portion of the genome contains a 303-nt untranslated region (UTR) which includes a potential short AUG-initiated ORF (nt 103–110), beginning with a Kozak sequence (5′-UCUAUGA-3′). The viral RNA-dependent RNA replicase include ORF1a (nt 304–12,357) and ORF1b (nt 12,315–20,357). Structural proteins encoding genes were S (nt 20,354–24,697), E (nt 25,846–26,094), M (nt 26,105–26,893), and N (nt 26,906–28,054), respectively. Non-structural protein encoding genes were ORF3a (nt 24,816–25,031), ORF3b (nt 25,125–25,859), and ORF7 (nt 28,029–28,265), respectively. The 3′end of the genome contains a 275-nt untranslated sequence and a poly(A) tail. The octameric sequence 5′-GGAAGAGC-3′ occurs upstream from the poly(A) tail.

Genomic Characteristics

The S gene of TGEV HQ2016 was 4,344-nt in length, predicted to a encode protein of 1,447 amino acids. A site of 6-nt deletion was observed in the S gene of TGEV HQ2016 at nt 1,123–1,128, which causes two amino acids shorter at this site than in strains of Virulent Purdue, AHHF, TS, Miller M6, JS2012, Attenuated H, and H16 (**Figure 2A**). A other site of 3-nt deletion was detected at nt 2,387–2,389 of the S gene in attenuated H, H16, and AHHF, while it was not found in strain TGEV HQ2016 and other strains (**Figure 2A**). In the Virulent Purdue, Miller M6, JS2012, and TS strains, amino acid 585 is serine, whereas in the TGEV HQ2016, it is alanine (**Figure 3**). Amino acids at 32, 72, 100, 184, 208, 218, 389, 403, 418, 487, 562, 590, 649, 675, 815, 951, 1,109, and 1,234 of TGEV HQ2016 S protein are same to those of the Purdue subgroup strains, especially the three viruses from the United States, and HE-1, HX, AYU, WH-1, SHXB, SC-Y from China, but differ from those of the Miller subgroup strains (**Figure 3**). The structural proteins of E, M and N were 249-nt, 789-nt and 1,149-nt in length and predicted to encode proteins of 82, 262, and 382 amino acids, respectively (**Table 3**), and there was no deletions or insertions compared with other TGEV reference strains.

The replicase genes contained ORF1a and ORF1b, which were12,054-nt and 8,037-nt in length, predicted to encode proteins of 4,017 amino acids and a protein of 2,680 amino-acid, respectively (**Table 3**). There were a common 43-nt region (nt 12,315–12,357) between ORF1a and ORF1b, and a "slippery site" (5′-UUUAAAC-3′, nt 12,322–12,328) which allows the ORF1a translation termination site to be bypassed and an additional ORF, ORF1b to be read. Nucleotide sequence analysis indicated that there were no major deletions or insertions presented in replicase genes both in any Purdue and Miller TGEV strains. ORF3a and 3b of TGEV HQ2016 are216-nt and 735-nt in length, predicted to encode a protein of 71 amino acid and a protein of 244 amino acid, respectively (**Table 3**). Previous research had demonstrated the presence of two deletions in the TGEV ORF3a/b gene in the Miller subgroup (5), a 16-nt deletion and a 29-nt deletion were observed in the strains of Miller subgroup in this study (**Figure 2B**), but no deletions were detected in the ORF3a/b genes of TGEV HQ2016 and other Purdue strains. The ORF7 gene of TGEV HQ2016 was 237-nt in length and predicted to encode a protein of 78 amino acid, which contains the common PP1c-binding motif 5′-RVIFLVI-3′. No deletions or insertions presented in ORF7 of TGEV HQ2016. The recombination analysis showed that no recombination event has ever occurred in TGEV HQ2016. Complete sequence alignment of 5′ and 3′-UTR regions, there was no deletions or insertions were found in strain HQ2016. The ORF initiated by short AUG beginning within the Kozak sequence (TCTATGA) in 5′ NTR regions, and the octameric sequence of "GGAAGAGC" at upstream of the 3′ end poly(A) tail, which could be found in all strains.

Phylogenetic Tree and Homology Analysis

The complete genomic sequence of TGEV HQ2016 was compared with those of 19 TGEV reference strains. Phylogenetic

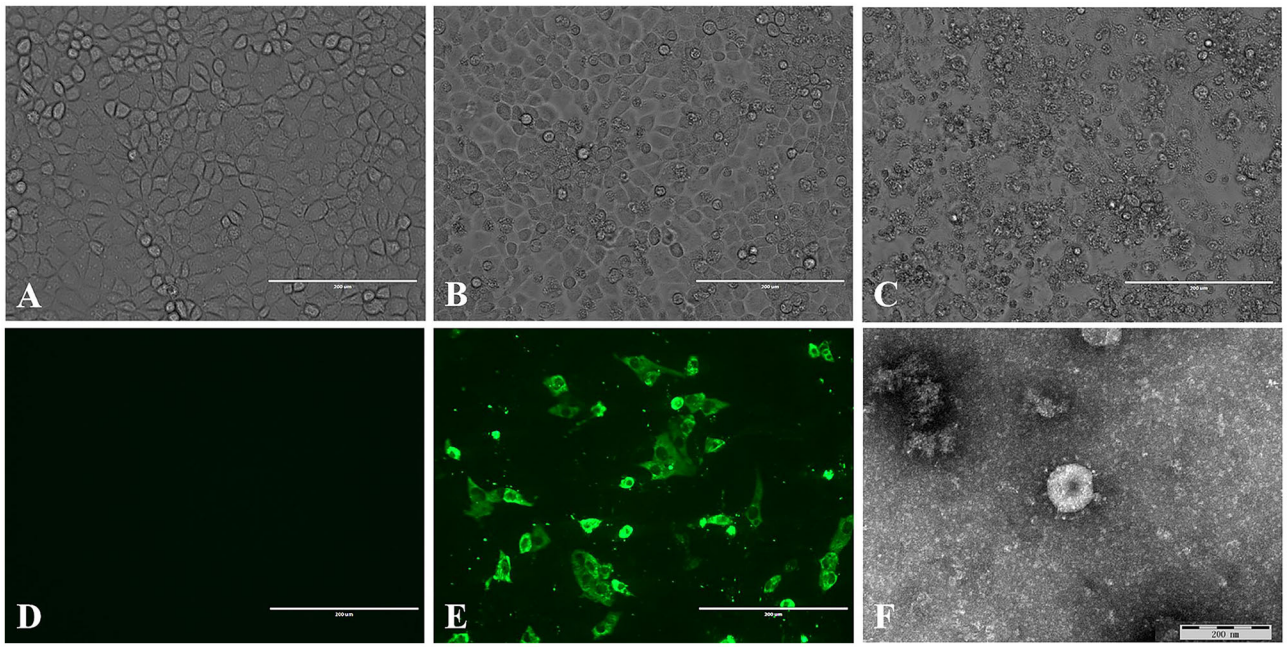

FIGURE 1 | Isolation and identification of the TGEV HQ2016 strain. **(A)** Control (uninfected) PK-15 cells. **(B)** Cytopathic effect (CPE) induced by TGEV HQ2016 after infected 24 h in the PK-15 cell line. **(C)** Cytopathic effect (CPE) induced by TGEV HQ2016 after infected 36 h in the PK-15 cell line. **(D)** IFA identification of control (uninfected) PK15 cells. **(E)** IFA identification of TGEV HQ2016 infected PK15 cells. **(F)** Electron microscopy observation of TGEV HQ2016.

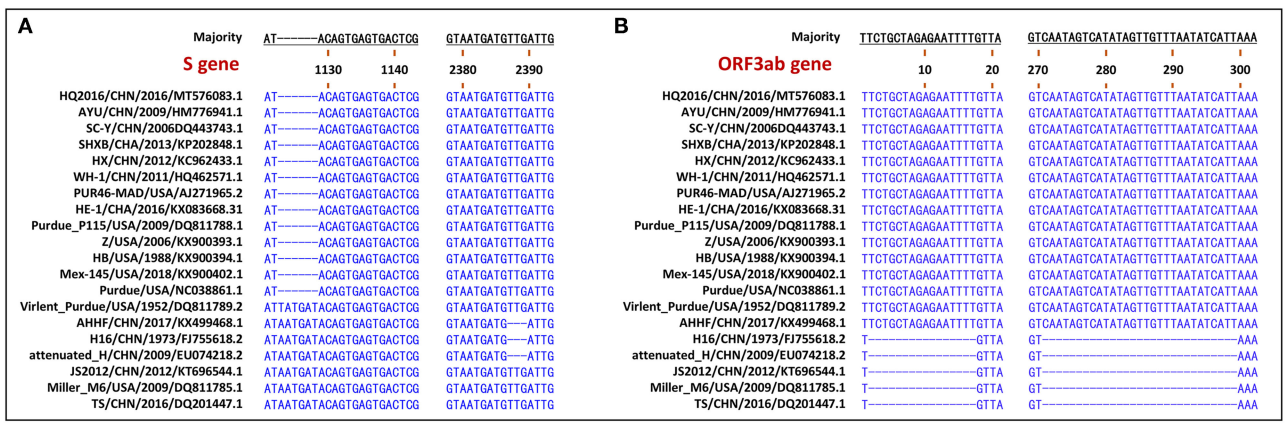

FIGURE 2 | Visualization of genomic deletion regions in the 20 TGEV strains. **(A)** deletion regions of S gene. **(B)** deletion regions of ORF3ab gene.

trees based on the complete genome (**Figure 4**) divided the TGEV strains into the Purdue and Miller genotypes (5). The TGEV HQ2016 strain clustered in the Purdue subgroup, together with SHXB, Purdue, Purdue P115, PUR46-MAD, WH-1, AYU, SC-Y, HX, HE-1, Z, HB, Mex145, Virulent Purdue, and AHHF, whereas the Miller subgroup included TS, JS2012, Miller M6, Attenuated H, and H16. Thus, TGEV strain HQ2016 is closely related to the Purdue strains and more distantly to the Miller strains. The strains of Purdue subgroup appear to share a common ancestor.

To investigate the homology of TGEV HQ2016 with other TGEVs, the nucleotide and predicted amino acid sequences of structural proteins and non-structural proteins were compared (**Table 4**). The results shown that structural proteins (S, E, M, N) and non-structural proteins (replicases 1a and 1b, ORF 3a and 3b, ORF 7) of TGEV HQ2016 shared greater identity with Purdue strains (**Table 4**), identity of predicted amino acid sequence identity in ORF1a was 98.7–100%, in ORF1b was 98.6–100%, in S protein was 97.1–100%, in ORF3a was 88.3–100%, in ORF3b was 97.1–100%, in E protein was 91.5–98.8%, in M

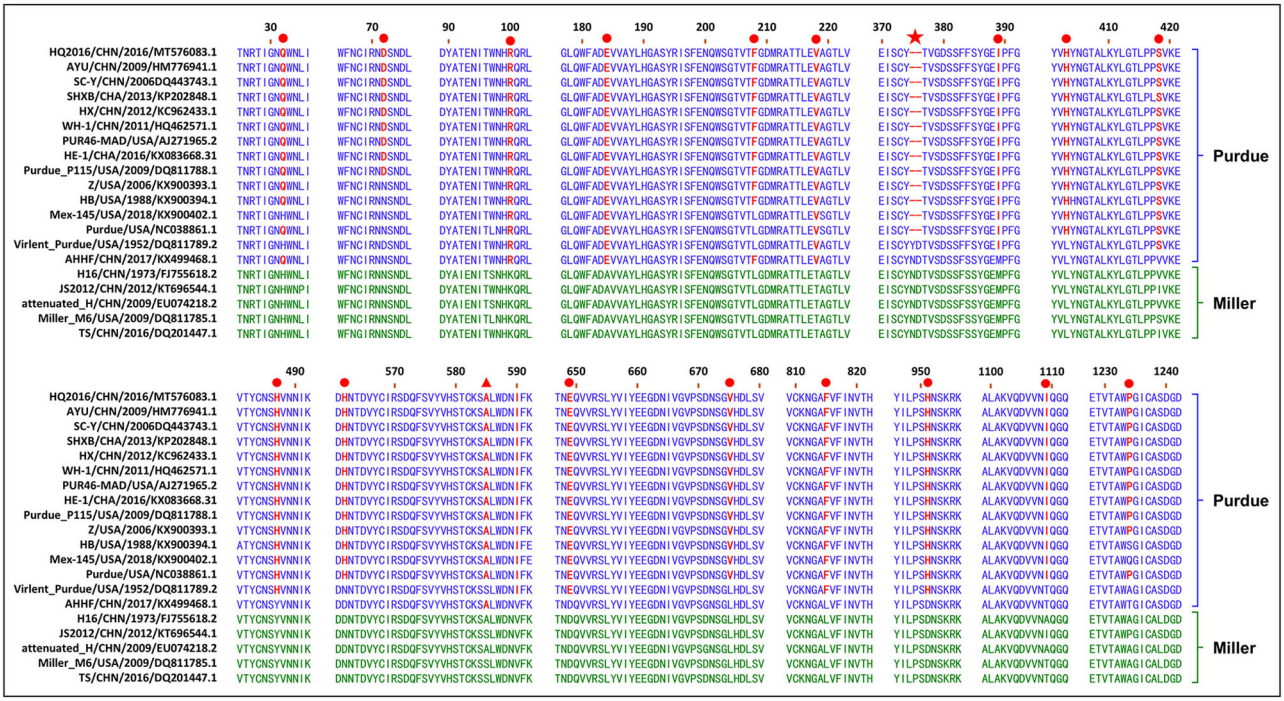

FIGURE 3 | Alignment of partial deduced amino acid sequence of S protein compared with strain TGEV HQ2016. (▲) indicates amino acid 585, (★) indicates 6-nt deletion in the S gene, (•) indicates amino acids of the Purdue subgroup strains include TGEV HQ2016 are different from those of Miller subgroups strains.

protein was 97.3–99.6%, in N protein was 98.2–100%, in ORF7 was 93.6–100%.

Clinical Signs in TGEV HQ2016 Inoculated Piglets

To evaluate the pathogenicity of TGEV HQ2016 in piglets, 12 newborn piglets were used without colostrum. The piglets were active and fleshy before inoculation, with normal fecal consistency. Mild diarrhea and loss of appetite were observed in the piglets of the TGEV HQ2016 inoculated group after 12 h. Severe depression, loss of appetite, vomiting, and yellow and white watery diarrhea appeared in the TGEV HQ2016 inoculated group after 48 h. After 72 h, all the piglets in TGEV HQ2016 inoculated group suffered watery diarrhea and were seriously dehydrated. None of the piglets inoculated with TGEV HQ2016 died within the 84 h of the experimental period, and the control piglets showed no vomiting or diarrhea. The body temperatures, body weight changes, clinical symptoms, and fecal scores of both groups are shown in **Figure 5**. The body temperatures and body weight changes were significantly lower in the piglets of the TGEV HQ2016 inoculated group after 72 h. The clinical symptoms and fecal scores increased continuously for 24 h after TGEV HQ2016 inoculated and differed significantly from those in the control group.

Histopathological Observations

All the piglets were sacrificed after virus challenged 84 h. Pathological changes were mainly observed in the intestinal tracts (jejunum and ileum) of the TGEV-HQ2016-challenged piglets. The whole intestinal tracts, in which yellow watery contents had accumulated, were transparent, thin walled, and gas distended. No lesions were observed in any other organs of the TGEV HQ2016 inoculated piglets or in the organs in the negative control piglets, indicating that the intestinal tract is the target organ of TGEV infection. In a microscopic examination, villus atrophy, degenerate mucosal epithelial cells, and necrosis were observed in both the jejunum and ileum tissues of the TGEV HQ2016 inoculated piglets, but not in those of the control piglets, as shown in **Figure 6**. An immunohistochemical examination showed TGEV antigen in the cytoplasm of the epithelial cells in the atrophied villi of the segments of jejunum and ileum tissues from the piglets inoculated with TGEV HQ2016, but no reactivity in either the jejunal or ileal tissues of the control group, as shown in **Figure 6**.

Viral Loads in Fecal Samples and Intestinal Tissues of TGEV HQ2016 Inoculated Piglets

Because TGEV caused diarrhea and intestinal damage in the newborn piglets, we collected rectal swabs and intestinal samples from them to investigate the viral shedding in the TGEV HQ2016 inoculated piglets. White and yellow watery feces were present in the TGEV HQ2016 inoculated piglets from 48 h after virus challenged. As shown in **Figure 7**, the TGEV viral RNA was detected with quantitative RT-PCR (19). The TGEV levels in the fecal samples were 5–10 $\log_{10}$ RNA copies/g at 12–84 hpi, indicating that TGEV HQ2016 infected and

TABLE 3 | Length of amino acids in the predicted structural and non-structural proteins of TGEV strains.

Strain	ORF1a	ORF1b	S	ORF3a	ORF3b	E	M	N	ORF7
SHXB	4017	2678	1447	71	244	82	262	382	78
Purdue P115	4017	2678	1447	71	244	82	262	382	78
PUR46-MAD	4017	2678	1447	71	244	82	262	382	78
WH-1	4017	2678	1447	71	244	82	262	382	78
AYU	4017	2678	1447	71	244	82	262	382	78
Purdue	4017	2678	1447	71	244	82	262	382	78
HX	4017	2678	1447	71	244	82	262	382	78
HE-1	4017	2678	1447	71	244	82	262	382	78
SC-Y	4017	2678	1447	71	244	82	262	382	78
Z	4017	2678	1447	71	244	82	262	382	78
HB	4017	2678	1447	71	244	82	262	382	78
Mex145	4017	2678	1447	71	244	82	262	382	78
Virulent Purdue	4017	2678	1449	71	244	82	262	382	78
AHHF	4017	2678	1448	71	244	82	262	382	78
TS	4017	2678	1449	65	244	82	262	382	78
JS2012	4017	2678	1449	65	244	82	262	382	78
Miller M6	4017	2678	1449	65	244	82	262	382	78
Attenuated H	4017	2678	1448	65	244	82	262	382	78
H16	4017	2678	1448	65	244	82	262	382	78
HQ2016	4017	2678	1447	71	244	82	262	382	78

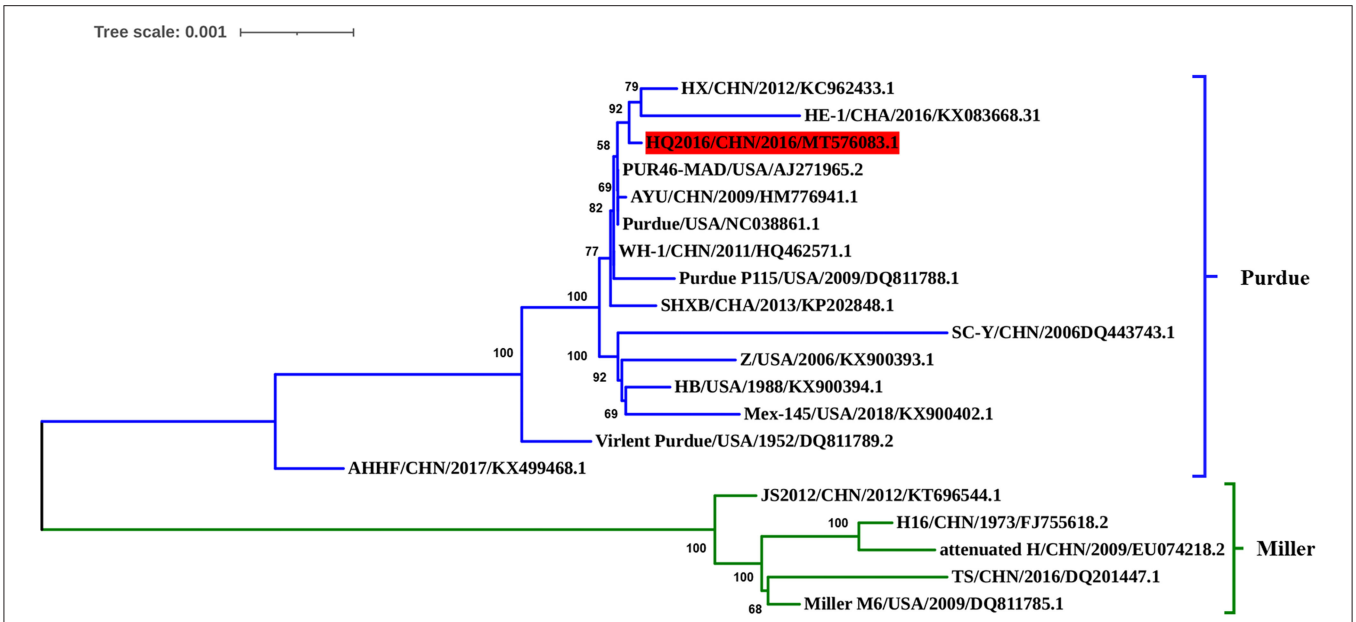

FIGURE 4 | Phylogenetic analysis of the complete genome sequences of the strain HQ2016, other TGEV reference strains. TGEV HQ2016 belongs to the Purdue cluster of TGEV, not the Miller cluster. Complete genome were aligned used Clustal W program which have trimed both 3′ and 5′ ends gaps between TGEV genomes Phylogenetic tree was constructed using the neighbor-joining method with the MEGA 6.0 program. The optimal tree with the sum of branch length = 0.02540989 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tajima-Nei method.

reproduced in these challenged piglets. At the end of the challenge experiment, samples of duodenum, jejunum, ileum, caecum, and colon were collected for viral RNA detection. At 84 hpi, the viral level was highest in the jejunum (7.21 ± 0.11 $\log_{10}$ RNA copies/g), and then (in decreasing order) in the ileum (6.51 ± 0.31 $\log_{10}$ RNA copies/g), cecum (6.28 ± 0.39

TABLE 4 | Nucleotide and amino acid sequence identities (%) of TGEV HQ2016 strain compared with other 19 TGEV strains.

	ORF1a	ORF1b	S	ORF3a	ORF3b	E	M	N	ORF7
SHXB	99.9/99.9	100.0/100.0	100.0/100.0	100.0/100.0	99.9/99.6	99.2/97.6	99.7/99.2	99.9/99.7	99.3/97.4
Purdue P115	99.9/99.9	100.0/100.0	99.9/99.9	100.0/100.0	99.9/99.6	99.6/98.8	99.9/99.6	99.9/99.7	100.0/100.0
PUR46-MAD	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	99.6/98.8	99.9/99.6	100.0/100.0	100.0/100.0
WH-1	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	99.9/99.6	99.6/98.8	99.9/99.6	100.0/100.0	100.0/100.0
AYU	99.9/99.9	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	99.6/98.8	99.7/99.2	100.0/100.0	100.0/100.0
Purdue	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	100.0/100.0	99.6/98.8	99.9/99.6	100.0/100.0	100.0/100.0
HX	99.9/99.9	100.0/100.0	99.9/99.9	100.0/100.0	100.0/100.0	99.6/98.8	100.0/100.0	100.0/100.0	100.0/100.0
HE-1	99.9/99.7	99.8/99.7	99.9/99.8	100.0/100.0	100.0/100.0	98.8/98.8	99.5/98.5	99.9/99.7	99.8/98.7
SC-Y	99.5/99.2	99.8/99.8	99.7/99.5	100.0/100.0	99.9/99.6	99.6/98.8	99.7/99.2	99.9/99.7	100.0/100.0
Z	99.9/99.8	99.9/99.9	99.6/99.0	99.1/98.6	99.9/99.6	99.2/98.8	99.7/99.2	99.8/99.7	100.0/100.0
HB	99.9/99.9	100.0/100.0	99.7/99.4	100.0/100.0	99.9/99.6	99.6/98.8	99.7/99.2	100.0/100.0	100.0/100.0
Mex145	99.9/99.8	99.9/99.9	99.7/99.2	99.5/98.6	99.9/99.6	99.2/98.8	99.7/99.2	99.9/99.7	100.0/100.0
Virulent Purdue	99.9/99.7	100.0/100.0	99.5/99.1	99.5/98.6	99.7/99.2	99.2/97.6	99.7/99.2	99.7/99.7	100.0/100.0
AHHF	99.5/99.5	100.0/100.0	98.9/98.6	100.0/100.0	99.9/99.6	99.6/98.8	99.7/99.2	100.0/100.0	100.0/100.0
TS	98.8/98.7	99.0/98.6	98.3/98.1	87.0/89.5	98.5/96.3	98.4/95.1	98.0/96.9	98.1/98.2	96.8/93.6
JS2012	99.0/99.1	99.0/99.7	98.6/98.3	88.0/88.7	98.8/97.1	98.4/95.1	98.2/97.7	98.2/98.4	96.8/93.6
Miller M6	99.0/99.1	99.1/99.6	98.3/97.1	88.0/88.3	98.9/97.5	98.0/93.9	98.2/97.7	98.2/98.4	96.6/93.6
Attenuated H	98.9/98.9	99.0/99.6	98.0/97.7	87.5/88.7	98.8/97.1	96.8/91.5	98.1/97.3	98.1/98.4	96.8/93.6
H16	98.9/98.9	99.0/99.6	98.2/97.9	88.0/88.7	98.9/97.5	97.6/93.9	98.1/97.3	98.2/98.4	96.8/93.6

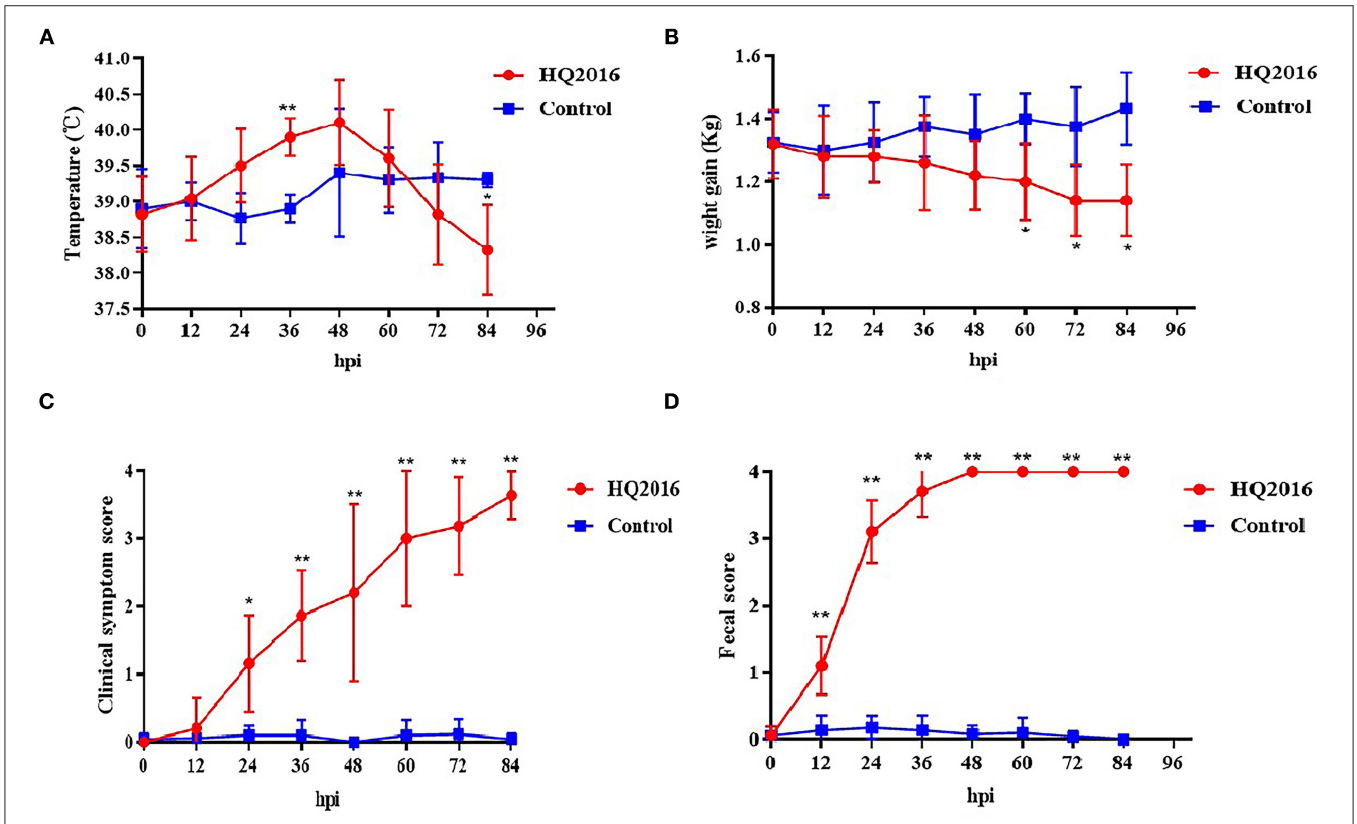

FIGURE 5 | The clinical symptom in the piglets. **(A)** The temperature changes in different groups. **(B)** The body weight changes in different groups. **(C)** The clinical symptom scores in different groups. **(D)** The fecal scores in different groups. Data are shown as mean standard (*$p < 0.05$, **$p < 0.01$).

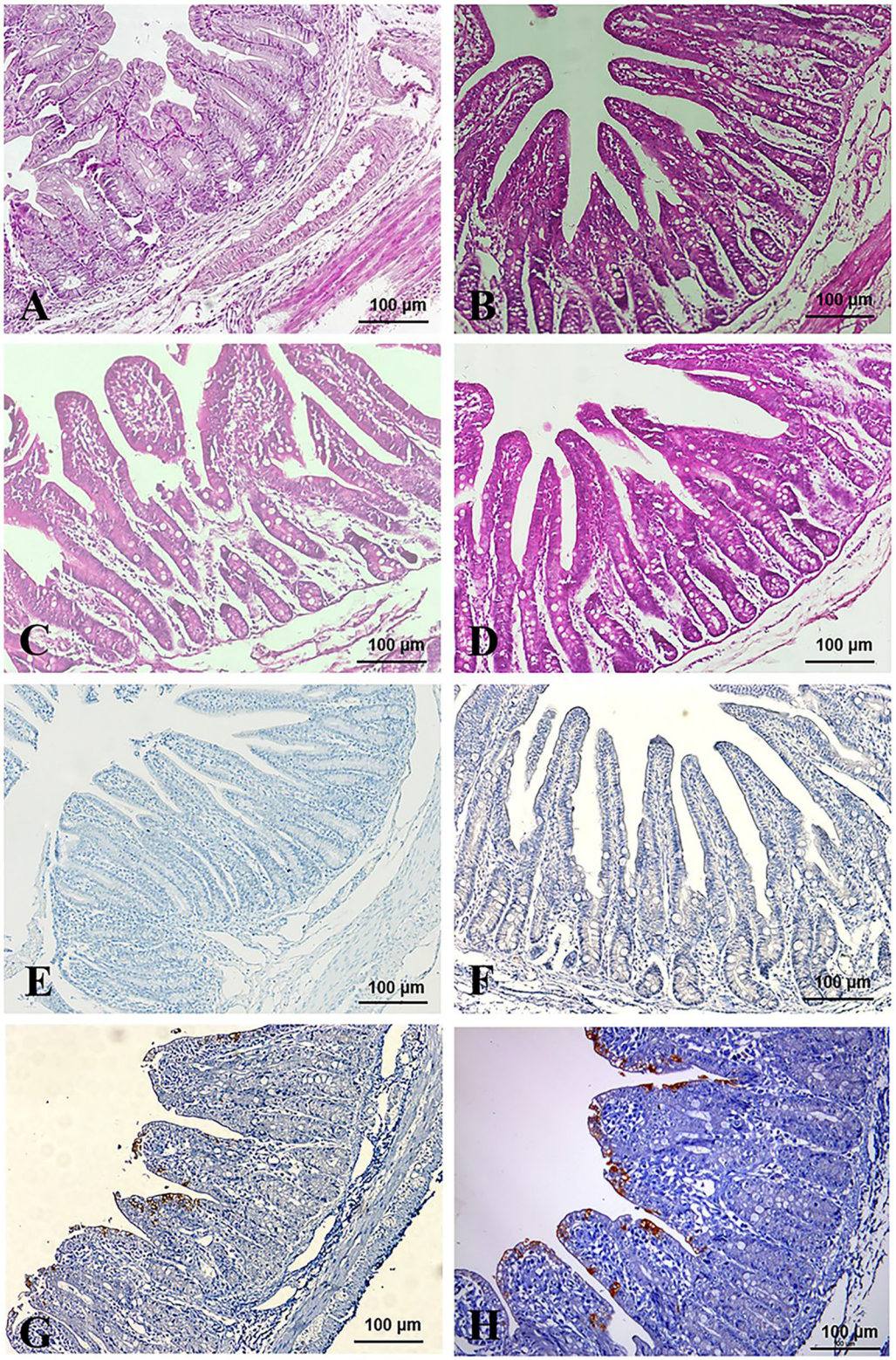

FIGURE 6 | Pathological changes and IHC assays of TGEV HQ2016-inoculated piglets. **(A,B)** H.E staining for jejunum and ileum tissue section of control piglets. **(C,D)** H.E staining for jejunum and ileum tissue section of TGEV HQ2016 challenged piglets. Villus atrophy, degenerate mucosal epithelial cells, and necrosis. **(E,F)** IHC assays for jejunum and ileum tissue section of control piglets. **(G,H)** IHC assays for jejunum and ileum tissue section of TGEV HQ2016 challenged piglets. Positive cells presented in the epithelial cells in the atrophied villi of the segments of jejunal and ileal tissues from the piglets.

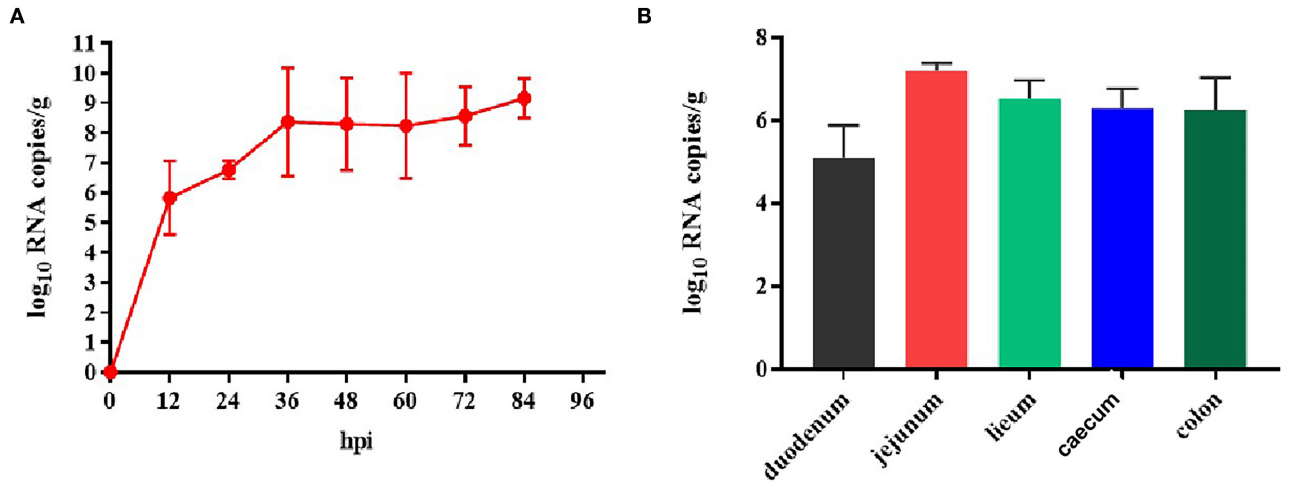

FIGURE 7 | Reproduction of watery diarrhea and viral shedding in newborn piglets inoculated with TGEV HQ2016 via oral feeding. **(A)** Quantification of viral RNA levels of fecal samples of piglets inoculated with TGEV HQ2016. **(B)** Quantification of viral RNA levels in intestine tissues of piglets at 84 h inoculated with TGEV HQ2016.

$\log_{10}$ RNA copies/g), colon (6.23 ± 0.55 $\log_{10}$ RNA copies/g), and duodenum (5.09 ± 0.61 $\log_{10}$ RNA copies/g). These results confirm that TGEV HQ2016 infected the piglets and invaded their intestinal tissues.

DISCUSSION

TGEV is an enteropathic coronavirus that infects pigs, and was first reported in the USA in the 1940s, after which spread throughout the world (1–3). TGEV causes significant diarrhea, vomiting, and dehydration in suckling piglets, with a high mortality rate (10). In recent years, mixed infections of TGEV with other swine diarrhea virus have occurred frequently, causing serious economic losses in the pig industry (1). In this study, a natural strain of TGEV, HQ2016, was successfully isolated from piglets intestinal samples, which collected from swine-raising farms in northeast China. In the farms, sows did not receive any vaccination for preventing diarrhea and piglets developed clinical symptoms including vomiting, diarrhea, rapid weight loss and dehydration. After experimental infection, piglets showed the characteristic clinical symptoms (diarrhea and vomiting) of TGE from 12 h after TGEV HQ2016 inoculated until the end of the experiment. A histopathological analysis showed villous atrophy, together with mucosal epithelial cells degeneration and necrosis, in the jejunum and ileum, and virus-positive cells were present in the villous epithelial cells in the jejunum and ileum by IHC. These results demonstrate that TGEV HQ2016 was replicated and had pathogenicity in enterocyte, is a natural, transmissible, enteric pathogenic porcine coronavirus. Viral nucleic acid of TGEV was detected on rectal swabs as early as 12 h after viral challenge, which indicated that virus infected the intestine and released to intestinal content, as described previously in infections with TGEV (6, 20). At 84 h of TGEV HQ2016 inoculated, we found a high level of viral RNA in jejunum, ileum, caecum and colon, which is similarity with the report previously (5), but there was no obviously pathological changes and TGEV antigen presence in caecum and colon epithelial cells (which is not shown in the results of this study), this result suggested that caecum and colon contained virus but epithelial cells had not yet been infected. Virus-positive epithelial cells and presence of virus in intestines indicated that TGEV HQ2016 prefers to infect small intestinal epithelial cells and replicate, caused pathological changes in the small intestinal epithelial cells, and then necrotic epithelial cells released the virus into the intestinal contents, and finally excreted through the large intestines. This finding may provide a proof for the study of host cell infection and transmission mechanism in coronavirus.

Traditional TGEVs can be divided into two clusters, the Purdue and Miller groups (4, 5, 7, 12, 21). In this study, we sequenced the entire genome of TGEV HQ2016, and a phylogenetic analysis placed TGEV HQ2016 in the Purdue cluster, indicating that it is more distantly evolutionarily related to the Miller cluster. Additionally, sequence alignment result showed two large deletions in ORF3a/3b that occur in the strains of the Miller cluster are not found in TGEV HQ2016 or the Purdue cluster, this may be considered to a marker of distinguishing the Purdue and Miller cluster of TGEV. Phylogenetic analysis shown that TGEV HQ2016 is closely related to with strains PUR46-MAD, Purdue, WH-1, AYU, which have the same ancestor, and this is consistent with the results of homology comparison. Nucleotide and predicted amino-acid sequence homology comparison shown the structural and non-structural proteins of TGEV HQ2016 is very similar to PUR46-MAD, Purdue, AYU and WH-1. These data suggest that TGEV HQ2016 might be had the same origin with WH-1 and AYU strains in China and more similar with Purdue and PUR46-MAD from USA.

The 5′- and 3′-UTRs of CoVs are critically important for viral replication and transcription (5, 22, 23). The "slippery" heptanucleotide sequence and a pseudoknot structure are both critical for viral RNA synthesis and are involved in ribosomal frame shifting (24). A complete sequence analysis indicated that no deletions or insertions are present in the 5′- or 3′-UTR regions of TGEV HQ2016, and that it contains both the slippery sequence and pseudoknot structure. These sequence data suggest that the replication and transcription mechanisms of TGEV HQ2016 are conserved, as reported previously (5, 21, 25).

CoVs attach to their host cells via the S protein, which is the major immunogenic protein of the virus and stimulate the host to produce antibodies with neutralizing activity (26). There are at least four main antigenic sites on the S protein, designated A, B, C, and D (4, 27, 28). The A/B sites (amino acids 506–706) are the major antigenic sites and have been mapped. Single-amino-acid changes in the S protein might affect its antigenicity or virulence (4–6). A mutation at amino acid 585 in the main major antigenic sites A/B of the S protein of TGEV HQ2016 causes a serine to alanine change, which also occurs in the PUR46-MAD, Purdue, Purdue P115, WH-1, AYU, HX, HE-1, SHXB, SC-Y, Z, HB, Mex145, AHHF, H16, and Attenuated H strains, but not in the JS2012, Miller M6, TS, or Virulent Purdue strains. This mutation may significantly influence receptor binding or the virus interactions with neutralizing antibodies, significantly affecting their antigenicity, this is also considered to be a marker of attenuation (6). There was a 6-nt deletion detected in the TGEV HQ2016 S gene, as in the rest of the Purdue cluster, except for the Virulent Purdue and AHHF strains. A 6-nt deletion (nt 1,123-1,128) in the S gene was considered a trait of the TGEV strains in the Purdue cluster (5). This 6-nt deletion in the S gene was also considered to play a role in viral attenuation (6). The S gene is also a hypervariable region in the TGEV genome, and amino acids 32, 72, 100, 184, 208, 218, 389, 403, 418, 487, 562, 590, 649, 675, 815, 951, 1,109, and 1,234 of TGEV HQ2016 are identical among the viruses in the Purdue cluster, but differ from those in the Miller cluster. These changes of amino acid in S gene may be related to the changes of virus virulence, which needs to be discussed in follow-up research. Except for S gene, ORF3a/3b genes were considered to affect the variation between attenuated and virulent strains (12). However, there are some uncertainties about the effects of deletions in TGEV ORF3a/3b on viral virulence (1, 28–31). In our study, homology analysis shown that HQ2016 and attenuated strains PUR46-MAD (4, 32) had highly identity. PUR46-MAD was generally considered an attenuated strain of TGEV, which derivative of Purdue P115, and both were derived from the strain virulent Purdue after highly passage in cell culture (4, 12, 25, 32, 33). TGEV HQ2016 used in our infected experiment was only 10th passage in cell culture. Therefore, we think that the virulence of HQ2016 might be reduced by highly passage in cell culture in the future, as previously reported for PUR46-MAD. 6-nt deletion or amino acid mutations in S gene might reduce the virulence of TGEV HQ2016 through the highly passage, this need to be confirmed in future studies. This hypothesis needs to be confirmed in future studies and facilitate the development of an attenuated vaccine for TGEV.

In conclusion, a epidemical strain of TGEV, HQ2016, was isolated from swine-raising farms in northeast China. Typical clinical signs, pathologic alterations and histological changes associated with TGE were observed in piglets inoculated with the TGEV HQ2016 strain. Phylogenetic analysis of whole genome, nucleotide and amino acid sequence homology analysis of the structural proteins and non-structural proteins indicated that TGEV HQ2016 belongs to the Purdue cluster, and it might be had the same origin with WH-1 and AYU strain in China and more similar with Purdue strains from USA. These results provide essential information for further understanding the evolution of TGEV and will facilitate future investigations into the molecular pathogenesis of TGEV.

DATA AVAILABILITY STATEMENT

The datasets generated in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, MT576083.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Experiment Ethical Committee of Heilongjiang Bayi Agricultural University.

AUTHOR CONTRIBUTIONS

DY: formal analysis and writing—original draft. ZY: methodology and validation. ML: methodology. YW: data curation. MS: writing and picture editing. DS: supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Key Research and Development Program of China (2017YFD0501604-5), Natural Science Foundation of Heilongjiang Province of China (C2018049) and Postdoctoral Science Foundation of Heilongjiang Province of China (LBH-Z19220).

ACKNOWLEDGMENTS

We also thank International Science Editing for editing the English text of a draft of this manuscript (http://www.internationalscienceediting.com).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 04 March 2021
doi: 10.3389/fvets.2021.605832

Molecular Characterization of the Nsp2 and ORF5 (ORF5a) Genes of PRRSV Strains in Nine Provinces of China During 2016–2018

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OPEN ACCESS

Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 13 September 2020
Accepted: 09 February 2021
Published: 04 March 2021

Citation:
Yin B, Qi S, Sha W, Qin H, Liu L, Yun J, Zhu J, Li G and Sun D (2021) Molecular Characterization of the Nsp2 and ORF5 (ORF5a) Genes of PRRSV Strains in Nine Provinces of China During 2016–2018. Front. Vet. Sci. 8:605832. doi: 10.3389/fvets.2021.605832

Porcine reproductive and respiratory syndrome virus (PRRSV) causes a highly contagious disease and brings huge economic losses to commercial pork production worldwide. PRRSV causes severe reproductive failure in sows and respiratory distress in piglets. To trace the evolution of PRRSV in pigs with respiratory diseases in some regions of China, 112 samples were collected from nine provinces in China during 2016–2018. All samples were detected by RT-PCR and analyzed by the Nsp2/ORF5 (ORF5a)-genes-phylogeny. Sequence analysis and recombination analysis were conducted on the Nsp2/ORF5 (ORF5a) genes of the identified strain in the study. The RT-PCR result shown that the positive rate of PRRSV was 50.89% (57/112). Phylogenetic analysis showed that the identified PRRSV strains were all NA genotype and belonged to lineage 1, 3, and 8. The Nsp2 gene of identified PRRSV strains exhibited nucleotide homologies of 53.0 ∼ 99.8%, and amino acid homologies of 46.8 ∼ 99.7%. The ORF5 gene of identified PRRSV strains exhibited nucleotide homologies of 82.4 ∼ 100%, and amino acid homologies of 79.6 ∼ 100%. Sequence analysis revealed that a discontinuous 30-amino-acid deletion (positions 481 and 533–561) and a 131-amino-acid discontinuity deletion (positions 323–433, 481, and 533–551) in Nsp2 of PPRSV isolates; all identified strains in this study may be wild strains, and most identified strains may be highly virulent strains. Sequence analysis of ORF5 and ORF5a revealed that the mutation sites of GP5 were mainly concentrated in the signal peptide and epitopes region, while the mutation sites of ORF5a were mainly concentrated in the transmembrane and the intramembrane region. The recombination analysis indicated that there may be multiple recombination regions in identified strains, and the recombination pattern was more complex. This study showed that the prevalent PRRSV strain in some regions of China was still HP-PRRSV, while NADC30 strain also occupied a certain proportion; different types of PRRSV strains showed different patterns and variation in China. This study suggested that the monitoring of PRRSV prevalence and genetic variation should be further strengthened.

Keywords: porcine reproductive and respiratory syndrome virus, Nsp2 gene, ORF5 gene, ORF5a gene, genetic evolution

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a major threat to the global swine industry, causing significant economic losses each year. The causative agent is PRRS virus (PRRSV), a member of the *Arteriviridae* family, order *Nidoviridales*. PRRSV is a single positive-strand RNA virus with a genome length of ∼15.4 kb (1, 2). The PRRSV contains at least 10 open reading frames (ORFs), which are ORF1a, ORF1b, ORF2a, ORF2b, ORF5a, and ORF3 ∼ 7 from the 5' to the 3' untranslated regions (UTR) (3, 4). ORF1a and ORF1b are cleaved into at least 13–16 non-structural proteins (Nsps) by a complex proteolytic cascade (3).

PRRSV was first reported in commercial pigs by the United States in 1987 (5), and the disease quickly spread worldwide with frequent break outs. PRRSV is still considered a highly contagious disease in the pig industry and creates huge economic losses (1, 6, 7). PRRSV was divided into two genotypes: the European genotype (type I) and North American genotype (type II) (3). There are three main subtypes of PRRSV (type II) isolates in Chinese pig populations: classical PRRSV (type II) including CH-1a, S1, and BJ-4; highly pathogenic PRRSV (HP-PRRSV) including JXA1, HuN4, and TJ; and NADC30-like PRRSV including JL580, CHsx1401, and HNjz15 (8). The genetic characteristic of HP-PRRSV isolates have a discontinuous 30-amino-acid deletion in Nsp2, and NADC30-like PRRSV isolates have a discontinuous 131-amino-acid deletion in Nsp2 (9, 10). PRRSV has mutated in the epidemic process to produce new strains due to the high frequency of gene mutation and recombination, in which new strains often have stronger environmental adaptations. The above factors have made the PRRSV epidemic more complicated, and it also brings great difficulties to disease prevention (11, 12). Nsp2 and ORF5 (ORF5a) are highly variable and ORF5 is associated with the neutralizing epitope (13, 14). They are usually used as target genes for PRRSV molecular epidemiological surveillance.

This study intends to reveal the prevalence and genetic evolution of PRRSV during 2016–2018 in different regions of China. The current study used the Nsp2 and ORF5 (ORF5a) genes to analyze the genetic evolution of the identified PRRSV strains. Our aim is to provide a theoretical basis for further monitoring of genetic variations of PRRSV in China.

METHODS

Sampling

In total, 112 samples of the lung or lymph node tissues from pigs with respiratory diseases were collected between 2016 and 2018 in nine provinces or municipalities of China, including Heilongjiang, Jilin, Liaoning, Hubei, Jiangsu, Jiangxi, Zhejiang, Hebei, and the Inner Mongolia Autonomous Region. The lung or lymph node tissues were ground to powder with liquid nitrogen and diluted with three volumes of phosphate-buffered saline (PBS). The samples were centrifuged at 5,000 × g for 15 min at 4°C and the supernatants were transferred to a 1.5 mL tube. The genomic RNA was extracted from the supernatant using a commercial TIANamp Stool RNA Kit (Tiangen Biotech Co., Ltd, Beijing, China). The viral cDNA was synthesized using Moloney murine leukemia virus (RNaseH-) reverse transcriptase (Novoprotein Scientific Inc., Shanghai, China) in conjunction with six-random-nucleotide primers. The extracted genomic RNA and cDNA was stored at −80°C.

PCR Detection and Sequencing of PRRSV Strains

The primer of ORF5 full-length gene (including complete ORF5a gene) can be found in the report by Cao et al. (15). A pair of primers of Nsp2 gene were designed based on the alignment of published PRRSV genome sequences obtained from the NCBI GenBank database. Primer information is shown in **Table 1**. The amplification reactions were carried out in a 25 μL reaction volume containing 12.5 μl of EmeraldAmp® PCR Master Mix (2×Premix) (TaKaRa Biotechnology Co., Ltd., Dalian, China), 0.5 μM of the forward primer, 0.5 μM of the reverse primer, 1 μL of cDNA, and an appropriate volume of double-distilled (dd) H_2O. The cycling parameters of ORF5 gene were: 36 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. The cycling parameters of Nsp2 gene were: 35 cycles of 95°C for 30 s, 57.6°C for 30 s, and 72°C for 3 min, followed by a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis in a 1% agarose gel under UV light, and the samples with positive results were recorded. After the amplification, products were purified using the AxyPrep DNA Gel Extraction kit (A Corning Brand, Suzhou, China), and cloned into pGM-T Vector (TaKaRa Biotechnology Co., Ltd., Dalian, China). Each fragment was sequenced at least three times. All nucleotide

TABLE 1 | Primers specific for ORF5 and Nsp2 genes of PRRSV.

Target gene	Primer name	Primer sequence(5′-3′)	Amplified length(bp)
ORF5/ORF5a	ORF5-F	GTTTTAGCCTGTCTTTTTGCC	731
	ORF5-R	TATATCATCACTGGCGTGTAGG	
Nsp2	Nsp2-nF	GAAGGGAATTGTGGTTGGCA	2 175 ∼ 2 568
	Nsp2-nR	AGACCCAGAAAACACACCCA	

TABLE 2 | The results of PRRSV sample positive rate in China between 2016 and 2018.

Region	Province	Positive rate	Total
Northeast China	Heilongjiang	60.46% (26/43)	52.3% (34/65)
	Jilin	0% (0/1)	
	Liaoning	36.36% (8/22)	
Central China	Hubei	11.11% (1/9)	11.11% (1/9)
East China	Jiangsu	100% (5/5)	85.7% (12/14)
	Jiangxi	100% (7/7)	
	Zhejiang	0% (0/2)	
North China	Inner Mongolia	44.44% (4/9)	43.5% (10/23)
	Hebei	42.86% (6/14)	
Total			50.89% (57/112)

sequences generated in this study have been submitted to the GenBank database.

Phylogenetic and Sequence Analysis of PRRSV Strains

For the phylogenetic analysis, the Nsp2 and ORF5 (ORF5a) genes of PRRSV reference strains were retrieved from the NCBI nucleotide database as reference sequences. Detailed information and the GenBank number of PRRSV reference strains is shown in **Supplementary Table 1**. To construct phylogenetic trees, nucleotide sequences of the target gene using the ClustalX alignment tool in the MEGA 6.06 software (16). Neighbor-joining phylogenetic trees were constructed with 1,000 bootstrap replicates and the remaining default parameters in the MEGA 6.06 software. The generated phylogenetic tree was annotated using the online software ITOL (https://itol.embl.de/) (17). The PRRSV-identified strains and reference strains were analyzed by MegAlign program in DNASTAR™ 5.06 software. Nucleotide/amino acid homology of Nsp2 and ORF5 (ORF5a) genes of PRRSV-identified strains, and reference strains were gained using the Pairwise/Multiple Align function in Geneious Prime software.

Recombination Analysis

When RDP, GENECONV, BootScan, MaxChi, Chimera, SiScan, and 3eq methods were used to detect potential

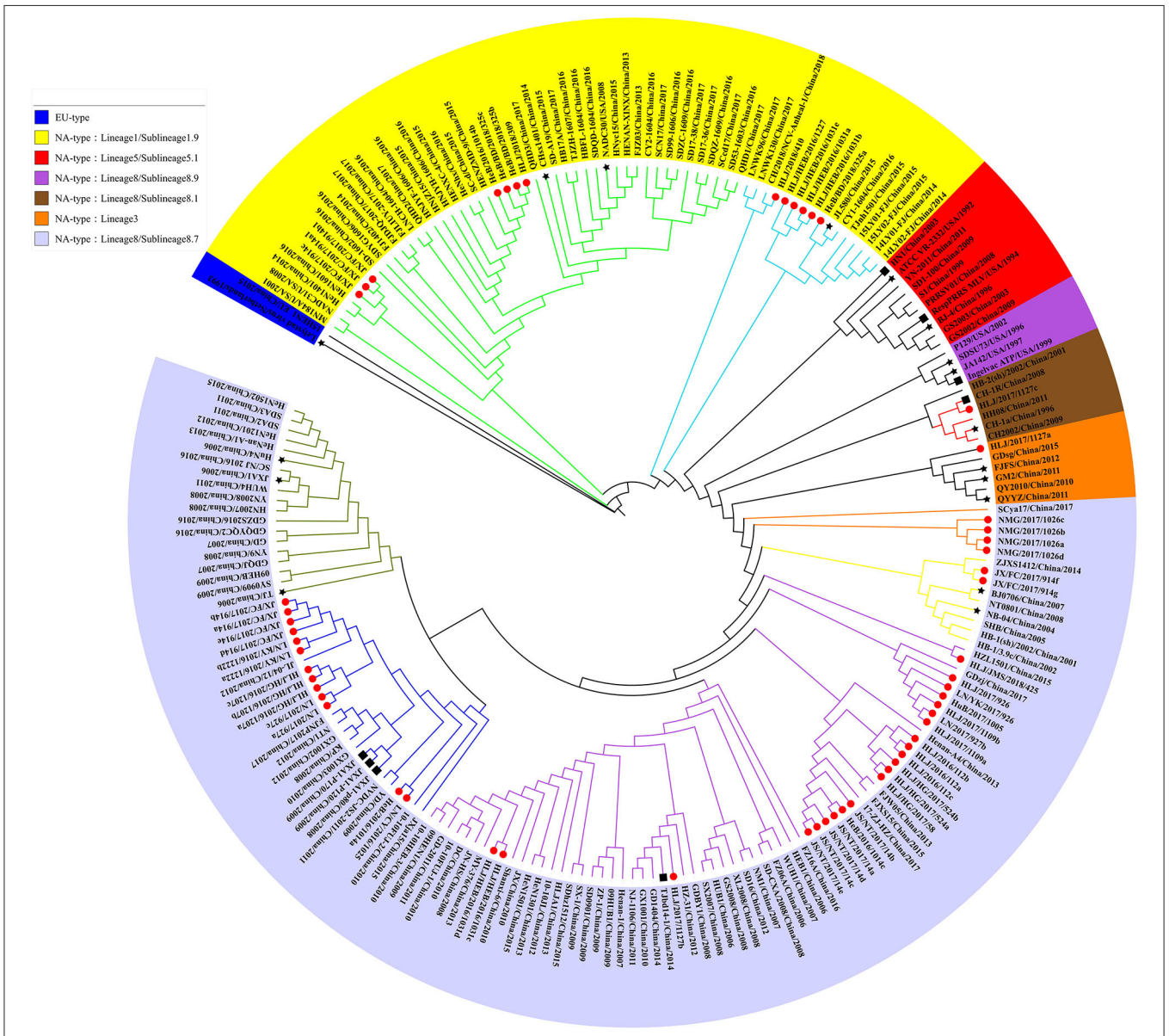

FIGURE 1 | Phylogenetic analysis based on Nsp2 gene. The red circle diagram represents PRRSV strains identified in our study, black star diagram represents PRRSV reference strains, and the black square diagram represents PRRSV vaccine strains.

recombinant events, five or more methods were identified as gene recombination and $P < 0.05$ in RDP4.0 software. The strains in this event were determined to be recombinant strains. In addition, the detected recombination events were further confirmed by SimPlot 3.5.1.

RESULTS

Detection and Analysis of PRRSV

In this study, 112 samples from nine provinces or municipalities of China were detected for PRRSV by RT-PCR. Of these tested samples, 50.89% (57/112) were positive for PRRSV. The infection rate of PRRSV was the highest in East China (85.71%, 12/14), including Jiangsu, Jiangxi, and Zhejiang province, and the lowest in Central China (11.11%, 1/9), including Hubei province. The infection rate of PRRSV in Northeast China was 52.3% (34/65), including Heilongjiang, Jilin, and Liaoning province, while the infection rate of PRRSV in North China was 43.5% (10/23), including Hebei province and the Inner Mongolia Autonomous Region (**Table 2**). Although the sources of the samples in different regions were non-uniform, the results showed that the presence of PRRSV was severe in some parts of China.

Phylogenetic Analysis of PRRSV

In 2010, Shi et al. conducted a systematic classification of type II PRRSVs based on ORF5 gene, and classified the

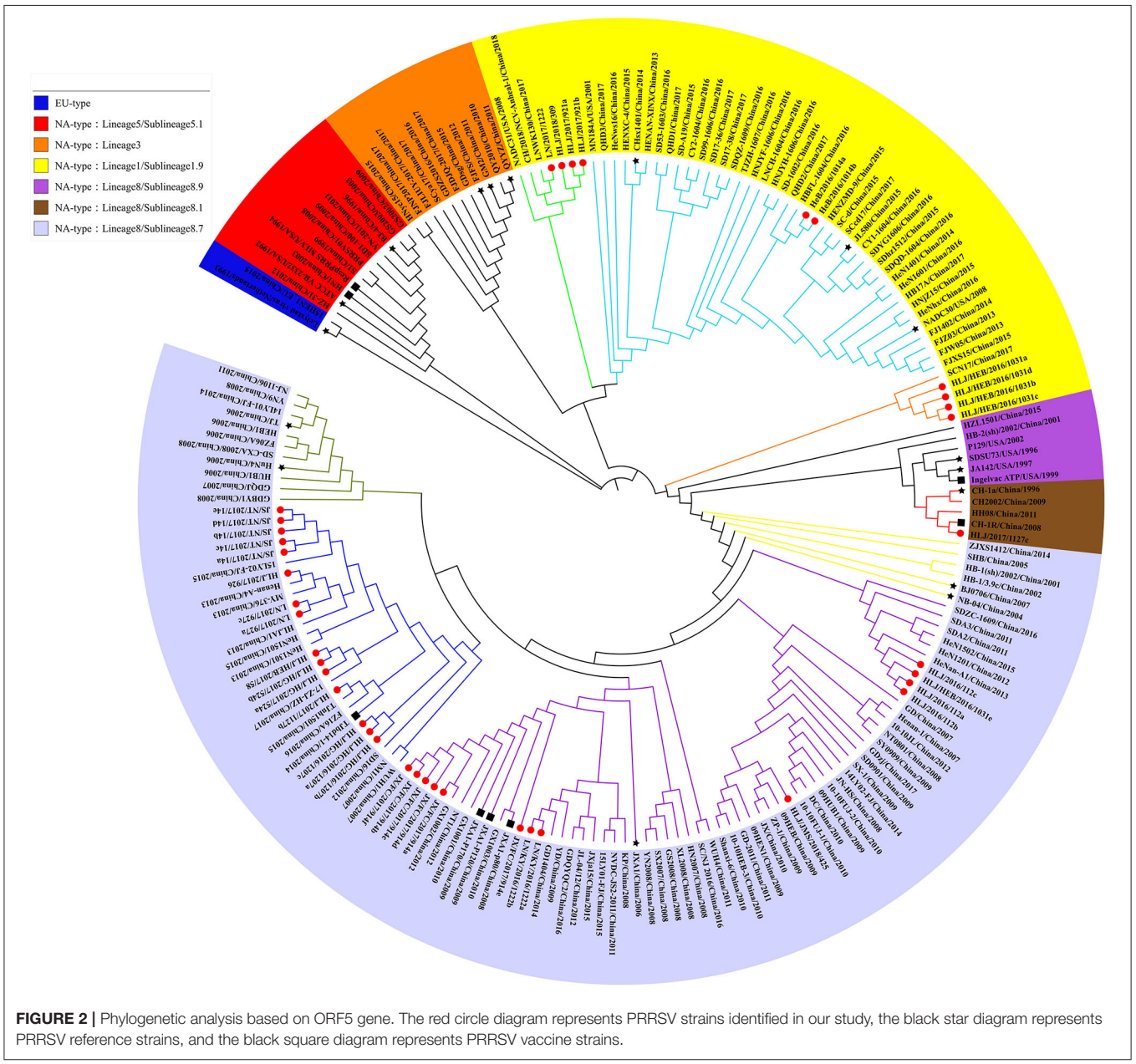

FIGURE 2 | Phylogenetic analysis based on ORF5 gene. The red circle diagram represents PRRSV strains identified in our study, the black star diagram represents PRRSV reference strains, and the black square diagram represents PRRSV vaccine strains.

PRRSVs into nine Lineage and 37 sublineage (18). Referring to the reported genotyping study by Shi et al., all the previously identified lineage reference sequences were also clustered in the same lineage in the phylogenetic tree of this study. The Nsp2 and ORF5-genes-based phylogenetic analysis revealed that all 56 PRRSV strains identified in this study belong to the North American genotype and were distributed in lineage 1, 3, and 8, among which the proportion of identified strains is the highest in sublineage 8.7 (73.2%, 41/56) and the lowest in lineage 3 (1.8%, 1/56) (**Figures 1**, **2**). In addition, some identified strains were distributed differently in phylogenetic trees constructed with different genes. For instance, HeB/2016/1014a and HLJ/HEB/2016/1031(c, d) belong to the sublineage 8.7 while HLJ/HEB/2016/1031e and JX/FC/2017/914c belong to lineage 1 of the phylogenetic tree based on Nsp2 gene. However, the distribution of the identified strains was reversed in

TABLE 3 | Nucleotide and deduced amino acid homologies analysis based on Nsp2 gene (%).

		Identified strain				Reference strain					
		Sublineage 8.1	**Lineage 3**	**Lineage 1**	**Sublineage 8.7**	**VR-2332**	**CH-1a**	**JXA1**	**NADC30**	**QYYZ**	**JA142**
Sublineage 8.1	nt	100	82.5	62.3 ~ 69.7	76.6 ~ 88.5	85.9	98.8	89.0	65.2	81.9	92.6
	aa	100	78.0	55.3 ~ 63.7	73.1 ~ 84.3	81.7	98.4	85.1	59.7	76.2	89.0
Lineage 3	nt		100	61.9 ~ 67.1	77.3 ~ 88.4	76.1	82.6	88.5	63.9	74.7	79.6
	aa		100	55.9 ~ 62.0	73.8 ~ 84.7	70.6	77.8	85.7	58.7	70.5	74.3
Lineage 1	nt			72.5 ~ 99.6	**53.0**[a] ~ 73.5	63.4 ~ 68.3	62.2 ~ 69.7	63.5 ~ 73.4	76.5 ~ 93.9	56.1 ~ 62.5	62.2 ~ 67.7
	aa			66.0 ~ 99.5	**46.8**[a] ~ 69.3	56.8 ~ 62.9	55.3 ~ 63.7	57.3 ~ 69.3	72.0 ~ 92.0	49.1 ~ 55.6	55.9 ~ 61.7
Sublineage 8.7	nt				77.8 ~ **99.8**[b]	69.1 ~ 80.3	76.5 ~ 88.5	85.5 ~ 98.8	54.9 ~ 65.8	65.1 ~ 77.0	73.0 ~ 84.9
	aa				74.7 ~ **99.7**[b]	63.4 ~ 74.3	72.6 ~ 84.2	84.3 ~ 97.9	50.1 ~ 60.8	59.6 ~ 71.6	68.3 ~ 80.1
All strain	nt					63.4 ~ 85.9	62.2 ~ 98.8	63.5 ~ 98.8	54.9 ~ 93.9	56.1 ~ 81.9	62.2 ~ 92.6
	aa					56.8 ~ 81.7	55.3 ~ 98.4	57.3 ~ 97.9	50.1 ~ 92.0	49.1 ~ 76.2	55.9 ~ 89.0

Mark a at the top right for the lowest value, Mark b at the top right for the highest value.

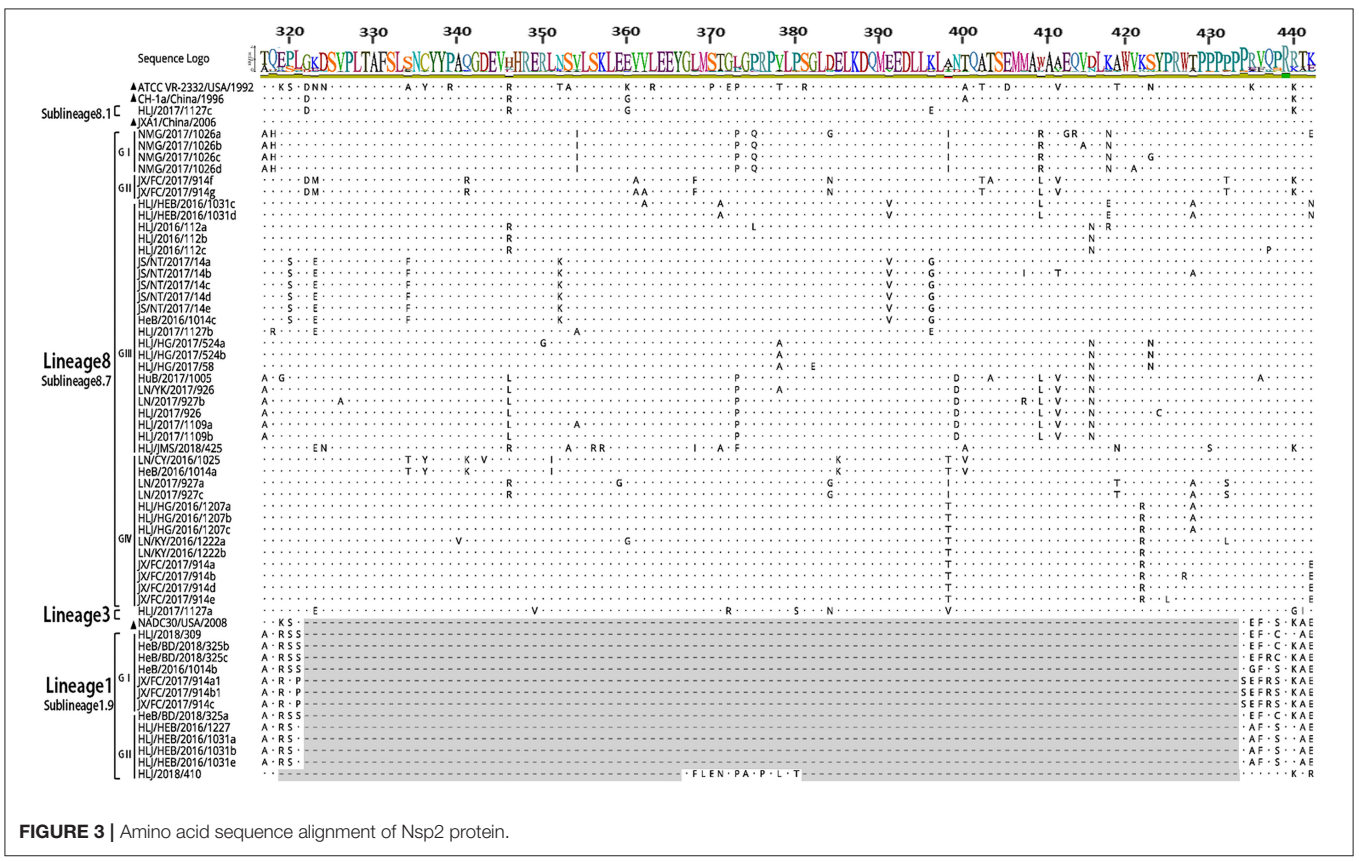

FIGURE 3 | Amino acid sequence alignment of Nsp2 protein.

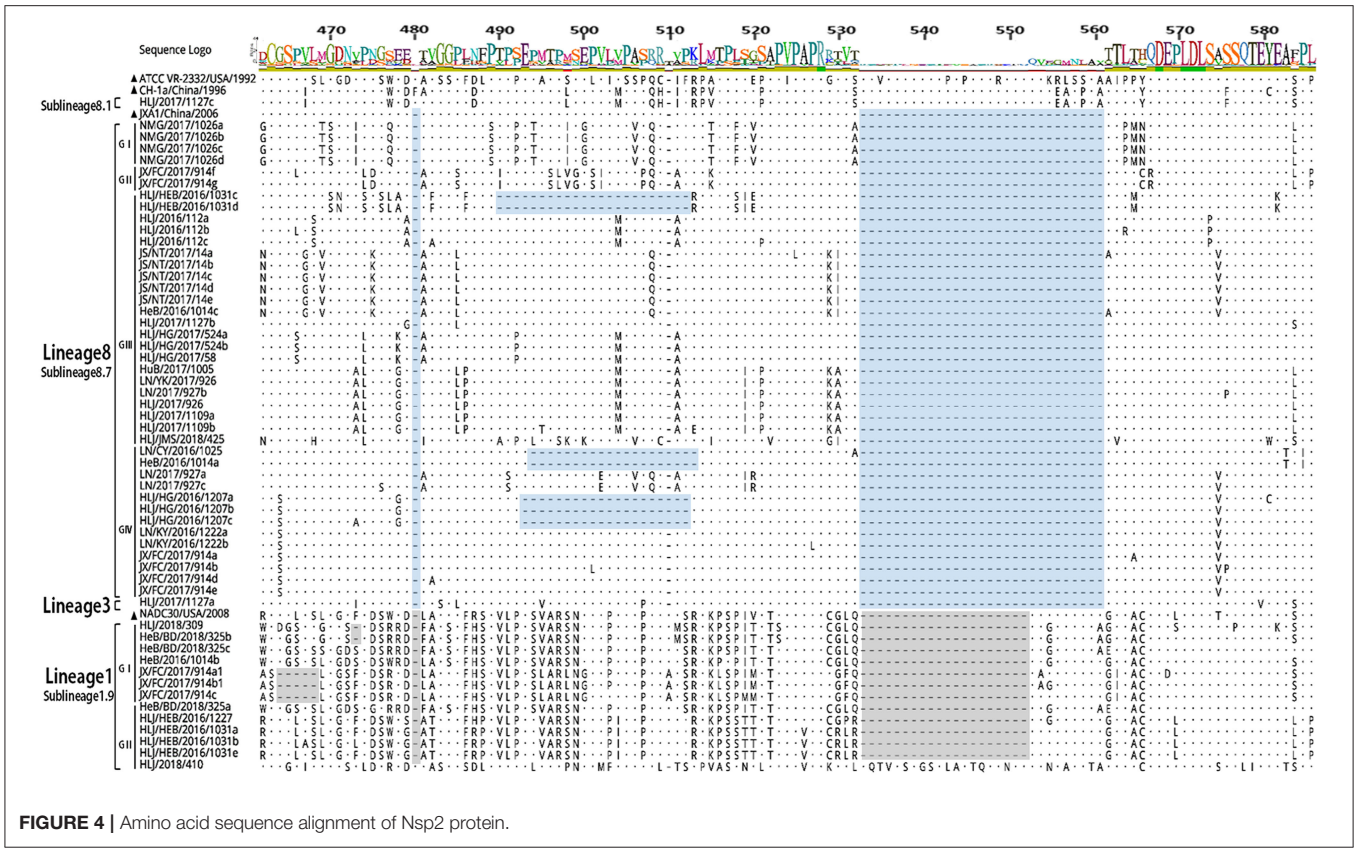

FIGURE 4 | Amino acid sequence alignment of Nsp2 protein.

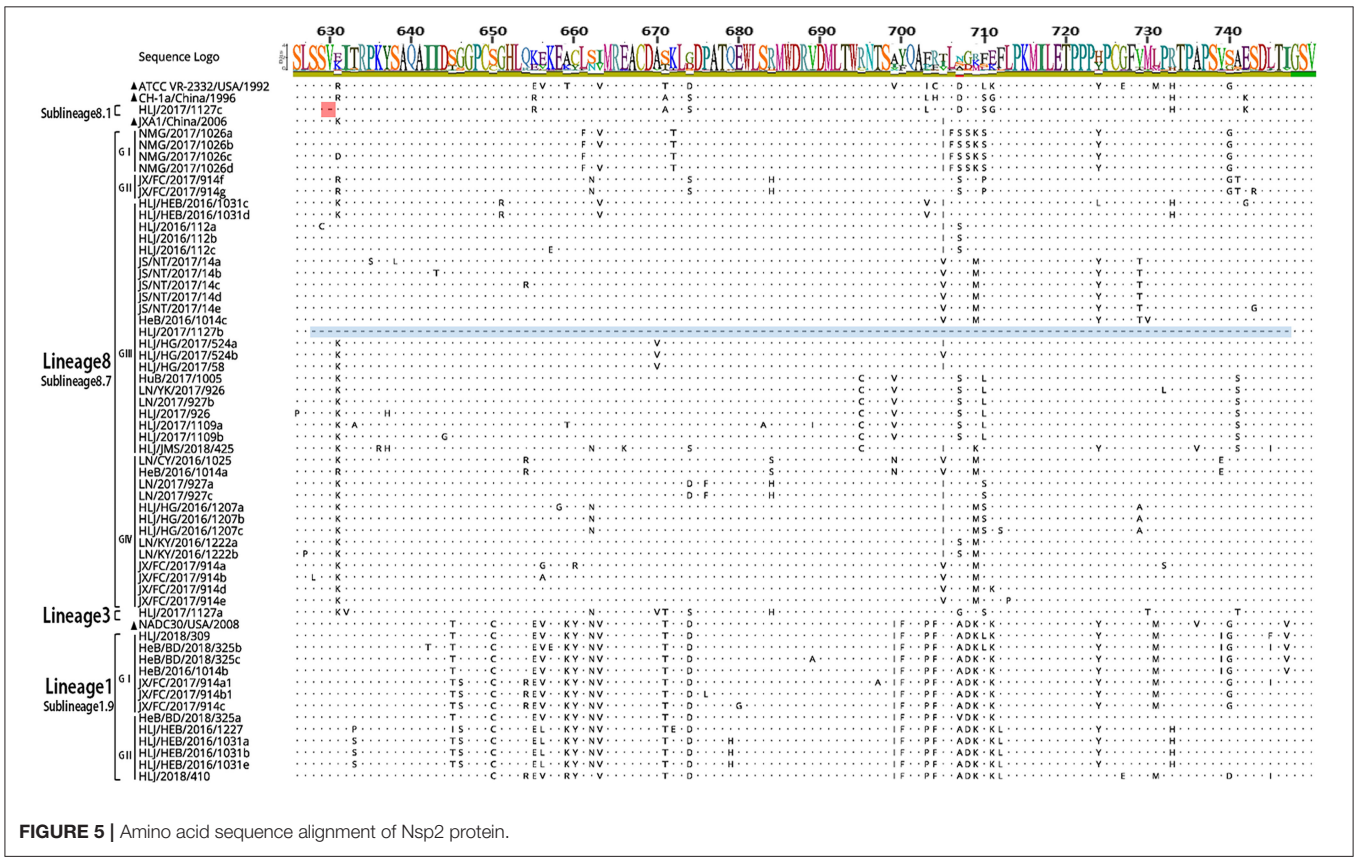

FIGURE 5 | Amino acid sequence alignment of Nsp2 protein.

the phylogenetic tree constructed with the ORF5 gene. With the rapid growth of sequence deposition into the databases, it would be complicated for the diversity of PRRSV sequences.

Sequence Analysis of PRRSV

Sequence Analysis of Nsp2 Genes of PRRSV

The 56 Nsp2 genes and 39 ORF5 (ORF5a) of PRRSV were successfully sequenced. The detailed GenBank number of the 56 PRRSV strains is shown in **Supplementary Table 2**. A sequence comparison of the Nsp2 genes revealed nucleotide homologies of 53.0 ∼ 99.8% and deduced amino acid homologies of 46.8 ∼ 99.7% among the 56 PRRSV strains. Along with the reference strain, its nucleotide and amino acid homologies compared with HP-PRRSV JXA1 strain was the highest (63.5 ∼ 98.8% and 57.3 ∼ 97.9%), and its nucleotide and amino acid homologies compared with NADC30 strain was the lowest (54.9 ∼ 93.9% and 50.1 ∼ 92.0%) (**Table 3**). In addition, the nucleotide and amino acid homologies of the identified strain in sublineage 8.1 and lineage 3 were higher than that of some identified strains in sublineage 8.7 from the same lineage. For example, HLJ/2017/1127c and HLJ/2017/1127b exhibited nucleotide and amino acid homologies of 76.6 and 73.1%, respectively. However, HLJ/2017/1127c and HLJ/2017/1127a exhibited nucleotide and amino acid homologies of 82.5 and 78.0%. The nucleotide and amino acid homologies of HLJ/2017/1127a and the representative strain QYYZ of lineage 3 was lower than the representative strain JXA1 of the 8.7 sublineage. This further suggests HLJ/2017/1127a is likely to be generated by recombinant strains in lineage 3 and sublineage 8.7. The classical PRRSV (type II) ATCC VR-2332 was used as the reference standard; the identified strain HLJ/2017/1127c had the same mutation pattern with vaccine strain CH-1R, which lacked a V at position 630 aa in the Nsp2. The identified strains of sublineage 8.7 and lineage 3 all showed a discontinuous 30-amino-acid deletion (positions 481 and 533–561) that conforms to the classical deletion mutation pattern of the HP-PRRSV-like strain. Excluding HLJ/2018/410, all strains identified in lineage 1 showed 131-amino-acid discontinuity deletion (positions 323–433, 481, and 533–551), that conforms to the classical deletion mutation pattern of the NADC30-like strain (**Figures 3–6**). The prevalent PRRSV strain in some regions of China was still HP-PRRSV, while NADC30 strain also occupied a certain proportion.

Sequence Analysis of ORF5 Genes of PRRSV

A sequence comparison of the ORF5 genes revealed nucleotide homologies of 82.4 ∼ 100% and deduced amino acid homologies of 79.6 ∼ 100% among the 39 PRRSV strains. The nucleotide and amino acid homologies compared with JXA1 strain was the highest (84.9 ∼ 99.7%, 84.1 ∼ 99.0%), and its nucleotide and amino acid homologies compared with QYYZ strain was the lowest (81.9 ∼ 84.6%, 80.1 ∼ 86.6%) (**Table 4**). The mutation sites of ORF5 were mainly concentrated in the signal peptide and epitopes region. GP5 virulence-related sites showed that nine of the 39 identified strains had mutated at the position 13th aa

FIGURE 6 | Amino acid sequence alignment of Nsp2 protein.

(R→ Q). A total of 12 identified strains of sublineage 8.7 and lineage 1 had mutated at position 151 aa (R→ K), The 137th aa of all identified strains was conservative and was S (**Figure 7**). It is observed that all identified strains may be wild strains, and most identified strains may be highly virulent strains in the study.

Sequence Analysis of ORF5a Genes of PRRSV

A sequence analysis of the ORF5a genes among the 39 PRRSV strains exhibited nucleotide homologies of 84.0 ∼ 100% and deduced amino acid homologies of 80.8 ∼ 100%. The nucleotide and amino acid homologies compared with JXA1 strain was the highest (87.2 ∼ 99.4%, 84.6 ∼ 100%), and its nucleotide and amino acid homologies compared with QYYZ strain was the lowest (70.5 ∼ 79.5%, 67.3 ∼ 78.8%) (**Table 5**). All the ORF5a proteins of the identified strains encoded 46 aa, and HLJ/2017/1127c exhibited high sequence similarity compared with CH-1a strain, with only a few point mutations, such as $M^{8} \rightarrow I^{8}$, $F^{20} \rightarrow L^{20}$, and $S^{42}/F^{42} \rightarrow I^{42}$; A part of identified strains had two amino acid mutations in the transmembrane region, $G^{12}/V^{12} \rightarrow A^{12}$, $R^{24} \rightarrow C^{24}$, and two amino acid mutations in the intramembrane region, $Q^{36} \rightarrow R^{36}$, $Q^{38} \rightarrow R^{38}/P^{38}$. Compared with NADC30, some of the strains identified had one amino acid mutation in the transmembrane region, $V^{26}/I^{26} \rightarrow T^{26}$, and one amino acid conformity mutation in the intramembrane region, $S^{42} \rightarrow F^{42}/L^{42}$ (**Figure 8**). This shows that the mutation sites of ORF5a were mainly concentrated in the transmembrane region and the intramembrane region.

Recombination Analysis

The recombination analysis of Nsp2 gene showed that there were five potential recombination events (**Table 6**). The recombination analysis of Nsp2 gene showed that the recombinant strains in event 1 and 2 were produced by recombination of lineage 1 and sublineage 8.7 (**Supplementary Figure 1**). The high frequency mutation and recombination make the virus gain more genetically diverse (19). This recombination pattern is the most common in PRRSV recombinant strains in China, and animal tests have confirmed that the virulence of some recombinant strains is higher than the prototype strain NADC30 (9). The identified strain HLJ/2017/1127a in recombinant event 3 was produced by recombination of lineage 3 and sublineage 8.7 wild strain. The main parental strain was FZ06A, while the minor parental strain was QYYZ. Previous studies have shown that the low virulence prototype strain QYYZ even became highly virulent after recombination with the vaccine strain derived from HP-PRRSV (20). The identified strain JS/NT/2017/14b in recombinant event four showed that the main parental strain HLJ/2017/1127b belongs to subline 8.7, while the minor parental strain JS/NT/2017/14a belongs to subline 8.7. Three recombinant strains in recombinant event five were from the same origin as JS/NT/2017/14b, but the recombinant sites are different. The recombination analysis of ORF5 genes showed that the recombinant event included four recombinant strains of lineage 1 (**Table 7**). The main parental strain CY1-1604 belongs to lineage 1, while the minor parental strain GS2008 belongs to sublineage 8.7 (**Supplementary Figure 2**). Combined with the recombination analysis of Nsp2 gene, the identified strain HLJ/HEB/2016/1031 (a, b) was also recombined in ORF5 gene, which indicated that there may be multiple recombination regions in identified strains, and the recombination pattern was more complex.

DISCUSSION

Since the outbreak of HP-PRRSV in 2006, PRRSV has been widely spread across the world. In previous studies, the positive rate of PRRSV was shown to be 55.21% (7,490/11,3567) in 29 provinces of China in 2012–2015 (21). In Central and Southern China, there was a positive rate of 50.62% (530/1,047) of PRRSV among 257 pig farms (22). In our study, the total positive rate of PRRSV was 50.89% (57/112) in nine provinces of China from 2016 to 2018, which was in accordance with the above scholars. PRRS is one of the most prevalent and threatening infectious diseases in Chinese pig farms.

Nsp2 and ORF5 (ORF5a) genes have the highest variability in PRRSV genome and are used as main target genes for PRRSV genetic variation. Phylogenetic tree analysis showed that all the 56 PRRSV strains identified in this study belong to the North American genotype and were distributed in lineage 1, 3, and 8 according to Shi et al. (18). In this study, the HP-PRRSV strain accounted for the highest proportion of epidemic strains in China; the NADC30-like strain had increased gradually, which was in accordance with the results of Gao et al. (23). However, some studies have shown that the NADC30-like strain in some regions of China have replaced the HP-PRRSV strain, and has become a new dominant strain (22). The rising infection rate of HP-PRRSV and NADC30-like strains may lead to a significant decrease in the effective protection rate of vaccines on pig farms.

The sequence alignments of Nsp2 gene revealed that the identified strain HLJ/2017/1127c in subline 8.1 had a high similarity with vaccine strain CH-1R, and existed a V deletion in the 630aa, suggesting that the identified strain may be a vaccine strain or a recombinant strain of a vaccine strain. Sequence alignments identified a discontinuous 30-amino-acid deletion (positions 481 and 533–561) and a 131-amino-acid discontinuity deletion (positions 323–433, 481, and 533–551) in Nsp2 of PPRSV isolates. JX/FC/2017/914(c, a1, b1) had the same deletion pattern as PRRSV strains HeN1401 and HeN1601, isolated by Zhang et al. (24). The recombinant analysis of the two epidemic strains revealed that HeN1401 and HeN1601 strains were generated by the recombinant weak vaccine strains TJbd14-1 and NADC30 (24). This further suggests that the identified strain JX/FC/2017/914(c, a1, b1) may also be a recombinant strain.

The GP5 protein sequences of different subline strains showed high similarity with the representative strains of the subline. The mutation sites of GP5 were mainly concentrated in the signal peptide and epitopes region. But some identified strains also have some amino acid consistent mutations in immune-related regions. Allende et al. found that nine amino acid site mutations may be closely related to the virulence of the PRRSV and that two sites (13 and 151 aa) were located in GP5 protein. The

TABLE 4 | Nucleotide and deduced amino acid homologies analysis based on ORF5 gene (%).

		Identified strain			Reference strain					
		Sublineage 8.1	Sublineage 8.7	Lineage 1	VR-2332	CH-1a	JXA1	NADC30	QYYZ	JA142
Sublineage 8.1	nt	100	90.9 ~ 94.0	84.9 ~ 91.0	91.0	98.7	94.4	87.1	84.4	93.4
	aa	100	87.1 ~ 89.6	81.1 ~ 88.6	89.1	97.5	90.5	85.1	81.6	94.0
Sublineage 8.7	nt		91.4 ~ **100**[b]	**82.4**[a] ~ 92.2	86.4 ~ 89.2	91.9 ~ 95.0	94.9 ~ 99.7	83.1 ~ 86.1	81.9 ~ 84.4	88.1 ~ 91.0
	aa		91.0 ~ **100**[b]	**79.6**[a] ~ 91.5	84.6 ~ 89.1	89.6 ~ 92.0	93.5 ~ 99.0	83.6 ~ 87.1	80.1 ~ 83.1	88.1 ~ 90.5
Lineage 1	nt			85.9 ~ **100**[b]	84.7 ~ 87.7	85.6 ~ 91.9	84.9 ~ 92.5	88.2 ~ 94.0	83.6 ~ 84.6	85.4 ~ 90.2
	aa			85.1 ~ **100**[b]	83.1 ~ 87.1	83.6 ~ 91.0	84.1 ~ 90.5	89.6 ~ 94.0	81.6 ~ 86.6	85.1 ~ 91.0
All strain	nt				84.7 ~ 91.0	85.6 ~ 98.7	84.9 ~ 99.7	83.1 ~ 94.0	81.9 ~ 84.6	85.4 ~ 93.4
	aa				83.1 ~ 89.1	83.6 ~ 97.5	84.1 ~ 99.0	83.6 ~ 94.0	80.1 ~ 86.6	85.1 ~ 94.0

Mark a at the top right for the lowest value, Mark b at the top right for the highest value.

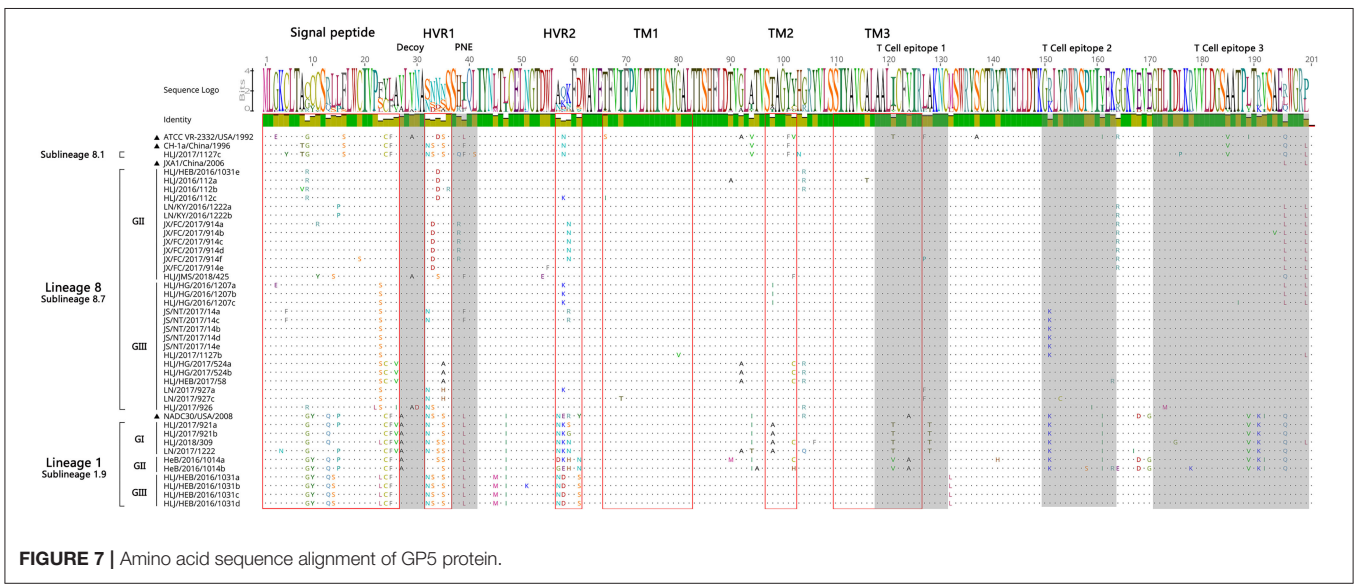

FIGURE 7 | Amino acid sequence alignment of GP5 protein.

TABLE 5 | Nucleotide and deduced amino acid homologies analysis based on ORF5a gene (%).

		Identified strain			Reference strain					
		Sublineage 8.1	Sublineage 8.7	Lineage 1	VR-2332	CH-1a	JXA1	NADC30	QYYZ	JA142
Sublineage 8.1	nt	100	87.8 ~ 90.4	87.2 ~ 90.4	80.1	97.4	91.0	88.5	74.4	83.3
	aa	100	82.7 ~ 88.5	84.6 ~ 86.5	75.0	94.2	88.5	84.6	69.2	80.8
Sublineage 8.7	nt		89.7 ~ **100**[b]	**84.0**[a] ~ 91.0	76.9 ~ 81.4	89.7 ~ 92.9	93.6 ~ 99.4	83.3 ~ 88.5	70.5 ~ 74.4	76.9 ~ 80.8
	aa		86.5 ~ **100**[b]	**80.8**[a] ~ 86.5	71.2 ~ 76.9	84.6 ~ 90.4	92.3 ~ 100	80.8 ~ 84.6	67.3 ~ 71.2	75.0 ~ 80.8
Lineage 1.9	nt			88.5 ~ **100**[b]	77.6 ~ 81.4	89.7 ~ 92.0	87.2 ~ 90.4	91.0 ~ 97.4	75.0 ~ 79.5	78.2 ~ 81.4
	aa			92.3 ~ **100**[b]	80.8 ~ 84.6	88.5 ~ 92.3	84.6 ~ 86.5	94.2 ~ 98.1	73.1 ~ 78.8	78.8 ~ 86.5
All strain	nt				76.9 ~ 81.4	89.7 ~ 97.4	87.2 ~ 99.4	83.3 ~ 97.4	70.5 ~ 79.5	76.9 ~ 83.3
	aa				71.2 ~ 84.6	84.6 ~ 94.2	84.6 ~ 100	80.8 ~ 98.1	67.3 ~ 78.8	75.0 ~ 86.5

Mark a at the top right for the lowest value, Mark b at the top right for the highest value.

GP5 protein of high virulence strains generally were shown as R^{13} and R^{151} (25). Wesley et al. showed that the 137aa of GP5 protein can distinguish the attenuated vaccine strain (A^{137}) and the wild strain (S^{137}) (26). Therefore, the above three amino acid sites are often used to predict the virulence of PRRSV strains. The sequence analysis of GP5 protein showed that only one mutation pattern ($R^{13} \rightarrow Q^{13}$ and $R^{151} \rightarrow K^{151}$) existed in this study. Nine identified strains had mutated at position 13 aa,

TABLE 6 | Recombination analysis of Nsp2 gene.

Recombination event	Recombinant strains	Main parental strain	Minor parental strain	Recombinant breakpoint	Recombination analysis method
1	HeB/2018/325a HLJ/2016/1031a HLJ/2016/1031b HLJ/2016/1031e HLJ/HEB/1227	HENZMD-9	10-10FUJ-2/China	18-2043 (18–1591)	RDP ($P = 1.22 \times 10^{-18}$) GENECONV ($P = 9.49 \times 10^{-28}$) BootScan ($P = 1.78 \times 10^{-28}$) MaxChi ($P = 4.14 \times 10^{-26}$) Chimaera ($P = 6.68 \times 10^{-8}$) SiScan ($P = 9.44 \times 10^{-27}$) 3seq ($P = 3.61 \times 10^{-8}$)
2	JX/2017/914c JX/2017/914a1 JX/2017/914b1	HENZMD-9	10-10FUJ-1/China	654–2600 (654–2015)	RDP ($P = 6.81 \times 10^{-40}$) GENECONV ($P = 3.43 \times 10^{-34}$) BootScan ($P = 6.79 \times 10^{-28}$) MaxChi ($P = 7.71 \times 10^{-20}$) Chimaera ($P = 4.31 \times 10^{-22}$) SiScan ($P = 2.28 \times 10^{-25}$) 3seq ($P = 2.27 \times 10^{-14}$)
3	HLJ/2017/1127a	FZ06A	QYYZ	1892–2730 (1742–2442)	RDP ($P = 2.08 \times 10^{-5}$) GENECONV (NS) BootScan ($P = 6.97 \times 10^{-5}$) MaxChi ($P = 6.41 \times 10^{-18}$) Chimaera ($P = 9.90 \times 10^{-16}$) SiScan ($P = 1.48 \times 10^{-8}$) 3seq ($P = 7.66 \times 10^{-15}$)
4	JS/NT/2017/14b	HLJ/2017/1127b	JS/NT/2017/14a	732–2227 (732–2077)	RDP ($P = 4.54 \times 10^{-7}$) GENECONV ($P = 4.83 \times 10^{-6}$) BootScan ($P = 7.00 \times 10^{-8}$) MaxChi ($P = 2.44 \times 10^{-11}$) Chimaera (NS) SiScan ($P = 7.43 \times 10^{-12}$) 3seq ($P = 3.88 \times 10^{-12}$)
5	JS/NT/2017/14d JS/NT/2017/14c JS/NT/2017/14e	HLJ/2017/1127b	JS/NT/2017/14a	561–2534 (561–2267)	RDP ($P = 1.75 \times 10^{-5}$) GENECONV ($P = 2.27 \times 10^{-4}$) BootScan($P = 1.16 \times 10^{-5}$) MaxChi ($P = 6.46 \times 10^{-8}$) Chimaera (NS) SiScan ($P = 7.01 \times 10^{-17}$) 3seq ($P = 8.06 \times 10^{-9}$)

NS, No significant P-value.

TABLE 7 | Recombination analysis of ORF5 gene.

Recombination event	Recombinant strains	Main parental strain	Minor parental strain	Recombinant breakpoint	Recombination analysis method
1	HLJ/HEB/2016/1031a HLJ/HEB/2016/1031b HLJ/HEB/2016/1031c HLJ/HEB/2016/1031d	CY1-1604	GS2008	249–580 (249–580)	RDP ($P = 1.92 \times 10^{-7}$) GENECONV ($P = 2.51 \times 10^{-6}$) BootScan ($P = 1.72 \times 10^{-6}$) MaxChi ($P = 2.60 \times 10^{-10}$) Chimaera ($P = 3.00 \times 10^{-9}$) SiScan ($P = 6.72 \times 10^{-12}$) 3seq ($P = 2.29 \times 10^{-12}$)

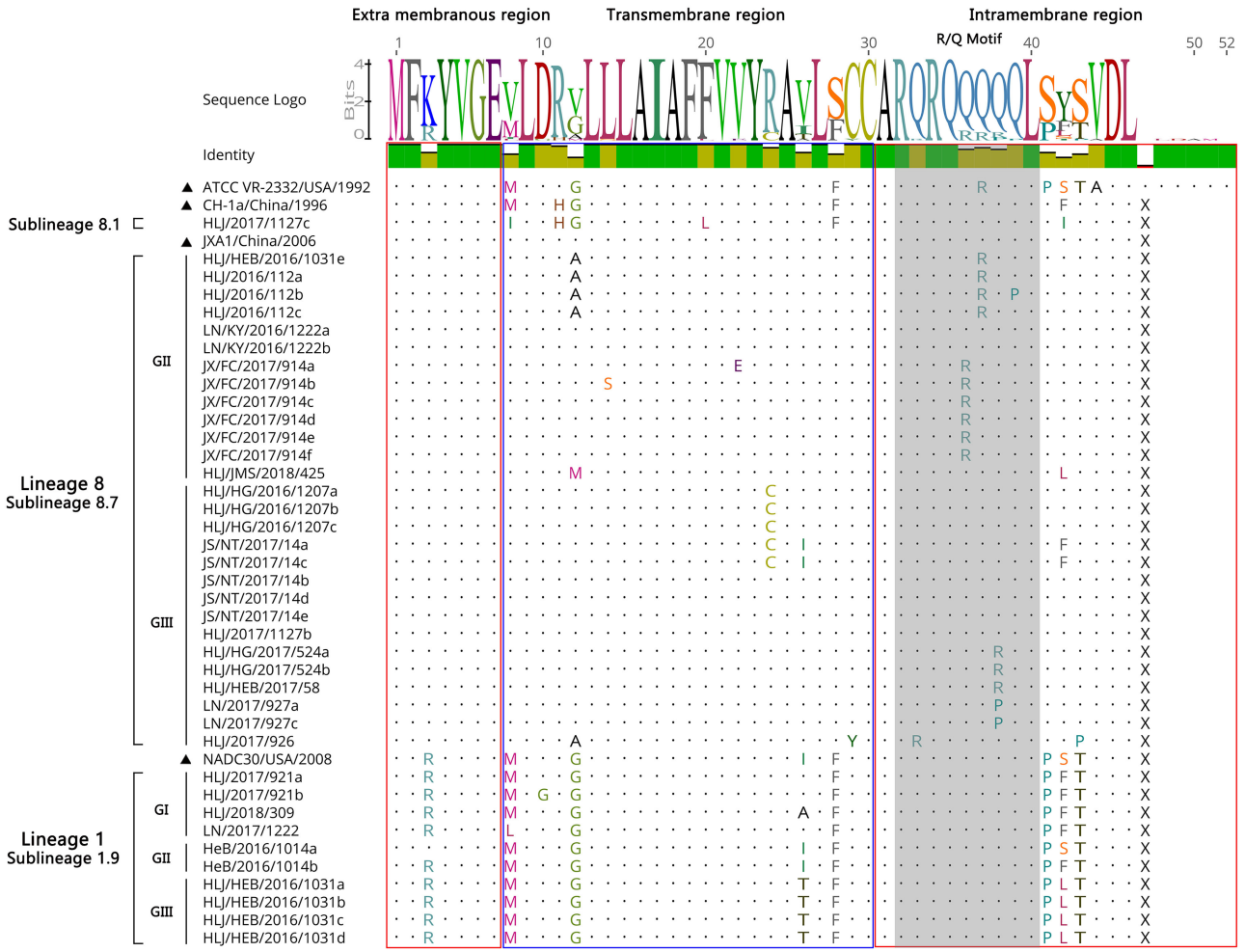

FIGURE 8 | Amino acid sequence alignment of GP5a protein.

and 12 identified strains mutated at position 151 aa. In addition, the 137 aa of all identified strains is S. The results suggest that all identified strains may be wild strains, and most identified strains may be highly virulent strains in nine provinces of China during 2016–2018.

Studies have shown that ORF5a protein is essential for viral viability and infectivity (27, 28). There is fairly limited information available on current genetic variations of PRRSV ORF5a gene (29). Therefore, this study explored the genetic variation of ORF5a gene of PRRSV epidemic strains in China by molecular biological methods. The ORF5a protein generally encoded 46–51 amino acids of which ORF5a protein of PRRSV strains encoded 46 amino acids in lineage 1 and 8, and 51 amino acids in lineage 3 and 5 (30). All the ORF5a proteins identified in this study encoded 46 amino acids. Compared with the reference strain, the identified strains also showed high sequence similarity, and the mutation sites of ORF5a were mainly concentrated in the transmembrane region and the intramembrane region, while the other region was highly conserved. Our study demonstrated the existence of multiple different strains in the same region and extensive genetic mutation of PRRSV in China from 2016 to 2018.

The recombination analysis indicated that there may be multiple recombination regions in identified strains, and the recombination pattern was more complex. At present, many studies have shown that PRRSV strain in lineage 1 is prone to recombinant mutation, and some of the recombinant strains are more virulent than others (10, 19). Although the recombination pattern of the virus identified in this study is in accordance with that reported by some previous scholars, the change of pathogenicity of PRRSV by gene recombination is not absolute. This study only carried out partial gene (Nsp2 and ORF5) recombination analysis without the virus isolation and whole genome sequences of PRRSV, so the recombination of whole genome sequences is more complicated and different.

CONCLUSION

This study showed that PRRSV infection was prevalent in nine provinces of China from 2016 to 2018, and the prevalent PRRSV strain in most regions was still HP-PRRSV, while the NADC30 strain also occupied a certain proportion. There was a discontinuous 30-amino-acid deletion (positions 481 and 533–561) and a 131-amino-acid discontinuity deletion (positions 323–433, 481, and 533–551) in Nsp2 of PPRSV isolates. All identified strains in this study may be wild strains, and most identified strains may be highly virulent strains. This study identified highly homologous HP-PRRSV variants with distinct genetic mutation, which contributes to further analyzing the epidemics and evolution of PRRSV in the field.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

DS and GL conceived the study. BY, SQ, and JZ analyzed the data. BY and SQ wrote the manuscript for submission. WS, HQ, LL, and JY participated in the design of the study, performed the data collection and analysis, and commented on the manuscript. All authors approved the final manuscript.

FUNDING

This work was supported by the Science and Technology Development Project of Jilin Province (20190301007NY), the Heilongjiang Province Postdoctoral Science Foundation of China (LBH-Q16188), and Heilongjiang Bayi Agricultural University Support Program for San Heng San Zong (TDJH201804).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.605832/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 04 March 2021
doi: 10.3389/fvets.2021.651978

Serological Evidence of Exposure to Peste des Petits Ruminants in Small Ruminants in Rwanda

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The status of Peste des Petits Ruminants (PPR) in Rwanda is unknown, despite its prevalence in neighboring countries. A cross-sectional sampling of goats and sheep was carried out in five districts of Rwanda located closer to neighboring countries endemic to PPR. Serum samples were analyzed using a commercial ELISA, to detect antibodies to PPR virus (PPRV). Sixty-eight samples [14.8, 95% Confidence Interval (CI): 11.7–18.4] were seropositive for PPR, of which 17.4% (95% CI: 11.6–24.6; 25/144) were from sheep, whereas 13.6% (95% CI: 10.0–17.9; 43/316) were from goats. Seropositivity ranged from 8.9 to 17.3% (goats) and from 10.5 to 25.8% (sheep) in sampled districts. Seropositivity was slightly higher in males than females in both goats (15.7 vs. 12.4%) and sheep (17.7 vs. 17.1%), and were significantly marked in goats and sheep aged more than 15 months (goats: 17.9, 95% CI: 12.9–24.0; sheep: 22.2, 95% CI: 14.1–32.2) than those between 6 and 15 months (goats: 6.1, 95% CI: 2.5–12.1; sheep: 9.3, 95% CI: 3.1–20.3). Sampling was non-randomized and results are not representative of the true prevalence of PPR antibody in small ruminants. Thus, data does not allow to fully discuss the findings beyond the presence/absence certitude and the comparisons made must be interpreted with caution. The presence of specific antibodies to PPRV may, however, be linked to one or a combination of following scenarios: (1) prevalence and persistence of PPRV in sampled regions which would cause low level of clinical cases and/or mortalities that go unnoticed; (2) introduction of PPRV to herds through movements of livestock from neighboring infected countries, and/or (3) events of disease outbreaks that are underreported by farmers and veterinarians. In addition to strengthen veterinary surveillance mechanisms, further studies using robust sampling methods and integrating livestock and wildlife, should be carried out to fully elucidate PPR epidemiology in Rwanda.

Keywords: small ruminants, Rwanda, PPR, seroprevalence, transboundary diseases

OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
Richard Anthony Kock, Royal Veterinary College (RVC), United Kingdom
Arnab Sen, University of North Bengal, India

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *11 January 2021*
Accepted: *09 February 2021*
Published: *04 March 2021*

Citation:
Shyaka A, Ugirabe MA and Wensman JJ (2021) Serological Evidence of Exposure to Peste des Petits Ruminants in Small Ruminants in Rwanda. Front. Vet. Sci. 8:651978. doi: 10.3389/fvets.2021.651978

INTRODUCTION

Livestock diseases are recognized global threats to food supply and to livestock industry specifically (1). Peste des Petits Ruminants Virus (PPRV) is a member of the family *Paramyxoviridae*, genus *Morbillivirus*, species *Small ruminant morbillivirus* (2). It primarily affects goats and sheep, but also other domestic animals such as cattle, pigs and camels as well as various wildlife ungulates (3, 4),

through contact with infected animals, or indirectly through fecal and/or mucosal secretions (5). The disease caused, Peste des Petits Ruminants (PPR), is highly contagious and is characterized by acute clinical signs in goats and sheep, as well as in wild ruminants (6–8). PPR is associated with a case fatality rate of 15.5% (8) that can reach up to 80–100% in naïve herds (9). PPR is recognized as the most widely distributed infectious disease of domestic small ruminants and wildlife ungulates, and is endemic in most countries of Africa, Middle East and Asia (10). It can negatively impact countries' economy and increase poverty in rural settings where small ruminants are mostly concentrated. In fact, PPR-associated losses are estimated at USD 1.2–1.7 billion annually and a third of this financial burden occurs in Africa (10). In addition, PPR constitutes a growing challenge to biodiversity and wildlife conservation (8, 11).

PPR has affected most countries in East Africa since the last 5 decades and confirmation of the first outbreak in Sudan in 1971–1972 (12), followed by further outbreak reports from Ethiopia in 1989–1990 (13). In Uganda, the major PPR outbreak was reported in 2006–2008, in Karamoja region along with a similar report in neighboring Kenya (14, 15). However, previous reports had suggested presence of PPR through seroprevalence studies carried out in the 1980s in Uganda and Kenya (16), in the 2000s in Uganda (17) and an outbreak reported in Uganda in 2003 (18). In addition, antibodies to PPRV were also detected in Ugandan wildlife in 2004 (19), probably as a consequence of spillover of the virus from livestock. Tanzania had its first confirmation of PPR in 2008 (20), with retrospective serological evidence of earlier circulation (17) and PPR is currently considered endemic, including in Kagera and Kigoma regions close to Rwanda (21). In neighboring Burundi, a first outbreak of PPR occurred in December 2017 to February 2018 (22), but retrospective serological analysis detected antibodies to the virus in samples collected in early 2017. A more recent study highlighted circulation of PPRV in livestock and wildlife living in eastern DRC and western Uganda (19). This brief history shows that PPR has had endemic events in various regions of east Africa surrounding Rwanda, with periodic outbreaks and circulation of the virus in various susceptible animals including detection in wildlife. Phylogenetic analyses of circulating viruses, showed that PPRV lineage II, III, and IV are prevalent in DRC, Uganda, Tanzania, Kenya, and Burundi (22–26).

Rwanda status vis-à-vis PPR is unknown (27). In fact, there has never been any empirical study to establish the prevalence of PPR in the country, despite its occurrence in the neighboring countries (9, 19, 22, 24, 28–31). The presence of this disease in the regional countries, transboundary movements of livestock passing through official and non-official entry/exit points and important wildlife species constitute potential factors for PPRV introduction in the country. In order to strengthen prevention mechanisms against PPR in Rwanda toward eradication of PPR by 2030, it is important to establish systems of surveillance as part of the stage 1 or "Assessment stage" of the Global Strategy for the Control and Eradication of PPR (10). An efficient surveillance stage would give insights on whether the disease is present and passes unnoticed, and provide information for the next steps toward the elimination of PPR. Such surveillance mechanisms must adopt strategies for the control of PPR in susceptible domestic and wild animals in Rwanda, in order to establish presence, circulation and persistence of the virus. This study investigated the prevalence of specific antibodies to PPRV and aimed at providing baseline data that can be used by concerned regulatory bodies and stakeholders to scale up PPR investigation in Rwanda and set up adequate prevention measures.

MATERIALS AND METHODS

Ethical Approval

Ethical approval for this study was obtained from the College of Agriculture, Animal Sciences and Veterinary Medicine, University of Rwanda (Ethical approval reference: 025/17/DRIPGS). Approved consent forms were distributed and signed prior to the interviews and sampling of animals. Sampling of animals was done following the protocols in conformity with the World Organization for Animal Health (OIE) Terrestrial Animal Health Code 2012 (use of animals in research and education).

Area Description and Study Design

Samples were collected from Bugesera, Kirehe, and Nyagatare districts of the eastern Province in Rwanda, and from Gicumbi and Musanze districts of the Northern Province (**Figure 1**). The five sites sampled are relatively close to borders with Uganda to the north, Tanzania to the east and Burundi to the south.

This cross-sectional study was conducted during a period of 3 months, from January to March 2019. Non-probability convenience samples were collected in farms located in the study area, under guidance of local veterinarians. In addition, a small questionnaire was used to collect information related to animal sampled, herd management and general animal health at the farm and its surrounding. Goats and sheep, apparently healthy, non-vaccinated against PPR and having more than 6 months of age, were recruited into the study. To accurately estimate the age of goats and sheep, the age dentition method was used, according to methods described elsewhere (32, 33). To calculate the sample size, we used a recommended formula for estimating the adequate sample size in prevalence study (34). Thus, assuming a large and homogenous population size with an estimated 50% prevalence (P) that optimizes the sample size, with a confidence level set at 95% and ±5% precision, the formula $n = \frac{z^2 P(1-P)}{d^2}$ recommended 385 samples. Finally, 460 blood samples were drawn from jugular veins of apparently healthy goats and sheep, using Vacutainer needles and sterile plain tubes. Samples were allowed to clot overnight in order to maximize sera collection, which were harvested following a centrifugation at 3,000 rpm for 5 min. The sera were then stored at −20°C until screening was done.

Screening of the Samples

The screening for the presence of PPR was done to detect antibodies to the nucleoprotein of Peste the Petits Ruminants virus, using a commercial competitive ELISA (cELISA) kit (ID screen® PPR competition, IDvet Genetics, Grabels, France) according to the manufacturer's instructions. The sensitivity and

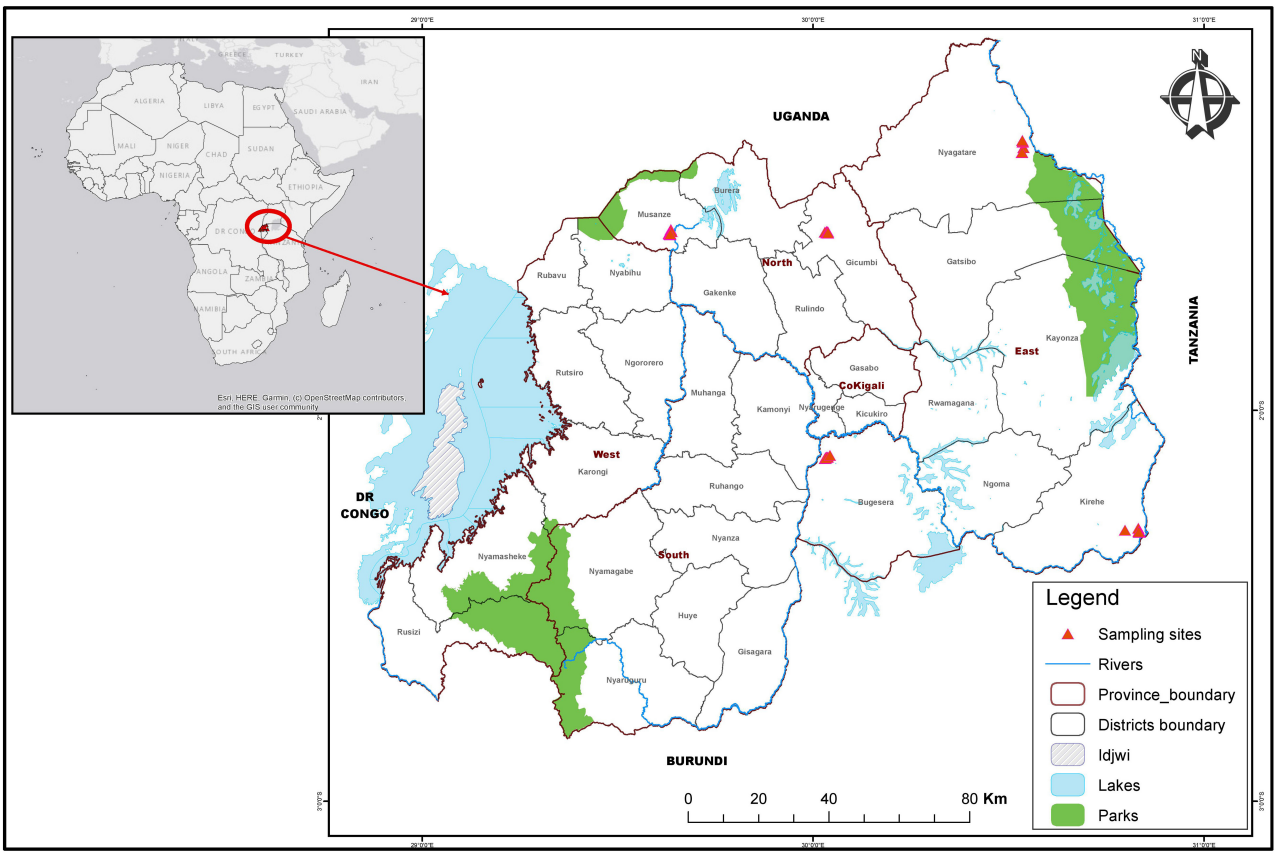

FIGURE 1 | Map of Rwanda districts highlighting sampling sites in East and North areas. The map shows administrative boundaries of districts and provinces. The red triangles highlight sampling sites in Nyagatare, Kirehe and Bugesera districts of the Eastern Province as well as Musanze and Gicumbi districts in the Northern Province.

specificity of this cELISA in sheep and goats, is estimated at 94.5 and 99.4%, respectively, compared to virus neutralization assay (35). The optical densities (ODs) were read using a Thermo Scientific™ Multiskan™ FC Microplate Photometer at a wavelength of 450 nm and the results were expressed as sample positivity percentage (S/N %). Samples were considered positive if the S/N % were ≤50%, negative if ≥60% or doubtful if it was between 50 and 60%. Since the sampled small ruminants had no clinical signs of PPR, doubtful results were finally considered as negative.

Statistical Analyses

The true prevalence in positive animals and herds was estimated by adjusting the apparent prevalence obtained from cELISA results to the sensitivity and specificity of the test, as described by Rogan and Gladen (36). In addition, in order to test for independence between two variables, univariable analysis was done using chi-square test. All the statistical analyses were carried out using R Statistical Software (version 3.6.3; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Characteristics of Study Respondents

The descriptions below for study participants and characteristics of farms and small ruminants sampled can be found in **Table 1**.

In total, this study reached 57 households distributed in the 5 districts targeted by this research. Participants were composed of 19 females and 38 males (**Table 1**). The age of respondents ranged between 18 to 87 in females and 16 to 83 in males, with a mean of 44 and 48 years, respectively.

Considering sampled households, 35 of the 57 interviewed (61.4%), reported that small ruminants were managed under a zero-grazing method, in which animals were stall-fed on grasses and food residues. In the remaining herds, 13 and 9 farmers reported to apply open-grazing and semi-zero grazing systems, respectively, in which the small ruminants were allowed to graze freely or go around grazing and get a supplement of food residues once back home. Of the 57 farms targeted, the biggest share (36 out of 57, representing 63.2%) was owning goats, whereas 9 (15.8%) farms had only sheep and 12 (21.1%) had both sheep and goats housed together at farm level. In interviewed farmers, majority (47 of the 57, 82.5%) reported that their animals

TABLE 1 | Characteristics of study respondents and farms sampled ($n = 45$).

Variables		Frequency (%)
District	Nyagatare	6 (10.5)
	Kirehe	18 (31.6)
	Bugesera	7 (12.3)
	Gicumbi	12 (21.1)
	Musanze	14 (24.6)
Gender	Female	19 (33.3)
	Male	38 (67.7)
Age quintiles (years)	<20	5 (8.8)
	21–30	3 (5.3)
	31–40	11 (19.3)
	42–50	17 (29.8)
	>50	21 (36, 8)
Education	No formal education	17 (29.8)
	Primary	28 (49.1)
	Secondary	12 (21.1)
Experience in animal husbandry (years)	<1	1 (1.8)
	1–5	24 (42.1)
	6–10	8 (14.0)
	>10	24 (42.1)
Types of small ruminant owned	Goats only	36 (63.2)
	Sheep only	9 (15.8)
	Goats and Sheep	12 (21.1)
Farming system	Zero-grazing	35 (61.4)
	Semi-zero-grazing	9 (15.8)
	Open grazing	13 (22.8)
Small ruminants disease history	Occurrence of abortions at sampled farms	19 (33.3)
	Occurrence of death at sampled farms	24 (42.1)
Small ruminants disease history in neighboring farms	Report of abortions in neighboring farms	12 (21.1)
	Report of death in neighboring farms	21 (36.8)

were obtained from local livestock markets and others (10/57, making up 17.5%) through various donations. Interestingly, all farmers reported not to observe any quarantine period prior to introduction of new animals in their herds.

Sampled Animals and Occurrence of Small Ruminant Diseases

On a period of 12 months, interviewed farmers reported occurrence of abortions in 19 of their farms, representing 33.3%, whereas 12 farmers (21%) indicated occurrence of abortion incidences in neighboring farms. According to the 19 farmers who experienced abortions, 18 cases occurred in goats whereas 1 case concerned sheep. Also, of the 12 abortion occurrences observed in neighboring farms, all were reportedly observed in goats. Moreover, 24 farms (42.1%) highlighted occurrence of death involving small ruminants at their own farms in the past 12 months, whereas 21 (36.8%) reported events of small ruminant deaths in neighboring farms (**Table 1**).

In total, 316 goats (201 females and 115 males) and 144 sheep (82 females and 62 males) were sampled. Based on age dentition, the goats were classified into three main categories of age: between 6 and 15 months (115 goats), 1.5–3 years (105 goats) and those being more than 3 years (96 goats). Similarly, age estimation in sheep showed that 54 were between 6 and 15 months, 53 were between 1.5 and 3 years, whereas 37 were over 3 years (**Table 2**).

Seroprevalence of PPR

A total of 14.8% (68/460) samples from small ruminants, including 17.4% (25/144) from sheep and 13.6% (43/316) from goats were seropositive for antibodies to PPRV (**Table 3** and **Supplementary Figure 1**). After adjusting to the test specificity and sensitivity, the overall animal-level estimated true prevalence was 15.1% (95% CI: 12.0–18.9), whereas species-level estimated true prevalence was 13.9% (95% CI: 10.3–18.3) and 17.8% (95% CI: 12.2–25.3) in goats and sheep, respectively. Of the 57 farms sampled, 35 had at least one animal seropositive (61.4, 95% CI: 48.4–72.9), giving an estimated farm-level true prevalence of 64.8% (95% CI: 50.9–77.0).

DISCUSSION

Sheep and goats represented 26% of 58,580 metric tons of red meat that was produced in Rwanda in 2017 (37) and this figure is expected to raise to meet growing population. Small ruminants are mainly raised for income generation through sales, but also for meat, wool and manure used in crop fields.

Information generated from this study show that diseases affecting small ruminant and causing deaths and/or abortions are prevalent in the sampled regions. However, due to inadequate veterinary services penetration in rural Rwandan regions, characterized by widespread of less qualified veterinary paraprofessionals (VPP), inadequate veterinary supervision of the VPP (38, 39) and unavailability of supporting laboratory services, diseases that caused abortions were not clearly identified and/or communicated to farmers. PPR is known to cause abortions at all stages of the pregnancy (40). Among possible differential diagnosis, Rift Valley Fever (RVF), another disease that causes abortions in affected animals (41), must be taken into consideration as a possible factor associated to the episodes reported by farmers. In fact, Rwanda has had its first outbreak of RVF declared in 2018 (42) and cases were mainly identified in the eastern region of the country, part of our study area.

This study is the first one to report seroprevalence of PPR in Rwanda. Our laboratory analyses indicated an estimated overall true prevalence of 15.1% (95% CI: 12.0–18.9) and seropositivity of 13.9% (95% CI: 10.3–18.3) and 17.8% (95% CI: 12.2–25.3) in goats and sheep, respectively. These findings of PPRV-specific antibodies circulating in goats and sheep sampled in various areas, constitute evidence of exposure to the disease. Further studies are needed to provide more insights on the epidemiology of PPR in Rwanda. For instance, other investigations should

TABLE 2 | Characteristics of small ruminants sampled.

Characteristics		Goats	Sheep	Total
Sex	Male	115	62	177
	Female	201	82	283
	Total	316	144	460
Age	6–15 months	115	54	169
	1.5–3 years	105	53	158
	>3 years	96	37	133
	Total	316	144	460

TABLE 3 | Seroprevalence of PPR in Small Ruminants according to various disease risk factors.

	Risk factors	Goats				Sheep			
		Total No. of samples	No. of positive samples	Sero-prevalence %	95% CI	Total No. of samples	No. of positive samples	Sero-prevalence %	95% CI
District	Nyagatare	75	13	17.3	(9.6–27.8)	21	4	19.0	(5.4–41.9)
	Bugesera	59	8	13.6	(6.0–25.0)	44	6	13.6	(5.2–27.4)
	Kirehe	70	9	12.9	(6.1–23.0)	29	5	17.2	(5.9–35.8)
	Musanze	56	8	14.3	(6.4–26.2)	31	8	25.8	(11.9–44.6)
	Gicumbi	56	5	8.9	(3.0–19.6)	19	2	10.5	(1.3–33.1)
	Total	316	43	13.6	(10.0–17.9)	144	25	17.4	(11.6–24.6)
Sex	Male	115	18	15.7	(9.5–23.6)	62	11	17.7	(9.2–29.5)
	Female	201	25	12.4	(8.2–17.8)	82	14	17.1	(9.7–27.0)
	Total	316	43	13.6	(10.0–17.9)	144	25	17.4	(11.6–24.6)
Age	6–15 months	115	7	6.1	(2.5–12.2)	54	5	9.3	(3.1–20.3)
	1.5–3 years	105	17	16.2	(9.7–24.7)	53	12	22.6	(12.3–36.2)
	>3 years	96	19	19.8	(12.4–29.2)	37	8	21.6	(9.8–38.2)
	Total	316	43	13.6	(10.0–17.9)	144	25	17.4	(11.6–24.6)
Farming system	Zero grazing	155	18	11.6	(7.0–17.7)	67	14	20.9	(11.9–32.6)
	Semi-zero grazing	37	6	16.2	(6.2–32.0)	20	2	10.0	(1.2–31.7)
	Open grazing	124	19	15.3	(9.5–22.9)	57	9	15.8	(7.5–27.9)
	Total	316	43	13.6	(10.0–17.9)	144	25	17.4	(11.6–24.6)

provide more information on nation-wide prevalence in susceptible domestic and wildlife animals, risk factors associated to PPR prevalence and phylogenetic characterization of circulating viruses in an attempt to determine origin, spread and distribution of various virus lineages and risk factors in Rwanda.

Based on laboratory data from this cross-sectional study, we certainly can confirm the exposure of goats and sheep to PPRV in sampled regions. The comparisons of prevalence with regional findings, should be undertaken carefully. In addition, this study has estimated prevalence of PPR in goats and sheep using non-probability sampling methods. Therefore, it does not allow generalizing the findings at country and small ruminant population level.

PPR is a well-known disease in the region as shown by endemic as well as epidemic events having been reported in Uganda, Tanzania, Burundi and Democratic Republic of Congo (9, 19, 22, 24, 28, 29, 43). In addition to reports of major outbreak in the region, various retrospective serological analyses, showed positive antibodies to PPRV and confirmed the prevalence of the virus and its circulation before occurrence of all recent outbreaks in above countries (17, 19, 22). Therefore, Rwanda Veterinary Services should strengthen active surveillance mechanisms in order to fully investigate prevalence of the disease and adopt prevention measures before occurrence of large outbreaks in the country.

Future studies on PPR in Rwanda should depict a clearer picture of the epidemiology of the disease in Rwanda. For example, due to limitations inherent to this study, some unanswered questions were for example, the difference in the distribution of PPR across various regions and possible contribution of animal age, sex, and husbandry to the occurrence of PPR.

Our data suggests a correlation between the age of the sampled animals and seroprevalence status within age groups. In fact, small ruminants of more than 15 months were more affected than younger ones. This finding is in conformity with other studies on PPR (28, 44) and can be explained by the facts that older animals have had more exposure time to the virus, especially if this is endemic in the region. In addition, older animals tend to move far from their home in search of greener pastures and water bodies. Some study participants (10 of the 57 interviewed, **Table 1**) reported that their small ruminants were acquired through livestock donating initiatives. Therefore, we cannot rule out the possibility that the livestock were seropositive to PPRV, when gifted to farmers. The presence of antibodies in younger animals is however suggestive of recent virus circulation in Rwanda and this should be investigated further.

Phylogenetic investigations showed that the virus lineages II, III and IV are circulating in the region. Based on limited available sequences, the lineage III seems predominating in western Uganda, eastern DRC and Burundi with sequence similarities in some countries such as DRC and Burundi (19). In addition, the 2017 outbreak of PPR that occurred in various regions in Burundi, followed introduction of Boer goats from potentially infected regions of Uganda and the goats were transported through Tanzania suggesting transboundary movements as possible route of PPRV transmission (22). The regional virus circulation, added to report of PPRV at wildlife-livestock interface in Kabale, Kisoro and eastern DRC; regions close to Rwanda (19), puts an emphasize on the role of movements of livestock across transnational boundaries as well as the role of wildlife animals in the circulation and maintenance of PPRV in the region. Our findings suggest that PPR is prevalent in Rwandan regions close to neighboring countries and areas with recent PPR outbreak events. Rwanda is located in an area characterized by large livestock as well as wildlife populations. In addition, the region is known for important livestock trade between regional countries including Rwanda, and eastern part of DRC (45). The presence of livestock and wildlife and trade movements across countries, could have contributed to the introduction of PPR in north and west parts of Rwanda. Last but not least, Rwanda has experienced large movements of returning citizens from neighboring countries in 1994 and from Tanzania in 2007 as well as Burundian refugees in 2015 (46–48). These movements of people and their livestock could have contributed to the introduction of PPR in various regions of Rwanda. Molecular epidemiology studies and analysis of transboundary livestock movements could shed more insights on PPR epidemiology in Rwanda and the region.

As a preliminary report, this study has several limitations. First, although PPR has never been declared in Rwanda, samples were taken from places relatively close to the borders of the country with countries with known reports of PPR in past years. Due to possible more intense transboundary livestock movements in sampled areas than in other parts of the country, the prevalence found in this study may not necessarily reflect a country-large situation. To minimize this bias, the calculation of needed sample size, assumed a prevalence of 50% which is a condition that maximizes the sample size. Secondly, sampling methods were non-randomized and only a small number of farms was reached. Therefore, without assumption of a homogenous population, the data presented may not be representative of the entire population of small ruminant farmers. Therefore, the current findings do not allow to calculate the true prevalence, analyse the risk factors or to compare prevalence across the study areas. Third, cross-sectional surveys are not suitable for detection of rare, non-endemic diseases such as PPR with an unknown status in Rwanda.

Further studies are needed to collect representative evidence, informative for the eradication and control programs. In this regard, comprehensive studies using probabilistic sampling methods are recommended to investigate PPR at wildlife-livestock interface in Rwandan regions neighboring DRC, Uganda, Tanzania and Burundi. Such studies would help to follow up the occurrence of disease events in small ruminants, and would retrospectively collect evidence of possible endemicity of PPR. This is justified by the possibility of regional circulation of the virus along with livestock transboundary movements as hypothesized by previous studies (19, 22). Rwanda is home to natural parks and forests which may serve as PPRV hotspots at the interface of livestock and wildlife. Therefore, future studies in Rwanda and the region, must take into consideration the livestock and wildlife components, in order to fully understand the epidemiology of PPR.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical approval for this study was obtained from the College of Agriculture, Animal Sciences and Veterinary Medicine, University of Rwanda (Ethical approval reference: 025/17/DRIPGS). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AS contributed to conceptualization, laboratory analysis, data curation, investigation, methodology, and writing original draft. MU participated in conceptualization, investigation, methodology, and manuscript editing. JW contributed to conceptualization, funding acquisition, supervision, review of the manuscript, and editing conception and design of the study. All authors contributed to the article and approved the submitted version.

FUNDING

This study was funded by the Swedish Research Council (Grant No. 2018-03956).

ACKNOWLEDGMENTS

We would like to thank Mr. Elysé Ndizeye and Mr. Justin Rucamihigo for their participation in data collection during this study. We would like to thank the Ministry of Agriculture and Animal Resources through the Rwanda Agriculture and Animal Resources Development Board (RAB) for their support in accessing the National Veterinary Laboratory facilities. We are grateful to Dr. Karin Alvåsen from Swedish University of Agricultural Sciences, for valuable discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.651978/full#supplementary-material

Supplementary Figure 1 | Percentage Inhibition (PI) distribution based on competitive ELISA results (ID screen® PPR competition, IDvet Genetics, Grabels, France). The distributions are shown by sampled locations (District). Positive samples have below 50% PI and are shown in the graphs with a dark dashed black line.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

SYSTEMATIC REVIEW
published: 21 April 2021
doi: 10.3389/fvets.2021.620347

Non-antibiotic Approaches for Disease Prevention and Control in Nursery Pigs: A Scoping Review

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Swine producers are encouraged to practice antibiotic stewardship by reducing their use of antibiotics belonging to classes of medical importance to humans. We conducted a scoping review of non-antibiotic approaches in the form of products or management practices that might prevent or control disease and thus reduce the need for antibiotics in nursery pigs. Our objectives were to systematically describe the research on this broad topic for the North American context, identify specific topics that could feasibly support systematic reviews, and identify knowledge gaps. A search of multiple databases identified 11,316 articles and proceedings for relevance screening. From these, 441 eligible clinical trials and observational studies were charted. The majority were clinical trials (94%). Study results from EU countries were mostly communicated through journal articles, whereas study results from the USA were mostly communicated through conference proceedings. Interventions and health outcomes were diverse. The two most frequent intervention categories were feed additives and piglet vaccines. The three most frequent outcomes reported were diarrhea, mortality, and indices of vaccine immunity. There were 13 specific topics comprising various feed additives and vaccines that might feasibly support systematic reviews. There were relatively few studies in which interventions were compared with antibiotic comparison groups and relatively few studies evaluating management practices.

Keywords: antibiotic stewardship, disease control, disease prevention, non-antibioic approaches, nursery pigs, scoping review

OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
Christine Unterweger, University of Veterinary Medicine Vienna, Austria
Edgar Garcia Manzanilla, Teagasc, Ireland

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *22 October 2020*
Accepted: *19 February 2021*
Published: *21 April 2021*

Citation:
Wisener LV, Sargeant JM, O'Sullivan TL, O'Connor AM, McEwen SA, Reist M and Churchill KJ (2021) Non-antibiotic Approaches for Disease Prevention and Control in Nursery Pigs: A Scoping Review. Front. Vet. Sci. 8:620347. doi: 10.3389/fvets.2021.620347

INTRODUCTION

Antimicrobial resistance (AMR) poses a serious threat to advances in modern human medicine, livestock health and production, and animal welfare (1, 2). There are limited or few alternative treatment options in patients infected with pathogens resistant to medically important antibiotics, particularly those with resistance to critically important antibiotics (3). The World Health Organization (WHO), the World Organization for Animal Health (OIE), and the Food and Agriculture Organization of the United Nations (FAO) regard antimicrobial use (AMU) in as a significant driver of AMR in humans and animals alike (2, 4, 5). In a tripartite "One Health" approach, these major global institutions have called for a worldwide effort to reduce inappropriate and unnecessary AMU in all sectors (2, 4, 5). The WHO and the OIE have categorized antibiotic classes according to their importance for human and animal health, respectively

(3, 6). Categorization of antibiotic classes is useful for prioritization of strategies to limit AMR, such as antibiotic stewardship. To help achieve this goal, the WHO has published guidelines recommending certain restrictions on the use of medically important antibiotics in non-human sectors (7). Food production sectors use antibiotics of importance to humans that are the same or belong to the same antibiotic class as those used in human medicine for animal disease treatment or prevention in vulnerable individuals or groups (3, 5, 8).

Worldwide, nations are heeding the call for reduced antibiotic use in food production through regulation and industry guidelines. For example, in the USA, the US Food and Drug Administration Center for Veterinary Medicine promotes prudent use of livestock antibiotics belonging to classes of importance to humans by requiring they be administered with veterinary oversight and be limited to the purpose of assuring animal health. The use of medically important antibiotics in healthy animals for growth promotion was prohibited by 2017 in the USA (9). In Canada, similar regulations enacted by December 2018 eliminated the over-the-counter use of antibiotics belonging to classes of medical importance to humans by requiring veterinary oversight for administration of these antibiotics by injection or by addition to feed or water (10). In the European Union (EU), the use of antibiotics in feeds for growth promotion has been banned since 2006 (11). In their systematic review of antibiotic use in swine production from 2000 to 2017, Lekagul et al. (12) reported that there was geographical variation in antibiotic use by types of diseases. Choice of antibiotic was dependent upon the common pathogens associated with age-specific diseases and upon route of administration, typically oral in-feed medication in nursery pigs. Lekagul et al. (12) concluded that medically important antibiotics are still commonly used worldwide for disease prevention and control in swine production, particularly in modern commercial swine production during the suckling piglet and nursery pig stages.

Scoping review methodology is used to systematically map the literature with regard to the extent, range, and nature of existing research of a particular topic area (13, 14). Scoping reviews are also useful as preliminary "reconnaissance" to assess the feasibility of undertaking a full systematic review of a specific topic and to identify gaps in the existing research (13, 14). While scoping reviews are descriptive and broad in nature, systematic reviews aim to address a specific research question by using explicit systematic methods to collate all the evidence that fits pre-specified eligibility criteria while minimizing bias (13, 15). To help inform antibiotic stewardship goals, a scoping review of non-antibiotic approaches to nursery pig health could help researchers advance the knowledge of alternative approaches by indicating topics that could be subject to formal systematic reviews or merit further research. In addition, a scoping review could illuminate current gaps in research on non-antibiotic approaches to nursery pig health for swine industry professionals, swine researchers, and research funding agencies.

The objectives of this scoping review were three-fold: (i) to examine and describe the volume, range, and nature of research on non-antibiotic approaches for disease prevention and control in commercial nursery pig production; (ii) to identify specific topics where available research literature may support systematic reviews; (iii) to identify knowledge gaps in the primary literature on the effectiveness of various non-antibiotic approaches. Summarizing the literature regarding intervention effectiveness was not an objective for this scoping review.

MATERIALS AND METHODS

This scoping review followed the framework for scoping reviews as outlined by Arksey and O'Malley (13) using the PRISMA-ScR (i.e., Preferred Reporting Items for Systematic Reviews and Meta-Analyses Extension for Scoping Reviews) guidelines for reporting scoping reviews (16). The registered protocol can be located through UoG Atrium https://atrium.lib.uoguelph.ca/xmlui/handle/10214/12929.

Our review question was as follows: What are the volume and nature of the primary research literature published between 2000 and 2018 that evaluated non-antibiotic interventions (i.e., products such as vaccines or feed additives and management practices such as weaning methods or biosecurity) to prevent or control bacterial and viral illnesses in nursery pig production in North America and regions or countries with similar production conditions? Viral illnesses were included because we presumed that preventing viral infections may reduce secondary bacterial illnesses.

Expert Stakeholder Engagement

As the volume of literature on the broad topic of non-antibiotic approaches for nursery pig health was potentially very extensive, we sought expert opinion to refine our research question and help to inform our search strategy and study eligibility criteria. More specifically, the stakeholder engagement served six objectives: (i) to identify the countries or regions with similar commercial swine production practices to those of North America, (ii) to select the swine production stage(s) for which non-antibiotic approaches could most effectively reduce total AMU in swine production, (iii) to solicit opinions on the importance of various antimicrobial types to antimicrobial stewardship in the swine industry (e.g., antibiotics belonging to medically important classes used to prevent or control swine diseases, anticoccidials, and anthelmintics), (iv) to identify the health and production outcomes of greatest importance to swine producers, (v) to identify the most important non-antibiotic interventions in the form of specific products (e.g., vaccines, feed additives, medications, or supplements) or specific management practices (e.g., weaning practices, biosecurity, housing, feed type, and restricted feeding); and (vi) to identity any additional outcomes or interventions that we had not included on our initial lists. We also asked the stakeholders to recommend other experts who should be consulted.

The expert opinion was gathered through an anonymous online survey (Qualtrics XM). The respondents selected and ranked options from a list for a particular question and/or responded in an open-ended format. Expert stakeholders who were consulted included representatives within provincial or national Canadian swine industry associations, representatives

TABLE 1 | Data platform and database information sources used in the scoping review search on non-antibiotic approaches to reduce the need for antibiotics in nursery pig production.

Data platform	Databases
ProQuest	Agricultural and environmental science AGRICOLA and TOXLINE
	Biological science database (MEDLINE and TOXLINE), dissertations and theses Guelph, ProQuest dissertations and theses
ProQuest	AGRICOLA
PubMed	PubMed (not MEDLINE)
Web of science	Science citation index, conference proceedings citation index–science
Web of science	MEDLINE
AASV	Annual meeting proceedings
AASV	International pig veterinary congress (biannual meetings) proceedings

in provincial agriculture departments, and Canadian scientists engaged in swine agriculture and/or swine veterinary research. We assumed that the responses from stakeholders of Canadian institutions would be representative of experts from the USA and other countries with commercial swine production. Stakeholders' most common responses and suggested additional responses were used to inform the parameters of the search, eligibility criteria, and data items. Ethics approval for this survey was not required, as the results were used strictly to inform the review process and not reported as a finding of the review.

Eligibility Criteria

Eligible information sources were from North America, EU countries, Australia, and New Zealand, available since 2000, as these sources of evidence were most likely to reflect current commercial swine production systems most similar to those of North America. Eligible publication types included English-language journal articles, conference proceedings, theses, and technical reports. In addition to electronic databases, proceedings were sourced from the American Association of Swine Veterinarians (AASV) Annual Meeting and Pre-conference seminar from 2000 to 2018 and the International Pig Veterinary Society Congress biannual meetings from 2000 to 2016. We included research reports that reported a challenge trial, a clinical trial (i.e., a controlled trial), or observational study of a modifiable intervention, and we reported a health or production outcome in nursery pigs. For purposes of this review, "interventions" also included modifiable risk factors. Consistent with our stakeholder engagement results, interventions related to breed or genetic improvement were not included. Our stakeholder engagement also informed our *a priori* selected outcomes of interest. These included health outcomes of bacterial or viral infections significant to swine health in North America, treatment costs, and measures of performance. Studies evaluating toxicities or parasitic infections were excluded. Non-English information sources were excluded. Quasi-experimental intervention studies designed as "before period" vs. "after period" of the intervention application were excluded.

Information Sources

To aid in the development and validation of the search, we checked that 25 known relevant citations are included. The primary reviewer (LW) performed the database search from March 27, 2018, to April 19, 2018, using multiple databases hosted by the data platforms of ProQuest, Web of Science, and PubMed (**Table 1**). Though the database CAB Direct was originally targeted for inclusion, due to technical difficulties, it was not used. The search for proceedings was conducted manually by the primary reviewer and a second reviewer working independently subsequent to obtaining access to the online AASV Swine Information Library through a membership, September 20, 2018.

Search

The database search was filtered by language (English), date of publication (published between 2000 and date of search in 2018), and by location filters for eligible countries if available. Additional filters were applied as were allowable within the data platform. These included source and document type (i.e., article, proceedings paper, meeting abstract, and thesis), subject or research areas, and Medical Subject Headings (MeSH) or qualifiers. The search terms were limited to the title or abstract in the PubMed and Web of Science platforms or "anywhere except full text" for the ProQuest platform databases. Management of the identified citations was as follows: first, they were imported into the reference manager software EndNote (Clarivate Analytics, Philadelphia, United States); second, they underwent exact match deduplication in EndNote; and third, they were imported into the systematic review software DistillerSR (Evidence Partners, Ottawa, Canada) where they underwent further deduplication based on close matches.

The database search strategy included a string of the population term groupings (e.g., weanling, nursery pig, and starter pig), one of two intervention term groupings, and an outcome term grouping (e.g., health, diarrhea, or growth) with each grouping connected by the Boolean operator "AND" (**Table 2**). There were two intervention term groupings, one for interventions of interest in the form of a product (e.g., vaccine, feed supplement, or plant extract) and another for interventions in the form of a management practice (e.g., antibiotic-free and late weaning as defined by the study authors, or disinfection). Within each term grouping, terms were combined by the use of the Boolean operator "OR." The search was conducted by the primary reviewer (LW) in consultation with a University of Guelph research librarian.

The online proceedings identified as being potentially relevant based on subject headings and titles were entered onto a Microsoft Excel (2010) spreadsheet for tracking of further screening decisions. Relevant full-text proceedings were entered into the DistillerSR database. Proceedings that were duplicates of journal articles were removed.

Selection of Sources of Evidence

Our review team consisted of veterinary epidemiologists, one of whom acted as the primary reviewer, a topic expert in swine research (TOS), and two trained MSc epidemiologists who acted

TABLE 2 | Search terms for non-antibiotic approaches to reduce the need for antibiotics in nursery pig production.

Groupings	Search terms
Population terms	(Piglet* OR weaner* OR "weaning pig*" OR "weanling pig*" OR "weaner pig*" OR "weaned pig*" OR "weaner stage" OR "weaner phase" OR "nursery pig*" OR "young pig*" OR "younger pig*" OR "early-weaned pig*" OR "late-weaned pig*" OR "nursery-age*" OR "naïve pig*" OR "starter pig*" OR "neonate pig*" OR "neonatal pig*" OR "suckling pig*")
Product intervention terms	(Antibiotic* OR antimicrobial* OR vaccin* OR immunization OR "sow vacc*" OR "dam vacc*" OR "gilt vacc*" OR "sow immunization" OR "dam immunization" OR "gilt immunization" OR "trace mineral*" OR "essential mineral*" OR "mineral source*" OR "mineral form*" OR Zinc*OR vitamin* OR "dietary acid*" OR "organic acid*" OR "dietary fatty acid*" OR "medium chain fatty acid*" OR acidif* OR "feed enzyme*" OR fermentable OR fermented OR "plant extract*" OR herbal OR seaweed OR spice OR phytogenic OR "dietary lysine" OR "dietary tryptophan" OR lactoferrin OR lysozyme OR L-glutamine OR nutraceutical* OR neutraceutical* OR supplemental OR "dietary supplement*" OR "diet supplement*" OR "feed supplement*" OR "dietary additive*" OR "diet additive*" OR inulin OR oligosaccharide* OR polysaccharide* OR mannan* OR B-glucan* OR probiotic* OR prebiotic* OR synbiotic* OR "direct-fed microbial*" OR "competitive exclusion" OR yeast OR "Saccharomyces cerevisiae" OR "essential oil*" OR "fish meal" OR "blood meal" OR "spray-dried" OR immunoprophylaxis OR immunotherapeutic* OR "egg-yolk antibod*" OR "IgY antibod*" OR bacteriophages OR "antimicrobial peptide*" OR "bovine colostrum" OR "epidermal growth factor*" OR "rare earth" OR clay OR "natural alternative*" OR homeopath*)
Management intervention terms	("natural pig*" OR "organic swine" OR "organic pig*" OR "natural conditions" OR "non-conventional" OR "antibiotic-free" OR "weaning practice*" OR "weaning method*" OR "weaning procedure*" OR "weaning regime*" OR "weaning system" OR "conventional weaning" OR "weaning age" OR "early weaning" OR "late wean*" OR "age at weaning" OR "creep feed*" OR "stocking" OR crowding OR overcrowding OR "floor space" OR "feeder space" OR "housing system*" OR "housing design*" OR "housing environment*" OR "housing type" OR ventilation OR "air quality" OR co-mingling OR "mingl*" OR "mixed litter" OR mixing OR "batch system" OR "batch management" OR biosecurity OR "sanit*" OR "disinfect*" OR "cleaning" OR hygiene OR "all-in-all-out" OR "pig flow" OR "disease eradication" OR "disease control*" OR "multi-site" OR "liquid feed" OR "liquid diet*" OR "pellet*" OR "low protein" OR "decreased protein" OR "restricted protein" OR "protein restrict*" OR "protein nutrition" OR "protein level" OR "protein source" OR "dietary protein" OR "restricted feed*" OR "feed restrict*" OR "control fed" OR "quality assurance" OR education)
Outcome terms	(health OR immun* OR diarrhea OR diarrhea OR scours OR "colibacillosis" OR "fecal score" OR "clinical response*" OR "clinical parameters" OR "fecal shedding" OR "fecal shedding" OR morbidity OR mortality OR performance OR growth OR "daily weight gain" OR "average daily gain" OR "G:F" OR "gain-to-feed" OR "feed conversion" OR "feed intake" OR "ADG" OR ADFI OR "lightweight gain" OR productivity)

A search string included the population terms and either the product intervention terms, or the management intervention terms plus the outcome terms connected by the Boolean operator "AND".

as second reviewers. Pre-testing of the relevance screening form was conducted on 100 citations based on title and abstracts. Pre-testing of the data charting was conducted on 25 full-text articles. Using forms created in DistillerSR, two independent reviewers screened and charted the data. Any disagreements were resolved by consensus or a third reviewer. After pre-testing, the level 1 relevance screening form did not change.

However, due to the large volume of literature identified after the first level of relevance screening, three additional relevance screening levels were applied to the titles and abstracts to refine the selection of relevant citations to the literature that was most pertinent to our research question (**Appendix 1**, level 1–4 relevance screening forms). Level 2 screened by eligible countries or regions by first author address. Level 3 screened by study type and information regarding the challenge pathogen or antigen that was collected, but then challenge trials were excluded from further screening and data charting. Level 3 also screened by pig type (i.e., included only conventional or specific pathogen-free pigs). Level 4 screened by intent of the intervention (i.e., included non-antibiotic interventions for viral or bacterial infections, excluded interventions for mycotoxins and soy allergens) and by eligible diseases (i.e., excluded reportable or rare diseases such as classic swine fever, Aujeszky's disease and foot and mouth disease, and outcomes only of public health impact such as swine hepatitis E virus and methicillin-resistant *Staphylococcus aureus*). At level 4, the articles that only reported a performance outcome without any health outcomes of interest were excluded from data charting. Health outcomes of interest were defined *a priori* and included clinical outcomes [i.e., mortality all-cause, diarrhea, respiratory disease and non-specific morbidity defined as non-diarrheal, non-respiratory non-specific morbidity (e.g., pyrexia, removals, or unthriftiness), or other morbidities such as lameness], surrogate health outcomes such as shedding of clinically important pathogens [i.e., S*almonella* spp., *Campylobacter* spp., enterotoxigenic *Escherichia coli* (ETEC *E. coli*)], and measures of specific and non-specific immunity to vaccines or bacterins (**Table 3**). In summary, articles that were selected for data charting reported research on non-antibiotic approaches to improve health outcomes of important viral and/or bacterial infections in conventional or specific pathogen-free nursery pigs in North America, EU countries, the UK, New Zealand, or Australia. The additional relevance screening levels 2–4 were a protocol deviation intended to focus the data charting on studies that addressed our research question.

Data Charting Process

Data charting of full-text articles (i.e., journal articles, technical reports, theses, and conference proceedings) was conducted by both the primary reviewer and a second reviewer working independently using a form in DistillerSR (**Appendix 2**, Data charting form). Any disagreements were resolved by consensus or a third reviewer. Data were charted at the individual study level.

TABLE 3 | Description of data charting items for relevant journal articles, technical reports, proceedings, or theses.

Variable	Description of items
General study characteristics	
Study design	Clinical trial (i.e., experimental or field-based trial under conditions of natural exposure), challenge trial (i.e., deliberate exposure to a pathogen or antigen under the control of the investigator), observational study
Study location	Country and region where the study was conducted as stated in the article or if not stated, first author address
Year of publication	Year of publication or year of proceeding
Farm setting	Population farm setting (i.e., experimental research farm, commercial farm, or unclear)
Detailed trial or observational study characteristics	
Specific pig population in which the intervention was given	Specific population based on production stage included dams, suckling piglets, nursery pigs
Purpose[a] of the intervention as stated in the title or objective statement	Disease prevention (i.e., no pre-existing health problems or known exposures), disease treatment (i.e., individual pigs or groups in whole or part or the farm were known to have clinical disease or exposure to viral or bacterial pathogens. In addition, some studies included performance (e.g., feed intake, growth or body weight, feed efficiency)
Non-antibiotic interventions in the form of a product or management practice or risk factor studied	*Products*: Piglet vaccines, maternal vaccination, non-antibiotic feed or water additive including the addition of specific dietary components, non-antibiotic medication (e.g., any medication, vitamin, mineral, antibodies, etc. administered directly to an individual). Combination products used as interventions that contained both an antibiotic [e.g., Zinc Oxide (ZnO) plus an antibiotic] were excluded. *Management:* Feeding regime as amount or schedule (e.g., protein level, creep feeding, restricted feeding); diet type or format (e.g., pelleted vs. mash, fermented feeds, complexity of feeds); weaning method or weaning stage as defined by the authors (e.g., early vs. late); biosecurity (e.g., comingling, mixing, introductions, animal movements); housing, flooring or feeders (e.g., animal density, feed troughs and water supply factors, flooring); air quality; producer education
Comparison groups	No treatment or conventional practice comparison, placebo or sham, different level or form of treatment, antibiotic and/or ZnO[b]
Health outcomes of interest reported	Mortality (i.e., piglet deaths in absolute terms, deaths per time period, excess deaths, or other metric); clinical diarrhea (e.g., scours, fecal consistency, or fecal score); clinical respiratory disease; non-diarrheal, non-respiratory non-specific morbidity (e.g., fever, removals or unthriftiness) or other morbidities such as lameness; treatment for illnesses or antibiotic use; pathology or lesions; fecal shedding of specific swine pathogens; measures of specific and non-specific immunity and infection (i.e., serology, cell mediated immunity, viremia, PCR, immune markers such as acute-phase proteins, or tumor necrosis factor (TNF)
Other outcomes measured	None, performance outcomes (i.e., feed intake, growth or body weight, or feed efficiency), farm economics or treatment costs, diet digestibility, gastrointestinal microflora, gastrointestinal morphology
Study size	Number of study subjects in each study at the hierarchical level of the analysis (e.g., individual, pen or group, herd or farm)

[a] *Studies in which the purpose included both prevention and treatment were counted as disease control in results.*
[b] *Some studies compared a non-antibiotic intervention group to a zinc oxide comparison group while other studies compared a zinc oxide treatment group to a no-treatment control group, antibiotic or other treatment comparison group.*

We focused the data charting on clinical trials and observational studies that reported a health outcome of interest in the nursery stage of production. However, for studies that reported a health outcome of interest, additional data charting related to other health outcomes and non-health outcomes was completed. A further protocol deviation included an additional question regarding the stage of production at which the intervention was applied (e.g., reproduction, suckling, or nursery).

Data Items

We charted data for the publication type and for the following study characteristics: study design, study location and year of publication or conference year, study size, and farm settings of the study population (i.e., experimental farm vs. commercial farm). In addition, we charted data for the following: production stage(s) of animals receiving the intervention, purpose of intervention as disease prevention, and/or treatment, and/or performance; specific intervention evaluated; comparison group(s); health outcomes of interest reported; other outcomes reported; and study size and the hierarchical level at which the outcome was measured (i.e., individual, group/pen, or herd/farm) (**Table 3**). Data were charted using preselected response options with an added text box for additional responses or clarification for the interventions, comparator groups, and outcomes reported (**Appendix 2**).

In this review, interventions such as non-antibiotic medication, vitamin, mineral, or antibody given directly to individual animals *via* injection or oral bolus were charted as a "medication," whereas the same intervention given to groups of animals *via* feed was charted as a "feed additive." There were two data charting options related to measures of immunity as an outcome, one specifically for vaccine immunity and another for non-vaccine immunity.

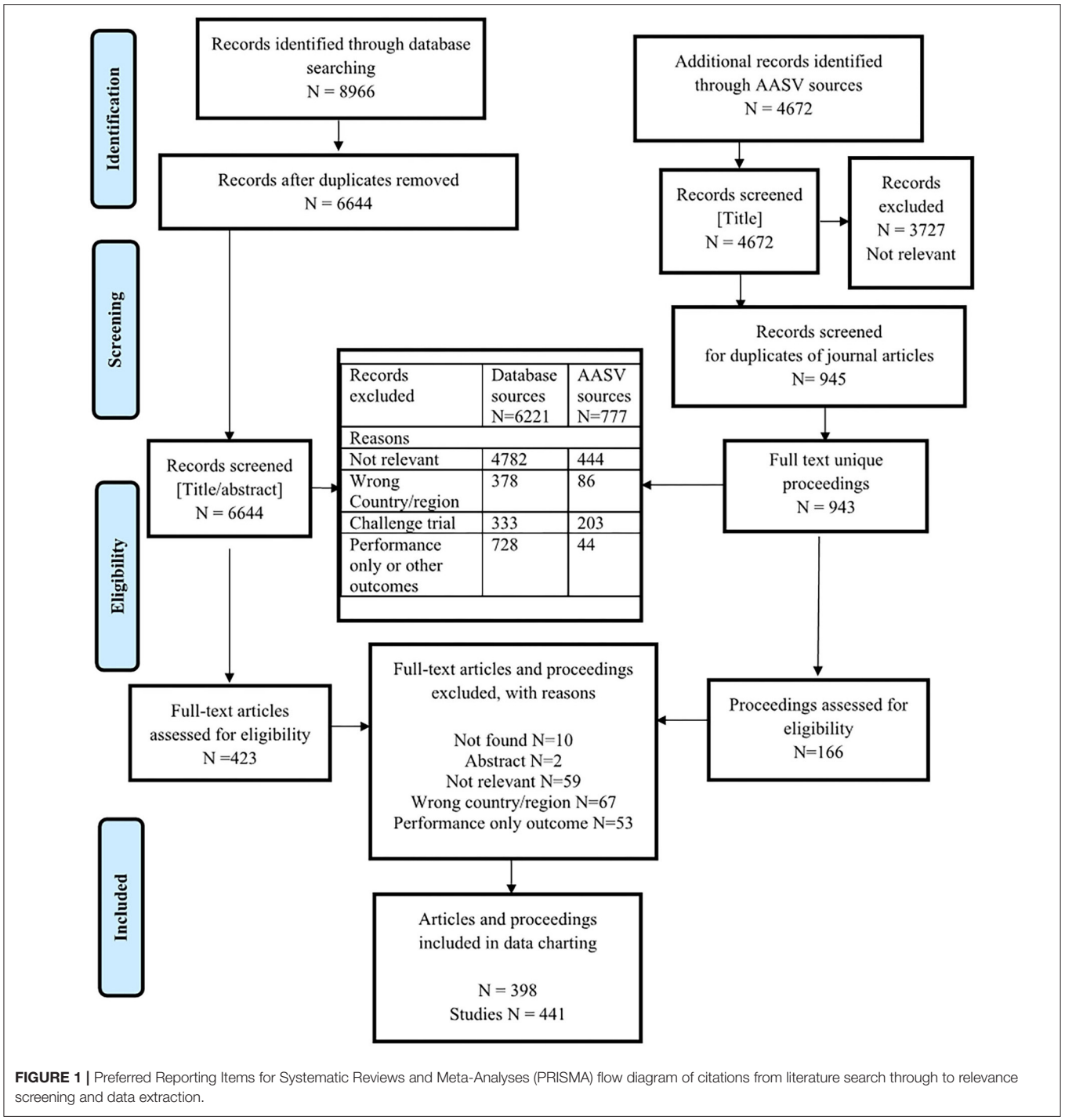

FIGURE 1 | Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram of citations from literature search through to relevance screening and data extraction.

Data charted regarding the purpose(s) of the intervention were based on information in the title or objective statement. Prevention was selected if the herd or group of pigs showed no clinical or subclinical evidence of disease or infection, whereas treatment was selected if the pigs as individuals or groups in part or whole showed evidence of infection, or were known to be exposed. Although we charted data for disease prevention and/or treatment, in the results, we reported disease control for those studies that described an intervention given for both purposes, treatment and prevention (i.e., the intervention was given to groups of pigs assumed to comprise both healthy and clinically or subclinically affected or exposed pigs). Definitions for disease prevention, treatment, control, and pig performance were based on those provided by the United States Government Accountability Office (GAO) (17) and the American Veterinary Medical Association (18).

Comparison groups that were not clearly stated as "no treatment" or "conventional practice" were charted as

comparison groups that received a "different form or level" of the intervention or exposure. Studies could be charted with multiple types of comparison groups (i.e., both a "no treatment" control group and "different form or level" if additional comparison groups received various levels or forms of the treatment or exposure). Zinc oxide (ZnO) was charted as an intervention when it was the study intervention of interest and charted as a comparison group when another non-antibiotic intervention of interest was compared with zinc treatment.

To meet our second objective of identifying specific intervention topics that could be combined for systematic reviews, we considered only clinical trials with clinically important outcomes. These included mortality; non-diarrheal, non-respiratory non-specific morbidity, or other morbidities such as lameness; diarrhea or fecal score; respiratory disease; and treatment for illness or antibiotic use. Thus, not all health outcomes of interest were considered as clinically important outcomes. The criteria for selection of topic areas as potentially extensive enough for systematic reviews were arbitrarily set at a minimum of 10 clinical trials reporting a similar intervention in the same population (e.g., amino acids in nursery pig feed, specific piglet vaccines, and/or dam vaccines) and one or more clinically important outcomes, though not necessarily the same clinically important outcome among all clinical trials for the specific topic area.

Synthesis of Results

Data charted in DistillerSR were entered into a database in Stata 15.1 (College Station, Texas, USA). These data were summarized descriptively and presented in the form of tables and figures in accordance with our stated data charting scheme. So as to emphasize the research with the highest evidentiary value, we present detailed results for articles that reported clinical trials and observational studies (19). For the challenge trials, we presented only details of the types of challenges evaluated as obtained during level 3 screening.

RESULTS

Expert Stakeholder Engagement

A total of 73 experts were invited to respond to the survey, of which 33 responded (45%). We incorporated the stakeholder input into our search strategy and data charting items. There were no suggested publications that were not identified through our search.

Selection of Sources of Evidence

There were 11,316 unique citations screened for eligibility: 6,644 were from the database search and 4,672 were from the gray literature proceedings of the AASV Swine Information Library sources (**Figure 1**). Two proceedings that were duplicates of journal articles were removed (**Figure 1**). A total of 536 challenge trials were identified at level 3 screening of database sources and at full-text screening of proceedings. A description of the types of challenge agents is presented in **Appendix 3**. A total of 772 journal articles and proceedings that described clinical trials or observational studies but only reported a performance outcome or other outcome without reporting any health outcome of interest were excluded at level 4 screening of the database sources and full-text screening of proceedings. In total, 589 citations (5%) describing clinical trials or observational studies and reporting a health outcome of interest were screened for eligibility based on full text. Among these, 398 were eligible for data charting. Thirty-four eligible articles (8.5%) described one or more studies; in total, there were 441 relevant clinical trial or observational studies included for data charting.

Characteristics of Sources of Evidence

The majority of eligible studies were clinical trials ($n = 414$, 94%). The remainder were observational studies ($n = 27$, 6%). The observational studies were conducted exclusively using animals living in commercial farm settings, whereas the clinical trials were conducted using animals living in experimental farm settings ($n = 206$, 50%) and in commercial farm settings ($n = 182$, 44%). The farm settings were unclear for 26 (6%) clinical trials, of which 20 were reported in proceedings.

The majority of the studies were conducted in EU countries or the UK ($n = 284$, 64%) followed by the USA ($n = 110$, 25%). There were 17 studies conducted in Australia or New Zealand. The five EU countries with the greatest numbers of studies were Spain, Denmark, Poland, Germany, and France. The five specific states of the USA with the greatest numbers of studies were Minnesota, Iowa, North Carolina, Illinois, and Nebraska. Among the 27 observational studies, 19 were conducted in EU countries and five were conducted in Canada. Among the 414 clinical trials, 265 were conducted in EU countries or the UK, 108 were conducted in the USA, and 25 were conducted in Canada.

The body of eligible studies ($n = 441$) was composed of published articles ($n = 297$, 67%) and proceedings ($n = 144$, 33%). The 284 studies reported from EU countries and the UK were mostly communicated through published articles ($n = 212$, 75%), whereas the 110 studies reported from the USA were mostly communicated through proceedings ($n = 60$, 55%).

The annual number of included studies reported in published articles increased in an approximately linear trend since 2000, whereas the number of proceedings varied every other year in accordance with the alternate year schedule of the International Pig Veterinary Society Congress and the number of accepted proceedings at the congress and the Annual Meeting of the AASV (**Figure 2**).

Synthesis of Results for Clinical Trials and Observational Studies

The stated purpose(s) of the intervention as disease prevention, disease control, and/or performance varied according to the farm settings among the 414 clinical trials and 27 observational studies. There were no studies that evaluated an intervention for the clearly stated purpose of treatment of individual sick pigs.

Studies where the purpose of the intervention was for disease control ($n = 134$) were conducted primarily on commercial farms ($n = 122$, 91%), whereas when the purpose of the intervention as prevention ($n = 250$), studies were conducted on both

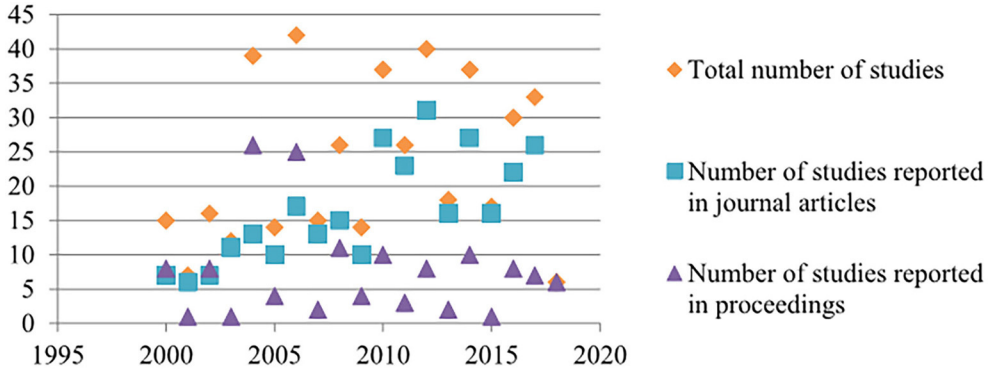

FIGURE 2 | Annual number of included studies by study type from journal articles from 2000 to 2017 and proceedings from 2000 to 2018.

commercial farms ($n = 75$, 30%) and research farms ($n = 153$, 61%). Among the 414 clinical trials, 238 (57%) evaluated an intervention for the purpose of prevention of which the majority were an evaluation of vaccines in piglets and/or dams ($n = 167$, 70%). Although all included studies ($n = 441$) reported a health outcome of interest in nursery pigs, many clinical trials ($n = 238$, 57%) and some observational studies ($n = 5$, 19%) also reported performance as a purpose of the intervention.

Non-antibiotic Interventions or Risk Factors

All eligible studies measured a health outcome in nursery pigs; however, interventions were applied to one or more specific populations based on production stage comprising dams, suckling piglets, or nursery pigs. Some studies reported the application of the intervention to more than one population. Among the total populations described in the eligible studies ($n = 553$), interventions were most commonly applied to nursery pigs ($n = 406$, 73%), followed by suckling piglets ($n = 86$, 16%), and dams ($n = 61$, 11%). Some studies reported the application of the intervention to all three populations ($n = 27$).

We charted data for 11 different categories of interventions or risk factors (**Figure 3**). Among the 414 clinical trial studies, there were 495 interventions described (**Appendix 4**). The two categories of interventions most frequently reported were feed additives ($n = 179$) and nursery or suckling piglet vaccination ($n = 160$) (**Figure 3**). Together, these two categories accounted for 68% of all interventions. The least common categories were air quality ($n = 3$) and producer education ($n = 4$). Details of all specific interventions for clinical trials are presented in **Appendix 4**; and further details for these specific interventions regarding comparison groups and health outcomes reported are available upon request. Among the 27 observational studies, there were 84 interventions or risk factors described (**Table 4**). The two categories that were most frequently studied were biosecurity ($n = 19$) and vaccinations of dams ($n = 14$).

Interventions Evaluated as Comparison Groups

Among the clinical trials and the observational studies, there were 672 comparison groups described. The most frequently reported comparison group was "different form or level" of the intervention or exposure ($n = 328$, 49%), followed by a no treatment control group ($n = 221$, 33%). The clinical trials also described comparison groups that received placebo ($n = 84$, 13%), antibiotics ($n = 24$, 4%), ZnO ($n = 8$, 1%), or a combination product containing an antibiotic and ZnO ($n = 3$, <1%). Note that some studies compared a non-antibiotic intervention group with a ZnO comparison group, whereas other studies compared a ZnO treatment group with a no treatment control group, or antibiotic or other treatment comparison group.

Among the 27 clinical trials that included an antibiotic comparison group, 21 investigated various feed additives, three investigated vaccinations to control *Mycoplasma hyopneumoniae*, one investigated a feed type, one investigated housing at the time of weaning, and one investigated producer education in the form of individual pig care training vs. standard metaphylactic antibiotic use in cases of nursery pig morbidities (20) (**Appendix 4**). Among the 179 clinical trials that investigated feed additives, 10 included a ZnO comparison group (**Appendix 4**).

Outcomes Measured

We charted data for nine health outcomes of interest. Among the clinical trials and observational studies, there were 729 reported outcomes (**Figure 4**). The three most commonly reported outcomes included clinical diarrhea ($n = 188$, 26%), mortality ($n = 158$, 22%), and vaccine immunity ($n = 140$, 19%). Immunity to vaccines included measures of specific immunity ($n = 140$) (e.g., serology and/or pathogen recovery or identification with PCR, and cell-mediated immunity) and in five studies also included non-specific immunity. Reporting of measures of immunity to a vaccine, with or without reporting other outcomes, was common among vaccine clinical trials ($n = 118$). A total of 43 (36%) vaccine clinical trials only reported measures of immunity without reporting any clinically important outcomes. These trials were conducted on research farms ($n = 18$), commercial farms ($n = 20$), and farm settings in which it was unclear ($n = 5$). Other outcomes reported included treatment for illness or antibiotic use ($n = 60$, 8%). Antibiotic treatments were typically

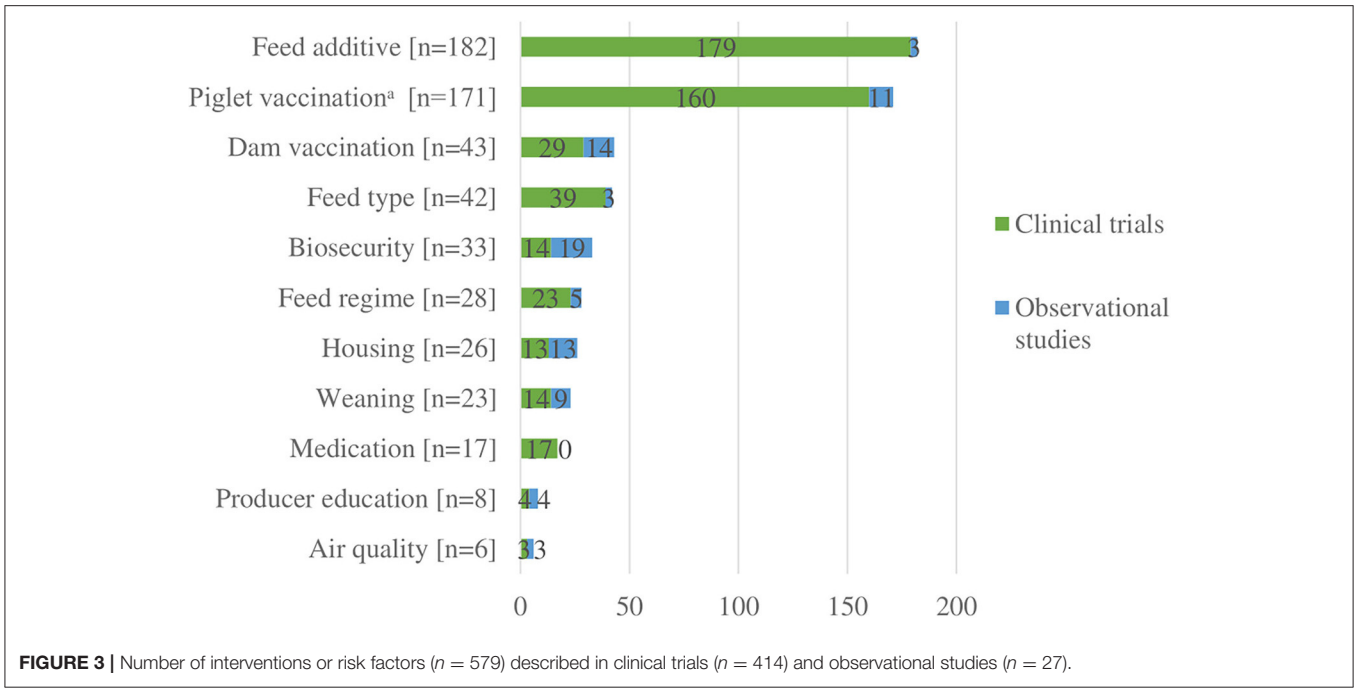

FIGURE 3 | Number of interventions or risk factors ($n = 579$) described in clinical trials ($n = 414$) and observational studies ($n = 27$).

TABLE 4 | Risk factors described in observational studies ($n = 27$).

Risk factors[a] ($n = 84$)	Risk factor details
Biosecurity ($n = 19$)	All-in-all-out vs. continuous flow ($n = 7$), mixing/cross fostering ($n = 5$), internal and external biosecurity ($n = 5$), air space separation ($n = 2$), piglet movement between stages ($n = 3$), infection control ($n = 4$), dead pig removal ($n = 1$)
Vaccination of dams ($n = 14$)	Porcine reproductive and respiratory syndrome virus (PRRSV) ($n = 4$), Porcine Circovirus type 2 (PCV2) ($n = 3$), Enterotoxigenic *Escherichia coli* (ETEC) ($n = 3$), rotavirus ($n = 1$), not clear or unspecified ($n = 3$)
Housing ($n = 13$)	Pen floor type ($n = 4$), space allowance/pig density ($n = 4$), use of bedding ($n = 2$), drinker type ($n = 2$), climatic and temperature conditions ($n = 3$), age of buildings ($n = 1$), indoors vs. outdoors ($n = 1$)
Vaccination of piglets[b] ($n = 11$)	PRRSV ($n = 4$), PCV2 ($n = 4$), not clear, or unspecified ($n = 5$)
Weaning ($n = 9$)	Weaning age ($n = 8$), mixing at weaning or weaning management ($n = 3$)
Feed regime ($n = 5$)	Restricted feeding ($n = 4$), creep feeding ($n = 3$), starter diet protein content restriction ($n = 1$)
Producer education ($n = 4$)	Experience level of manager/producer/worker ($n = 3$), Education level of manager/producer/worker ($n = 2$)
Feed type ($n = 3$)	Pelleted nursery feed ($n = 1$), feed composition quality ($n = 1$), level of soybean and canola ($n = 1$)
Feed additive ($n = 3$)	Zinc product (e.g., ZnO) ($n = 3$)
Air quality ($n = 3$)	Ventilation ($n = 3$)

Categories of risk factors presented in order of decreasing frequency.
[a]*Risk factors included modifiable exposures regardless of the positive or negative impact of the exposure on an outcome.*
[b]*Piglet vaccination includes suckling piglet or nursery pig vaccination.*

for diarrhea when specified. The metric used for treatment for illness or antibiotic use varied (e.g., number of treatments, percent treated animals, treatment incidence calculated on an animal daily dose basis, and farm-level antibiotic use). Less commonly reported outcomes included non-diarrheal, non-respiratory, non-specific morbidity (e.g., fever, removals, or unthriftiness) or other morbidities such as lameness ($n = 55$, 8%) and pathogen shedding ($n = 54$, 7%). The remainder of the reported health outcomes of interest included non-vaccine immunity ($n = 32$, 4%), which comprised studies that measured specific immunity ($n = 21$), non-specific immunity ($n = 10$), or both ($n = 1$). Presence of pathological lesions (e.g., lung lesions at necropsy or injection site lesions) ($n = 26$, 3.5%) and clinical respiratory disease ($n = 16$, 2%) also were reported (**Appendix 4**).

Among the 441 included clinical trials and observational studies, all of which reported a health outcome of interest, the most commonly reported additional outcomes included performance outcomes such as growth ($n = 297$, 67%), feed efficiency ($n = 197$, 45%), and feed intake ($n = 186$, 42%).

Study Size

Among both clinical trials and observational studies, the study size varied widely from the smallest study using six individuals to evaluate an autogenous vaccine (21) to the largest

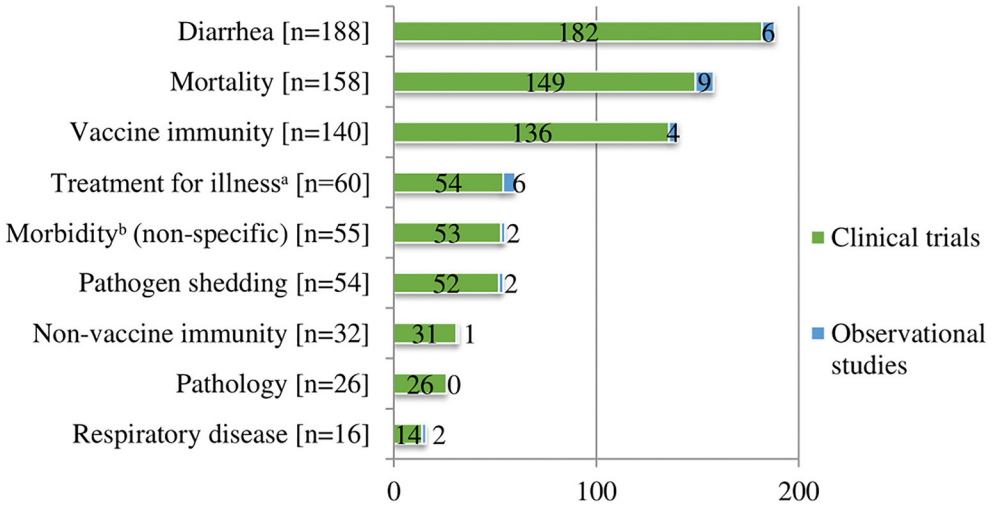

FIGURE 4 | Number of health outcomes of interest[a] ($n = 729$) described in clinical trials ($n = 414$) and observational studies ($n = 27$).

TABLE 5 | Study size[a] of clinical trials and observational studies at the hierarchical level of the data analysis.

	Number of studies	Range of study sizes	Experimental settings	Commercial settings	Unclear settings
Individual	405	9–3,31,592	200	179	26
Group/pen/room	113	2–653	63	45	5
Herd	37	3–1,513	0	37	0

[a]*Study size was the number of study subjects included in the analyses.*
Some studies measured outcomes at multiple levels.
For some studies some outcomes were measured at the individual level but performance was measured at the pen level.

study using 331,592 individual pigs to evaluate the impact of a producer education program on nursery pig mortality (22) (**Table 5**). Some studies measured health outcomes at the individual level but performance outcomes at the pen level. Most studies measured outcomes at the individual level regardless of the level of intervention allocation (**Table 5**). Among the observational studies alone, studies varied from 160 to 3,736 individuals.

Material for Potential Systematic Review Questions

There were 13 interventions evaluated in clinical trials that met our inclusion criteria for studies that could feasibly support systematic reviews; these included feed additives (e.g., amino acids, diet acidification, organic acids, fiber, phytobiotics, pre-biotics, probiotics, egg yolk antibodies, and ZnO), vaccination of piglets [e.g., porcine circovirus 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), and *M. hyopneumoniae*], and vaccination of dams (e.g., PCV2) (**Appendix 4**).

Knowledge Gaps

The selection of knowledge gaps identified discretionary items based on the authors' opinions. We found that there were relatively few studies in which a non-antibiotic intervention was compared directly with an antibiotic comparison group. Among the vaccine clinical trials, approximately one third failed to report a clinically important outcome.

DISCUSSION

This scoping review of non-antibiotic approaches for disease prevention or control that may reduce the need for antibiotics in nursery pigs relevant to the North American context identified a large body of literature with considerable breadth and depth. Since most of the studies described in this ScR were conducted in the EU or the UK, we may have not captured a body of knowledge on this broad topic for the North American context. The breadth of this literature was reflected in the diversity of interventions or risk factors evaluated, whereas the depth of this literature was reflected in the number of specific topic areas with similar studies that might feasibly support systematic reviews.

Clinical and policy decisions are generally regarded as best guided by the interpretation of findings of multiple studies evaluating the same research question rather than the findings of a single study, which is a random event from a distribution of possible results (23). A summary of multiple relevant studies in the form of a systematic review can provide a credible summary

of the primary literature (23). However, a third of the literature included in this scoping review was sourced from proceedings that present at least three challenges to systematic reviews. First, proceedings obtained through the AASV Swine Information Library were available exclusively to members and thus would not be available to review teams through database searches. Second, these proceedings databases were not searchable through word string searches, which are an efficient method to search the literature on a specific topic. Third, these proceedings were not peer-reviewed and often were short; thus, the quality of research reporting in proceedings may be insufficient for inclusion into systematic reviews. Brace et al. (24), in an evaluation of the quality of reporting of vaccine trials at veterinary conferences, concluded that it would be difficult to assess validity from the information provided in most conference proceedings. Although there was apparently considerable depth in this body of literature, without the inclusion of proceedings, the depth may actually be considerably less. Interestingly, the majority of research from the USA on this broad topic was available only through proceedings. Similarly, Brace et al. (24) reported that only 6% of 89 proceedings presented on swine vaccines at the AASV annual conference from 1988 to 2003 were later published as full articles. Although assessing the proceedings-to-publication ratio from AASV conferences was not our objective, our findings of only two duplicate journal articles with conference proceedings suggest the ratio is still low. This may represent a lost opportunity for the communication of research.

We chose to focus our data charting on controlled clinical trials. We did not include quasi-experimental trial design (i.e., before and after intervention comparisons), as this design does not provide an equal evidentiary value to clinical trials (19). Though challenge trials serve an important purpose in providing proof of concept prior to field trials under natural exposures, challenge trials tend to report more favorable outcomes compared with clinical trials of the same intervention (25). We found that the body of literature in this review was dominated by clinical trials with comparatively few observational studies. This may reflect the comparative ease of conducting and study design appropriateness of clinical trials vs. observational studies on swine farms.

This scoping review identified a wide variety of vaccines and feed additives, which together comprised the majority of interventions evaluated in clinical trials. There was a dearth of clinical trials that evaluated management interventions such as biosecurity and infection control, feed or nutrient restrictions, housing, and weaning. This may reflect the difficulty of evaluating these types of interventions in clinical trial settings and/or the difficulty in sourcing funding for trials of these interventions (26). Biosecurity was the most frequently studied intervention among the relatively low number of observational studies identified by this scoping review. Thus, the depth of research available for synthesis on management practices was far less than for other interventions.

Despite the approximately equal number of studies conducted on research farms vs. commercial farms, there was a strong predominance of research with the purpose of preventing disease on research farms, whereas research with the purpose of disease control was predominately conducted on commercial farms. Variables that may impact the outcome of a trial, such as prior disease-free status, could potentially be better controlled on research farms than commercial farms, whereas the field conditions of commercial farms, such as an existing disease problem, provides a better setting to truly test the effectiveness of an intervention under "natural commercial" conditions (27). However, it is unknown to what extent the research vs. commercial farm settings impact external validity in swine research.

Among the studies identified through this scoping review, approximately half reported a comparison group that was a different form or level of the intervention itself. Traditional systematic reviews and meta-analyses are not just based on studies with similar interventions, populations, and outcomes but also on similar comparison groups. In the absence of sufficient studies with similar comparison groups, combining studies with the same outcome through network meta-analysis may prove useful. Network meta-analysis allows comparisons of interventions that may not have been directly compared in head-to-head trials by mathematically evaluating both direct and indirect comparison evidence of multiple interventions and comparisons (28). If we had restricted our scoping review to studies that compared a non-antibiotic intervention group with an antibiotic intervention group, our review would have been very limited: first, because most of these studies evaluated a feed additive intervention, and second, because there were relatively few feed additive intervention studies with an antibiotic comparison group. In a body of literature that describes non-antibiotic approaches to improve the health of nursery pigs, this lack of comparisons with antibiotics may represent a knowledge gap for decisions about antibiotic alternatives. Where it may be appropriate to use an antibiotic comparison group, the results could potentially demonstrate the superiority or at least the non-inferiority of a non-antibiotic intervention.

Approximately one third of vaccine clinical trials did not report a clinically important outcome, though they did report measures of immunity. The lack of reporting clinically important outcomes when they could have been measured reduces our opportunity to build a body of evidence best suited for clinical decision making. Clinically important outcomes as determined by guidelines, clinicians, patients, or the researcher provide the best evidence for inclusion in systematic reviews, whereas indirect outcomes such as measures of immunity provide a lower quality of evidence (29–33). To enhance research efficiency, future vaccine clinical trials should report clinically important outcomes.

Beyond describing the body of literature pertaining to non-antibiotic approaches that may reduce the need for antibiotics for disease prevention or control in nursery pigs, an additional objective of this review was to identify specific topic areas where there may be sufficient literature to support systematic reviews. We identified 13 specific topic areas with a minimum of 10 clinical trials that may feasibly support systematic reviews. These topic areas were composed of various vaccines and feed additives. Although we listed the ZnO as an intervention for which there may be sufficient material to support an SR, we do

not recommend knowledge synthesis for this intervention given that concerns regarding AMR co-selection with the use of ZnO in swine (34). Though this scoping review identified numerous specific topic areas that might be feasibly combined in systematic reviews, similarity among comparison groups and choice of outcome would need to be carefully considered. Nevertheless, systematic reviews of these topic areas for nursery pigs, if not already conducted, could provide a useful synthesis of existing knowledge. Some systematic reviews of related topics have been conducted (35–39); however, none of these systematic reviews pertained exclusively to health outcomes in nursery pigs.

In determining the specific topic areas with sufficient similar studies to support systematic reviews, we used an arbitrary number of 10 similar clinical trials with some commonality of the intervention and population. Technically, a minimum of two studies are all that is needed for combination in a meta-analysis if those studies are similar enough to combine in a meaningful way (40). However, having additional studies provides an opportunity to explore between-study variability, which in turn impacts the interpretation and meaning of the meta-analysis (40), (41).

There were potential limitations that may have impacted the comprehensiveness of this scoping review. First, we may have missed some articles if we did not include all possible terms for each of the many non-antibiotic interventions included in the search. Systematic reviews for specific interventions should maximize comprehensiveness by including all possible terms. Second, we may have overlooked some interventions or outcomes if they did not appear in the title or abstract. Third, we accessed bibliographic sources available through the University of Guelph data platforms and two conference proceedings available through the AASV library. We may have missed additional published articles available through other databases, and unpublished studies generated by companies testing products or proceedings from other conferences. Fourth, our search using the CAB Direct platform was unsuccessful due to technical difficulties. Without the additional studies identified through that platform, our search may not have been as comprehensive as we had intended. The coverage provided by CAB Abstracts was found to be excellent in a comparison of nine databases for veterinary journals (42), so it is possible that the CAB platform contained relevant articles that our search did not identify. Finally, due to limited resources, we could not include all possibly relevant sources of gray literature such as symposia proceedings. We chose to focus on the gray literature of North America.

In addition to limitations to comprehensiveness, this review may have two additional limitations. First, we may have misclassified the purpose of the intervention for disease control by including in this category pig herds or groups that also had a known exposure to an infection and not solely groups that contained clinically ill or infected pigs as defined by AVMA and GAO reports. Finally, we did not assess inherent biases of included studies such as lack of appropriate randomization of clinical trials, lack of concealment or blinding, loss to follow-up, or selective outcome reporting. Any systematic reviews of non-antibiotic approaches that may reduce the need for antibiotic prevention or control in nursery pig production should include a risk of bias assessment (43).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

LW coordinated the project, acted as the primary reviewer for relevance screening and data charting, analyzed data, interpreted the results, and wrote the manuscript drafts. JS oversaw the work, piloted the relevance screening and data charting forms, assisted with the interpretation of results, reviewed manuscript drafts, and approved the final manuscript. AO'C and SM provided guidance for interpretation of the results, commented on the manuscript drafts, and approved the final manuscript. TO'S provided guidance for the stakeholder survey and interpretation of the results, commented on the manuscript drafts, and approved the final manuscript. MR and KC conducted relevance screening and data charting as a second reviewer, commented on manuscript drafts, and approved the final manuscript version. All authors contributed to the article and approved the submitted version.

FUNDING

Funding support for this scoping review was provided by the Ontario Ministry of Agriculture, Food, and Rural Affairs and through an Ontario Veterinary College PhD Fellowship and the New Directions Research Program 2016-17 (ND2015-2772).

ACKNOWLEDGMENTS

We thank our stakeholders for their invaluable guidance. We also thank our research librarian, Ms. Ali Versluis, for her help.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.620347/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 07 May 2021
doi: 10.3389/fvets.2021.650866

Genetic Characterization of Feline Parvovirus Isolate Fe–P2 in Korean Cat and Serological Evidence on Its Infection in Wild Leopard Cat and Asian Badger

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 08 January 2021
Accepted: 15 April 2021
Published: 07 May 2021

Citation:
Kim YJ, Yoon S-W, Jang JH, Jeong DG, Lee BJ and Kim HK (2021) Genetic Characterization of Feline Parvovirus Isolate Fe–P2 in Korean Cat and Serological Evidence on Its Infection in Wild Leopard Cat and Asian Badger. Front. Vet. Sci. 8:650866. doi: 10.3389/fvets.2021.650866

Feline parvovirus (FPV) is a small, non-enveloped, single-stranded DNA virus that infects cats. We recently isolated a feline parvovirus Fe–P2 strain from a dead stray cat in Iksan, 2017. Its partial genomic sequence (4,643 bases) was obtained, and phylogenetic analysis based on the VP2 nucleotide sequence showed that the FPV Fe-P2 strain was closely related to the FPV isolate Gigucheon in cat, 2017 (MN400978). In addition, we performed a serum neutralization (SN) test with the FPV isolates in various mammalian sera. These were from raccoon dog, water deer, Eurasian otter, Korean hare, leopard cat, and Asian badger, which were kindly provided by Chungnam Wild Animal Rescue Center. Notably, serological evidence of its infection was found in Asian badger, *Meles leucurus* (2/2) and leopard cat, *Prionailurus bengalensis* (5/8) through SN tests, whereas there was no evidence in raccoon dog, water deer, Eurasian otter, and Korean hare based on the collected sera in this study. These findings might provide partial evidence for the possible circulation of FPV or its related viruses among wild leopard cat and Asian badger in Korea. There should be additional study to confirm this through direct detection of FPVs in the related animal samples.

Keywords: feline parvovirus, feline panleukopenia, leopard cat, Asian badger, serum neutralization

INTRODUCTION

Feline parvovirus (FPV) is a single-stranded DNA virus which is a variant of *Carnivore protoparvovirus 1*, belonging to the genus *Protoparvovirus* within the family *Parvoviridae*. A range of serious condition (often lethal disease, inducing vomiting, enteritis, diarrhea, and acute lymphopenia) in young animal is closely involved the *Carnivore protoparvovirus 1*.

FPV is the main causative agent for feline panleukopenia, which can also be caused by canine parvovirus (CPV) variants, CPV-2a, 2b, and 2c (1, 2). CPV-2 can only infect dogs, whereas its variant can infect cats (1, 3). Thus, parvovirus members

of *Carnivore protoparvovirus 1* might be one of the host range variants (4). Also, mink enteritis virus (MEV) and raccoon parvovirus (RaPV) are included in that.

FPV can infect not only domestic cats, but also other species such as raccoons, foxes, and minks (5). Previous findings have reported the detection of FPV-sequences in tissues of the African wild cat and in feces of both cheetahs and honey badgers (6). In Italy, FPV was detected in red foxes (*Vulpes vulpes*) (2.8%, 7/252) and Eurasian badgers (*Meles meles*) (10%, 1/10), and in Portugal, parvovirus DNA was detected in Egyptian mongoose (57.8%), red fox (78.9%), and stone marten (75%) (7, 8). Although FPV can infect diverse animal species, it is difficult to confirm the FPV-infection cases due to the genetic similarity and cross-reactivity between FPV and CPV (3).

The parvovirus contains two open reading frames. One is the codes for non-structural proteins (NS1 and NS2) and the other is the codes for structural viral proteins (VP1 and VP2). Several amino acid changes in the structural protein VP2, its major capsid protein, were associated with host specificity and antigenic properties for the parvovirus (9–11). Based on VP2 gene analysis, there were three genetic clusters (G1, G2, and G3) of FPV around the world. In Korea, FPVs belonging to both the G1 and G2 clusters were found (12). In Korea, 2% of 200 cats were FPV-positive in Seoul (11). To date there has been no study about the possible interspecies transmission of FPV among wild mammals in Korea.

In this study, a recent feline parvovirus Fe-P2 strain was isolated from a fecal swab from a stray cat carcass found in Iksan, 2017. Using this isolate, its genomic sequence was obtained, and serum neutralization (SN) tests were performed with various sera from several wild mammals rescued by the Chungnam Wild Animal Rescue Center between 2016 and 2018. Through these experiments, this study provide evidence for the possible interspecies transmission of FPV (or its related viruses) among wild carnivores in Korea.

MATERIALS AND METHODS

Virus Isolation

A fecal swab was collected from a dead stray cat found in Iksan, Korea, 2017. The swab was transported to the lab with virus transport medium (Noble Bio. Co., Ltd., Hwasung, Korea). The transport medium containing the fecal swab was centrifuged at 3,000 g for 20 min at 4°C, and the supernatant was filtered through a 0.22 μm syringe filter, MF-Millipore™ Membrane Filter (Merck, Darmstadt, Germany). The filtered supernatant was inoculated on the monolayer of CRFK cells and incubated for 1 h, followed by phosphate buffered saline (PBS) washing. The inoculated cells were further incubated for 7 d with DMEM, supplemented with 2.5% fetal bovine serum (FBS) and cytopathic effect (CPE) was observed after two blind passages (**Figure 1A**). After freezing and thawing, the supernatant was aliquoted for further testing.

Polymerase Chain Reaction and Sequencing

DNA was extracted from the virus stock using QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). First, PCR targeting VP2 was performed by using primer sets: 555-F† 5′-CAGGA AGATATCCAGAAGGA-3′ and 555-R† 5′-GGTGCTAGTTG ATATGTAATAAACA-3′ (13).

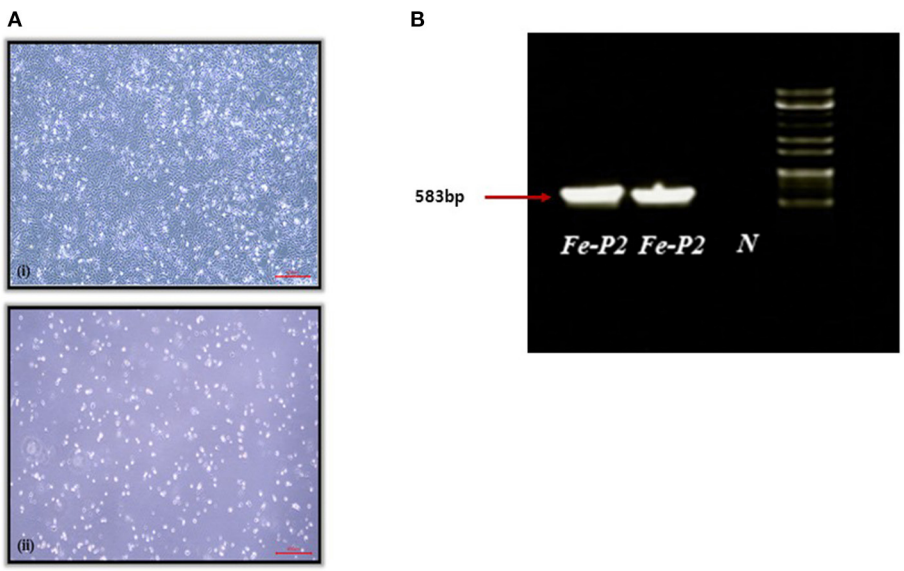

FIGURE 1 | (A) Cytopathogenic effect of feline parvovirus (FPV) Fe-P2 on cell culture. (7 days); (i) Uninfected cell control, (ii) CRFK infected with FPV Fe-P2, **(B)** Screening of clinical samples by PCR assay using CPV-2a and b primers. A single DNA amplicon of 583 bp is shown after using the CPV-2a and b primer pair, Fe-P2, FPV isolate; N, negative control.

A PCR 583 bp amplicon (**Figure 1B**) was obtained and further sequenced by Cosmogenetech, Seoul, Korea. Genomic sequencing was performed using the designed primers based on the reference FPV genomic sequence (MN683826) and PCR-positive sequences. Primer information is presented in **Table 1**.

Genetic Analysis

Phylogenetic analysis based on the nucleotide sequences showed that the detected feline parvoviruses belonged to the genera Protoparvovirus. The obtained genomic sequences of the FPV Fe-P2 strain were further analyzed with related sequences from GenBank using BioEdit (14) and MEGA version 7.0 (15) tools. Phylogenetic trees based on the genomic sequences and VP2 were drawn with the maximum likelihood method, with 1,000 replicates of bootstrap sampling and using the Kimura 2-parameter model using MEGA version 7.0. Genomic sequence data generated in this study have been deposited in GenBank under the accession number MN683826.

Serum Neutralization Test

A total of 109 sera from wild mammals, including raccoon dog, water deer, Eurasian otter, Korean hare, leopard cat, and Asian badger, were obtained from rescued wild animals from the Chungnam Wild Animal Rescue Center. The information on the animal species and collection dates is presented in **Table 2**.

For the SN test, the sera samples were first inactivated at 56°C for 30 min. The inactivated sera were two-fold diluted from an initial dilution of 1:10 with DMEM in 96 well-plates. The diluted sera were then mixed with the same volume of FPV Fe–P2 isolate in 200 Tissue Culture Infective Dose ($TCID_{50}$)/50 μl. The mixture was incubated for an hour at 37°C and 100 μl of that were transferred to the CRFK monolayers with 5% FBS in a 96 well-cell culture plate, followed by incubation at 37°C for 4–5 days. Then, the plates were examined for CPE.

TABLE 1 | Primers used for genotyping and sequence analysis.

Primer	Sequence (5′ → 3′)	Sense	Position (Fe–P2)
555-F[a]	CAGGAAGATATCCAGAAGGA	+	3,841–3,860
555-R[a]	GGTGCTAGTTGATATGTAATAAACA	–	4,423–4,399
FPVin-F4	GGCAATTGCTCCCGTATT	+	2,465–2,482
FPVin-R4	AGCCATGTTTCCTTTAACTGCAG	–	2,915–2,893
FPV-F3	CAGAATCTGCTACTCAGCC	+	3,085–3,103
FPV-R5	ACCAACCACCCACACCAT	–	4,823–4,806
NSF-1	CTGGCAACCAGTATACTG	+	115–132
NSR-1	GCTTGTGCTATGGCTTGAGC	–	1,357–1,338

[a]*Primers for amplification of the 583 bp products represent a parvovirus-specific VP2 gene (6).*

TABLE 2 | Wild animal sera information for the serum neutralization test.

Animal species*	Collected year	No. of sera
Raccoon dog (*Nyctereutes procyonoides koreensis*)	2016–2018	96
Asian badger (*Meles leucurus*)	2016–2017	2
European Otter (*Lutra lutra*)	2018	1
Leopard cat (*Prionailurus bengalensis*)	2018	8
Korean hare (*Lepus coreanus*)	2017	1
Water deer (*Hydropotes inermis*)	2018	1
	Total sera	109

**A total of six mammalian species: Asian badger (Meles leucurus), leopard cat (Prionailurus bengalensis), water deer (Hydropotes inermis), Eurasian otter (Lutra lutra), raccoon dog (Nyctereutes procyonoides koreensis), and Korean hare (Lepus coreanus).*

RESULTS

Genetic Characterization of the Feline Parvovirus Fe–P2

The partial genomic sequence of 4,643 bases of FPV Fe–P2 strain was obtained in this study. Two main open reading frames (ORFs) encoding VP1 and VP2 were deposited as complete coding sequences. As a determinant of the host range of parvoviruses, it appears to be the minority amino acids of the capsid protein that determine the ability of the virus to replicate in different hosts (3). The host-specific amino acid position in VP2; 80, 93, 103, 297, 300, 305, 323, 564, and 568 (6) were compared with other FPVs and CPVs. All the amino acids were well-conserved among FPVs including the Fe–P2 strain, although these differed from CPV-2a, 2b, and 2c strains (**Table 3**). The partial genomic sequence-based phylogenetic tree was drawn with other strains of *Carnivore protoparvovirus 1* (**Figure 2A**). Based on the phylogenetic relationship, it is suggested that the FPV Fe-P2 strain was not related to the recently reported FPV from wild raccoon dogs (GenBank Accession No. MF069445 and MF069447) in Canada (16). Phylogenetic analysis based on the VP2 nucleotide sequence showed that the FPV Fe–P2 strain belonged to the G1 cluster (**Figure 2B**). The FPV Fe–P2 in this study was closely related to a feline-specific FPV strain (GenBank Accession No. MN400978), which was reported previously in Korea (**Figure 2B**), showing 99.16% sequence identity.

Serum Neutralizing Antibodies Against FPV Fe-P2 in Leopard Cat and Asian Badger

In the result of the SN test against FPV Fe-P2 with a total of 109 sera from wild mammals (section Serum Neutralization Test), the neutralizing antibodies were evident in the sera of Asian badgers and wild leopard cats, showing SN titer, 80–1,280 (**Table 4**). Five out of eight leopard cats and all the Asian badgers (two samples) were seropositive with the SN test. The other wild mammals, including 96 Raccoon dogs, one water deer, one Eurasian otter, and one Korean hare did not have SN titers more than 20 against FPV Fe–P2. The SN titers in Asian badger were 80 and 320, while that in leopard cats were between 80 and 1,280.

DISCUSSION

FPV-infected cats older than 6 weeks develop symptoms from subclinical level to sudden death within 12 h (5). FPV-infectious disease is characterized by severe panleukopenia and enteritis, which is also associated with high mortality and morbidity (3, 5, 17). Multiple, epizootic outbreaks of FPV infection in most unvaccinated cats were reported in Australia between 2014 and

TABLE 3 | Amino acids positions for host-specificity between dogs and cats.

Virus strain	Accession number	Amino acid VP2								
		80	93	103	297	300	305	323	564	568
Feline parvovirus Fe-P2*	MN683826*	K	K	V	S	A	D	D	N	S
Feline parvovirus (FPV)	HQ184189	K	K	V	S	A	D	D	N	S
	AB054226	K	K	V	S	A	D	D	N	S
	KP081409	K	K	V	S	A	D	D	N	S
	KJ415112	K	K	V	S	A	D	D	N	S
Canine parvovirus (CPV)-2a	EU009201	R	N	A	A	G	Y	N	S	R
	KT156829	R	N	A	A	G	Y	N	S	R
Canine parvovirus (CPV)-2b	FJ977077	R	N	A	A	G	Y	N	S	R
	EF599097	R	N	A	A	G	Y	N	S	R
	EU009206	R	N	A	A	G	Y	N	S	R
Canine parvovirus (CPV)-2c(a)	EF599098	R	N	A	A	D	Y	N	S	R

**The partial genomic sequence of 4,643 bases from Feline Parvovirus Fe-P2 (MN683826).*

2018 (2). In Korea, FPV infection was found in 2% of cats in Seoul (11), and FPVs belonging to the G1 and G2 clusters were circulating (12). In this study, an FPV isolate Fe–P2 from 2017 was included in the G1 cluster, and amino acids of the VP2 protein indicate that the isolate would have the same host specificity as other FPVs. Observing no close relationship with recent novel strains of FPV from wild raccoon dogs in Canada (16), the FPV isolate Fe–P2 in this study might be one of the strains circulating in Korea. In addition, the amino acids positions for host-specificity of Fe–P2 strain were the same as other feline parvoviruses rather than canine parvoviruses. There were no apparent genetic differences between Fe-P2 strain and other FPVs. Although there should be additional screening on the prevailing FPV genotypes in Korea, we used this isolate to screen the serological evidence of FPV infection in wild animals.

As we can collect sera of six wild animals from the Chungnam Wild Animal Rescue Center, we tried to screen SN antibodies using the recent FPV isolate, Fe–P2 in this study. As expected, no SN antibodies were observed in Korean hare and water deer, which could be regarded as negative controls for the SN test in this study. Notably, two wild mammals, leopard cat and Asian badger were seropositive in the SN test using the FPV isolate Fe–P2 with 109 sera of wild mammals from Korea. The SN titers were notable due to relatively high titers, 80 and 1,280 in leopard cats and 80 and 320 in Asian badgers.

Parvoviruses have shown consistently evolution over many years at a faster rate than other DNA viruses. Also, a variety of wild animals have increasingly detected as parvoviruses hosts (8). FPV has been known to infect other species like raccoon dogs, foxes, minks, African wild cats, cheetahs, and honey badgers (5, 6). Furthermore, FPV was still frequently found in wild animals like otters, minks, and martens (18, 19). The FPV-positive rate in Canadian otters was almost 30%. Hence, it was suggested that otters might be a principal maintenance host for FPV enabling viral persistence and serving as a source for other susceptible species (18). In this study, the serum from otter had SN titer <20 in the SN test against FPV Fe-P2 strain. However, since only one otter sample was tested, there may be a possibility for FPV infection in the species, additional screening would provide more detailed information.

In this study, five out of eight leopard cats were seropositive against the FPV Fe–P2 strain, which may indicate they were infected by FPV previously. This is not surprising as FPV had already been detected in leopard cats in Taiwan and Vietnam (20, 21). However, seropositive Asian badgers (two samples) in the SN test against FPV Fe–P2 strain, were identified in this study for the first time. While FPV was detected in honey badger (*Mellivora capensis*) in South Africa (6), in this study we report the serological evidence of FPV infection in Asian badgers (*Meles leucurus*), which belong to a different subfamily that had never been reported before. Thus, this study might provide partial evidence for the possible circulation of FPV or its related viruses among wild leopard cat and Asian badger in Korea.

As a variant of *Carnivore protoparvovirus 1*, FPV had wide range of host animals. Evidence for FPV infection in a diverse range of wild mammals indicated that FPV has been circulating not only in cats but also in other mammals, with sporadic interspecies transmission. In this study, we could suggest Asian badgers and wild leopard cats as potential hosts for FPV infection in Korea. However, conventional parvoviruses may have observed cross-reactivity through high virus-neutralizing (VN) antibodies titers between FPV and CPV (22), thus we cannot exclude the possible infection of CPVs in those animals. There should be additional study to confirm this through direct detection of FPVs in the related animal samples. Therefore, it would be more feasible that there was serological evidence of FPV or its related viruses in Asian badgers and wild leopard cats in Korea.

Thus, FPV and its related viruses may circulate in the wild life hosts. The leopard cats and Asian badgers in this study were

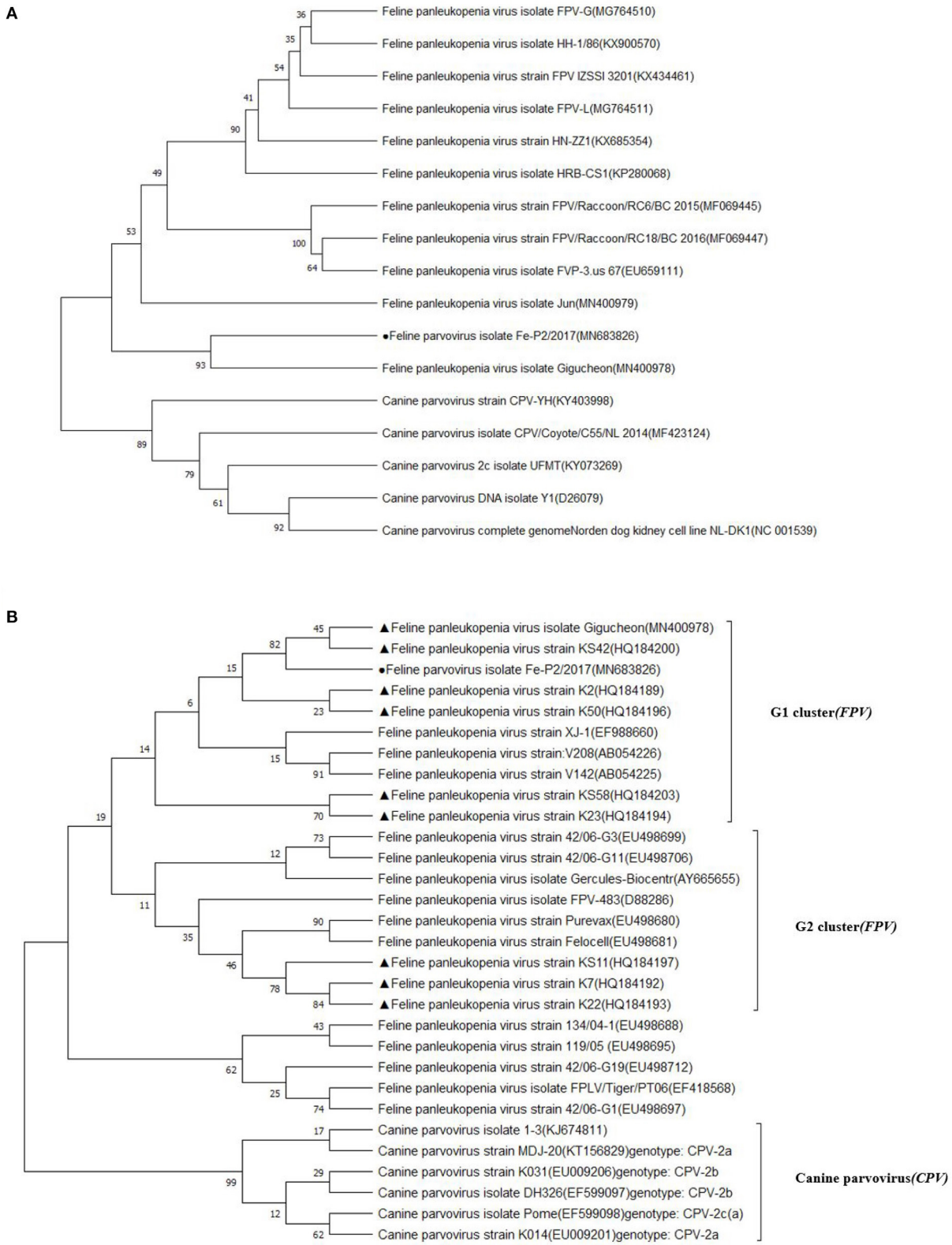

FIGURE 2 | (A) Phylogenetic tree based on partial genomic sequences; black solid circle indicates the FPV Fe-P2 virus. The tree produced by the maximum likelihood method using MEGA 7.0 software shows the phylogenetic relationship between the 5 canine parvovirus strains and 12 feline parvoviruses. **(B)** Phylogenetic tree based on VP2 nucleotide sequences; black solid circle indicates the Fe-P2 virus, and black solid triangles indicate viruses isolated in Korea. The tree produced by the maximum likelihood method using MEGA 7.0 software shows the phylogenetic relationship between the 6 canine parvovirus strains and 24 feline parvoviruses.

TABLE 4 | Serum neutralization test results.

Animal species	Year	SN-positive/total sera*	SN titer of the positive
Raccoon dog (*Nyctereutes procyonoides koreensis*)	2016–2018	0/96	<20
Leopard cat (*Prionailurus bengalensis*)	2016–2018	5/8	80, 1,280, 1,280, 1,280, 1,280
Asian badger (*Meles leucurus*)	2016–2017	2/2	80, 320
Korean hare (*Lepus coreanus*)	2017	0/1	<20
Water deer (*Hydropotes inermis*)	2018	0/1	<20
European otter (*Lutra lutra*)	2018	0/1	<20
	Total (sera)	7/109	

**Five out of eight leopard cats and two Asian badgers were seropositive with the SN test.*

rescued animals from human populated regions, which means that they were near human habitat. Therefore, a vaccination or a control policy against FPV and its related viruses should be considered not only for household cats, but also for wild animals.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because we used the archived sera which were collected for veterinary treatment from the rescued wild animals by Chungnam Wild Animal Rescue Center in Korea.

AUTHOR CONTRIBUTIONS

HK and JJ: conceptualization. YK and S-WY: methodology. JJ: resources. DJ: data curation. YK and HK: writing—original draft preparation. BL, S-WY, and DJ: writing—review and editing. BL and S-WY: supervision. HK and DJ: funding acquisition. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by grants from the KRIBB Initiative program (Grant No. KGM4691814) and supported by the BioNano Health-Guard Research Center, funded by the Ministry of Science and ICT (MSIT) of Korea as a Global Frontier Project (grant No. H-GUARD_2013M3A6B2078954). This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2020R1A6A1A06046235).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

SYSTEMATIC REVIEW
published: 07 May 2021
doi: 10.3389/fvets.2021.630960

Global Comprehensive Literature Review and Meta-Analysis of Brucella spp. in Swine Based on Publications From 2000 to 2020

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OPEN ACCESS

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: 18 November 2020
Accepted: 06 April 2021
Published: 07 May 2021

Citation:
Gong Q-L, Sun Y-H, Yang Y, Zhao B, Wang Q, Li J-M, Ge G-Y, Chen Z-Y, Shi K, Leng X, Zong Y and Du R (2021) Global Comprehensive Literature Review and Meta-Analysis of Brucella spp. in Swine Based on Publications From 2000 to 2020. Front. Vet. Sci. 8:630960. doi: 10.3389/fvets.2021.630960

Background: Brucellosis, a zoonotic disease, infects various hosts, including swine and humans. It has reemerged in recent years as a public health concern, and current studies on brucellosis infection in swine have been conducted worldwide. However, no meta-analyses of global brucellosis infection in swine have been published. The aim of this study was to provide an overview of *Brucella* species (spp.) in swine worldwide and the factors associated with its persistence.

Results: We searched seven databases for published epidemiological studies on brucellosis in pigs, including the Chinese National Knowledge Infrastructure, Wanfang Data, SpringerLink, ScienceDirect, Web of Science, the VIP Chinese Journal Database and PubMed. We selected 119 articles published from January 1, 2000 to January 3, 2020 for inclusion in the meta-analysis and analyzed the data using a random-effects model. Funnel plots and Egger's test showed significant publication bias in the included studies. The results of the sensitivity analysis showed that our study was relatively stable and reliable. The prevalence of brucellosis in swine was 2.1% (95% CI: 1.6–2.6), of which the highest infection rate, which was found in Europe, was 17.4% (95% CI: 11.1–24.9). The prevalence in feral pigs (15.0%, 95% CI: 8.4–23.2) was higher than that in domestic pigs (1.1%, 95% CI 0.2–2.5). The prevalence in high-income countries (15.7%, 95% CI 8.0–25.3) was significantly higher than that in middle- (0.8%, 95% CI 0.5–1.1), and low-income countries (0.1%, 95% CI 0.0–0.2). The prevalence was highest in finishing pigs at 4.9% (95% CI 0.9–11.0), and lowest among suckling pigs at 0% (95% CI 0.0–0.5).

Conclusion: The *Brucella* prevalence in pig herds currently is distributed widely throughout the world. In some countries, swine brucellosis may be a neglected zoonotic disease. We recommend long-term monitoring of the prevalence of brucellosis in domestic and wild pig herds. Attention should also be paid to animal welfare on intensive pig farms; controlling the breeding density may play an important role in reducing the spread of brucellosis among pigs.

Keywords: brucellosis, brucella suis, meta-analysis, prevalence, swine

INTRODUCTION

Brucellosis is a serious zoonotic disease caused by the *Brucella* species (spp), which occurs worldwide, especially in developing countries (1, 2). Although some developed countries have achieved freedom from animal brucellosis, it has reemerged in Japan, Australia and some European countries (Germany, Finland, Austria, Belgium and Italy) during the past 3 years (3–7). Brucellosis has been found in more than 170 countries in six major regions of the world (8). More than 500,000 new human infections are estimated to occur every year and more than 850 million pigs are infected with *Brucella* spp. (9, 10). At present, the prevalence of swine brucellosis varies widely worldwide, with the highest rates in America, North Africa and southern Europe (11, 12). In South America, the positive rate of swine brucellosis antibodies is 9%, and some countries in the European Union have no swine brucellosis while other countries have a positive antibody rate of 22.7%. In China, the positive rate of swine brucellosis antibodies in some areas is 10% (13–15). The prevalence of the disease varies among different regions, but the overall prevalence has been on the rise since the 1990s, which has had a considerable impact on the health of humans and animals and on the economy (16).

In addition to *Brucella suis*, there are 12 *Brucella* spp. currently (*Brucella* ovis, *Brucella* abortus, *Brucella* canis, et al.) and other strains without standing in nomenclature (17). Most of these species mainly infect specific hosts. Although it has been reported that pigs can be infected with different types of *Brucella* besides *Brucella suis* (18), *B. suis* is responsible for brucellosis in pigs. *Brucella suis* is composed of five biovars referred to as 1 through 5 (19, 20). Among them, *Brucella suis* biovars 1, 2 and 3 cause brucellosis in domestic swine, cattle, sheep and even human beings. Although *Brucella suis* is less harmful than *Brucella melitensis* and *Brucella abortus*, brucellosis in pigs caused by it often leads to chronic infection that is not easily detected (21). It may infect the surrounding livestock and other animals, increasing its epidemic scope and widening its range of infection (22). Most human infections derived from swine are caused by *Brucella suis* biovars 1 and 3 (23–27), which easily infect humans through direct exposure, particularly abattoir workers, farmers and veterinarians (28, 29). To date, there is no effective vaccine for *Brucella* (30). More importantly, the antimicrobial resistance of *Brucella* is emerging in brucellosis endemic regions of the world, such as China, Malaysia, Iran, Qatar and Egypt (31). Therefore, we should pay greater attention to its ongoing spread worldwide.

Pigs play a key role globally in providing animal protein in animal husbandry production. Pork is the most consumed land-animal meat, accounting for more than 36% of the world's meat intake, and has maintained a steady growth over the past few decades (32). Brucellosis was once considered to be one of the main diseases affecting the pig industry. In many countries, especially those in the developing world, pig production is usually housed in low biosecurity environments (32). However, as far as we know, systematic analyses of the overall prevalence of brucellosis in pigs worldwide, are scarce. Hence, we conducted a systematic review and meta-analysis of *Brucella* spp. infection worldwide to analyze the pooled prevalence of brucellosis in pigs and to assess potential risk factors associated with brucellosis prevalence.

Abbreviations: WHO, World Health Organization; LPS, Lipopolysaccharide; S-LPS, Smooth lipopolysaccharide; CNKI, Chinese National Knowledge Infrastructure; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; OIE, Office International Des Epizooties.

MATERIALS AND METHODS

Search Strategy

Six databases were used to search the published research literature related to porcine brucellosis, including PubMed, ScienceDirect, SpringerLink, Web of Science, CNKI, Wanfang Data, and the VIP Chinese Journal Database. We retrieved all papers on worldwide *Brucella* spp. infection in swine that were published from January 1, 2000 to January 3, 2020 (the actual sampling dates in those publications were from 1980 to 2019).

In PubMed, the search terms and formulas used were "("*Brucella suis*" [MeSH] OR *Brucella melitensis biovar suis*) AND ("Swine"[MeSH] OR Suidae OR Pigs OR Warthogs OR Wart Hogs OR Hog, Wart OR Hogs, Wart OR Wart Hog OR Phacochoerus)." In ScienceDirect, we used the terms, "*Brucella suis,*" "swine," "pig" and "prevalence." In SpringerLink, we used the terms "*Brucella suis*" and "pigs." In Web of Science, we used the keywords "*Brucella suis*," "Swine" and "prevalence" to search for the "TOPIC" (the article topic). We used the term "*Brucella*" (in Chinese) or "*Brucella* spp." (in Chinese) or "Brucellosis" (in Chinese) in the CNKI database. In Wanfang Data, we used the terms "*Brucella*" (in Chinese) and "pigs" (in Chinese), or "*Brucella* spp." (in Chinese) and "pigs" (in Chinese), or "Brucellosis" (in Chinese) and "pigs" (in Chinese). The types of articles found in Wanfang Data were limited to "papers in journals, degree theses and conferences." The search formulas used in the VIP Chinese Journal Database consisted of "Title" or "keywords" = "*Brucella*" (in Chinese) or "Brucellosis" (in Chinese) and "pigs" (in Chinese). The search strategies and search restrictions are reported in **Supplementary Material 1**. We used different keywords ("*Brucella suis*," "brucellosis," "swine," "pigs," "prevalence" and "epidemiological investigation") in each database for search verification; however, no additional qualified studies were found. Endnote (version X9.3.1) was used to catalog the articles retrieved.

Eligible studies were selected in accordance with the following criteria (inclusion criteria):

- The subjects of the research must be swine.
- The study's aim must be to investigate the prevalence of *Brucella suis* in swine.
- Data must include information on the number of examined pigs and the number of *Brucella suis*-positive pigs.
- The study must be published in Chinese or English.

Articles with the following characteristics were excluded:

- Articles that did not match the titles and abstracts (see inclusion criteria).
- Repetition of articles or data.
- The hosts were not swine.
- The article was not research study.
- Unable to access the article's full text.
- Published before 2000.
- The article had one or more internal data conflicts.
- The number of samples was <30.

TABLE 1 | Normal distribution test of the original rates and the different transformations of the original rates.

Conversion form	W	P
PRAW	0.448	< 2.2e−16
PLN	NaN	NA
PLOGIT	NaN	NA
PAS	0.653	2.238e−15
PFT	0.647	1.653e−15

PRAW, original rate; PLN, logarithmic conversion; PLOGIT, logit transformation; PAS, arcsine transformation; PFT, double-arcsine transformation; NaN, meaningless number; NA, missing data.

Data Extraction and Quality Assessments of the Publications

The four reviewers used standardized data collection forms to extract data that were consistent with the criteria to qualify for inclusion in the meta-analysis (33). Any differences between the reviewers or uncertainty about the quality of the research were resolved through the intervention of the lead author (QLG). The following information was reported: first author, the sampling year, the year of publication, income level, geographical region of the study, detection method, age, gender, collection season, feeding mode, pig classification, total number of pig samples and the number of samples that tested positive for *Brucella*.

The quality of the publications was graded using a scoring approach (34). We scored each study, and assigned a score of 5 when the information was described in greater detail (i.e., random sampling, detection method used, sampling method, sampling year and analyses of four or more factors). All the papers were assigned 0-5 points based on the standards. The quality of the papers with 3, 4 or 5 points was considered to be high, papers with a score of 2 points were considered to be average and those with 0 or 1 point were considered to be of low quality.

Statistical Analysis

Based on a large number of studies, all calculations, including the prevalence of *Brucella* spp. in swine were performed using R software (version 3.5.2). We chose the double-arcsine transformation (PFT) to perform the rate conversions (**Table 1**), based on these results and those of previous studies (35). The formula of PFT was as followed:

$$t = \arcsin\{\mathrm{sqrt}[r/(n+1)]\} + \arcsin\{\mathrm{sqrt}[(r+1)/(n+1)]\}$$
$$se(t) = \mathrm{sqrt}[1/(n+0.5)]$$
$$p = [\sin(t/2)]^2$$

Note: t: transformed prevalence; r = positive number; n = sample size; se = standard error.

We used forest plots to visualize the results of the analyses and to evaluate the heterogeneity between the studies. Heterogeneity was calculated using Cochran's *Q*-test, the I^2 statistic and the χ^2 test ($P < 0.05$), and the cutoff value for the I^2 statistic was 50%. These two methods were used to examine the degree of statistical significance of the heterogeneity between the selected studies. We used a random effects model for the meta-analysis when heterogeneity was apparent in the selected articles (36). The funnel plot, trim and fill method and Egger's test were used to evaluate the studies for publication bias. Studies have shown that different subgroups may generate different funnel plots because of prevalence changes over time (37). Therefore, a funnel plot and forest plot were used for further evaluation of each subgroup. A sensitivity analysis was conducted to check whether any one study would have a significant impact on the estimates (38).

Heterogeneity between studies is an important indicator in meta-analyses; thus, an accurate assessment of heterogeneity is necessary to finding the key for preventing *Brucella* spp. infection in pigs worldwide. In order to examine the potential sources of heterogeneity, we analyzed the research data using subgroup analyses and univariate regression analysis to identify factors predictive of heterogeneity. The investigated factors consisted of geographical region (comparisons between Asia and other regions), the period of data collection (2006 to 2010 compared to 2000 or before, 2001 to 2005, 2011 to 2015 and 2016 or later), income (comparisons of high- with low- and middle-incomes), detection methods (comparison of the RBPT & TAT with other serological or molecular biology-based methods), season (comparisons of summer with spring, autumn and winter), gender (comparison of boars with sows), pigs' age classifications (comparisons of suckling pigs with finishing, growing and weaning pigs), feeding modes (comparison of extensive farms with intensive farms), pig classification (comparison of feral with domestic pigs) and quality of studies (comparisons of high-quality studies with average-quality studies). This meta-analysis was performed in accordance with the PRISMA guidelines (**Supplementary Material 2**) (39–41). A correlation analysis was performed for each subgroup by detection method and country in order to track the source of heterogeneity. The heterogeneity of the covariates is represented by R^2. Our meta-analysis does not include a review agreement and is not registered in the Cochrane database. The code in R for this meta-analysis was presented in **Supplementary Material 3**.

RESULTS

Search Results and Quality of the Eligible Studies

A total of 2,530 studies were retrieved from the seven databases. We conducted the meta-analysis with 119 studies based on our

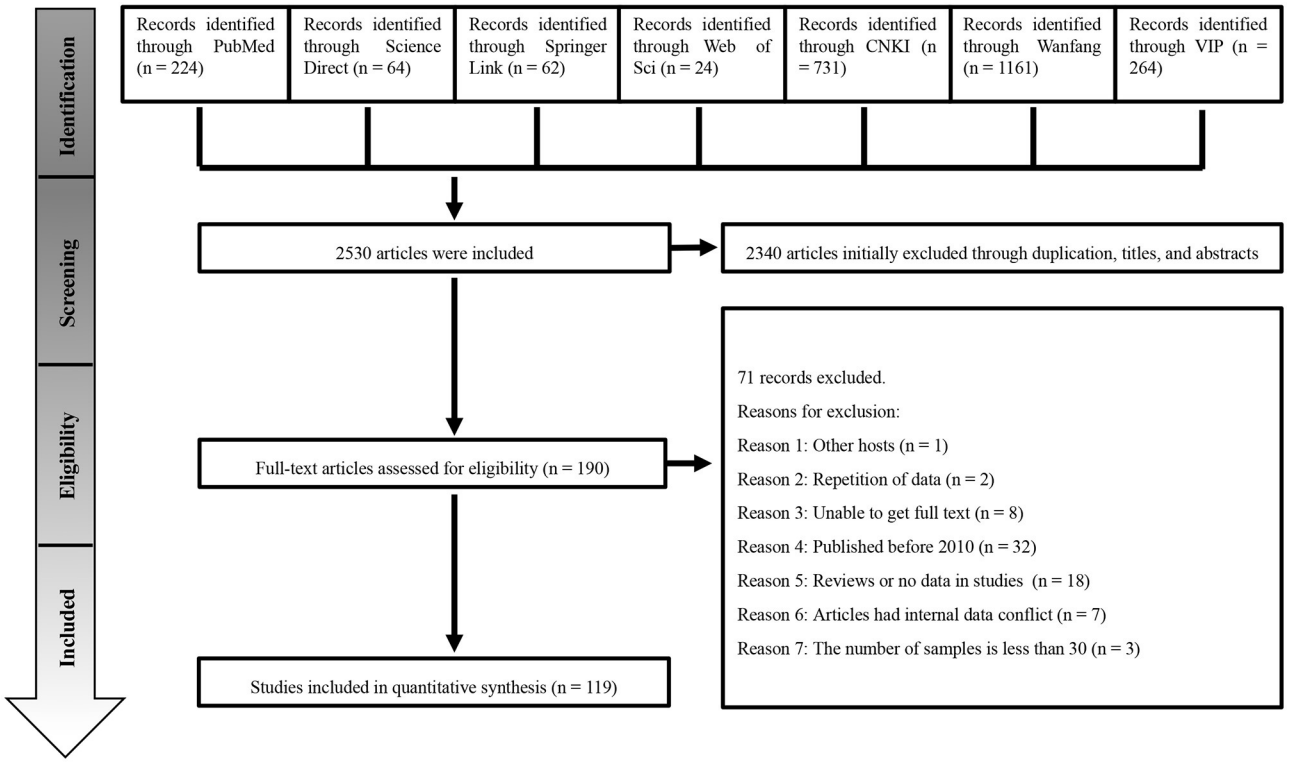

FIGURE 1 | Flow diagram of the search strategies and selection of studies.

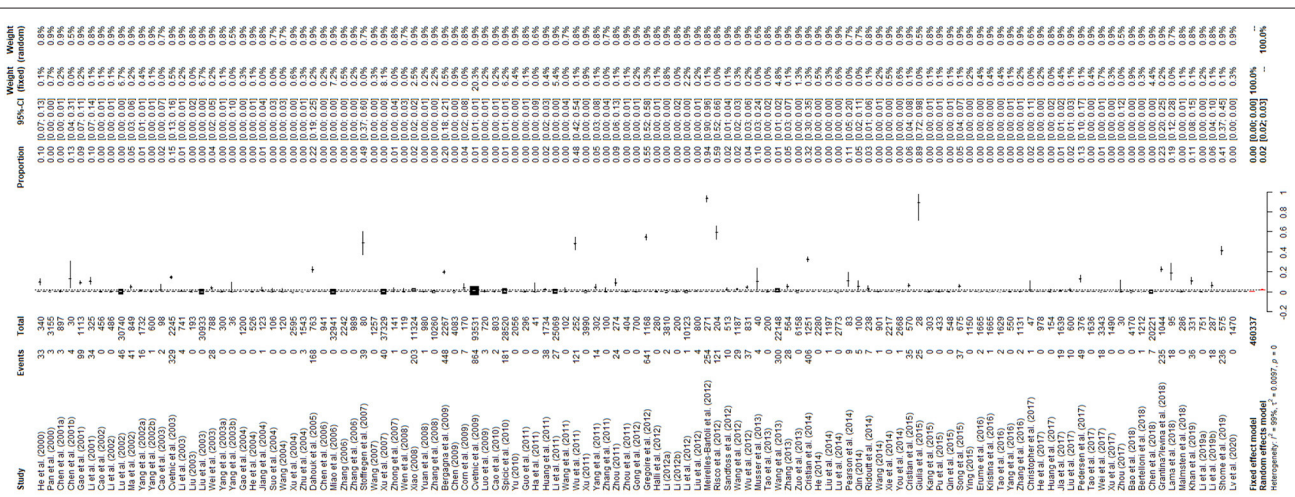

FIGURE 2 | Forest plot of the worldwide prevalence of *Brucella* spp. The length of the horizontal line represents the 95% confidence interval, and the diamonds represent the summarized effect.

inclusion and exclusion criteria (**Figure 1**). The included articles consisted of 41 high-quality publications (4 or 5 points), 78 average-quality publications (2 or 3 points) and no low-quality publications (0 or 1 point; **Supplementary Materials 4**, **5**).

Results of Publication Bias

The results of the forest plot showed a high degree of heterogeneity between studies (I^2 = 99.3%, P = 0; **Figure 2**). The funnel plot showed that the graph was asymmetric, indicating the possibility of publication bias or small study effects (**Figure 3**). Egger's test showed significant publication bias in the included studies (P < 0.05; **Supplementary Materials 6**, **7**). The results of the trim and fill method showed that some studies were filled, indicating publication bias or small study effects (**Supplementary Material 8**). In addition, we evaluated

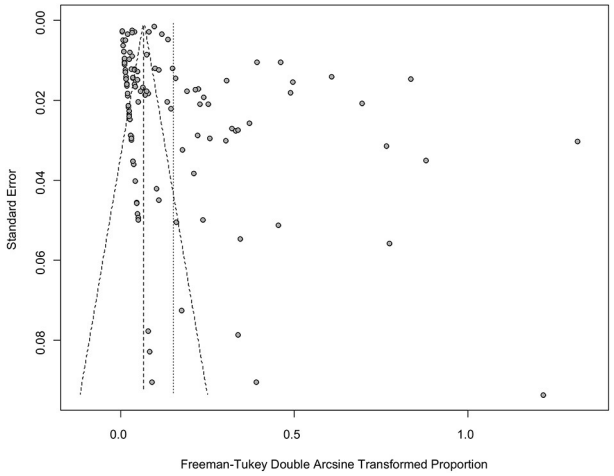

FIGURE 3 | Funnel plot with pseudo 95% confidence intervals for the publication bias test.

publication bias in all subgroups using funnel plots (**Supplementary Materials 9–18**).

Results of Sensitivity Analyses

The results of the sensitivity analysis showed that when a study was omitted, the analysis of the remaining studies yielded the same results as the previous analysis. Therefore, the results of our systematic review and meta-analysis were relatively stable and reliable (**Supplementary Material 19**).

Meta-Analysis of *Brucella* spp. in Swine worldwide

Our meta-analysis included five global geographic regions, namely Africa, America, Asia, Europe and Oceania. The pooled prevalence of *Brucella* spp. in swine worldwide was 2.1% (95% CI: 1.6–2.6; **Table 2**). Among the regional subgroups, the highest prevalence occurred in Europe, which was 17.4% (95% CI: 11.1–24.9; **Table 2**). Among the countries, Brazil had the highest rate of 93.73% (95% CI 90.5–96.3; **Table 2**), followed by Spain, with a rate of 59.3% (95% CI: 52.5–66.0; **Table 3**).

We conducted a subgroup analysis of sampling years, income level, detection method, season, gender, age, feeding mode, pig classification and quality of studies to explore their influence on the prevalence of *Brucella* spp. in swine. Among them, regions, sampling years, income level, detection method, age of pigs, pig classification and quality of the study were identified as risk factors for *Brucella* spp. infection in pigs ($P < 0.05$; **Table 2**). The combined prevalence of *Brucella* spp. in sampling years 2006 to 2010 was 2.7% (95% CI: 1.1–4.8; **Table 2**), which was higher than the other four periods. The estimate of prevalence in the high-income group was 15.7% (95% CI: 8.0–25.3; **Table 2**), which was higher than that of the low- and middle-income groups. In the detection methods subgroup, the CFT showed a prevalence of 26.5% (95% CI: 0.0–80.3; **Table 2**). The point estimate of the prevalence of *Brucella* spp. in pigs during the summer was the highest at 11.1% (95% CI: 0.0–63.0; **Table 2**). Compared with the other ages of the pigs, the prevalence of *Brucella* spp. among the finishing pigs (4.9%, 95% CI: 0.9–11.0; **Table 2**) was higher than that of the growing pigs, suckling pigs and weaning pigs. The prevalence of *Brucella* spp. in feral pigs (15.0%, 95% CI: 8.4–23.2) was significantly higher than that of domestic pigs. The subgroup analysis by quality of study showed the prevalence of *Brucella* spp. in swine was higher in the studies of high quality (5.0%, 95% CI: 3.5–6.7; **Table 2**). The heterogeneity of each subgroup was explained by detection method (the covariate), which ranged from 0–79.25% (R^2-method), and countries (the covariate), which was 60.97–97.04% (R^2-country).

DISCUSSION

Brucellosis is a zoonotic infectious disease caused by *Brucella*. It is the main cause of infertility, low litter size and miscarriage among sows (42) and an occupational hazard for farmers, slaughterhouse workers and veterinarians (43, 44). The OIE, WHO and Food and Agriculture Organization of the United Nations have classified brucellosis as one of the most important neglected occupational hazards in the world (45, 46). It has had a significant economic impact on the livestock industry and other industries (47). The prevalence of brucellosis plays an important role in the development of the world's pig herds (48). Therefore, we conducted the first meta-analysis to examine the prevalence of brucellosis in pig herds around the world and found that it was unevenly distributed among pigs.

Brucellosis in swine has been widely distributed worldwide for a long time, but in some high-income countries, including Canada, Australia, New Zealand and other countries, the eradication of brucellosis in animal husbandry has been successfully achieved (49, 50). Brucellosis has not been reported in domestic pigs in Belgium since 1969 (51). The United States has implemented reforms in pig management since 1950 to eliminate brucellosis in livestock, and efforts have been made to solve the problem of brucellosis infection in wild animals with almost complete eradication of it in livestock populations (52). However, in our study, the highest prevalence rates were found in America and Europe, and the prevalence rates in the high-income countries were higher than those in middle- and low-income countries. We attribute these results to several factors. First, most of the samples tested in these countries were feral or domestic pigs in contact with wild boars. This was confirmed in the subgroup analysis of pig classification, which showed the prevalence of feral pigs was significantly higher than that of domestic pigs. In recent decades, the population of wild boars has increased rapidly in the United States, which is caused mainly by natural population dynamics, and brucellosis has been reported in wild boars in 14 states (53). In Belgium, the increase in the wild boar population and the prevalence of brucellosis has led to an increased risk of infection in outdoor pig farms (54). Therefore, overabundance of wildlife is considered to be an important factor in the transmission of brucellosis between wildlife and livestock (55). After the first isolation of the *Brucella suis* biovar 2 strain from boars killed by hunters

TABLE 2 | Pooled worldwide prevalence of *Brucella* spp. by region.

	No. studies	No. tested	No. positive	% (95% CI*)	Heterogeneity			Univariate meta-regression		Joint analysis*	
					χ^2	*P*-value	I^2 (%)	*P*-value	Coefficient (95% CI)	R^2-method	R^2-country
Regions*											
Africa	3	3,661	39	1.7% (0.0–6.7)	111.64	< 0.01	98.2%				
America	9	2,570	382	16.5% (1.3–42.8)	1,580.03	0.00	99.5%				
Asia	90	320,164	1,429	0.5% (0.3–0.7)	4,029.49	0.00	97.8%	< 0.001	−0.295 (−0.3378 to −0.253)	0.00%	68.49%
Europe	15	133,621	3,575	17.4% (11.1–24.9)	7,805.04	0.00	99.8%				
Oceania	2	321	16	6.0% (0.5–16.0)	6.73	< 0.01	85.1%				
Sampling years											
2000 or before	41	81,246	922	0.7% (0.3–1.4)	2,252.12	0.00	98.2%				
2001 to 2005	42	140,647	1,182	0.7% (0.4–1.1)	2,182.60	< 0.01	98.1%				
2006 to 2010	35	55,798	1,095	2.7% (1.1–4.8)	4,111.44	0.00	99.2%	< 0.001	0.075 (0.039 to 0.112)	12.16%	67.09%
2011 to 2015	31	32,998	374	0.6% (0.1–1.3)	1,154.60	< 0.01	97.4%				
2016 or later	12	8,965	82	0.7% (0.0–2.0)	289.89	< 0.01	96.2%				
Income level*											
Low	2	3,330	3	0.1% (0.0–0.2)	0.27	–	–				
Middle	95	445,062	3,093	0.8% (0.5–1.1)	7,877.36	0.00	98.8%				
High	22	11,945	2,345	15.7% (8.0–25.3)	3,438.12	0.00	99.4%	< 0.001	0.297 (0.256 to 0.338)	19.42%	70.65%
Detection method*											
CFT	6	2,174	295	26.5% (0.0–80.3)	1,682.83	< 0.01	99.7%				
ELISA	11	35,170	1,216	9.4% (2.1–21.0)	3,647.44	0.00	99.7%				
PCR	3	623	101	16.3% (5.0–32.2)	41.30	< 0.01	95.2%				
RBPT	55	173,023	1,846	1.4% (0.8–2.1)	5,727.35	0.00	99.1%				
RBPT & TAT	23	105,223	162	0.2% (0.1–0.4)	479.88	< 0.01	95.4%	< 0.001	−0.152 (−0.206 to −0.103)	0.00%	66.46%
TAT	17	20,121	275	0.5% (0.0–1.4)	312.29	< 0.01	94.9%				
Others	26	112,102	3,169	11.0% (6.1–17.2)	6,830.99	0.00	99.6%				
Season*											
Spring	11	3,716	69	2.6% (0.0–3.8)	226.43	< 0.01	95.6%				
Summer	5	1,163	258	11.1% (0.0–63.0)	1,300.31	< 0.01	99.7%	0.077	0.195 (−0.021 to 0.411)	36.17%	97.04%
Autumn	6	1,452	12	0.7% (0.0–3.8)	46.65	< 0.01	89.3%				
Winter	6	3,444	21	1.2% (0.1–3.3)	63.17	< 0.01	92.1%				
Gender											
Boars	23	12,737	1282	7.9% (2.4–15.9)	3,594.80	0.00	99.4%	0.314	0.056 (−0.053 to 0.165)	75.00%	82.75%
Sows	29	51,698	1288	5.1% (2.8–8.1)	3,208.62	0.00	99.1%				
Age of pigs											
Finishing pigs	14	5,778	321	4.9% (0.9–11.0)	684.93	< 0.01	98.1%				
Growing pigs	18	31,454	411	2.1% (0.2–5.2)	1271.98	< 0.01	98.7%				

(Continued)

TABLE 2 | Continued

	No. studies	No. tested	No. positive	% (95% CI*)	Heterogeneity			Univariate meta-regression		Joint analysis*	
					χ^2	*P*-value	I^2 (%)	*P*-value	Coefficient (95% CI)	R^2-method	R^2-country
Suckling pigs	9	1,917	7	0.0% (0.0–0.5)	15.01	0.05	46.7%	0.045	−0.120 (−0.237 to −0.003)	79.25%	87.44%
Weaning pigs	5	1,015	28	1.0% (0.0–14.2)	98.67	< 0.01	95.9%				
Feeding mode											
Extensive	12	34,083	555	2.5% (0.4–5.9)	1,823.79	0.00	99.2%	0.065	0.076 (−0.005 to 0.156)	6.50%	68.57%
Intensive	35	55,196	468	0.5% (0.1–1.2)	1,790.10	0.00	98.9%				
Pig classification											
Domestic pigs	21	131,196	1,504	1.1% (0.2–2.5)	2,397.46	0.00	99.5%				
Feral pigs	21	9,186	2,085	15.0% (8.4–23.2)	1,838.57	0.00	99.0%	< 0.001	0.277 (0.197 to 0.357)	0.00%	74.40%
Quality level											
Middle	78	203,157	1,201	1.0% (0.6–1.3)	4,344.03	0.00	98.2%				
High	41	257,180	4,240	5.0% (3.5–6.7)	11,773.05	0.00	99.7%	< 0.001	0.117 (0.078 to 0.157)	9.21%	60.97%
Total	119	460,337	5,441	2.1% (1.6–2.6)	16,698.88	0.000	99.3%				

CI, Confidence interval; Joint analysis*, Joint analysis with prevalence of detection methods and provinces in China; R^2, Proportion of between-study variance explained.*

Region: Africa: India, Uganda; America: Brazil. USA; Asia: China. India; Europe: Belgium, Croatia, Finland, Germany, Italy, Latvia, Sweden; Oceania: Australia.*

Method: CFT: Complement fixation test; ELISA: Enzyme linked immunosorbent assay; PCR: Polymerase chain reaction; RBPT: Rose Bengal plate test; RBPT&TAT: Rose Bengal plate test and Tube agglutination test; TAT: Tube agglutination test.*

Season: Spring: Mar. to May.; Summer: Jun. to Aug.; Autumn: Sep. to Nov.; Winter: Dec. to Feb.*

Income level: High: Developed Country; Middle: Developing Country; Low: Least Developed Country.

TABLE 3 | Estimated pooled seroprevalence of *Brucella* spp. by country and region worldwide.

Countries	No. studies	Region	No. tested	No. positive	% Prevalence	% (95% CI)
Australia	2	Oceania	321	16	6.0%	0.5–16.0
Belgium	1	Europe	1,168	641	54.9%	52.0–57.7
Brazil	1	America	271	254	93.7%	90.5–96.3
China	89	Asia	319,589	1,193	0.3%	0.2–0.5
Croatia	3	Europe	124,296	1,374	3.5%	1.3–6.8
Egypt	1	Africa	331	36	10.9%	7.7–14.5
Finland	1	Europe	280	0	0.0%	0.0–0.6
Germany	1	Europe	763	168	22.0%	19.2–25.0
India	1	Asia	575	236	41.0%	37.1–45.1
Italy	5	Europe	5,328	915	22.6%	6.7–44.3
Spain	1	Europe	204	121	59.3%	52.5–66.0
Latvia	1	Europe	1,044	235	22.5%	20.0–25.1
Sweden	1	Europe	286	0	0.0%	0.0–0.6
Switzerland	1	Europe	252	121	48.0%	41.9–54.2
Uganda	2	Africa	3,330	3	0.1%	0.0–0.2
USA	8	America	2,299	128	8.7%	2.0–19.2
Total	119		460,337	5,441	2.1%	1.6–2.6

in 1994 (56), *Brucella suis* biovar 2 has been isolated from wild boars in many countries (57–61). Studies have reported that brucellosis among wild boars is widely distributed all over the world (51, 56, 57, 62–65). Second, different modes of feeding in different countries have led to different prevalence rate. Most developed countries mainly focus on intensive farming, while countries with lower incomes focus mainly focus on extensive farming. The prevalence of disease in countries with intensive farming is higher than that in countries with extensive farming, which has been confirmed in several studies (66–68). The increase in herd size results in higher stocking density and worse farm sanitation, thereby promoting the spread of *Brucella* among animals after abortion and parturition (69, 70). We recommend long-term monitoring of wildlife to implement preventive measures before an outbreak of brucellosis. Intensive farms need to control breeding density, pay attention to animal welfare, improve the prevention and control of epidemics and optimize the breeding environment to avoid the large-scale spread of disease. It is worth noting that among the studies we included, only a few on swine brucellosis were conducted in low-income countries. This may indicate that swine brucellosis has been overlooked in these countries and regions. Therefore, although our results show that the prevalence of brucellosis in low-income countries is lower than that of other countries, this finding may be due to these countries' neglect of surveillance and detection of brucellosis. Likewise, the farms in high-income countries are more capable of strengthening their detection of brucellosis, thus, showing a relatively high prevalence. We infer that the actual global infection rate of swine brucellosis may be higher. Although the disease has been controlled or eliminated in some developed countries (such as Canada, New Zealand, Australia and the majority of northern European countries) (71), brucellosis remains an intractable public health problem in poor and underdeveloped countries, especially in the Middle East (72). While strengthening the surveillance and prevention of swine brucellosis in high-incidence areas, we should also continue to strengthen the surveillance in lower incidence areas to prevent widespread infection of swine brucellosis.

The 2006-2010 prevalence of brucellosis was higher than that of the other sampling years. First, as reported in the included studies, an outbreak of swine brucellosis in Jaboticabal, Brazil in 2006 increased the prevalence to 93.7% (73). At the same time, brucellosis was found in Italy after collecting samples from pig farms with breeding problems for serological analysis. Furthermore, detection of suspected cases (non-random sampling) may overestimate the local prevalence of brucellosis (74, 75). Second, during this period, many countries began to analyze the situation of *Brucella* infection in feral pigs. Among the included articles, there were 21 studies on the prevalence of wild boars, and the prevalence of wild boars between 2006 and 2010 was 22.3% (881/3956). There were also studies on isolated *Brucella suis* biovar 2 from wild boars because of the substantial increase in the number of feral pigs (58–60, 76). Moreover, several *Brucella* outbreaks occurred in Germany due to infection of domestic pigs by feral pigs (77). After 2010, the prevalence of brucellosis gradually declined because the OIE proposed controls for *Brucella* farms, giving priority to the development of food safety standards for future animal production (78). Although the prevalence of brucellosis has shown a downward trend, its control should be continued.

The 119 selected studies that were analyzed included five main methods of brucellosis detection: CFT, ELISA, PCR, RBPT and TAT ($P < 0.001$; **Table 2**). We used detection method as a covariate to perform joint analysis with other risk factors, and the range of heterogeneity explained by the detection method was 0-100.00%, implying that different detection methods had a greater effect on some subgroups.

The analyses of the age and sex of pigs showed that the prevalence of finishing pigs was higher than that of the pigs in the other age groups, and the prevalence of sows was lower than

that of the boars. This finding is mainly due to the adult males' contacts with these matrilineal groups during the mating season, while females live in matrilineal groups (60). The prevalence observed in the finishing pigs was higher than that in the other age groups, which was due to the higher involvement of the finishing pigs (79). Although the prevalence of boars was higher than that of sows, no significant differences were found between them, indicating that the relationship between the two animals warrants further examination. We recommend controlling the breeding density; planning a reasonable breeding process may play a positive role in the reduction of the spread of brucellosis in pigs.

The prevalence of brucellosis in the summer was higher than that in the other seasons, but the difference was not significant (**Table 2**). As far as we know, no research has shown a strong correlation between breeding season and prevalence of swine brucellosis. Studies have shown that the dryness of summer may lead to lack of food and water, increasing the trajectory coverage of animals (80). The correlation analysis showed that countries explained 97.04% of heterogeneity in the seasons subgroup. These results can be interpreted in the context of the world's vast territory with different countries having different characteristics during summer. Meta-analyses showed that the incidence rates of *Brucella* in cattle and deer were higher in hotter and more humid areas (81, 82). Therefore, we speculate that a similar phenomenon occurs in pigs with brucellosis. Thus, efforts to prevent epidemics should be increased in hot and humid areas to create a healthy environment for livestock and reduce the occurrence of disease.

Our study included 41 high-quality articles and 78 average-quality articles. We found that random sampling and detailed descriptions of sampling methods were not included in some of the articles by examining those of average quality. These findings may reflect sampling bias. We recommend that researchers provide detailed descriptions of their sampling and data collection methods to improve the reliability of the data.

This meta-analysis had the advantages of a long-time span, wide coverage and clear methods of analyses, yet it has some limitations. First, the language of the selected articles was limited to English or Chinese, and therefore, qualified articles in other languages might have been overlooked. Second, the articles were obtained from seven databases, which might have excluded qualified articles from other databases. Third, the inadequate information provided by the included studies (e.g., brucellosis classification and geographical factors) might have led to publication bias or other biases in the subgroups (**Supplementary Figures 3–12**). Fourth, some risk factors were examined in a small number of studies and samples, which might have resulted in small study effects leading to unstable results. We recommend that researchers conduct large-scale studies because the results of small-scale studies are often not representative of the population. Fifth, the research we have included covers only 14 countries, and some of those countries (e.g., Brazil) had few relevant reports. For these countries, we only presented data to reflect global trends, and the results presented are for reference only. The lack of articles from some countries might have led to inaccurate estimates of the prevalence of swine brucellosis in those countries. Prevalence surveys of *Brucella* spp. in more countries are recommended to clarify the true prevalence of swine brucellosis worldwide. Sixth, this study was not registered; however, it was carried out strictly in accordance with the PRISMA guidelines.

In conclusion, the *Brucella* infection rate in pig herds is distributed widely throughout the world. In addition, *Brucella* is common among wild boars in developed countries. Therefore, we suggest carrying out long-term detection of *Brucella* in wild animals and implementing reasonable isolation measures between livestock and wild animals to reduce the chance of contact between them. In addition, countries that do not pay much attention to swine brucellosis should disseminate information about *Brucella* infection, and epidemiological investigations should be conducted as soon as possible to establish better control of the disease. The high prevalence of swine brucellosis will cause serious economic losses to herdsmen, and increase the risk of infection. Therefore, attention to animal welfare on intensive pig farms is crucial, and control of the breeding density may play an important role in reducing the spread of brucellosis in pigs. This study can provide a theoretical basis for researchers to explore control schemes for brucellosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

YZ and RD: idea contributions and funding. BZ, G-YG, Z-YC, and YY: data extraction. Y-HS: database establishment. QW and J-ML: data analysis. Q-LG: writing – original draft. Y-HS, KS, and XL: writing – review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the National Key R & D Program of China (2018YFD0500900) and the Scientific Research Planning Project of the Jilin Provincial Department of Education (JJKH20200364KJ).

ACKNOWLEDGMENTS

We thank the scientists and personnel of the College of Animal Science and Technology, Jilin Agricultural University and the College of Chinese Medicine Materials, Jilin Agricultural University, for their collaboration.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.630960/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

REVIEW
published: 24 May 2021
doi: 10.3389/fvets.2021.635155

Application of Volatilome Analysis to the Diagnosis of Mycobacteria Infection in Livestock

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Volatile organic compounds (VOCs) are small molecular mass metabolites which compose the volatilome, whose analysis has been widely employed in different areas. This innovative approach has emerged in research as a diagnostic alternative to different diseases in human and veterinary medicine, which still present constraints regarding analytical and diagnostic sensitivity. Such is the case of the infection by mycobacteria responsible for tuberculosis and paratuberculosis in livestock. Although eradication and control programs have been partly managed with success in many countries worldwide, the often low sensitivity of the current diagnostic techniques against *Mycobacterium bovis* (as well as other mycobacteria from *Mycobacterium tuberculosis* complex) and *Mycobacterium avium* subsp. *paratuberculosis* together with other hurdles such as low mycobacteria loads in samples, a tedious process of microbiological culture, inhibition by many variables, or intermittent shedding of the mycobacteria highlight the importance of evaluating new techniques that open different options and complement the diagnostic paradigm. In this sense, volatilome analysis stands as a potential option because it fulfills part of the mycobacterial diagnosis requirements. The aim of the present review is to compile the information related to the diagnosis of tuberculosis and paratuberculosis in livestock through the analysis of VOCs by using different biological matrices. The analytical techniques used for the evaluation of VOCs are discussed focusing on the advantages and drawbacks offered compared with the routine diagnostic tools. In addition, the differences described in the literature among *in vivo* and *in vitro* assays, natural and experimental infections, and the use of specific VOCs (targeted analysis) and complete VOC pattern (non-targeted analysis) are highlighted. This review emphasizes how this methodology could be useful in the problematic diagnosis of tuberculosis and paratuberculosis in livestock and poses challenges to be addressed in future research.

Keywords: diagnosis, livestock, mycobacteria, volatilome, veterinary

OPEN ACCESS

Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

Reviewed by:
Douwe Bakker, Independent Researcher, Lelystad, Netherlands
Wolfram Miekisch, University Hospital Rostock, Germany

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *29 November 2020*
Accepted: *08 April 2021*
Published: *24 May 2021*

Citation:
Rodríguez-Hernández P, Rodríguez-Estévez V, Arce L and Gómez-Laguna J (2021) Application of Volatilome Analysis to the Diagnosis of Mycobacteria Infection in Livestock. Front. Vet. Sci. 8:635155. doi: 10.3389/fvets.2021.635155

INTRODUCTION

Analysis of volatile organic compounds (VOCs) is an emerging research area in both human and veterinary medicine (1), which allows a non-invasive, fast, and economic diagnosis as well as identification of new biomarkers as alternative to current diagnostic techniques (2). VOCs are defined as a sub-category of small molecular mass substances within metabolites, which are characterized by its low boiling point and high vapor pressure (3, 4). VOCs are produced into the environment, allowing a direct measuring in the gas phase and offering a minimum sample handling, a non-invasive monitoring, and an easier sampling compared with other metabolites which have to be extracted from biological samples (5, 6). In this context, volatilome (or volatome) is the VOCs' signature produced by an organism (7–10).

The volatilome has a wide variety of uses and applications, such as diagnosis of infectious diseases (11) and neoplasia (12), distinction between vaccinated and non-vaccinated animals (13), monitoring of antibiotic treatment (14), differentiation of diet composition (15, 16), and even evaluation of reproductive parameters (17). Because VOCs are constantly emitted during metabolic processes, the detection of VOC profiles might enable the development of novel non-invasive diagnostic tools (7).

The identification of VOCs produced by pathogens, host–pathogen interactions, and biochemical pathways, either associated with homeostasis or pathophysiological responses, has become the volatilome into an approach of growing interest for the diagnosis of infectious diseases (18). Pathologic processes have the capacity to modify VOCs' patterns either by producing new volatile substances or by the metabolic consumption of VOC substrates that are normally present (19). Consequently, the diagnostic approach of VOC analysis provides two perspectives, the search of new biomarkers and the identification of biomarkers lost along a pathological process (1).

Infection of livestock by slow-growing mycobacteria, such as those grouped under *Mycobacterium tuberculosis* complex (MTBC), especially *Mycobacterium bovis*, as well as *M. avium* subsp. *paratuberculosis* (MAP), might take advantage of the development of faster and sensitive diagnostic techniques. Considering the growth requirements of these mycobacteria, as well as other factors associated with the host immune response after infection, diagnosis of mycobacterial infections becomes a challenge, especially in the livestock sector. The diagnosis of the infection by mycobacteria is currently based on different tedious, expensive, laborious, and time-consuming methodologies (20–22). Thus, the analysis of VOCs could be proposed as an innovative strategy to improve the diagnostic field of these infections (**Table 1**) supported by the fact that, historically, people suffering from tuberculosis had a characteristic breath smell (24). The research carried out in this context has used different biological matrices, such as serum (33, 34), breath (34, 35), feces (13, 28), and microbiological culture (36–39) to identify biomarkers related to diseases produced by mycobacteria.

Although the use of VOCs obtained from different biological samples to diagnose diseases is considered as a big hope with a promising future, now it remains at a developing stage (40). One of the main hurdles against the development of this new strategy is the lack of standardization between studies which often leads to non-comparable results (40, 41). Few detailed *in vivo* studies are available on the analysis of VOCs as a diagnostic tool for mycobacterial infection in animals. In light of these premises, the present review collects the available literature from the volatilome approach to evaluate the recent methodologies and procedures used as an attempt of improvement of the diagnosis of infection by mycobacteria in livestock, focusing on the infection by MTBC (*Mycobacterium bovis*) and MAP, to point out future research lines of interest to be implemented.

MYCOBACTERIA TARGET OF STUDY

Mycobacteria belong to the genus *Mycobacterium* which includes the MTBC, with all the causative species of human and mammal tuberculosis; the *M. avium* complex (MAC), which also comprises species of relevance in human and veterinary medicine, such as MAP; as well as environmental rapid and slow-growing non-tuberculous mycobacteria. These all are aerobic and immobile bacilli with specific growing conditions which include pathogenic, opportunistic, and saprophytic species (42, 43). While there are many species, such as *M. tuberculosis* and *M. bovis*, known for being the etiological agents of important human and animal diseases, rapid- and slow-growing non-tuberculous mycobacteria used to be minority species, which should be considered because of their interference with the currently established diagnostic strategies (44).

Mycobacterium tuberculosis Complex (*Mycobacterium bovis*)

MTBC is composed by a broad group of mycobacteria species characterized for its genetic proximity and its pathogenic ability of affecting humans, such as *M. tuberculosis* and *M. africanum*, and a wide variety of wild and domestic animals, such as *M. bovis* and *M. caprae*. *M. bovis* stands out for being the primary etiological agent responsible for bovine tuberculosis (bTB), also considered as the main cause of animal tuberculosis due to the multi-host character of this bacterium (45). Animal tuberculosis is a zoonotic disease with great impact on public health, agriculture, wildlife, and trade areas (20, 46). In this sense, although most cases reported as human tuberculosis are caused by *M. tuberculosis*, ∼30% of these cases are related to *M. bovis* infection (zoonotic tuberculosis) (28), especially in developing countries (47) where prevalence of livestock bTB becomes substantial (48–50). Despite huge efforts that are currently focused on the eradication of bTB, there are many difficulties mainly associated with the performance of the different diagnostic techniques as well as with other geographical and epidemiological conditions, which make it very difficult in endemic countries (51). Therefore, zoonotic tuberculosis is often under-reported, emphasizing the importance of providing appropriate diagnostic tools in livestock to reach the eradication of *M. bovis* and reduce zoonotic tuberculosis cases.

TABLE 1 | *In vivo* studies evaluating VOC analysis as a diagnostic tool for mycobacterial infection in animals.

Mycobacteria species and animal species	Matrix	Analytical technique	Kind of infection	Sensitivity	Specificity	References
Mycobacterium bovis						
Cattle	Serum	EN	Experimental	–	–	(23)
Badger	Serum	EN	Natural and experimental	–	–	(23)
Badger	Serum	SIFT-MS	Natural	88%	62%	(24)
Cattle	Exhaled breath	GC-MS EN	Natural	– 100%	– 79%	(25)
Cattle	Exhaled breath	ATD-GC-MS	Experimental	–	–	(26)
Cattle	Exhaled breath	GC-MS	Experimental	83.8–96.4%	97.4–99.2%	(18)
Cattle	Serum	EN	Natural	–	–	(27)
White-tailed deer	Feces	GC-MS	Experimental	78.6%	91.4%	(13)
Cattle	Feces	GC-MS	Experimental	83–100%	100%	(28)
Wild boar	Exhaled breath	GC-MS	Natural	100%	90%	(29)
Wild boar	Feces	GC-MS	Natural	100%	80%	(29)
Mycobacterium avium* subsp. *paratuberculosis						
Cattle	Serum	EN	Natural	–	–	(30)
Goat	Exhaled breath and feces	DMS	Experimental	–	–	(1)
Goat	Exhaled breath and feces	GC-MS	Experimental	–	–	(31)
Goat	Exhaled breath and feces	GC-MS	Experimental	Exhaled breath: 90.3% Feces: 86.6%	Exhaled breath: 81.8% Feces: 85.0%	(32)

ATD-GC-MS, thermal desorption–gas chromatography–mass spectrometry; DMS, differential mobility spectrometry; EN, electronic nose; GC-MS, gas chromatography–mass spectrometry; GC-GC-MS, two-dimensional gas chromatography–mass spectrometry; SIFT-MS, selected ion flow tube mass spectrometry.

Mycobacterium avium subsp. *paratuberculosis*

MAP is the causative agent of paratuberculosis (PTB) or Johne's disease, a chronic infection that affects the small intestine of ruminants resulting in a marked reduction of animal productivity (31) and sometimes in death (1). MAP is also believed to be related to Crohn's disease, a chronic bowel disease in humans, although this fact is yet to be defined (52–54).

The main importance of PTB comes from the great economic losses in animals due to reduced milk and meat yields as well as slaughter value (32). MAP diagnosis becomes a challenge because of its pathogenesis: while the main clinical signs are only present in the late progression of the disease, when the body condition is severely affected, the animals intermittently spread bacteria during a previous subclinical phase. These features result in a low sensitivity of the current direct (fecal culture and genome detection) and indirect (specific antibodies detection) diagnostic methods (55, 56) (**Table 2**). Hence, reliable and complementary diagnostic methodologies are of key importance to enhance the current diagnostic repertoire of techniques focused on the identification of infected animals so as to improve the sensitivity of the diagnosis.

ROUTINE DIAGNOSTIC TECHNIQUES AGAINST MTBC AND *M. avium* SUBSP. *paratuberculosis*

The diagnosis of mycobacterial infection is currently at the center of attention because, although well-established and reliable, it has its own limitations. Apart from being a tedious process, special consideration must be given to the lack of an optimal diagnostic sensitivity (**Table 2**) and the different variables which may interfere with the methods and techniques in use (2, 21). Therefore, an accurate and reliable diagnostic methodology of the infection by mycobacteria, or a combination of various strategies, is the cornerstone of their control (23).

Current *Ante-mortem* and *Post-mortem* Diagnostic Techniques Against MTBC

Field and *ante-mortem* surveillance tests against MTBC infection are mainly based on the detection of a delayed-type hypersensitivity response to the intradermal skin test (IST) through the inoculation of purified protein derivative from *M. bovis* (bPPD; tuberculin protein), and on quantifying the concentration of gamma interferon (IFN-γ) after culturing blood samples in the presence of tuberculin, in the case of IFN-γ assay test, a supplemental or confirmatory test (25). IST is the OIE prescribed test for international trade and is currently considered as the official diagnostic screening technique in many countries worldwide; it is the primary *ante-mortem* test to support control and eradication programs in different geographical areas (46), responsible for its effectiveness as it is compulsory in the slaughtering of those animals with a positive result (64). In Europe, the aforementioned information is regulated by the Council Directive 64/432/EEC. Although IST and IFN-γ assay have reasonable sensitivity and good specificity (**Table 2**), both techniques require a minimum of 48–72 h to obtain a result (21, 65) besides presenting other disadvantages and limitations. On the one hand, IST requires visiting the farm and restraint

TABLE 2 | Sensitivity and specificity parameters from conventional diagnostic techniques.

Technique	Sensitivity	Specificity	References
Mycobacterium bovis			
Intradermal skin test			
Caudal fold test	68–96.8%	96–98.8%	(46)
Cervical intradermal test	80–91%	75.5–96.8%	(46)
Comparative cervical test	55.1–93.5%	88.8–100%	(46)
Gamma interferon evaluation	74.00% 73–100%	≥99% 80–90%	(57) (46)
Microbiological culture	72.9–82.8%	97.1–100.0%	(58)
Serologic assays—ELISA			
From milk samples (MPB70+MPB83)	50.0%	97.5%	(59)‡
From sera samples (recombinant antigen cocktails)	40.6–93.1%	69.7–99.1%	(60)‡
From sera or plasma samples (recombinant antigen cocktails)	62.7–69.5%	97.2–98%	(IDEXX)†,‡
PCR			
IS6110	82.5–92.3%	94.3–99.0%	(58)
MPB70	94.59%	96.03%	(61)
Mycobacterium avium* subsp. *paratuberculosis			
Microbiological culture	23–70%	100%	(62)
Serologic assays—ELISA	7–94%	40–100%	(62)
PCR			
IS900	79.3–91.0%	88.3–93.9%	(63)

† *Mycobacterium bovis antibody test kit, IDEXX Laboratories Inc.*
‡ *ELISA results compared with culture or single intradermal comparative cervical test positive and negative status.*

of the animals twice, and a delicate and difficult administration and interpretation of skin results, which may vary due to differences in tuberculin doses, site of application (**Table 2**), and interpretation schemes (25, 27, 66); on the other hand, IFN-γ assay implies a complex laboratory methodology (18), a considerably more expensive price than a skin test (65, 67), and suffers from cross-reactivity with other related mycobacteria resulting in false-positive results (2). In addition, performance of these tests can be compromised by factors associated with the immune response and health status of the animal leading to a misinterpretation of the results. Development and use of a pre-screening test before field tests would be useful to reduce work efforts and diagnostic time (27).

Although microbiological culture is considered the gold-standard approach for the diagnosis of mycobacterial infection, it is characterized by a long incubation time to confirm the presence of mycobacteria (around 8–12 weeks) (2). Furthermore, the isolation of mycobacteria sometimes requires specific compounds such as mycobactin, a siderophore which determines the viability and growth of some mycobacteria species, as is the case of MAP; and, sometimes, additional steps such as decontamination. For all these reasons, culture becomes a tedious and laborious, although necessary, option in *M. bovis* diagnosis.

Other *in vitro* assays, such as serologic assays (ELISA) or PCR, have limitations associated with accuracy and execution that restrict their use (66). While ELISA sensitivity is affected by the delayed and irregular antibodies response in bTB (68), PCR is considered a postmortem diagnostic option with promising findings but still under development, focused on the search of markers that ensure diagnostic sensitivity (61) (**Table 2**). Therefore, the reliability of these tests depends on the stage of infection and, in addition, these require transporting of animal samples to the laboratory, which finally increases diagnostic time too (40), highlighting the interest on the availability of portable equipment.

Current *Ante-mortem* and *Post-mortem* Diagnostic Techniques Against *M. avium* subsp. *paratuberculosis*

The intermittent and sometimes low shedding of the mycobacteria as well as the irregular seroconversion in the subclinical phase of PTB (69, 70) gives a limited sensitivity to the *in vivo* diagnosis (32), currently based on serological assays (ELISA) and PCR from feces. Although ELISA has a limited sensitivity (**Table 2**), the irregular spread of bacteria *via* feces has raised serology as the most common technique used for the monitoring of PTB (71) due to its cheap and easy use. In addition, fecal shedding and immune response vary individually to a large extent (72). For example, the sensitivity of PCR methods can be affected by the variable bacterial load in samples and the co-purification of PCR inhibitors during DNA extraction (73). Therefore, and although the current combination between serology, vaccination (when regulation allows this option due to its possible interference on bTB eradication campaigns), and slaughtering constitutes a strategy with remarkable effectiveness, there is a need for diagnostic tests with higher sensitivity and

decreased processing time to reduce false-negative results and enable effective disease control strategies, as different authors have highlighted before (31, 32). Volatilome evaluation has been capable of discriminating MAP infection before clinical illness occurs, offering an early diagnosis and significant time savings (1). This could be considered as one of the main advantages against the current techniques in use.

In short, against the current situation, it would be of help to have an *ante-mortem* diagnostic methodology capable of detecting mycobacterial infection with repeatability, a good quality/price ratio, high sensitivity and specificity, and rapid detection and obtaining of results. VOC strategy could be a complementary option because it mostly fulfills these requirements, and it has been successfully used for mycobacterial diagnosis in many animal species (**Table 3**). In this sense, an initial approximation analyzing stable air as matrix has been proposed to evaluate MAP infection in cattle (74); however, due to the low number of infected animals included within each infected group, further studies are required to confirm the suitability of this approach.

IMPACT OF THE EXPERIMENTAL SETTING ON THE VOCs PROFILE

In vitro vs. *in vivo* Studies

Analysis of VOCs as a diagnostic option for mycobacterial disease has been evaluated both *in vivo* and *in vitro*. Compared with *in vivo* assays, the large number of existing *in vitro* studies, which basically consist in mycobacteria culturing, reveals the early stage of development where this research area stands (36–38, 75, 76). The reviewed literature in the present study suggests some drawbacks related to those *in vitro* studies.

First, mycobacteria growth, which as aforementioned requires several weeks or even months, is required to identify changes in the analysis of VOCs from microbiological culture to allow the distinction between negative and positive samples. In other words, although *in vitro* experiments can detect VOC changes related to different stages of the mycobacteria growth (76, 77), it still takes a long time to identify these changes, which is one of the main disadvantages linked to the current diagnostic methodology. Accordingly, researchers point to reduce the diagnostic time by avoiding the limiting step of culturing and suggesting other innovative techniques such as VOC measurement directly *in vivo* (32).

It is also important to highlight the low correlation existing between results obtained from cultured bacteria compared with those VOCs produced from other biological samples studied in *in vivo* experiments (40). For example, (32) detected two compounds only present above MAP cultures which were ranked among the top discriminating VOCs in their statistical analysis. However, in the comparison with their *in vivo* results, these two compounds tended to be in lower concentration in MAP-inoculated animals compared with non-inoculated animals. This situation emphasizes the caution required when adopting *in vitro* findings to *in vivo* conditions since the influence from the host, its microbiome and host–microbiome interactions (78), as well as the influence from environmental factors, such as diet, age, or drug use (40), needs to be considered. In addition, another hurdle of the *in vitro* settings is related to the different VOC profiles obtained depending on the substrate where the mycobacteria grow resulting in inconclusive findings (79).

The effectiveness of *in vivo* approach is supported by the results of many studies where VOCs from biological samples have been used to distinguish between infected animals with different mycobacteria species and non-infected animals (1, 18, 31, 80). Many different biological matrices such as serum, breath, or feces have been studied as a source of information for VOC analysis in this field, existing great differences between their nature and characteristics. This constitutes another problem in the comparison between *in vitro* vs. *in vivo* experiments, giving inconsistent results. When (31) compared *in vivo* results obtained from feces and breath samples with the *in vitro* VOC profiles obtained from different MAP strains' culture by (77), their conclusions were not very clarifying: from more than 100 substances detected in feces and breath, only 15 and 5 of them, respectively, were found in the bacterial *in vitro* pattern.

Experimental vs. Natural Infection

Another variable to consider when evaluating VOCs as an option for mycobacterial diagnosis in animals is the type of infection: natural or experimental. Although experimental infections are logically the most common and easy option for this kind of approximation, studies in naturally infected animals are of paramount importance. Experimental infections allow controlling different environmental conditions that may impact on the results, being the most studied option in the analysis of VOCs for mycobacterial diagnosis (**Table 1**). However, assays with natural infections are needed to validate the results obtained from any new diagnostic tool, such as volatilome analysis, in experimental settings. Along this review, only a single article has been found to include the analysis of VOCs from both experimentally and naturally infected animals (23). These researchers found that differences between negative and positive animals were more pronounced in the natural infection group than in the experimentally infected one. This fact highlights the importance of performing studies in field conditions in the future to compare with those with experimentally infected animals and to validate the results from the latter ones.

SPECIES UNDER STUDY

The analysis of VOCs has been used in many species for the diagnosis of mycobacterial infection. Livestock species are the most frequent ones, probably because of the importance and repercussion of bTB and PTB for farm animals. As expected, bovine is the most studied animal model with this innovative approach, followed by goats (**Table 1**). Our findings are consistent with the wide variety of diseases that have been tested through this methodology in cattle, such as bovine respiratory disease (11), mastitis (81), brucellosis (30), ketosis (82), or ketoacidosis (83, 84).

TABLE 3 | VOCs related to mycobacterial infection in different animal species (targeted analysis).

Mycobacteria species	Potential discriminatory compounds or type of compounds	Animal species	Matrix	Analytical technique	References
Mycobacterium bovis	2,3-Dimethyl, 1,3- pentadiene 1,3-Dimethylbutyl cyclohexane	Cattle	Exhaled breath	GC-MS	(25)
Mycobacterium bovis	>100 compounds (acetone, dimethyl sulfide, and 2-butanone as the most abundant)	Cattle	Exhaled breath	ATD-GC-MS	(26)
Mycobacterium bovis	4-Hydroxy-4-methyl-2-pentanone Benzaldehyde 1-Ethyl-2-pyrrolidinone α, α-Dimethyl-benzenemethanol **Nonanal**	Cattle (1 year old)	Exhaled breath	GC-MS	(18)
Mycobacterium bovis	Methylbenzene **Hexanal** 2-Methyl pyridine 2,4-Dimethyl pyridine 2-(1,1-Dimethoxy)-ethanol† **2-Ethyl-1-hexanol** Benzene acetaldehyde 3,7-Dimethyl-6-octenyl-(2E)-2-butanoate Acetophenone† 4-Methyl-phenol 2-Decanone† (–)-Beta-fenchol 1-Decanol **Indole** 3-(1,1-Dimethylethyl)-4-methoxy-phenol 1-Octadecanol 2-Dodecanone	White-tailed deer (12–18 months old)	Feces	GC-MS	(13)
Mycobacterium bovis	Thioether Thiophene Aldehyde Organosulfur (sulfone) Imine Pyridine derivative Amino acid Ketone Alcohol **Indole** Diterpenoid alkane Fatty acyl (amino acid derivative) Diterpene alcohol Dicarboxylic acid and derivative	Cattle (120–121 days old)	Feces	GC-MS	(28)
Mycobacterium bovis	Adult animals (>2 years): O-Cymene Juvenile animals (<12 months): Acetic acid, methyl ester **3-Methylpentane** Trichloromethane α-Methylstyrene Decane 4,6,8-Trimethyl-1-nonene 1,3-Bis(1,1-dimethylethyl)-benzene 2,5-Dimethylhexane-2,5-dihydroperoxide 2,5-Bis(1,1-dimethylethyl)-phenol Heptacosane 5-Butyl-5-ethylheptadecane 11-Decyl-tetracosane 11-(1-Ethylpropyl)-heneicosane 3-Ethyl-5-(2-ethylbutyl)-octadecane	Wild boar (juveniles, sub-adults, adults)	Exhaled breath	GC-MS	(29)
Mycobacterium bovis	Sub-adult animals (12–24 months): 10,18-Bisnorabieta-8,11,13-triene Juvenile animals (<12 months):	Wild boar (juveniles, sub-adults, adults)	Feces	GC-MS	(29)

(Continued)

TABLE 3 | Continued

Mycobacteria species	Potential discriminatory compounds or type of compounds	Animal species	Matrix	Analytical technique	References
	Acetone Toluene 2,6-Bis(1,1-dimethylethyl)-4-(1-methylpropyl)-phenol				
Mycobacterium avium subsp. *paratuberculosis*	1-Propanol **2-Butanone** **Acetone** Benzene 2-Methyl-butanal Ethylbenzene **Hexanal** **Nonanal** Styrene	Goat (21–55 weeks old)	Exhaled breath	GC-MS	(31)
Mycobacterium avium subsp. *paratuberculosis*	Pentane Hexane Heptane **Acetone** **2-Butanone** 2-Pentanone 2-Hexanone 2-Heptanone 3-Octanone 3-Methyl-2-butanone 3-Methyl-2-pentatone Methyl isobutyl ketone Isoprene **Methyl acetate** Dimethyl sulfide Dimethyl disulfide Furan 2-Ethylfuran 2-Methylfuran **3-Methylfuran** 2-Pentylfuran	Goat (21–55 weeks old)	Feces	GC-MS	(31)
Mycobacterium avium subsp. *paratuberculosis*	45 compounds. Top-3 (random-forest): **3-Methylfuran** 2,3-Butanedione **Methyl acetate**	Goat (2–3 weeks old)	Feces	GC-MS	(32)
Mycobacterium avium subsp. *paratuberculosis*	51 compounds. Top-3 (random-forest): **3-Methylpentane** **2-Ethyl-1-hexanol** 2-Methylpentane	Goat (2–3 weeks old)	Exhaled breath	GC-MS	(32)

ATD-GC-MS, thermal desorption–gas chromatography–mass spectrometry; GC-MS, gas chromatography–mass spectrometry; GC-GC-MS, two-dimensional gas chromatography–mass spectrometry.

† Statistically significant trends identified for vaccinated and infected animals but not in non-vaccinated and infected animals.

Consistent compounds between different assays are highlighted in bold.

Remarkably, diagnosis through volatilome has been also performed in wildlife, more specifically in deer (13), badger (23, 24), and recently in wild boar (29), with much effort put into the development of a better disease surveillance methodology on these species. Among laboratory animals, although outside the scope of this review, non-human primates have been used to study the mycobacteria species which usually affect humans, *M. tuberculosis* (80, 85), as well as the murine model, which has been also employed to assess the use of breath for mycobacterial infection (41).

The encouraging results obtained in these studies with different animal species highlight the great potential of this methodology in MTBC and MAP diagnosis. However, there is a lack of studies in other species of interest, such as the pig, an animal model with an increasing interest in biomedical research (86). Furthermore, the marked differences that exist among different animal species make feasible that different approaches may be necessary for each species. This review highlights the starting point where this new diagnostic approach stands and the necessity of further studies

and research before its setting up as an alternative routine or field technique.

BIOLOGICAL MATRICES

VOCs can be detected directly from different biological samples such as blood, serum, breath, feces, sweat, skin, urine, or vaginal fluids (13, 27, 87–89), opening up huge opportunities for this new diagnostic methodology. Although samples should be initially selected according to the disease and the pathogenesis of the agent, there are multiple options that allow collection of alternative samples. For example, the predominantly respiratory character of bTB would place exhaled breath as the most appropriate sample to study this disease. However, there are studies that show interesting results for the analysis of VOCs from *M. bovis*–infected animals using different biological matrices such as feces (13, 28) or serum (24, 27). A similar situation occurs with PTB. MAP is a mycobacteria characterized by causing digestive disorders, making feasible to find these alterations directly reflected in the fecal volatilome. Despite of this, exhaled breath (1, 31, 32) and serum (30) have given promising findings in different animal species.

The rationale for analyzing exhaled breath in a model of chronic intestinal infection or feces in a primarily respiratory disease is based on the hypothesis that they do not only contain substances originated from the airways or from the digestive system. These also contain metabolites released *via* the lung or the intestine but originated and related to the whole metabolic or health state of the subject (1).

The three most used biological samples for VOC analysis of mycobacterial diseases in animals are exhaled breath, serum, and feces (**Table 1**).

Exhaled Breath

The principle of using exhaled breath lies in its capability for discerning disease-related changes and biomarkers in the organism that are reflected into the breath through exchange *via* the lungs (25), because of its ability to cross the alveolar membranes before being exhaled (26). The use of exhaled breath offers several advantages because it is a non-invasive sample produced in ample supply, having the potential for direct, inexpensive, and eventually real-time monitoring (25, 90). Although in the literature it is considered as a sample that is relatively easy to obtain, its sampling methodology in animals is diverse, revealing a lack of standardization: from modified equine nebulization masks or nostril samplers for cattle, specific ventilators for mice or intubation for macaques, to automated alveolar sampling devices for goats. Furthermore, some factors can affect the sampling methodology, such as eructation in ruminants, which has been shown to significantly affect exhaled VOC profile (91). VOCs from breath are normally concentrated to sorbent materials, such as Tenax or Carbopack Y, Carbopack X, and Carboxen 1000 (18, 80), which simplify its transport and storage, and later these are used to quantify and evaluate the volatile substances with different analytical techniques.

Healthy and diseased animals have been successfully distinguished in mycobacterial infections by identifying volatile molecules in exhaled breath (**Table 3**). (18) performed breath collection and analysis in *M. bovis*–inoculated cattle with two strains obtaining good sensitivity and specificity: 83.8–96.4% and 97.4–99.2%, respectively, using the microbiological culture as reference technique. In addition, (25) reported the measurement of two VOCs from breath linked with *M. bovis* infection and other two VOCs associated with samples from negative individuals, obtaining sensitivity and specificity values of 100 and 79%, respectively.

The studies included in this revision evaluating exhaled breath in the context of mycobacteria infection highlight some important variables to be considered (41). The use of different animal species models, the *Mycobacterium* species and strain used, the infection phase, the breath volume collected, and the sorbent phases used to concentrate VOCs are factors that often differ between the existing assays. Considering all the aforementioned information, a comparison between the existing results is a challenge.

Feces

Feces are regarded as the most accessible sample for research (92). Considering that feces constitute the main media for eliminating metabolic products, these are an important source of information about the internal homeostasis (17). The reason for testing changes in VOCs in feces is based on the common assumption that any abnormality in the activity or composition of the intestinal microbiota and in the whole organism may alter the odor of this matrix (1), which is supported by studies from both human (93–96) and animal medicine (97–100). Consequently, examination of volatile fecal emission could be a very useful non-invasive diagnostic approach (1).

However, as a remarkable fraction of VOCs found in feces is generated by gut commensal microbiota (101), a well-matched control group and knowledge on these bacteria are necessary to identify VOC patterns of pathogenic conditions (31). Despite this shortcoming, using feces as matrix has many advantages; besides an easier sampling, it is not necessary to restrain the animals, eliminating the stressful situation that it implies. Moreover, and in contrast with human medicine, feces offer many different possibilities in terms of sampling protocol: per rectum, after sacrifice, using laboratory animal cages, or just after defecation are some options in veterinary research. The studies using feces reviewed in the present work highlight the existing heterogeneity between the published results (**Tables 1**, **2**). However, the obtained results have placed fecal volatilome analysis as an innovative diagnostic approach in the current research context for mycobacterial infections. In this sense, attention has been focused not only on the discrimination between infected and healthy individuals (1, 13, 31, 32) but also in the use of fecal VOC profile for other purposes, such as identification of vaccinated animals in white-tailed deer (13) and cattle (28).

Serum

Serum is the sample of choice in many studies because of its relative ease to obtain and store, and safe distribution (24). Blood or serum is the means of transport of many different

substances, compounds, and markers through the organism, existing a complex exchange with the lung or the intestine, among other systems (102). Alterations in VOCs from serum can be detected when a disease, an infection, or a pathologic condition occurs (103).

Serum has been used to distinguish the infection by *M. bovis* or MAP in different animal species through volatilome evaluation (**Table 3**) obtaining very interesting results. For example (30), were able to discriminate MAP and *Brucella* spp. infection in cattle through VOC analysis; and (27) reported an analyzing time of only 20 min to differentiate between bTB-infected and bTB-free bovine sera. However, and although blood and serum could be the most routine samples used in diagnostic field, its collection supposes a stressful situation as it is an invasive method that requires individual immobilization.

In conclusion, three different biological samples have been discussed as source of information in mycobacterial diagnosis in animals through volatilome analysis. Although interesting and useful findings have been shown, there is still a lack of homogeneity among many different study conditions. This often leads to non-comparable and inconsistent results. For example, despite studying the same pathogen (MAP), and using the same biological samples (exhaled breath and feces) and animal species (goats), contradictory conclusions can be found in the literature: while some showed that differences in VOC profiles were less pronounced from breath than those obtained in feces (31, 32), others suggest that volatilome evaluation from exhaled breath might be superior compared with the one from feces (1). In fact, the researchers usually acknowledge that their hypotheses should be verified by future studies, considering their findings as starting points (1, 32). Hence, no reliable comparisons or conclusions can be made with the available information, being advisable to carry out studies where the biological matrices are used simultaneously with the same methodological conditions.

INSTRUMENTAL TECHNIQUES

Some analytical instrumentation techniques allow VOC evaluation. Although gas chromatography with mass spectrometry (GC-MS) is referred very often as the "gold standard" for VOC analysis (104, 105), selected ion flow tube–mass spectrometry (SIFT-MS), proton transfer reaction mass spectrometry (PTR-MS), and secondary electrospray ionization mass spectrometry (SESI-MS) are other mass spectrometry–based options available for bacterial VOC analysis (40). Moreover, various types of ion mobility spectrometers (IMS), such as classical time of flight IMS (IMS-ToF), aspiration IMS (a-IMS), differential mobility spectrometers (DMS), field-asymmetric wave IMS (FAIMS), or multi-capillary column IMS-ToF (MCC-IMS-ToF) have been successfully used in identification of bacterial VOCs as well (22).

In the present review, different analytical techniques have been evaluated to assess VOCs as a diagnostic methodology for mycobacterial infection in animals (**Table 3**): different GC-MS modalities, various electronic nose (EN) models, and DMS, being the first two options by far the most frequent approaches. In this sense, as other researchers have previously indicated, the diverse methods of VOC collection and analytical systems that have been used are likely to have contributed to the results' variability (18). Supporting this context, each analytical method offers both advantages and limitations.

Gas Chromatography Coupled to Mass Spectrometry

GC-MS has become one of the most preferred options for marker identification of bacterial origin with a very good sensitivity (40). It has a huge potential for both identification and quantification of unknown VOCs from complex matrices (106, 107). Ellis et al. (18) found that 4-hydroxy-4-methyl-2-pentanone, benzaldehyde, 1-ethyl-2-pyrrolidinone, α,α-dimethyl-benzenemethanol, and nonanal were present in significantly greater concentration in *M. bovis*–infected animals than in control ones. Moreover, Bergmann et al. (31) found 16 and 3 VOCs in feces and breath, respectively, which provide detectable differences at any infection time between MAP-inoculated and non-inoculated animals. GC-MS has the capacity of detecting VOCs within a range of parts per billion range, or lower, with good reproducibility and linearity (22, 25). In other words, GC-MS not only seems to be the most suitable for bacterial biomarkers search; in fact, it is the most used technique to diagnose these infections (40). The present review highlights the usefulness of GC-MS as an analytic tool to evaluate VOC changes due to mycobacteria infection employing different biological samples such as exhaled breath or feces (**Table 3**).

In spite of these many advantages, GC-MS has also several drawbacks: most GC-MS equipment are still not implemented as a portable tool; it requires high levels of expertise, qualified personnel, and pre-concentration techniques; and it is currently an expensive instrumentation (40). Therefore, given the aforementioned cons and the significant sampling and analysis time that it implies, GC-MS is not suitable for being used in end-user or point-of-care sites (25, 40).

It is also worth mentioning that comprehensive two-dimensional GC-MS (GCxGC-MS) stands out for the possibility of analyzing VOCs coming from complex matrices (40) and for providing a more complex and unparalleled separation as well as three-dimensional chromatograms' visualization (108).

Electronic Nose

The electronic nose (EN) is an instrument based on chemical sensors combined with a pattern recognition system (109), able to detect different VOCs, such as odors, flavors, and vapors (110, 111). The main advantages of this methodology are the ease of use, its low price, and the rapid analysis time (27). Furthermore, EN methodology avoids sample transport to laboratory, positioning itself as one of the optimal techniques for pen-side use (27). However, it has problems with background separation, it does not identify substances detected, and sometimes its detection limit is high, giving insufficient sensitivity (31, 112).

The huge variety of applications where EN has shown effectiveness could be also considered as another of its strengths: versatility. In this sense, the reviewed information reveals the applicability of many types of EN sensors for different species

of mycobacteria diagnosis through VOC identification (**Table 3**). Despite the good and interesting results obtained, the ease of transport of this device has not been exploited in depth because most studies using EN has analyzed VOCs from serum (**Table 3**) and not from other types of samples, such as feces or exhaled breath. The aforementioned information enhances the importance of carrying out future studies using EN focused on non-invasive biological matrices which would permit to develop a portable tool. In this sense (25), used their GC-MS results to tailor an artificial olfactory system to detect bTB in cattle exhaled breath. Although their new system successfully identified all infected animals (100% sensitivity), it wrongly classified 21% of the non-infected individuals (79% specificity).

Other Minor Techniques

DMS is an IMS modality that has been occasionally used for volatilome assessment in mycobacterial infections (**Table 3**). This instrumentation has a lower cost, and it can be used alone or coupled with a GC column which acts as a pre-separation stage (40). Its relatively low price, robustness, reliability, and miniaturization turn IMS technology into one of the potential alternatives for portable VOC analysis in disease diagnosis (40). As with EN, one of its main drawbacks is its lack of capacity to identify specific VOCs (1). This analytical device used by (1) permitted to discriminate healthy from MAP-infected goats, noting a direct correlation among postmortem findings and *in vivo* measurements.

SIFT-MS is a quantitative technique for trace gas analysis based on the ionization of these volatile compounds by positive precursor ions along a flow tube. Although its main advantages are a rapid analysis time and a lower mass range, biological samples usually provide complex data which need computational assistance to be analyzed (24). Spooner et al. (24) applied multivariate analysis for the first time to SIFT-MS data to evaluate serum headspace analysis as a faster screening tool for *M. bovis* infection in badgers, obtaining a much faster diagnosis. However, the insufficient accuracy achieved (88% of true positive and 38% of false positive) makes this approach unsuitable as an alternative for conventional diagnostic techniques.

The existing differences between the reviewed analytical techniques suggest the importance of using methodologies, such as GC-MS, as a first-line analysis, with the objective to identify and define tentative biomarkers. Then, other approaches, such as IMS or EN, could be developed and adapted to a field or point-of-care use.

TARGETED ANALYSIS VS. NON-TARGETED ANALYSIS

The diagnosis of an infection using VOC analysis can be reached by identifying specific substances related to the pathologic process or by detecting significant alterations in the whole VOC profile. Most of the research has attempted to isolate unique VOC biomarkers (targeted analysis) (**Table 3**) that would indicate the presence of mycobacterial infection, with little work done investigating potential changes within the whole VOC profile (non-targeted analysis).

There are VOCs that can be present in many different situations, hampering to find a specific substance for a particular infection or process. This is the case of methyl-nicotinate, a compound that, although it is proposed as a *M. tuberculosis* biomarker (28), can be found in the breath of non-tuberculous smokers (113); it is used as a flavoring ingredient, and it is present in coffee, various nuts, alcoholic beverages, and fruits (114, 115). In this sense, although tentative biomarkers have been associated with mycobacterial infection in both human (35, 37, 116, 117) and veterinary medicine (**Table 3**), the influence of different factors as well as the dynamic character of volatilome makes the identification of indicative or unique VOCs difficult (28). According to the literature, these factors may be related to host biological variables, environmental conditions, symbiotic and infectious microbe–host interactions, pathophysiological responses, the method of sample collection, and differences in analytical methods used for sample analysis (30, 85, 89). The bias induced by these factors is exemplified by the comparison of two studies which aimed to use exhaled breath VOCs as a source of information to diagnose *M. bovis* infection in cattle (18, 25); using the same animal species, pathogen, and biological sample, only two VOCs were consistent between both studies, highlighting the challenge that this approach supposes. However, along the present review, several VOCs have been pointed out due to its frequency and consistency between the included studies (**Table 3**); while nonanal, hexanal, 2-ethyl-1-hexanol, acetone, and 3-methylpentane were found to be present in both *M. bovis* and MAP infection, there were also compounds indicative of single infection. This is the case of indole, for *M. bovis*, and 2-butanone, methyl acetate, and 3-methylfuran for MAP, molecules that could be postulated as candidates for the discrimination between MTBC and MAP processes. The aforementioned VOCs were found to be consistent between different studies (13, 18, 28, 29, 31, 32), matrices (feces and exhaled breath), and animal species (cattle, goat, deer, and wild boar), which opens up a huge opportunity to use this approach as a diagnostic option for general mycobacterial infections and specific infections as well. Nonetheless, there is still no specific biomarker for mycobacterial infections, being priority to develop analytical methods adapted to volatilome characteristics which allow adequate identification and quantification of these molecules.

On the other hand, there are already studies in the literature which have used the entire VOC profile (non-targeted analysis) to successfully discriminate between diseased and non-infected animals (28). In this way, many research groups have highlighted the importance of considering the entire profile of VOCs released by specific pathogens and how these profiles can help discriminating between infecting pathogens, rather than relying on a limited number of biomarkers (targeted analysis) (118). However, non-targeted analysis does not identify compounds, making not feasible to gather information about the source of these compounds. In addition, other factors, such as feeding, environmental conditions, or metabolic variables, need to be fixed to draw conclusions from the results obtained using this methodology. In this sense, non-targeted analyses by EN or

DMS (1, 23, 27, 30), although showing volatilome potential, make difficult the comparison with other studies, underlining the importance of a proper VOC identification and quantification to obtain consistent results.

CONCLUDING REMARKS AND FUTURE PROSPECTS

In conclusion, although currently there is an important research trend that evidences the potential of VOCs emitted in mycobacterial infections in animals as a diagnostic tool, it is still in an initial phase and presents some difficulties. The number of *in vivo* assays which study the implementation of the analysis of VOCs for mycobacterial diagnosis in animal research is considered scarce. Furthermore, considering the lack of standardization, the dynamic nature of volatilome, the drawbacks and differences in the current methodology, and the use of biological matrices, inconsistent and non-comparable results are usually obtained. Thus, no singular biomarkers indicative of mycobacterial infections have been described to date. The high number of research groups that have studied this new approach worldwide contributes to the lack of standardization because they usually use different protocols, a reason that makes more difficult to reproduce their results.

In the authors' view, volatilome analysis is considered an innovative approach which is likely to become of interest as a complementary tool for current diagnostic methods; this approach is not presented as an alternative, at least to date, but it is considered a strategy that could offer significant and complementary advances. Although the strategies based on IST and serology have partly succeeded for control and eradication campaigns of MTBC and MAP, respectively, volatilome features could allow the development of an *ante-mortem*, portable, and non-invasive technique, possibly used as a field screening method able to improve sensitivity and specificity parameters as the collected data highlight (**Table 2**). In addition, the possibility of discrimination of highly related mycobacteria infections and the detection of infected subclinical animals stand as major ambitions. Further and thorough studies using several biological matrices with constant *in vivo* conditions are required to obtain robust results as well as reliable comparisons and check the consistency of this methodology between different assays before its implementation at field level. Against the previously described background, the development of analytical tools to obtain useful and robust information about potential VOC marker identification and quantification is considered of paramount importance. This will open new and complementary possibilities in the questioned diagnosis of mycobacterial infection and help to overcome the described drawbacks in the present revision.

AUTHOR CONTRIBUTIONS

JG-L and PR-H conceived and designed the review. PR-H analyzed the data and wrote the manuscript. JG-L, VR-E, and LA revised the manuscript. All authors read and approved the final manuscript.

FUNDING

JG-L holds a Ramón y Cajal contract of the Spanish Ministry of Economy and Competitiveness (RYC-2014-16735). PR-H was supported by a Semillero scholarship funded by the University of Córdoba.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 25 May 2021
doi: 10.3389/fvets.2021.616755

Thermal Image Scanning for the Early Detection of Fever Induced by Highly Pathogenic Avian Influenza Virus Infection in Chickens and Ducks and Its Application in Farms

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Highly pathogenic avian influenza (HPAI) is considered as one of the most devastating poultry diseases. It is imperative to immediately report any known outbreaks to the World Organization for Animal Health. Early detection of infected birds is of paramount importance to control virus spread, thus minimizing the associated economic loss. In this study, thermal imaging camera devices were used to detect change in the maximum surface temperature (MST) of chickens ($n = 5$) and ducks ($n = 2$) as an early indicator of experimental HPAI infection. The MST of both chickens and ducks increased at least 24 h before the manifestation of clinical signs of HPAI infection, depending on the severity of the infection. The basal MST was recorded for broiler chickens housed under small pen and normal farm conditions without intentional infection. A threshold cutoff of MST was established based on the circadian rhythm of normal MST. This study suggests that thermal imaging of chickens and ducks is a promising tool to screen any potential HPAI-infected flock in order to expedite HPAI diagnosis.

Keywords: thermal imaging, highly pathogenic avian influenza, early detection, circadian rhythm, farm application

OPEN ACCESS

Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

Reviewed by:
Daniel Marc, Le nouvel Institut national de recherche sur l'agriculture, l'alimentation et l'environnement en France INRAE, France
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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *13 October 2020*
Accepted: *30 April 2021*
Published: *25 May 2021*

Citation:
Noh J-Y, Kim K-J, Lee S-H, Kim J-B, Kim D-H, Youk S, Song C-S and Nahm S-S (2021) Thermal Image Scanning for the Early Detection of Fever Induced by Highly Pathogenic Avian Influenza Virus Infection in Chickens and Ducks and Its Application in Farms. Front. Vet. Sci. 8:616755. doi: 10.3389/fvets.2021.616755

INTRODUCTION

Highly pathogenic avian influenza (HPAI) is considered as one of the most devastating poultry diseases (1). HPAI virus infection in gallinaceous birds is associated with high mortality, often without any apparent clinical signs, imposing difficulties in controlling virus transmission prior to viral detection (2). Since 2003, seven HPAI outbreaks involving different clades of the H5 HPAI virus have occurred in South Korea; these outbreaks have been controlled by enhanced biosecurity measures and stamping-out policies (3, 4). For better implementation of preemptive measures, early disease detection and the subsequent reporting system should precede the virus spread (5). To date, the diagnosis of HPAI infection has mostly relied on passive surveillance upon suspicion and reporting by farmers or veterinarians. However, the absence of apparent clinical signs before sudden death poses difficulties in discriminating HPAI-induced death from daily mortality at the early stage of HPAI infection (5, 6).

A network of sensors refers to a variety of wireless micro-sensors attached to, or installed near,

target objects to collect behavioral, biological, or modal information of individuals or groups, followed by its transmission to a comprehensive analytic system for risk assessment (7). Many studies have demonstrated the application of sensors in the poultry industry to improve flock management through the detection of abnormal night vocalization (8), jumping and landing force in laying hens (9), and floor distribution of broilers (10) under laboratory conditions. A thermal imaging device (TID) comprises a network of sensors that detect radiant heat from single or multiple target objects. As a non-invasive tool, its potential use has been investigated in the human and veterinary fields and confirmed for the detection of fever induced by pathogen infections or an increase in maximum surface temperature (MST) caused by heat stress or ventilation problems (11–14).

In the present study, we examined the potential use of TID to detect changes in MST associated with HPAI infection in chickens and ducks before the manifestation of clinical signs. For practical application, the MST of broiler chickens was monitored by TID over 4 weeks to establish a threshold cutoff of MST during their housing in simulated small pen and real broiler farm conditions with no intentional infection. In addition, real-time data collection and analyses were performed with a central analytic system to activate an alarm system when the monitored MST of chickens exceeded the threshold setup as per the experimental infection data.

MATERIALS AND METHODS

Ethics Statement

All animal procedures performed in this study (permit number: KU18193) were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee (IACUC) of Konkuk University.

Viruses

All experiments with viable virus were conducted in biosafety level 3 and animal biosafety level (BL) 3 facilities at the Konkuk University. The HPAI H5N6 A/duck/Korea/ES2/2016(H5N6) strain was provided by the Animal and Plant Quarantine Agency, Korea, and propagated in 9–11-day-old specific pathogen-free (SPF) embryonated chicken eggs and stored at −70°C until further use.

Thermal Imaging Analysis

Thermal video was recorded using an SM080TIP camera (Somo Energy & Technology Co., Ltd, Korea, Emissivity 0.98). Real-time changes in MST were monitored throughout the animal experiment. The MST was considered as one pixel representing the highest temperature among 80 × 60 pixels at each time point, regardless of the body part and number of animals. The MST of every 5 min was automatically transferred to a laptop via a network. The collected data of every 5 min were analyzed using the Argus viewer software program (Somo Energy & Technology Co., Ltd), and used for 3 h interval analyses (36 5-min time points for 3 h).

Temperature Measurement Setup Under Normal Condition

First, 6-week-old SPF chickens and 5-week-old ducks were tested negative for influenza A virus using enzyme-linked immunosorbent assay (ELISA). To examine the compatibility of the TID in the experimental setup, five chickens and one duck, respectively, were monitored with an infrared camera in the BL2 facility at Konkuk University. The thermal images were taken into consideration for analysis based on the following criteria: (1) Which body area was the site for the recognition of the highest temperature? (2) Was there any difference in temperature monitoring between individual animals and groups of animals? (3) How does the distance between the TID and photographed animal affect the measured temperature?

Temperature Measurement According to HPAI Infection

The five chickens used for the TID configuration were moved to negative pressure isolators in BL3. A thermal imaging camera was installed and fixed at the door of the isolator (0.3 m from the chickens) to minimize the effect of the distance between the camera and target object. MST was monitored at 5-min intervals. Basal MST was recorded from 24 h pre-challenge. The chickens were then intranasally challenged with 100 μL of H5N6 HPAI virus suspension (dose of $10^{6.0}$ EID_{50}/bird). Changes in MST were compared with those reported before virus inoculation. Clinical signs and mortality were recorded daily, and swab samples were obtained at 40 h post-infection (hpi) to evaluate viral shedding. The lights were turned on and off every 12 h.

In addition, a separated duck experiment similar to the chicken experiment was conducted using two ducks. The ducks used for the TID configuration were intranasally challenged with 100 μL of two different doses of HPAI H5N6 virus ($10^{8.0}$ EID_{50}/bird, high dose; $10^{4.0}$ EID_{50}/bird, low dose) to differentiate between the MST changes caused by higher and lower dose infection (15). MST was measured in the same way as that in chickens. Clinical signs and mortality were recorded daily, and swab samples were collected to quantify viral shedding at 2, 4, 7, 10, and 14 days post-infection (dpi). The temperature and relative humidity (20 ± 1°C and 50% humidity) of the animal facility were constantly maintained throughout the experiments.

Virus Detection and Quantification

To investigate the replication of the virus inoculated in chickens and ducks, oropharyngeal and cloacal swab samples were collected as section Temperature Measurement According to HPAI Infection and re-suspended in 1 mL of sterile phosphate buffer solution. The suspension was centrifuged at 15,000 × g, and 200 μL of the supernatant was used for RNA extraction using MagNA Pure 96 DNA and Viral NA Small Volume Kit on a MagNA Pure 96 instrument (Roche Applied Sciences, Germany), according to the manufacturer's instructions. The amount of viral RNA was measured by real-time reverse-transcription quantitative polymerase chain reaction (RT-qPCR) and expressed as the cycle threshold (Ct) value (16).

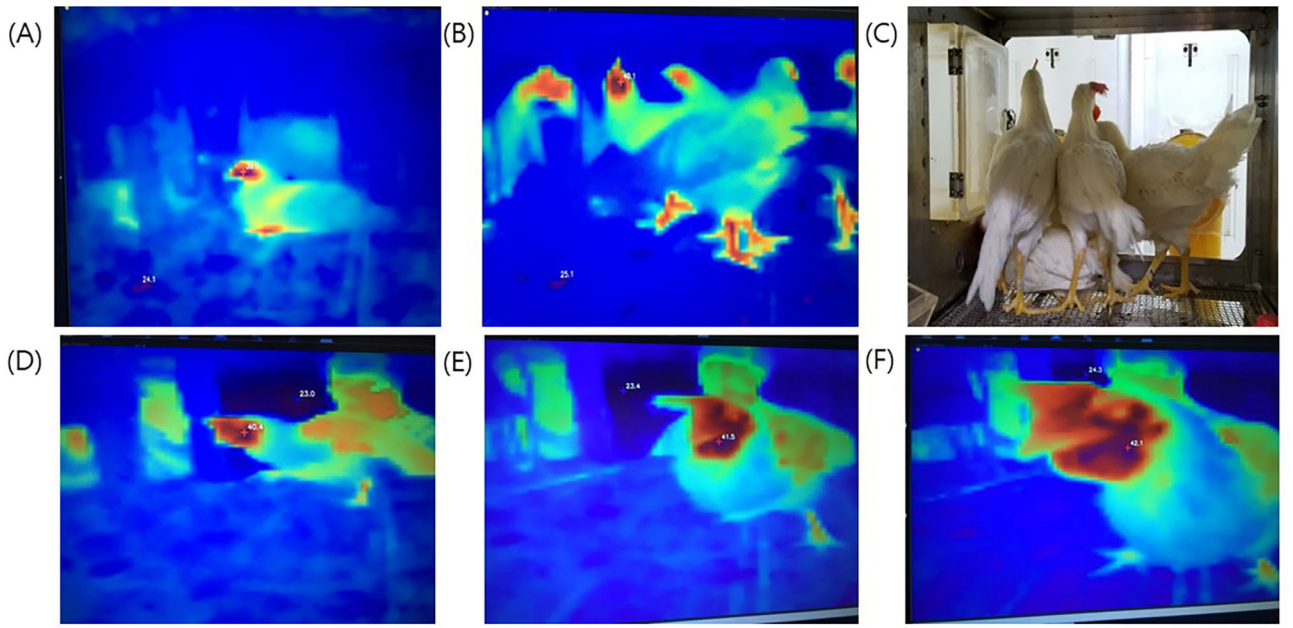

FIGURE 1 | Thermal images taken from different experimental set-ups. Top photos **(A–C)**: Temperature measurement site of one chicken or several chickens compared to the actual photographs. **(A)** One chicken; **(B)** several chickens; **(C)** actual photo. Bottom photos **(D–F)**: Differences in temperature measurement relative to distance. The shorter the distance, the higher was the measured temperature. **(D)** long distance, 40.4°C; **(E)** intermediate distance, 41.5°C; **(F)** short distance 42.1°C.

Maximum Surface Temperature Threshold Cut-Off Establishment in Actual Farm Setting

To establish a threshold cutoff for the MST in an actual farm setting, 30 1-day-old broiler chickens were raised in a 2 × 2 m pen for 4 weeks, thereby simulating a small-scale floor environment. For a larger scale environment, a broiler house with ∼20,000 birds was monitored for the entire rearing period. Under simulated farm conditions, a thermal imaging camera was installed at a height of 2 m from the ground at a vertical shooting angle. In the broiler farm, a thermal imaging camera was installed at a height of 1.5 m from the ground near the farm door at a shooting angle of ∼30° relative to the ground level (**Supplementary Figure 1**), unlike that in the case of the simulated farm. The temperature in a broiler house was changed from 33 to 23°C as per broiler growth and relative humidity was maintained at 50%. This monitoring was designed to set the threshold cutoff of the MST under farm conditions, considering environmental factors that affect MST, such as the circadian rhythm related to lighting.

Statistical Analysis

For the challenge study, the 24 h temporal temperature data before challenge were averaged to set the basal MST and compared to the average of temperatures recorded at 3 h intervals following infection using one-way analysis of variance (ANOVA) with a Bonferroni *post-hoc* test. Time points with asterisks denote statistically different time points after infection. $P < 0.05$ were considered statistically significant.

RESULTS

Temperature Measurement Under Normal Conditions

We investigated the possibility of temperature detection using a thermal imaging camera by monitoring chickens and ducks before they were subjected to virus challenge. The MST was detected at the head and legs of the chickens, and the beak, wings, and legs of the ducks (**Figure 1**, **Supplementary Figure 2**). There was no interference effect in MST monitoring upon examination of a single bird or multiple birds. In addition, it was confirmed that TID could successfully identify the highest temperature among multiple birds, although the only one pixel representing highest temperature was sent to the laptop.

Change in the Surface Temperature of Chickens After HPAI Virus Challenge

To determine the effect of viral infection on the MST of chickens, we monitored their MST starting from 24 h before virus inoculation. The basal MST before viral infection varied from 40.5 to 42.4°C at each time point, and the mean maximum surface temperature (MMST) over time for 24 h was 41.6°C (**Supplementary Figure 3**). After virus inoculation, the MST decreased by as low as 1.3°C until 24 hpi The MST started increasing from 26 hpi and peaked to 42.9°C from 27 to 36 hpi

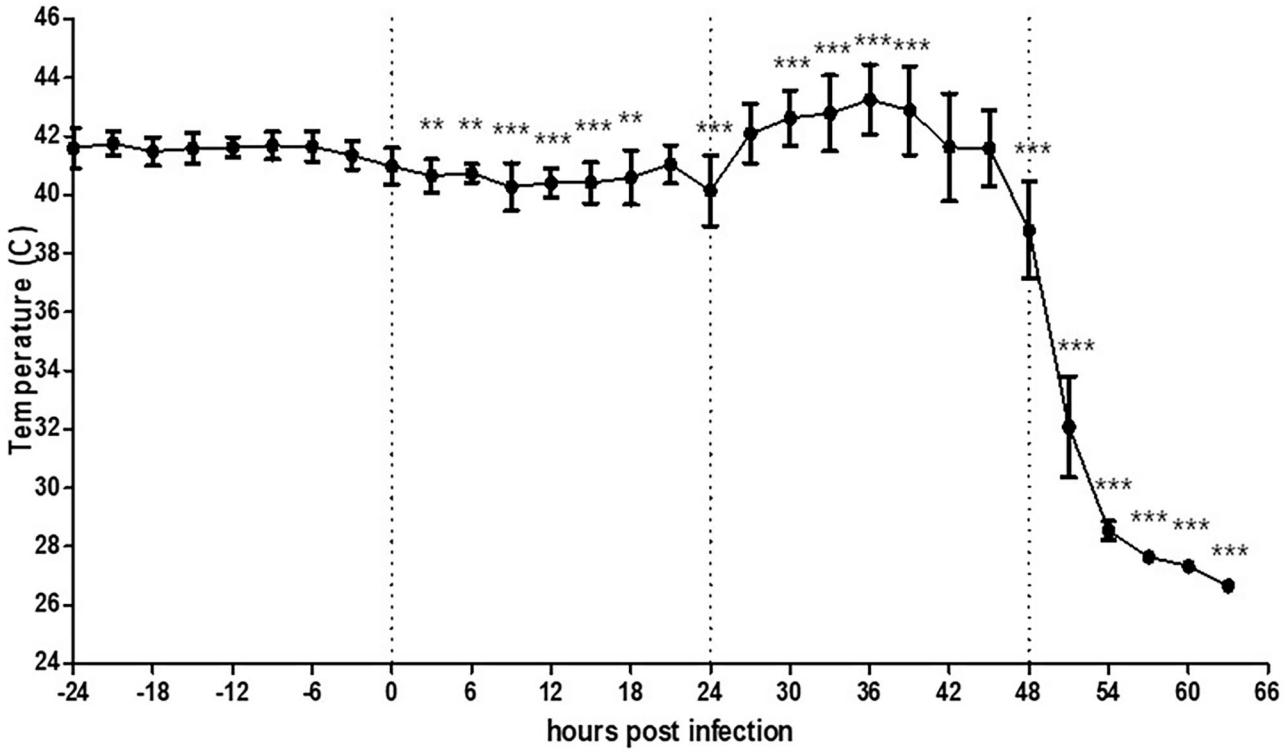

FIGURE 2 | Average temperature per 3 h interval before and after HPAI virus challenge in chickens ($n = 5$). Chickens were further monitored several more hours after death without removal. The error bars represent standard deviations of 36 5-min time points. Statistical significance was determined using ANOVA followed by Bonferroni *post-hoc* test. $^{***}P < 0.001$; $^{**}P < 0.01$.

(**Figure 2**), and suddenly decreased thereafter. This decrease in temperature was accompanied by lethargic behavior; the chickens finally died at 44 hpi. All chickens showed high viral shedding at 40 hpi from both the oropharynx (Ct = 25.7–30.5) and cloaca (Ct = 26.1–29.7).

Change in the Surface Temperature of Ducks After HPAI Virus Challenge

The recorded basal MST of the two ducks at 5 weeks of age varied from 36.6 to 39.9°C (MMST = 38.2°C) and 38.7 to 41.8°C (MMST = 40.2°C) at each time point (**Supplementary Figures 4**, **5**). This difference was not calibrated to body temperature because it had no effect on the monitoring of infection-related changes in MST. To demonstrate MST changes caused by either lethal or non-lethal infection of HPAI H5N6 virus, two different doses ($10^{8.0}$ EID_{50}/bird, high dose; $10^{4.0}$ EID_{50}/bird, low dose) were used for the duck experiment.

The duck challenged with the high dose of the virus was found dead at 105 hpi. The dead duck showed a similar trend of increased MST as that observed in the chickens. After virus inoculation, the MST decreased by as low as 1.0°C until 27 hpi and started increasing from 40 hpi and peaked to 40.3°C from 80 to 86 hpi, which was 2.1°C higher than the MMST before viral challenge (**Figure 3**). The duck showed no obvious clinical signs before death, but viral shedding was detected in both oropharyngeal (Ct = 26.8 at 2 dpi and 30.8 at 4 dpi) and cloacal swabs (Ct = 31.2 at 2 dpi and undetermined at 4 dpi).

The duck challenged with the low viral dose showed a bimodal temperature increase (42.8°C and 41.4°C, which was 2.6°C and 1.2°C higher than the MMST before viral challenge) and maintained its temperature below the basal MST after 39 hpi (**Figure 4**). Although the surviving duck showed no specific clinical signs, viral shedding was observed in oropharyngeal swabs (Ct = 31.6 at 2 dpi and undetermined at 4 dpi); this indicated that, viral infection was successfully performed.

Chickens Housed Under Farm Conditions

In the small pen of the simulated floor farm conditions, the background temperature was too high to allow the recording of the MST of the chickens until the brooder was turned off. Therefore, the recording started once the heat lamp was turned off. It was confirmed that the MST of the chickens could be normally recorded even in a much wider environment than that of a small cage (**Figure 5**, **Supplementary Figure 6**). In addition, the circadian rhythm of MST was successfully detected using the thermal imaging camera by controlling the light at regular intervals. However, the recorded MST was lower than the actual MST and varied greatly from 28.0 to 38.0°C, thereby necessitating the calibration of image analysis.

In the actual broiler farm condition, it was possible to normally shoot videos even when the brooder was

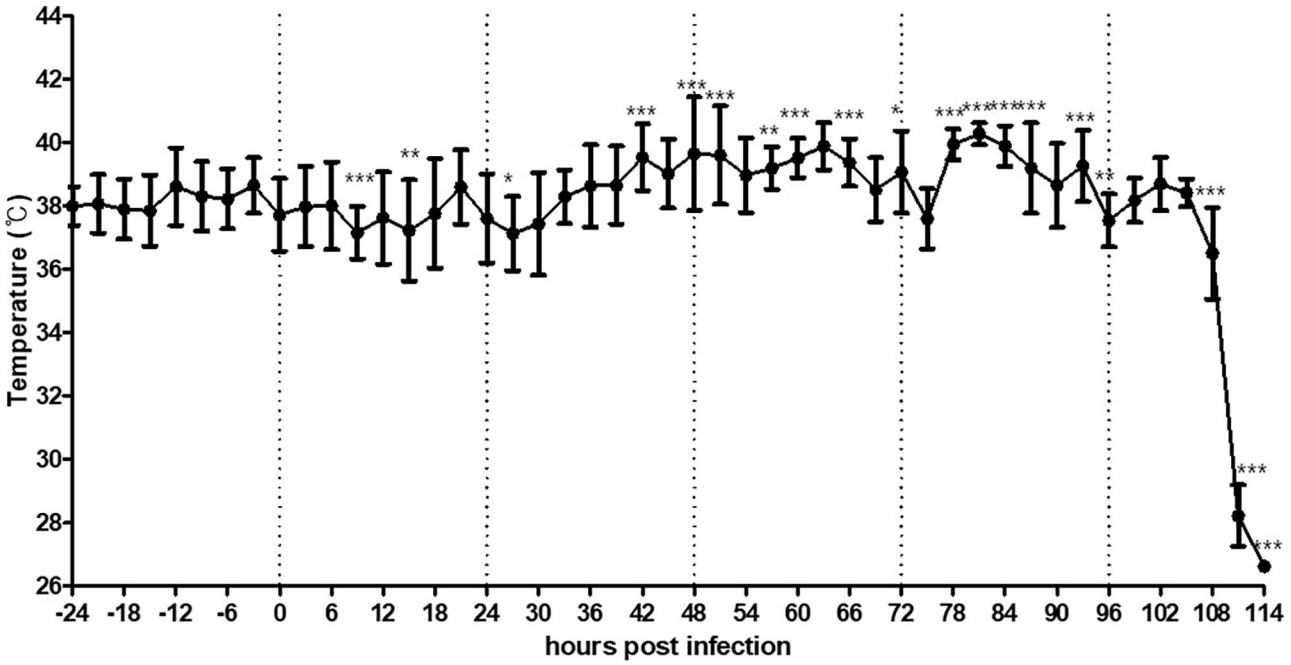

FIGURE 3 | Average temperature per 3 h interval before and after HPAI virus challenge in a duck intranasally exposed to the virus at a dose of $10^{8.0}$ EID_{50}/bird. Duck was further monitored several more hours after death without removal. The error bars represent standard deviations of 36 5-min time points. Statistical significance was determined using ANOVA followed by Bonferroni *post-hoc* test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

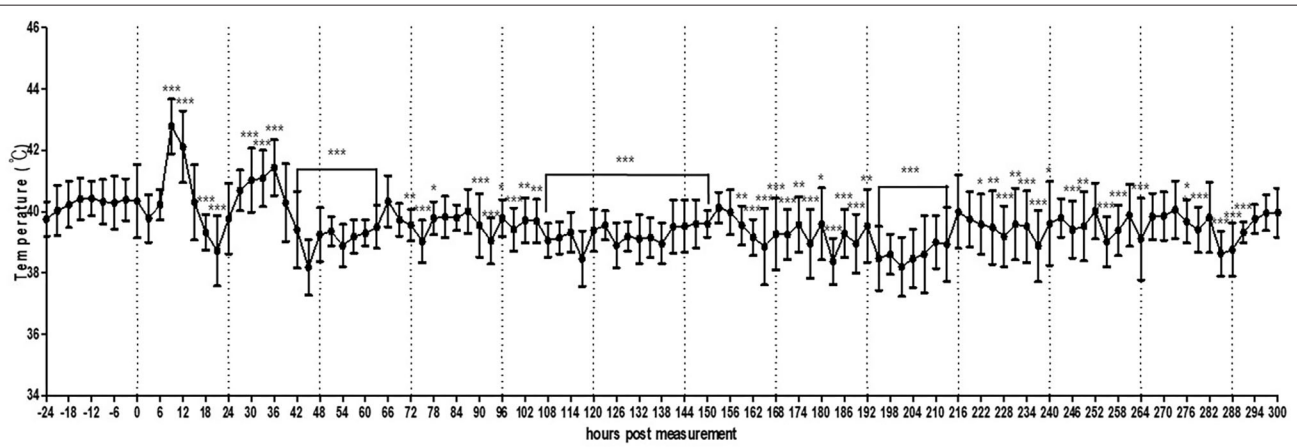

FIGURE 4 | Average temperature per 3 h interval before and after HPAI virus challenge in a duck intranasally challenged with virus at $10^{4.0}$ EID_{50}/bird dose. The error bars represent standard deviations of 36 5-min time points. Statistical significance was determined by ANOVA, followed by Bonferroni *post-hoc* test. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

turned on, as the brooder was installed on the ceiling and did not fall within the range of the thermal image. Consistent with the small pen simulation, the circadian rhythm of MST was observed under this actual broiler farm condition (**Figure 6**, **Supplementary Figure 7**). The temperature fluctuation was less than that in the small pen setting (39.0–42.0°C).

DISCUSSION

Thermal imaging cameras have been used for the screening of various infectious and non-infectious diseases in both human and veterinary fields, including the detection of severe acute respiratory syndrome (SARS) and coronavirus disease 2019 (COVID-19) (17, 18) in humans, bovine respiratory disease

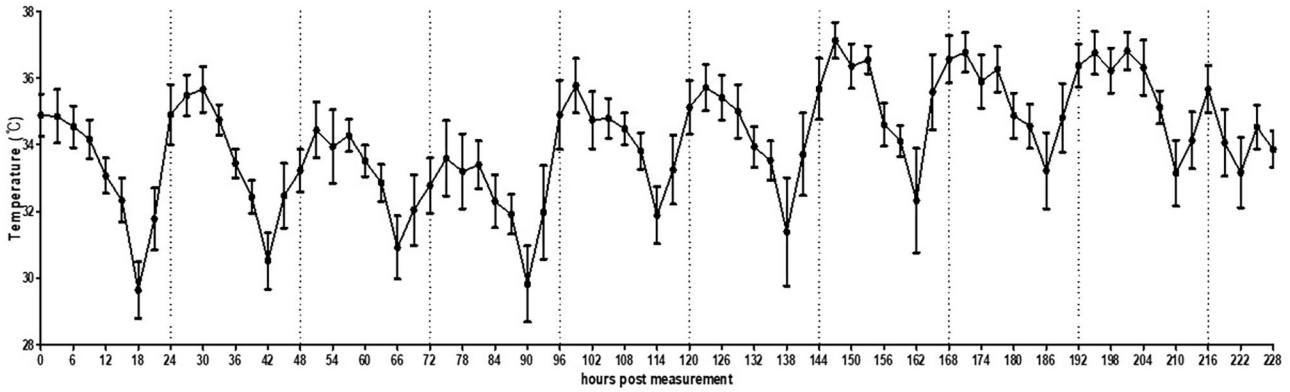

FIGURE 5 | Average temperature per 3 h interval of broilers ($n = 30$) housed in a small pen size (2 × 2 m). The error bars represent standard deviations of 36 5-min time points.

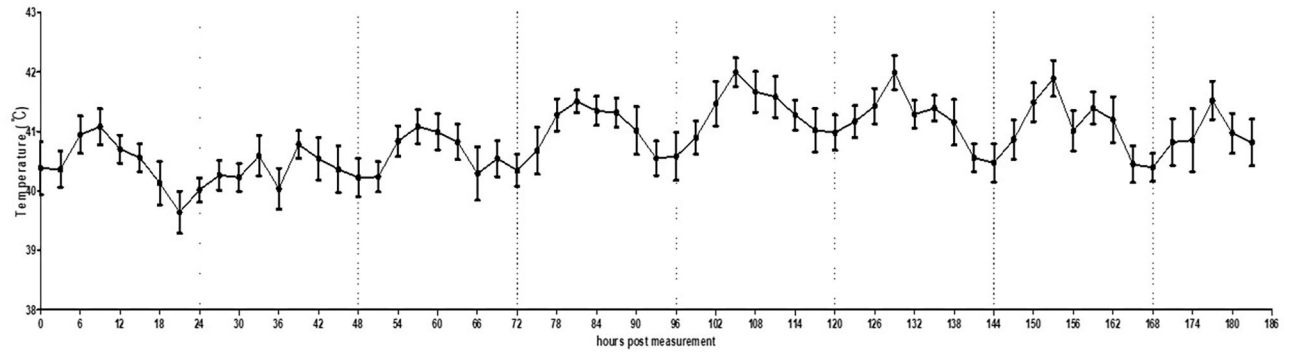

FIGURE 6 | Average temperature per 3 h interval of broilers housed in an actual farm. The error bars represent standard deviations of 36 5-min time points. Thermography was performed for 7 days.

and bumblefoot in animals (19–21). In general, two different types of thermal data loggers have been devised for animal use, one of which is directly attached to the body and the other, placed at a certain distance to sense or image the surface temperature. A study showed that the attached sensors were able to precisely detect HPAI infection under experimental setup conditions (22). However, limited battery capacity and low coverage of data sampling are the main hurdles associated with the practical use of this technique. On the other hand, thermal imaging cameras can measure the temperature at a distance. The measured temperature may vary depending on the distance and humidity because the temperature recognition method involves the analysis of the infrared rays emitted from the object. However, the camera-type sensor is easy to maintain, and the calibration of the actual temperature based on the distance from which the image is obtained, as a default step, could minimize the variations in measurements. In addition, the use of thermal imaging cameras would be beneficial for monitoring the level of population in terms of flock-based surveillance for chickens and ducks.

In the present study, thermal imaging cameras could successfully record the temperature of an individual or community of birds in cage and broiler farm conditions and maintain a track of the patterns in MST fluctuations. It is well-documented that the MST of both chickens and ducks inoculated with a range of HPAI virus rises and is accompanied with active virus shedding early during the infection (22, 23). Hence, we aimed to detect the increased MST following HPAI infection. The MST was measured at the heads and legs of chickens and ducks, presumably owing to the presence of relatively fewer hair over these areas. In addition, the detected temperature varied with the distance between the thermal imaging camera and the object (**Figure 1**). Therefore, we set the normal temperature standard by shooting at a fixed measurement position to take the image in a uniform distance from 24 h before viral challenge or by continuous temperature monitoring for the farm experiment.

Throughout the recording period, different patterns in MST change were observed depending on the species, dose of virus inoculation, and severity of clinical signs. At high viral doses, both chickens and ducks succumbed to infection and showed a slight decrease in MST, which is statistically significant increased thereafter. This effect may be attributed to the inability of the body to function normally after initial

infection, followed by fever related to the immune response. However, chickens showed lethargy a few hours before their death, whereas ducks did not show any clinical signs. As recording with thermal imaging cameras can detect statistically significant increase in MST at least 24 h before the death of an animal that the farmers usually recognize the infection, these results indicate that thermal imaging could serve as a useful strategy to rapidly predict HPAI-induced fever and minimize the response time to diagnosis or related preemptive measures. However, subclinical infection of ducks with low viral doses showed a different pattern than that of the lethal infection and might not be recognized until the subsequent virus spreads.

In the present study, we also recorded the pattern of change in basic temperature without intentional infection under farm conditions. Throughout the monitoring process, MST was not only influenced by disease conditions that induce fever but also by the circadian rhythm in a large number of birds under simulated and normal farm conditions. These circadian rhythm results will serve as the baseline under normal conditions, thereby allowing the tracking of temperature deviations induced by HPAI-induced fever. Thus, the alarming deviation in MST should be set in consideration of the daylight time and local temperature that affects the circadian rhythm of specific animals. In addition, the difference between the maximum and minimum temperatures in the simulated farm and actual farm conditions was very high, probably owing to the difference in the percentage of space used or shooting angle. The changes in the camera setting may have affected actual amplitude of minimum/maximum temperature within 24 h. Further vigorous experiments on the distance effect should be carried out to understand effects of shooting distance. Therefore, preliminary testing under normal conditions is essential for optimal calibration (for example, by comparing body temperature and TID; using more than one TID to monitor birds from different angles and making "stereo view" to apply triangulation).

Thermal imaging alone is inadequate for the diagnosis of any specific disease. However, the present study shows that thermal imaging can be used as an early monitoring tool for diseases such as HPAI. As a part of smart animal agriculture methods, thermal imaging could be applied across a wider range of applications for disease monitoring and detection of heat stress or ventilation problems for flock-based management.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of Konkuk University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

J-YN, K-JK, and S-HL gathered the data for the study and conducted the data analysis. J-YN prepared the manuscript. SY contributed to the interpretation of the results and manuscript preparation. J-BK contributed to the statistical analysis. D-HK coordinated the sample submission and testing. C-SS and S-SN conceived and supervised the study. S-SN reviewed, edited, and approved the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry, and Fisheries (IPET) through the Animal Disease Management Technology Development Program and by the Ministry of Agriculture, Food, and Rural Affairs (MAFRA) (Grant numbers: 318041-3 and 318032-3). The funders had no role in the study design, data collection and analysis, decision to publish this manuscript, or manuscript preparation.

ACKNOWLEDGMENTS

We thank SL solution Co., Ltd (Seoul, Korea) and Somo IR Co., Ltd (Seoul, Korea) for their assistance and technical contributions to this project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.616755/full#supplementary-material

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Conflict of Interest: J-YN, K-JK, J-BK, and C-SS were employed by KCAV Co. Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 31 May 2021
doi: 10.3389/fvets.2021.638820

Pan-Genome Analysis of *Vibrio cholerae* and *Vibrio metschnikovii* Strains Isolated From Migratory Birds at Dali Nouer Lake in Chifeng, China

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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: 07 December 2020
Accepted: 04 May 2021
Published: 31 May 2021

Citation:
Zheng L, Zhu L-W, Jing J, Guan J-y, Lu G-J, Xie L-H, Ji X, Chu D, Sun Y, Chen P and Guo X-J (2021) Pan-Genome Analysis of Vibrio cholerae and Vibrio metschnikovii Strains Isolated From Migratory Birds at Dali Nouer Lake in Chifeng, China. Front. Vet. Sci. 8:638820. doi: 10.3389/fvets.2021.638820

Migratory birds are recently recognized as *Vibrio* disease vectors, but may be widespread transporters of *Vibrio* strains. We isolated *Vibrio cholerae (V. cholerae)* and *Vibrio metschnikovii (V. metschnikovii)* strains from migratory bird epidemic samples from 2017 to 2018 and isolated *V. metschnikovii* from migratory bird feces in 2019 from bird samples taken from the Inner Mongolia autonomous region of China. To investigate the evolution of these two *Vibrio* species, we sequenced the genomes of 40 *V. cholerae* strains and 34 *V. metschnikovii* strains isolated from the bird samples and compared these genomes with reference strain genomes. The pan-genome of all *V. cholerae* and *V. metschnikovii* genomes was large, with strains exhibiting considerable individual differences. A total of 2,130 and 1,352 core genes were identified in the *V. cholerae* and *V. metschnikovii* genomes, respectively, while dispensable genes accounted for 16,180 and 9,178 of all genes for the two strains, respectively. All *V. cholerae* strains isolated from the migratory birds that encoded T6SS and *hlyA* were non-O1/O139 serotypes without the ability to produce CTX. These strains also lacked the ability to produce the TCP fimbriae nor the extracellular matrix protein RbmA and could not metabolize trimetlylamine oxide (TMAO). Thus, these characteristics render them unlikely to be pandemic-inducing strains. However, a *V. metschnikovii* isolate encoding the complete T6SS system was isolated for the first time. These data provide new molecular insights into the diversity of *V. cholerae* and *V. metschnikovii* isolates recovered from migratory birds.

Keywords: *Vibrio cholerae*, *Vibrio metschnikovii*, comparative genomics, migratory bird, pathogenic

INTRODUCTION

Vibrio is an abundant bacterial genus in oceans and comprises numerous species including pathogenic types such as *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio metschnikovii*. They are often observed in high abundance in marine products. *Vibrio* are halophilic and naturally found ubiquitously in marine settings, and thus raw seafood naturally harbors these microorganisms and is the main food source responsible for gastroenteritis caused by *Vibrio* spp. (1). Cholera is caused by *V. cholerae* that carry the cholera toxin and has resulted in seven pandemics throughout

human history. The seventh *V. cholerae* pandemic continues to present day, and exhibits evolved characteristics compared to previous pandemics, rendering it difficult to treat cholera disease outbreaks (2, 3). One commonality between the sixth and seventh pandemics has been observed to be associated with the CTXΦ bacteriophage that may have introduced the CTX toxin to *V. cholerae*. Nevertheless, the strains underlying the seventh pandemic did not directly evolve from those underlying the sixth pandemic (2). In contrast to *V. cholerae*, few reports have described characterizations of *V. metschnikovii*, with only some studies focused on detection and no reports on their pathogenicity.

Migratory birds have been considered potential vectors of *V. cholerae*, wherein colonization of their intestines may occur by ingesting water and marine animals infected with *V. cholerae* (4). Migratory birds travel over long distances and could carry pathogenic microorganisms from one region to another, spreading pathogenic microorganisms by excretion (5, 6). Therefore, migratory birds have been considered as important reservoirs of *Vibrio*, forming a fecal-food-mouth transmission route (3). Within intestinal environments, *Vibrio* are induced to evolve into treatment-resistant bacteria or pandemic strains due to stresses they are exposed to. Consequently, increased risk of bacterial spread and difficulty of bacterial source tracking occur due to the long migration distances of vector birds.

Consequently, it is important to analyze the pathogenicity of *V. cholerae* strains and whether they exhibit the potential to evolve into pandemic strains. The concept of a pan-genome was first proposed in 2005 and is a term that encompasses the sum of all genes of a species, including their core-genome, dispensable genome, and unique genome components (7). Importantly, the population-level evolutionary trends of a species can be evaluated by analyzing their pan-genome.

Migratory birds exhibiting malnutrition, wasting, diarrhea, and high mortality were observed in the Inner Mongolia region of China between 2017 and 2018. Dead birds comprised individuals from several species including *Larus ridibundus*, *Pluvialis squatarola*, *Tadorna ferrgina*, *Anas poecilorhyncha*, and *Aix galericulata,* in addition to other migratory birds. Moreover, 40 *V. cholerae* strains and 34 *V. metschnikovii* strains were isolated from the migratory bird epidemic materials and water environments. The *V. cholerae* strains were non-O1/O139 strains without the *ctxA/B* toxin, based on serotyping and PCR identification. To further investigate this die-off, we conducted a bacteriological examination again on migratory bird feces in Inner Mongolia, China, in 2019. However, *V. cholerae* strains were again not present in the samples, while only *V. metschnikovii* was present. In this study, we analyzed the core-/pan- genomes of *V. cholerae*/*V. metschnikovii* and compared them with previously sequenced strains to evaluate whether migratory birds harbor *Vibrio* spp. that have the potential to evolve into pandemic or drug-resistant strains. These analyses thus provide important data to inform the prevention and treatment of *V. cholerae* and *V. metschnikovii* infections and/or outbreaks.

Abbreviations: PCR, Polymerase chain reaction; MLST, Multilocus sequence typing; ARDB, Antibiotic Resistance Genes Database; VFDB, virulence factor database; CARD, Comprehensive Antibiotic Research Database; COG, Cluster of Orthologous Groups of proteins; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NR, Non-Redundant Protein Database. T6SS, Type VI secretory system; T3SS, Type III secretory system; CTX, cholera toxin.

MATERIALS AND METHODS

Sampling

Thirty-six samples (including 10 water samples, 2 aquatic plant samples, 19 epidemic material samples, and 24 feace samples) were taken from birds in 2018 and 2019. DNA was obtained from each fecal sample by stool DNA kit, and molecular method to determine the host source of feaces (8). The visceral organ and intestinal tract samples used for this study were collected aseptically from 19 freshly dead migratory birds (not corrupt). Samples were cultivated via spread plates in triplicate and directly cultivated onto selective thiosulfate citrate bile salts sucrose (TCBS) agar plates and incubated for 24 h at 37°C. Isolates were identified using PCR amplification of *ompW, infC, ctxA, hlyA,* and *chxA* genes (9–13). The primers and conditions used for PCR amplification are described in **Supplementary Table 1**. In addition, *V. cholerae* isolates were subjected to O1/O139 antigen serotyping using *V. cholerae* O antisera (Tianjin Biochip Corporation). Isolates were sub-cultured at 37°C in brain heart liquid (Qingdao Haibo, China) or on CHROM agar *Vibrio* plates (CHROM, Paris, France) unless otherwise specified. Green colonies were again confirmed using serology and PCR assays (see **Supplementary Table 1** for primer sequences and PCR conditions). Isolates identified as *Vibrio* were then stored in a −80°C freezer.

Extraction of Genomic DNA and Library Construction

Genomic DNA was extracted from isolates using the Bacterial Genomic DNA Extraction Kit (Omega). Harvested DNA was then quantified using a Qubit® 2.0 Fluorometer (Thermo Scientific). A total of 1 μg of DNA per sample was used as input material for DNA library preparations. Sequencing libraries were generated using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations. In addition, indexing oligonucleotides were added to sequences to identify each sample. Briefly, DNA samples were fragmented by sonication to a size of 350 bp, followed by DNA fragment end-polishing, addition of poly A-tails, and ligation with full-length adaptors for Illumina sequencing, followed by additional PCR amplification. Finally, PCR products were purified (AMPure XP system), and the size distribution of the libraries was analyzed using an Agilent 2100 Bioanalyzer and quantified using real-time PCR. The whole-genomes of strains were sequenced using an Illumina NovaSeq PE150 platform at the Beijing Novogene Bioinformatics Technology Co., Ltd.

Genome Assembly

Reads containing Illumina PCR adapters and low-quality reads were filtered from the dataset using readfq (vision 10). The remaining good quality paired reads were assembled into

scaffolds using the SOAP denovo (https://sourceforge.net/projects/soapdenovo2/files/SOAPdenovo2/), SPAdes (http://cab.spbu.ru/software/spades/), and ABySS (http://www.bcgsc.ca/platform/bioinfo/software/abyss) assemblers (14–17). The filtered reads were then used to close gaps in the scaffolds using Readfq with the options: –rq1 input_1.fq, –rq2 input_2.fq,–oq1 out_1.fq,–oq2 out_2.fq,–adp1 adapter_1.lst,–adp2 adapter_2.lst,– Q QUAL, PERCENT,–C QUAL, PERCENT,–N PERCENT,–alen INT,–amis INT,–dup,–gz,–check1 read1.check,–check2 read2.check.

Genome Feature Predictions

Genome component prediction included prediction of coding genes, repetitive sequences, non-coding RNAs, genomic islands, transposons, prophages, and clustered regularly interspaced short palindromic repeat sequences (CRISPRs). The Gene Mark program was used to identify coding genes, while interspersed repetitive sequences were predicted using the Repeat Masker (http://www.repeatmasker.org/). Tandem repeats were identified using the tandem repeats finder (TRF) program. Transfer RNA (tRNA) genes were predicted using tRNA scan-SE (18–21), while ribosomal RNA (rRNA) genes were identified using rRNAmmer (22). Small nuclear RNAs (snRNA) were predicted by BLAST searches against the Rfam database (23, 24). The Island Path-DIOMB program was used to predict genomic islands and the transposon PSI program was used to identify transposons based on the homologous blast method (25). Lastly, PHAST was used to predict prophages (http://phast.wishartlab.com/), and CRISPR Finder was used to identify CRISPRs (26, 27).

Gene Function Prediction

Four databases were used to predict gene functions, including the Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG) (28), and non-redundant (NR) protein databases (29–32). In addition, whole-genome BLAST searches were performed against the above-mentioned four databases using an *E*-value threshold of <1e-5 and a minimum alignment length percentage >40% as criteria (33). Secretory proteins were predicted using the Signal P database, and the prediction of Type I-VII proteins secreted by pathogenic bacteria was based on the EffectiveT3software program (34, 35). To evaluate pathogenic factors, pathogenicity, and drug resistance analyses were also conducted using the Virulence Factors of Pathogenic Bacteria (VFDB) and Antibiotic Resistance Genes Database (ARDB) databases (36, 37).

Comparative Genomic Analyses

Comparative genomic analyses were conducted including analysis of genomic synteny, the distribution of core- and strain-specific genes, and phylogenetic analysis of gene families. Genomic alignment between the sample and reference genomes was performed using the MUMmer and LASTZ tools while including 41 *V. cholerae* strains and 4 *V. metschnikovii* strains as reference genomes (see **Supplementary Table 2** for additional details) (38). The analysis of genomic synteny was based on alignment results. Core- and strain-specific genes were identified based on rapid clustering of similar proteins with the CD-HIT program while specifying a threshold of 50% pairwise identity and a 70% length difference cutoff. A Venn diagram was then used to show the relationships of core- and strain-specific genes among samples. The MUSCLE software program was used to align multiple single-copy core-encoded proteins identified by the core-/pan-genome analysis. The aligned sequences were then subjected to phylogenetic analysis using the TreeBeST program, a neighbor-joining tree reconstruction algorithm, and 1,000 bootstrap replicates.

Multilocus gene sequence typing was conducted based on sequence analysis of eight housekeeping genes (*adk, gyrB, mdh, metE, pgm, pntA, purM,* and *pyrC*) using previously described PubMLST protocols (https://pubmlst.org/databases.shtml). The nucleotide sequences for each locus were analyzed with the BioNumerics software program (version 7.6; Applied Maths, Belgium) and compared against published sequences on the PubMLST website. Sequence types (STs) were then determined on the basis of the eight locus allelic profiles.

Nucleotide Sequence

Accession Numbers

The 74 sequences of *V. cholerae* and *V. metschnikovii* were submitted to FigShare under the public doi: 10.6084/m9.figshare.14417870. And C16-2-29, M13F, M9D, M21D, M28D, and M29D uploaded Genbank database at the same time, accession number GCA_014281135.1, GCA_014305065.1, GCA_014267955.1, GCA_014267965.1, GCA_014305075.1 and GCA_014305185.1, respectively.

RESULTS

Genome Sequencing

A total of 40 *V. cholerae* and 34 *V. Metschnikovii* strains were identified in this study using PCR identification and comparison of gene sequences against the non-redundant (NR) protein database. All of the strains lacked the *ctxA, tcpA,* and *chxA* genes, and were also non-O1/O139 serotypes by PCR. All isolates were completely turbid after dripping *V. cholerae* O antisera and no agglutination occurred, the result was the consistent as the PCR, and it was identified as non-O1/O139 *V. cholerae* (**Table 1**).

Core- and Pan-Genomic Analysis

The *V. cholerae* pan-genomes were analyzed by dividing them into core and dispensable genomes. A total of 40 *V. cholerae* strains isolated from migratory birds were used in comparison against 41 reference *V. cholerae* genomes retrieved from GenBank. The number of core genes and the pan-genome sizes of the *V. cholerae* strains are shown in **Figure 1A** as a function of the number of genes within the genomes. The number of core genes plateaued when plotting the number of core genes against the reciprocal of the number of genomes included in the estimates. In contrast, the pan-genome size steadily increased with the addition of each additional genome, suggesting that *V. cholerae* exhibited a large pan-genome. Overall, a total of 18,310 pan-genes, 2,130 core-genes, and 16,180 dispensable genes were identified among all *V. cholerae* strain genomes (**Supplementary Table 3**). In this study, the dispensable genome

TABLE 1 | Characteristics of *Vibrio cholerae* and *Vibrio metschnikovii* isolates identified in migratory birds.

Isolate name	Sampling date	Species identification	Host	Sample location
C1F_2	2018.8.23	*V. cholerae*	*Larus ridibundus* (lung)	Chifeng, Inner Mongolia, China
C1C_1	2018.8.23	*V. cholerae*	*Larus ridibundus* (intestines)	Chifeng, Inner Mongolia, China
C1S_2	2018.8.23	*V. cholerae*	*Larus ridibundus* (kidney)	Chifeng, Inner Mongolia, China
C2XS_1	2018.8.23	*V. cholerae*	*Larus ridibundus* (kidney)	Chifeng, Inner Mongolia, China
C4C_1	2018.8.23	*V. cholerae*	*Himantopus* (intestines)	Chifeng, Inner Mongolia, China
C5G	2018.8.23	*V. cholerae*	*Pochard* (liver)	Chifeng, Inner Mongolia, China
C5G_R	2018.8.23	*V. cholerae*	*Pochard* (liver)	Chifeng, Inner Mongolia, China
C7F_1	2018.8.23	*V. cholerae*	*Larus argentatus* (lung)	Chifeng, Inner Mongolia, China
C8C_1	2018.8.23	*V. cholerae*	*Tadorna ferruginea* (intestines)	Chifeng, Inner Mongolia, China
C8C_2	2018.8.23	*V. cholerae*	*Tadorna ferruginea* (intestines)	Chifeng, Inner Mongolia, China
C11G_R	2018.8.23	*V. cholerae*	*Anas zonorhyncha* (liver)	Chifeng, Inner Mongolia, China
C11C_1	2018.8.23	*V. cholerae*	*Anas zonorhyncha* (intestines)	Chifeng, Inner Mongolia, China
C11S_2	2018.8.23	*V. cholerae*	*Anas zonorhyncha* (kidney)	Chifeng, Inner Mongolia, China
C18S_a	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (kidney)	Chifeng, Inner Mongolia, China
C18p_2	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (spleen)	Chifeng, Inner Mongolia, China
C18F_2	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (lung)	Chifeng, Inner Mongolia, China
C18x_2	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (heart)	Chifeng, Inner Mongolia, China
C18s_b	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (kidney)	Chifeng, Inner Mongolia, China
C18s_2	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (kidney)	Chifeng, Inner Mongolia, China
C18c_3	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (intestines)	Chifeng, Inner Mongolia, China
C18G_a	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (liver)	Chifeng, Inner Mongolia, China
C18F_b	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (lung)	Chifeng, Inner Mongolia, China
C18G_b	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (liver)	Chifeng, Inner Mongolia, China
C18c_a	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (intestines)	Chifeng, Inner Mongolia, China
C18x_b	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (heart)	Chifeng, Inner Mongolia, China
C19p_c	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (spleen)	Chifeng, Inner Mongolia, China
C19F_3	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (lung)	Chifeng, Inner Mongolia, China
C19c_a	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (intestines)	Chifeng, Inner Mongolia, China
C19c_B	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (intestines)	Chifeng, Inner Mongolia, China
C19G_b	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (liver)	Chifeng, Inner Mongolia, China
C19F_1	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (lung)	Chifeng, Inner Mongolia, China
C19S_b	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (kidney)	Chifeng, Inner Mongolia, China
C19x_a	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (heart)	Chifeng, Inner Mongolia, China
C19c_1	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (intestines)	Chifeng, Inner Mongolia, China
C19x_b	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (heart)	Chifeng, Inner Mongolia, China
C19G_a	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (liver)	Chifeng, Inner Mongolia, China
C19p_b	2018.8.30	*V. cholerae*	*Tadorna ferruginea* (spleen)	Chifeng, Inner Mongolia, China
C2W	2018.8.30	*V. cholerae*	Water	Chifeng, Inner Mongolia, China
C7W	2018.8.30	*V. cholerae*	Water	Chifeng, Inner Mongolia, China
C16_2_290	2017.8.4	*V. cholerae*	*Phalacrocorax*	Wuliangsuhai, Inner Mongolia, China
M4G_2	2018.8.23	*V. metschnikovii*	*Himantopus mexicanus* (liver)	Chifeng, Inner Mongolia, China
M5C_1	2018.8.23	*V. metschnikovii*	*Pochard* (intestines)	Chifeng, Inner Mongolia, China
M5C_2	2018.8.23	*V. metschnikovii*	*Pochard* (intestines)	Chifeng, Inner Mongolia, China
M7S_1	2018.8.23	*V. metschnikovii*	*Larus argentatus* (kidney)	Chifeng, Inner Mongolia, China
M7C_1	2018.8.23	*V. metschnikovii*	*Larus argentatus* (intestines)	Chifeng, Inner Mongolia, China
M9G_2	2018.8.23	*V. metschnikovii*	*Tadorna ferruginea* (liver)	Chifeng, Inner Mongolia, China
M11F_1	2018.8.23	*V. metschnikovii*	*Anas poecilorhyncha* (lung)	Chifeng, Inner Mongolia, China
M12C	2018.8.30	*V. metschnikovii*	*Pochard* (intestines)	Chifeng, Inner Mongolia, China
M13F	2018.8.30	*V. metschnikovii*	*Aix galericulata* (lung)	Chifeng, Inner Mongolia, China
M14Y	2018.8.30	*V. metschnikovii*	*Tadorna ferruginea* (pancreas)	Chifeng, Inner Mongolia, China

(Continued)

TABLE 1 | Continued

Isolate name	Sampling date	Species identification	Host	Sample location
M14Y_1	2018.8.30	*V. metschnikovii*	*Tadorna ferruginea* (pancreas)	Chifeng, Inner Mongolia, China
M17C_1	2018.8.30	*V. metschnikovii*	Migratory birds (intestines)	Chifeng, Inner Mongolia, China
M19C_2	2018.8.30	*V. metschnikovii*	*Tadorna ferruginea* (intestines)	Chifeng, Inner Mongolia, China
M19C_6	2018.8.30	*V. metschnikovii*	*Tadorna ferruginea* (intestines)	Chifeng, Inner Mongolia, China
M1W	2018.8.30	*V. metschnikovii*	Water	Chifeng, Inner Mongolia, China
M3W	2018.8.30	*V. metschnikovii*	Water	Chifeng, Inner Mongolia, China
MH3GW	2018.8.30	*V. metschnikovii*	Water	Chifeng, Inner Mongolia, China
MNW	2018.8.30	*V. metschnikovii*	Water	Chifeng, Inner Mongolia, China
MNW_3	2018.8.30	*V. metschnikovii*	Water	Chifeng, Inner Mongolia, China
M3X	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M7D	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M8D	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M9D	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M14D	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M15D	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M16X	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M19X	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M21D	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M26X	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M27D	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M28D	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M29D	2019.7	*V. metschnikovii*	*Larus ridibundus*	Chifeng, Inner Mongolia, China
M19_1W	2019.7	*V. metschnikovii*	Water	Chifeng, Inner Mongolia, China
M19_6WC	2019.7	*V. metschnikovii*	Aquatic plants	Chifeng, Inner Mongolia, China

was considered to comprise strain-specific genes. A heatmap was constructed to identify the distribution of dispensable genes among different *V. cholerae* strains. With the exception of the strain TSY216 genome that contained 984 dispensable genes, most other strains in the group C clade contained few dispensable genes.

To analyze the *V. metschnikovii* pan-genome, 34 *V. metschnikovii* strain genomes recovered from migratory bird isolates and four reference genomes of *V. metschnikovii* strains were used for comparative analysis. The size of the pan-genome steadily increased with the addition of each additional genome in the analysis (**Figure 1B**), suggesting that *V. metschnikovii* also exhibited a large pan-genome. A total of 10,530 pan-genes, 1,352 core-genes, and 9,178 dispensable genes were identified among all *V. metschnikovii* strains. Like the genomes of the *V. cholerae* strains, most *V. metschnikovii* genomes did not contain many dispensable genes, with the exception of strain M13F, whose genome contained 1,048 dispensable genes.

Phylogenetic Analysis

Phylogenetic analysis indicated the presence of similar branching patterns, wherein the *V. cholerae* genomes consistently grouped into two major clades. The first clade contained *V. cholerae* strains from the GenBank reference database and one strain (C16-2-29) from our study. All of the other reference strains, with the exception of strain NCTC30, were present in this clade. In the first clade, strains harboring the cholera toxin (CTX) were divided into group C, while the others in the first clade were considered as group A. All of the strains identified in this study, with the exception of strain C16-2-29 (Wuliangsuhai, 2017), were present in the second clade, which was designated as Group B (**Figure 2**).

MLST analysis of the pandemic *V. cholerae* strains was conducted using eight different loci (*adk, gyrB, mdh, metE, pgm, pntA, purM,* and *pyrC*). However, only seven different loci (*adk, gyrB, mdh, metE, pntA, purM,* and *pyrC*) were used for the MLST of the environmental *V. cholerae* strains (**Supplementary Table 3**), since *pgm* was not detected in the environmental strain genomes. With the exception of the C16-2-29 genome, most of the housekeeping genes of *V. cholerae* strains from migratory birds did not exhibit homology to known genotypes in the pubMLST database. In addition, the *pgm* gene was detected in the strain C16-2-29 genome, as observed for the pandemic strain genomes. ST69 was the common sequence typing classification for the pandemic strains, while the sixth pandemic strain, O395, exhibited an ST73 sequence type. ST69 was not observed for the *V. cholerae* strains derived from environments or migratory birds.

Phylognetically, the *V. metschnikovii* and *V. cholerae* strains were genetically highly distant. The *V. metschnikovii* genomes consistently grouped into two major clades, with the first comprising *V. metschnikovii* strains from different samples and

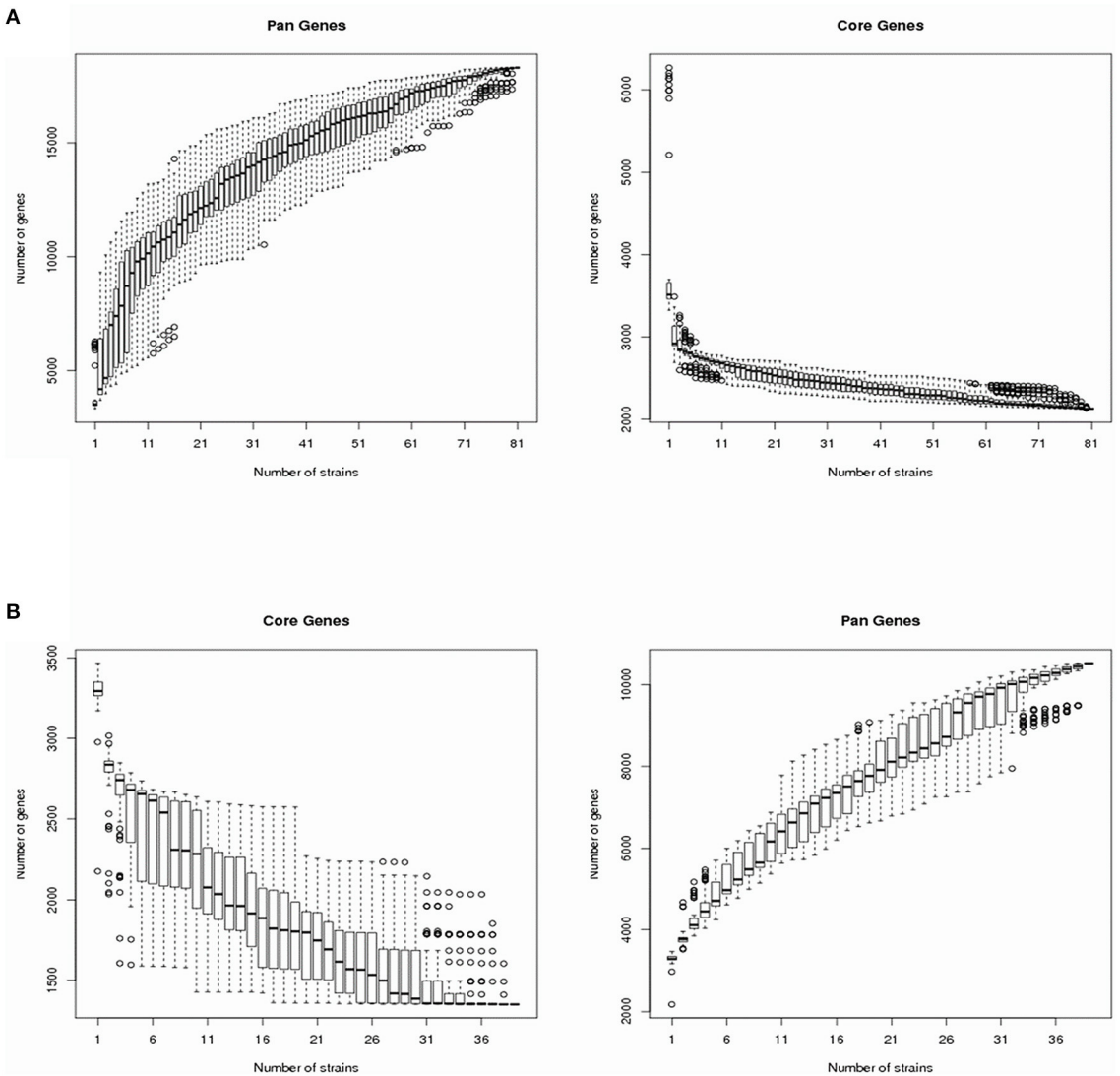

FIGURE 1 | *Vibrio cholerae* and *Vibrio metschnikovii* core-/pan-genome diversity curves. **(A)** The *V. cholerae* core- and pan-genomes are shown as a function of the number of genomes included in the counts. Boxes represent one standard deviation around the median number of genes within the subsets, while the whiskers indicate two standard deviations from the median. **(B)** The *V. metschnikovii* core- and pan-genomes are shown as a function of the number of genomes included in the counts. Boxes represent one standard deviation around the median number of genes within the subsets, while the whiskers indicate two standard deviations from the median.

the reference genomes. This first group was designated as Group D. The second clade comprised four strains (M21D, M28D, M9D, and M29D) designated as Group E (**Figure 3**). A *V. metschnikovii* MLST database has not been established yet, and thus the *V. metschnikovii* genomes were not subject to these analyses.

Comparison of Gene Functional Enrichment

Gene functions were predicted from the predicted genes and subjected to functional gene enrichment analysis. The *V. cholerae* core genes were enriched in numerous KEGG pathways including the "*E*," amino acid transport and metabolism, and "*T*," signal transduction mechanism pathways of the COG database. The numbers of genes within the "*T*," signal transduction mechanism pathway, were enriched in group C genomes relative to "*E*," amino acid transport and metabolism pathways. However, the other *V. cholerae* strain genomes exhibited opposite enrichment patterns (**Figure 4**).

To further investigate the functional differences encoded by the *Vibrio* genomes, we analyzed the "metabolism and environmental information processing" functional category encoded by different *V. cholerae* group genomes. Differences were observed in trimetlylamine oxide (TMAO) metabolism, in addition to two-component systems. Specifically, only group C genomes encoded complete TMAO systems. In addition, the genomes of the other groups lacked *TorA*, and these isolates are thus unable to metabolize TMAO. Differences

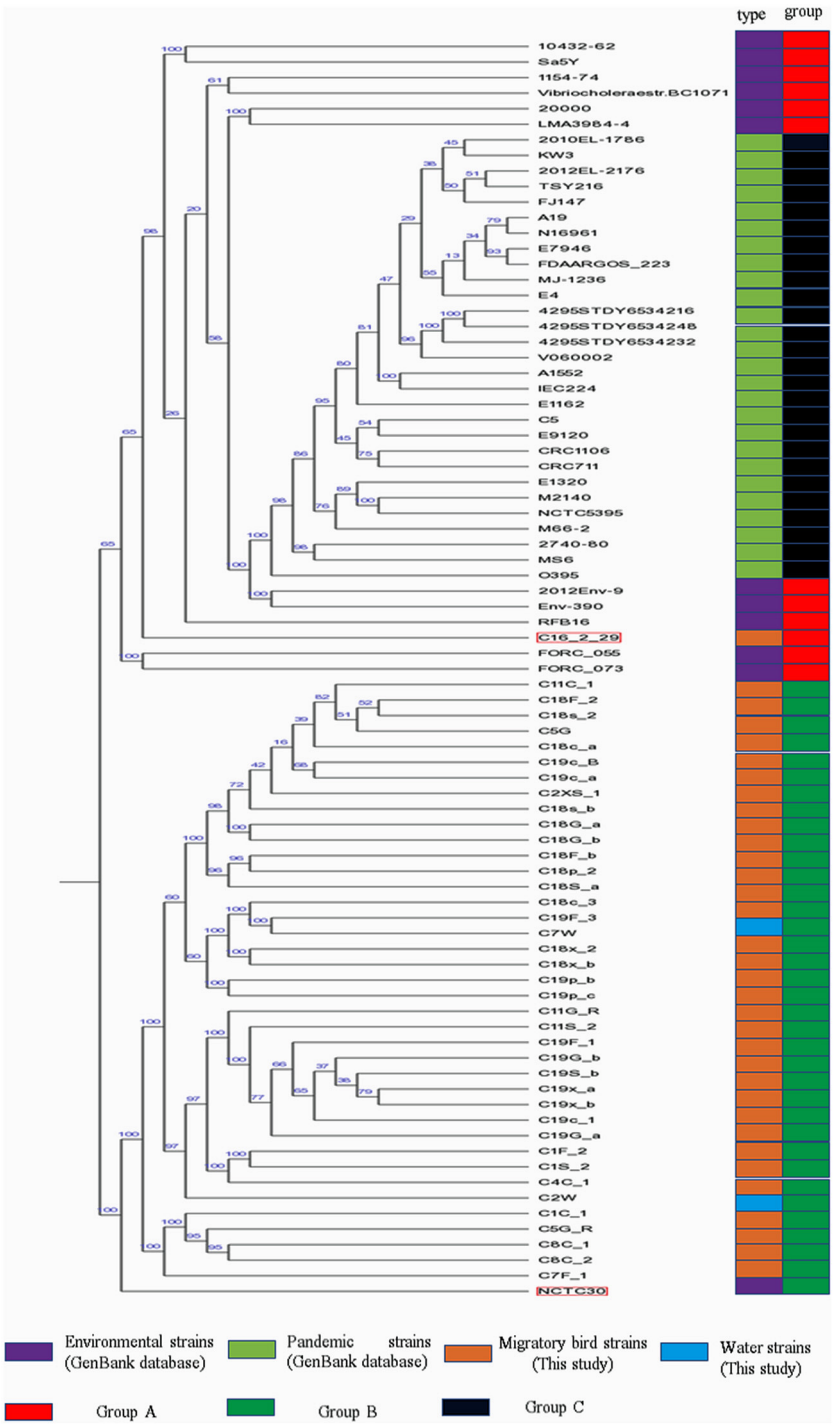

FIGURE 2 | Phylogenetic analysis of *V. cholera* strains. Different colored blocks are used to indicate types and groups of strains on the left of phylogenetic tree. The color legend is shown below the phylogenetic tree.

between the two groups of *V. metschnikovii* strain genomes were minimal, although all group genomes were particularly enriched in genes involved in the "*E*," amino acid transport and metabolism pathway.

Pathogenicity and Antibiotic-Resistance Potential

The *V. cholerae* genomes generated in this study only encoded partial RTX protein structures and not *rtxA*. In

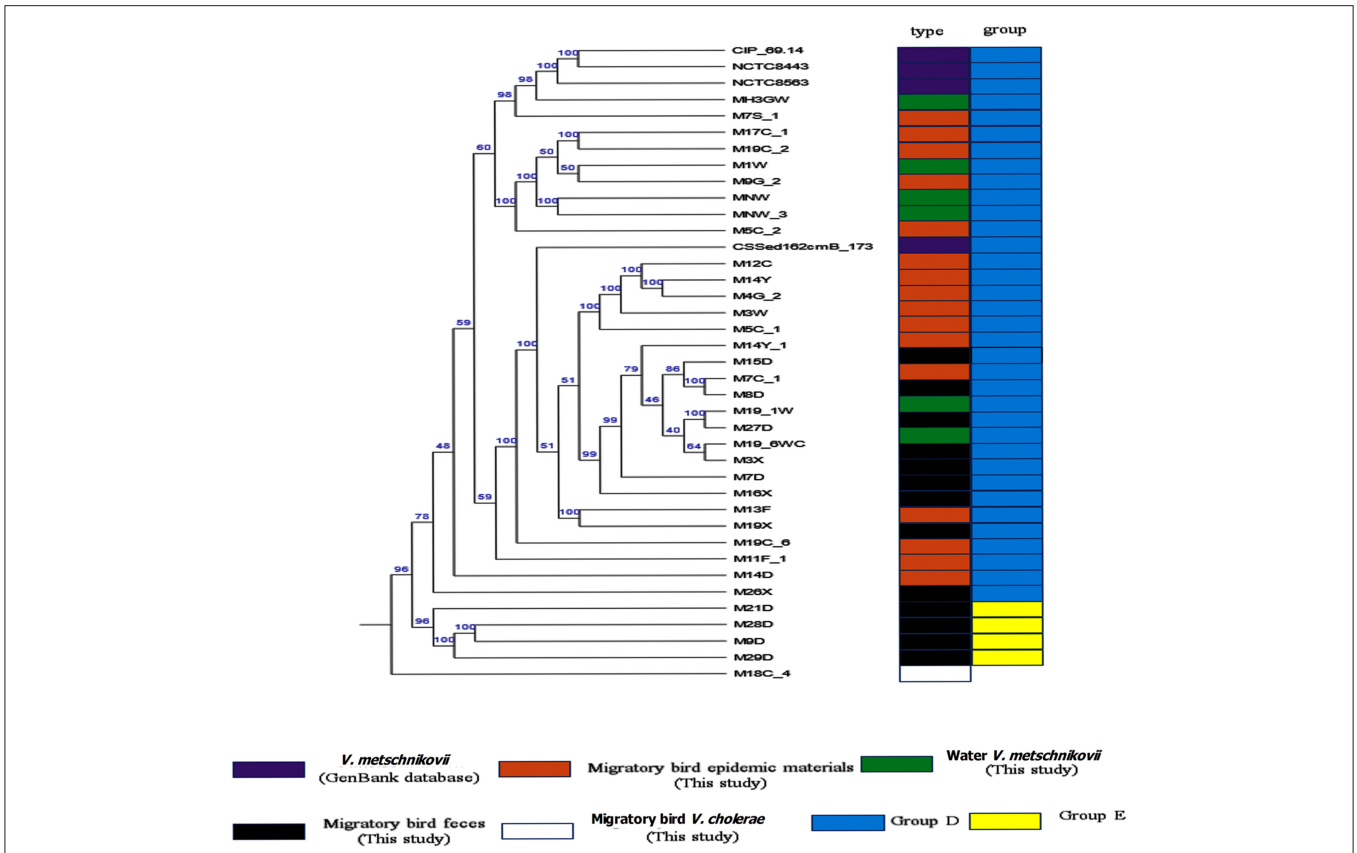

FIGURE 3 | Phylogenetic analysis of *V. metschnikovii* strain genomes. Different colored blocks are used to indicate types and groups of strains on the left of phylogenetic tree. The color legend is shown below the phylogenetic tree.

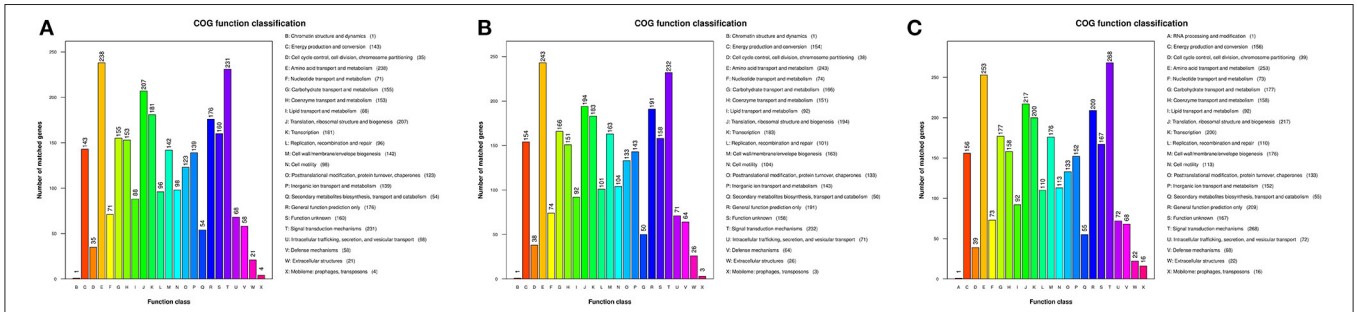

FIGURE 4 | Functional classification of *V. cholerae* core genome COG functions. **(A)** COG functional classification of the core genomes of reference environmental *V. cholerae* strains; **(B)** COG functional classification of the core genomes of migratory bird *V. cholerae* strains; **(C)** COG functional classification of the core genomes of *V. cholerae* pandemic strains retrieved from the GenBank database.

addition, all of the groups encoded complete type VI secretory systems (T6SS). The presence of drug resistance genes for *V. cholerae* in the genomes generated here exhibited low frequencies of resistance genes and the lack of plasmids (**Supplementary Table 4**). All *V. metschnikovii* strains lacked CTX, and RTX. However, the genomes of the four strains from migratory birds (M13F, M9D, M29D, and M28D) encoded a T6SS system. Among these, three strains (M9D, M29D, and M28D) lacked other genes involved in secreting proteins (e.g., *hcp* and *vgrG*), while only M13F encoded a complete T6SS system *V. metschnikovii.*

DISCUSSION

Vibrio cholerae acquired cholera toxin (CTX), which is the primary reason for their ability to cause global pandemics, and CTX acquisition occurred over a long evolutionary history (39).

CTX was likely introduced to *Vibrio* by the CTXϕ phage, while the toxin co-regulated pilus (TCP) is also a critical colonization factor of *V. cholerae* that serves as a receptor for CTXϕ (40). Other studies have indicated that AphA functions in a previously unknown step in the *ToxR* virulence cascade and helps activate the transcription of *tcpPH*. *TcpP/TcpH*, together with *ToxR/ToxS*, then activate *toxT* expression, ultimately resulting in the production of virulence factors like the cholera toxin and TCP (41–43). *Vibrio* strains isolated in this study exhibited unique combinations of virulence factor genes [e.g., *ctx*(-) *tcpA*(-) *hlyA*(+) *AphA*(+) *toxR*(+) *toxT*(-) *tcpP*(-) *tcpH*(-)], and thus they did not contain complete virulence factor regulatory networks based on the lack of *toxT*.

The group C genomes identified in this study also encoded the TMAO metabolic pathway, which differed from other *Vibrio* groups based on core-/pan-genome analysis. The TorR response regulator mediates TMAO induction of the *torCAD* operon expression and was intriguingly observed in all of the *V. cholerae* pandemic strains. With the exception of M66-2, all of the other strains in group C also encoded CTX. The original M66-2 strain did encode CTX, but CTX was knocked out of the strain in a previous study (44). The TMAO metabolic pathway exhibits a unique relationship with CTX production. Specifically, human intestines are anaerobic, and *V. cholerae*, as a facultative anaerobe, is able to grow by anaerobic respiration. CTX is a major virulence factor of *V. cholerae*, and its production is highly promoted during anaerobic growth using trimethylamine N-oxide (TMAO) as an alternative electron acceptor (45). The *V. cholerae* strains from environmental samples lacked the *torA* operon, which could lead to an inability to conduct TMAO respiration and thus produce CTX. However, the TMAO metabolic signaling pathway was not observed in the migratory bird *V. cholerae* strain genomes. Thus, it is likely that this metabolic pathway is not present in all *V. cholerae* strains. However, strain C16-2-29 shared very similar genomic attributes with the reference pandemic strains, unlike the other migratory bird *V. cholerae* strains. Specifically, the strain harbored the ability to encode a complete TMAO metabolic pathway, despite the apparent inability to produce CTX.

It should be noted that we were not able to exclude geographical factors that can influence bacterial evolution or differences in genomic contents. Strains that were isolated from the same host species but that exhibit differences in two-component systems will respond to external stresses differently. We speculated that conditionally pathogenic *V. cholerae* would unlike to evolve into a strain with CTX over a short period. Accordingly, the cholera pandemic strains have all been confirmed to be clonally related (2). The genomes of most strains belonging to group C contained fewer dispensable genes based on core-/pan-genomic analysis. A notable exception was the genome of TSY216, which contained more strain-specific genes. In addition to harboring two pairs of chromosomes, strain TSY216 also harbors a giant replicon that could explain its high strain-specificity relative to the others (46).

We additionally searched for other virulence genes in the genomes of *V. cholerae* from migratory birds by comparing them against the VFDB database. While the genomes contained some of the pathway components to produce the RTX toxin, they lacked the *rtxA* gene. *rtxA* deletion can cause a significant reduction in RTX toxin production (47). All strain genomes also encoded the T6SS system, while the *V. cholerae* genomes from migratory birds also harbored complete T6SS pathways based on KEGG analysis. T6SS was first discovered in *V. cholerae* in 2006 (48). Several of the *V. cholerae* groups encoded the T6SS system, and thus T6SS is probably a specific secretion system of *V. cholerae*. Only one of the three *V. metschnikovii* strain genomes contained the gene encoding the spike protein for T6SS. The *V. metschnikovii* strain encoding the spike protein of T6SS was isolated from migratory bird epidemic materials, while the *V. metschnikovii* strains that did not encode the spike protein of T6SS were isolated from healthy migratory bird feces in the second year. Importantly, T6SS without a spike protein does not confer pathogenic ability. Nevertheless, these results indicate that T6SS systems could have begun to evolve in *V. metschnikovii* strains. The M19X and M13F strains were closely related in the core genome phylogenetic analysis, although strain M13F did not encode a complete T6SS structure. Furthermore, strain M13F contained the HS I-I system that could activate T6SS, while M19X did not. Thus, different induction environments might activate the expression of HS I-I.

Phylogenetic analysis indicated that the *V. cholerae* strains from migratory birds (including strain NCTC30) were largely in group B, with the exception of C16-2-29. Strain NCTC30 was isolated from a World War One soldier with diarrhea (49). The strain harbored the T3SS-1 system, conferring it cytotoxicity (50). However, all of the migratory bird *V. cholerae* strains lacked the T3SS-1 system.

MLST analysis revealed a high degree of diversity among the strains that were evaluated. Indeed, these analyses indicated that the migratory bird strains harbored numerous new *V. cholerae* alleles and STs that have not been previously observed in databases. A total of seven different loci were used for MLST analysis of the environmental *V. cholerae* strains (excluding strain C16-2-29), while MLST analysis of the pandemic strains and C16-2-29 was performed using eight different loci (**Supplementary Table 2**). The combined phylogenetic analyses indicated that C16-2-29 was more closely related to the pandemic strains than to the strains isolated from the migratory birds.

In contrast to other migratory bird *V. cholerae* strains, C16-2-29 was classified within group A. The primary difference between the genome of this strain and other migratory bird *V. cholerae* strains was differences in the sequence of the Cbb3-type cytochrome oxidase. Accordingly, the genetic relationship between strain C16-2-29 and strains from group C was low. The COG group, "R," functional genes were specifically enriched in the C16-2-29 strain relative to the others. In addition, C16-2-29 harbored a complete biosynthesis pathway for siderophore group non-ribosomal peptides based on KEGG annotations, while the other strains did not. The C16-2-29 strain was isolated from the Wuliangsuhai area of the Inner Mongolia autonomous region of China in 2017, while the other migratory bird *V. cholerae* strains were isolated from the Chifeng area of the Inner Mongolia autonomous region of China in 2018. Consequently,

it is unclear whether differences in geographic location influence the evolution of *V. cholerae* migratory bird strain genomes.

Transmissible elements similar to plasmids were not found integrated into the *V. cholerae* and *V. metschnikovii* genomes from migratory bird strains, indicating little chance of transmitting and spreading resistance genes. Rather, inherent resistance mechanisms were identified on the genomic chromosomes. For example, efflux pump-based inhibition is likely to be a viable strategy to overcome antibiotic resistance for V. *cholerae* and *V. metschnikovii* strains present in migratory birds. An important pathogenic characteristic to identify is whether *V. cholerae* can form biofilms based on complete formation pathways. All of the strains analyzed here exhibited this capacity, except for the environmental strains and those from migratory birds. Moreover, the biofilm formation capacity of the *V. metschnikovii* strains was the same as the environmental strains and *V. cholerae* strains from the migratory birds, wherein the basement protein-forming gene *RmbA* was absent. Thus, biofilms formed by these strains might be thinner than those of group C, or they may not develop a three-dimensional structure (51, 52). In addition, CTXϕ phage has been observed in biofilms (53), although it is yet unclear whether a relationship exists between biofilm thickness and the presence of CTXϕ phage.

Overall, our study demonstrates that *V. cholerae* strains isolated from migratory birds do not exhibit genomic features consistent with an ability to cause pandemics, or otherwise be pathogenic. Specifically, these strains were identified as non-O1/O139 serotypes and only conditional pathogens. In addition, we document the first isolation of *V. metschnikovii* strains with complete T6SS pathways. Both *V. cholerae* and *V. metschnikovii* strains may cause intestinal discomfort in migratory birds through the activities of T6SS and hemolysin. In contrast, all strains with an ability to form biofilms were identified in aquatic plant or animal intestine environments.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the FigShare repository: https://doi.org/10.6084/m9.figshare.14417870.v2.

ETHICS STATEMENT

All migratory bird stool samples were collected under the supervision by the Wild Animal Sources and Diseases Inspection Station, National Forestry and Grassland Bureau of China, and did not cause any harm to the animals. All migratory bird epidemic material samples were provided by the local animal disease prevention and control center for bacteriological examination. The experimental protocol was established, according to the ethical guidelines of Helsinki Declaration and was approved by the Laboratory Animal Welfare and Ethics Committee of the Institute of Military Veterinary Science, the Academy of Military Medical Sciences (AMMS - 11 - 2020 - 11).

AUTHOR CONTRIBUTIONS

PC and X-JG conceived, directed, and carried out the study. L-WZ, DC, L-HX, JJ, YS, and G-JL prepared samples for sequence analysis. XJ, J-yG, and LZ acquired samples and analyzed the data. All authors have read and approved the final manuscript.

FUNDING

Publication costs are also funded by the National Key Research and Development Programme of China (No. 2016YFD0501305). Funding for study design, data collection, and data generation was provided by the National Science and Technology Major Project of China (Grant agreement 2018ZX10733402).

ACKNOWLEDGMENTS

We are grateful to members of Dali Nuoer Lake protected area, Chifeng city, Inner Mongolia autonomous region, China, which help for sampling.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.638820/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 15 June 2021
doi: 10.3389/fvets.2021.666254

A Retrospective Survey of the Abortion Outbreak Event Caused by Brucellosis at a Blue Fox Breeding Farm in Heilongjiang Province, China

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OPEN ACCESS

Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

Reviewed by:
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Kumaragurubaran Karthik, Tamil Nadu Veterinary and Animal Sciences University, India

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *09 February 2021*
Accepted: *06 April 2021*
Published: *15 June 2021*

Citation:
Zhou Y, Meng Y, Ren Y, Liu Z and Li Z (2021) A Retrospective Survey of the Abortion Outbreak Event Caused by Brucellosis at a Blue Fox Breeding Farm in Heilongjiang Province, China. Front. Vet. Sci. 8:666254. doi: 10.3389/fvets.2021.666254

Brucellosis is a common zoonosis in China, resulting in abortion in animals. Outbreaks of abortion in blue foxes caused by *Brucella* infection have rarely been reported. In the present study, 3–5 mL blood samples collected from the femoral veins of 10 abortuses of blue foxes were assessed by RBPT (Rose Bengal plate test) and SAT (serum tube agglutination test) to preliminarily investigate the source of infection for the clustering of abortion events at a blue fox farm in Heilongjiang Province. Screening experiments showed that all 10 blood samples were positive in the RBPT, while only eight blood samples out of the 10 were positive in the SAT. Subsequently, 10 tissue samples (spleen, lungs, stomach contents, and afterbirth) from the same 10 foxes were assessed using AMOS (acronym for *B. abortus*, *melitensis*, *ovis*, and *suis*)-PCR (polymerase chain reaction), and sequencing analysis was performed on amplification products to verify the results of the serology survey. Results showed a spectral band of ∼731 bp in these samples. BLAST showed sequences of AMOS-PCR products in this study to be 100% similar ($E = 0.0$) to sequences in *B. melitensis* strain from GenBank. These data preliminarily indicated that the blue fox's outbreak of abortion events was caused by brucellosis via the *B. melitensis* strain. Then 726 serum samples were tested by RBPT and SAT to determine the prevalence of brucellosis on the farm. A comprehensive epidemiological and reproductive status survey of the infected blue fox population was performed. The seropositive rate was found to be 67.90% (493/726) by RBPT and 41.32% (300/726) by SAT. The technicians had stopped feeding the foxes with chicken carcasses and instead fed them raw ground sheep organs (lungs, tracheae, placentae, and dead sheep fetuses) infected by *B. meliteneis* strains, and that this change in diet caused the outbreak of abortion events. The high abortion rate (55%) and low cub survival rate (65%) were the most distinctive features of the outbreak; these factors led to severe economic losses. Feeding cooked sheep/goat offal and strict breeding management is necessary for disease prevention.

Keywords: *Brucella melitensis*, abortion, reproductive, blue fox, goats (sheep) offal

INTRODUCTION

Brucellosis is a widespread zoonotic disease that is caused by bacteria and is categorized as a bacterial human disease (1). The World Organization for Animal Health (OIE) lists brucellosis as a multi-animal comorbidity (2), and brucellosis is a second-category animal infectious disease in China (3). The disease mainly affects the reproductive systems of animals (4, 5). Although 12 *Brucella* species have been identified, *B. melitensis*, *B. abortus*, and *B. suis* are the most common pathogens occurring in human and animal infections (6). Among domestic animals, cattle, sheep, and pigs are infected most frequently, and the disease can be transmitted to bison, elk, wild boars, foxes, hares, African buffalo, and reindeer (7). Brucellosis has caused huge economic losses in the animal husbandry and economic animal breeding industries worldwide (8, 9). The highest and lowest prevalence rates of brucellosis among different fox species were found in red fox (*Vulpes vulpes*) (100%) and hoary fox (*Lycalopex vetulus*) (9%), respectively (10). A study showed *Gardnerella vaginalis* to be the main pathogen that causes miscarriage in foxes in China; the seropositivity rate range of fox population in China is 0.9–21.9%, and in some farms it exceeds 75% (11). Canine distemper virus, pseudorabies virus, and *Staphylococcus aureus* are common pathogenic agents in the fox population (12), but there is no report of fox abortion caused by *Brucella* spp. Moreover, the incidence of brucellosis in China has continued to rise in recent years. Heilongjiang Province was designated a Type I brucellosis severe epidemic region due to the ongoing high incidence rate of animal brucellosis (13, 14). The animal husbandry industry is a main economic pillar of this province, and fox and raccoon breeding are the main sources of income for many farmers in this region. In March 2017, an outbreak of abortion of unknown origin occurred at a blue fox breeding farm in Heilongjiang Province, resulting in a high rate of abortion in pregnant blue foxes and causing serious economic losses. At present, serological techniques remains the mainstay for brucellosis diagnosis (15). These include the Rose Bengal Plate Test (RBPT), serum agglutination test (SAT), and complement-fixation test (CFT) (16–18). However, CFT is a technically complex test, and it requires good laboratory facilities and well-trained personnel to perform it accurately and maintain its reagents (19). Moreover, identification of *Brucella* sp. by conventional tests involves considerable time, risk of human infection, and expert interpretation, whereas PCR is fast, safe, and easy to interpret (20, 21). Previous works described a *Brucella* PCR assay that can distinguish *Brucella abortus* (biovars 1, 2, and 4), *Brucella melitensis* (biovars 1, 2, and 3), *Brucella ovis*, and *Brucella suis* (biovar 1) from each other (22). In this study, RBPT, SAT, and AMOS (*B. abortus*, *B. melitensis, Brucella ovis*, and *Brucella suis*)—PCR were used to determine the cause of the outbreak of abortions at a blue fox farm. Our investigation will provide important data for technical guidance in the prevention of blue fox brucellosis as well as promote better management of blue foxes in Heilongjiang province, China.

Abbreviations: RBPT, Rose-Bengal plate test; SAT, serum tube agglutination test; AMOS-PCR, B. abortus-*melitensis-ovis-suis* polymerase chain reaction.

METHODS

Serological Testing

Blood samples were collected from the femoral vein, 3–5 mL per blue fox. A total of 10 serum samples (HBF001–010) were collected from 10 female foxes that had miscarried during 15–20 days in April 2017, and 726 serum samples [65 male foxes, 34 male cub foxes (<1 year old), 564 female foxes, and 61 female cub foxes (<1 year old)] from the blue fox farm were collected in October 2017 to implement the epidemiological survey. Both the Rose Bengal plate test (RBPT) and the Serum Agglutination Test (SAT) were performed according to standard serological procedures (23). RBPT and SAT were used to diagnose human brucellosis (23). RBPT antigen (production batch number: 201701) and SAT antigen (production batch number: 201702) were purchased from Qingdao Yibang Bioengineering Co., Ltd.; brucellosis positive control serum (production batch number: 201702) and negative control serum (production batch number: 201701) were purchased from China Veterinary Drug Supervision Institute. Sperm samples collected from male foxes were preliminarily screened for quality by microscopic examination. Some medicines, including oxytetracycline, astragalus polysaccharides, Vitamin E, and other herbs, were used to treat the blue foxes.

AMOS-PCR

The 10 tissue samples (liver, spleen, lungs, stomach contents, and afterbirth) from the same 10 aborted blue fox fetuses were collected following biosafety regulations. DNA of all samples was extracted using a Qiagen genome DNA prepare kit (Qiagen, Germany) according to the manufacturer's instructions. Subsequently, AMOS-PCR was employed to discriminate the species/biovar of *Brucella* strains. Amplification and detection procedures were as previously described (24). Briefly, the concentration of the four primer pairs was 25 μM/L, and primer A 1 μL, primer M 1.5 μL, primer O 1.5 μL, primer S 1 μL, primer IS711 2 μL, Taq DNA polymerase 1.25 U, and DNA template 2 μL. Finally, sterilized double distilled water was added to a final volume of 50 μL. Amplification parameters: 94°C pre-denaturation 5 min; 94°C 1 min, 60°C 1.5 min, 72°C 10 min, for 40 cycles; final extension at 72°C for 10 min. Five microliter products and 1 μL loading buffer were uploaded to agarose gels to determine the sizes of products. The target gene size was 498 bp for *B. abortus* (bv. 1, 2, and 4), 731 bp for *B. melitensis*, 976 bp for *B. ovis*, and 285 bp for *B. suis* (bv. 1). Then, 10 AMOS-PCR products were sequencing using M primer (F) and comparison was performed using the Basic Local Alignment Search Tool (BLAST).

The Evaluation of Reproductive Performance in Female Blue Foxes

The breeding conditions, estrus rate, weak cub rate, abortion rate, disease occurrence, and medication use of the blue fox farm from 2017 to 2019 were investigated to determine the production performance impact of a female blue fox infected with *B. melitensis*.

TABLE 1 | Brucellosis epidemic situation as detected by serological tests in 726 serum samples from blue fox breeding farm.

Methods	Male	Male cub foxes	Female foxes	Female cub foxes	Total (%)
RBPT (%)	61.58 (40/65)	11.76 (4/34)	78.55 (443/564)	8.20 (5/61)	67.90 (493/726)
SAT (%)	38.49 (25/65)	0	48.58 (274/564)	1.63 (1/61)	41.32 (300/726)

RBPT, Rose Bengal plate test; SAT, serum tube agglutination test.

RESULTS

Serological Tests

In order to investigation the cause of the outbeak abortus event. First, ten samples from female foxes were collected and examined by RBPT and SAT. The RBPT results in all 10 serum samples from female foxes were positive. However, eight samples were positive for the SAT (titer 1:50, ++), while the two remaining samples were all suspect cases (titer 1:50, +) (**Supplementary Table 1**). A preliminary serological survey indicated that infection with *Brucella* spp. could be a cause of spontaneous abortion in blue foxes. Subsequently, for further survey the situation the infection in blue fax farming, a total of 726 serum samples were collected and detected by RBPT and SAT. The positive rate of the RBPT was 67.90% (493/726) (**Table 1**), and the positive rate of the SAT was 41.32% (300/726) (**Table 1**). The SAT titer in 125 samples was 1:25 + (**Table 1**). Finally, eight of the human staff of this far were screened for serum antibodies against *Brucella* infection in eight staff in this farming were performed, five staff members of the farm were diagnosed with brucellosis, while there were no brucellosis antibodies detected in the other three staff members. The obvious clinical symptoms (swollen testicles, bedridden, back pain, leg pain) were observed in five brucellosis patients. They frequently ground the raw internal organs of sheep/goat to feed the blue foxes.

AMOS-PCR Amplification

The AMOS-PCR showed that the expected 731 bp size amplified result was observed in three positive controls (*B. melitensis* M5; 6. *B. abortus* A19, and *B. suis* S2), and there were no bands in the negative control *E. coli* strain. Moreover, an expected 731 bp band was detected among four different tissue types in the samples from aborted fetuses, including spleen, lung, stomach contents, and fetal coats, consistent with the target gene fragment of *B. melitensis* strains (**Figure 1**). PCR product sequencing showed that sequences ∼700 bp in size were obtained from all 10 samples. Further BLAST showed that these sequences were 100% similar ($E = 0.0$, sort by percent identity as 100%) to sequences of *B. melitensis* strain hosted in GenBank (**Supplementary Figure 1**). This result further verified the results from serological tests as well as confirming that *B. melitensis* was the pathogen involved in the blue fox cluster of abortion events.

The Epidemiology Investigation

The farm began breeding blue foxes in 2014. In 2016, there were 2,000 female foxes and 110 male foxes, and the abortion rate was 5%. Blue foxes started mating in March 2017, and miscarriages occurred 10–40 days after pregnancy [in general, around 53 days (49–56) for the entire pregnancy]. Although the female fox's estrus rate was 85% (1,700/2,000) in that year, the miscarriage rate was 50% (850/1,700); weak cubs accounted for 5% (250/5,000), and the mortality rate of foxes reached 35% (1,750/5,000) (**Table 2**). After the brucellosis was diagnosed, oxytetracycline, astragalus polysaccharides, Vitamin E, and other herbs were used for treatment, but these had no effect. Therefore, only 18 brucellosis-positive female foxes were kept for breeding in 2018, and the remainder were eliminated. The investigation found that from August 2016 to November 2016, previously used chicken carcasses were replaced by raw ground sheep internal organs (lungs, tracheae, placentae, and dead fetuses) to feed breeding foxes, and clustering of female fox abortions occurred a few months later. After being infected, female blue foxes were without any obvious manifestations; however, reduced sperm counts and deformed sperm in male foxes were observed in microscopic examinations (unpublished).

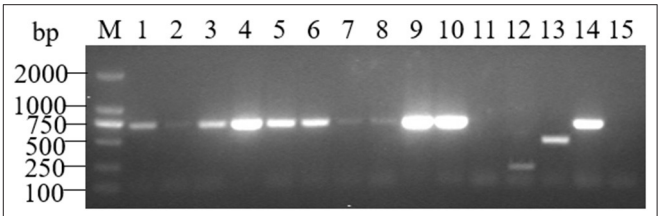

FIGURE 1 | AMOS-PCR typing of the sample from three aborted fetuses of the blue fox. M, marker, DL2000 bp; lane 1–3, spleen, lungs and stomach contents samples from HBF001; lane 4–6, spleen, lungs and stomach contents samples from HBF002; lane 7–8, spleen and lungs samples from HBF003; lane 9–11, Fetal coats samples from three aborted fetus (HBF001-3); lane 12, *B. suis* S2; lane 13, *B. abortus* A19; lane 14, *B. melitensis* M5; lane 15. Negative control, *E. coli*.

DISCUSSION

Brucellosis is one of the most important infectious causes of reproductive disorders in various species of animals (25). Various *Brucella* species are well-known causes of contagious abortion in cattle, sheep, goats, swine, and other animals (26). In the present study, both serological and AMOS-PCR methods confirmed that a *Brucella* spp. strain was the cause of the outbreak of abortion among blue foxes on this farm. Similarly, a previous study reported that brucellosis was found in a fox farm (27). Molecular tools can support the results from serological tests to avoid cross-reaction with other pathogens (28). AMOS-PCR results showed the presence of this special 731 bp band in many aborted fetuses' samples. Moreover, sequences from PCR products have 100% similarity to *B. melitensis* sequences from GenBank. These data

TABLE 2 | The reproduction profile of the blue fox in this farm during 2016–2018.

Years	Female no.	Estrus rate (%)	Abortion rate (%)	Survival rate (%)
2016	2,000	93.33	5	98
2017	2,000	85.00	50	65
2018	18	77.78	7.14	33.33

2017 is the year the infection occurred.

indicate that the outbreak at the blue fox farm was causing by the *B. melitensis* infected. A similar study showed that *B. melitensis* biovar 3 was the main pathogen responsible for cow and sheep abortion in China, and that this variant posed a human health risk (29). The seroprevalence of brucellosis in sheep and goat flocks was higher in eastern China, with 7.00% positive rate, than in any other region (30). Heilongjiang Province is one of the severe animal brucellosis epidemic regions in northern China (30). Moreover, ∼9% (56/621) of the samples from yaks were seropositive for *Brucella* tested via SAT at the Qinghai-Tibet Plateau, China (31). Similarly, the individual yak seroprevalence of brucellosis was 2.8% and herd level seroprevalence was 18.2% (32). Also, *Brucella* strains were isolated from the wildlife in China, such as blue sheep (*Pseudois nayaur*), yaks (*Bos mutus grunniens*), and Tibetan gazelle (*Procapra picticaudata*) (33). *B. melitensis* biovar 3 from the spleen of an Asian badger (*Meles leucurus*) showed a MLVA-16 genotype similar to that of isolates from local aborted sheep fetuses (34).

Our surveys showed that sporadic abortion events occurred in 5% of pregnancies on this farm during 2016. However, a >50% abortion rate was observed in 2017. The blue fox farm did not introduce new foxes during the period 2014–2017, and the breeding environment had not changed. The only changed factor was the feed for the blue foxes, where raw ground offal of sheep from the local slaughterhouse was used to feed the breeding foxes instead of chicken carcasses as used previously. Subsequently, an outbreak abortion event occurred during March and April in 2017. Moreover, serological screening showed that the seropositive rate of brucellosis in the fox breeding farm was 41.32% (300/726), being 38.49% (40/64) in male foxes and 48.58% (274/564) in female foxes. Moreover, five out of eight staff in this farm were diagnosed with brucellosis. This evidence indirectly showed that feeding the raw viscera of sheep infected with *Brucella* spp. were the main cause for the outbreak of abortion events on the blue fox breeding farm. Due to the high abortion rate (55%), low cub survival rate (65%), and human infections, this farm was closed at the beginning of 2019. The study showed that the highest-threat organs of ruminants are the lungs, and the trend analysis also highlighted the cattle intestine as a potentially high-threat organ (35). Moreover, our previous study reported that *B. melitensis* was obtained from dogs that were often fed with sheep offal (36). Moreover, hares have been considered as a possible source of *B. suis* biovar 2 outbreaks in domestic pigs via swill feeding with offal from hunted infected hares (37).

In order to identify the causative pathogen of blue fox abortion, we tried to isolate and cultivate *Gardnerella vaginalis* and other common abortion-related pathogens, but only a few *Staphylococcus* and *Streptococcus* strains were detected in abortion afterbirth. What we particularly regret is that our laboratory (Heilongjiang Bayi Agricultural University) did not meet the expected biosafety requirements necessary for bacteriological experiments, so *Brucella* strains isolation were not performed. Isolated *Brucella* from the (wild) red fox (*Vulpes vulpes*) (38, 39), gray fox (40), and tundra wolf (41) have been reported. Therefore, our conclusion is a reasonable explanation for this outbreak of abortion events. In addition, blue foxes infected by *Brucella* strains were without any obvious symptoms except the abortion after pregnancy at 10–40 days. This observation agrees with a previous report that *B. melitensis* in the adult ewe is generally asymptomatic and self-limiting within about 3 months. However, because the bacteria may enter and cause necrosis of the chorionic villi and fetal organs, abortion or stillbirths may occur (42, 43). Another study showed that brucellosis is essentially a disease of sexually mature animals, the preferred site being the reproductive tracts of males and females. If the animal is not pregnant, the infected animal may be without clinical symptoms and may have a negative serological reaction. However, if such an animal becomes pregnant, the production of the simple carbohydrate erythritol in the fetus and its membranes causes rapid multiplication of bacteria in the uterus, and this is likely to end in abortion (44). In this study, a 77.78% (14/18) estrus rate was recorded in blue foxes after infection by *B. melitensis*. In comparison with 2016, the estrus rate had declined; the abortion rate was 10 times higher than previously, and the survival rate of the pups dropped significantly. *B. melitensis* primarily affects the reproductive tracts of sheep and goats, and the infection is characterized by late abortion, stillbirth, a weakened fetus, and to a lesser extent orchitis and infection of the accessory sex glands and impaired fertility in males (45). The stillbirths and weakened fetuses in this case resulted in economic losses. The infected staff member often participated in the offal grinding, and thus the specific source of infection needs further investigation. *B. melitensis* infects mainly sheep and goats and other animals, resulting in an important zoonosis that has a significant effect on the husbandry economy and the public health of many developing countries.

Our study has several limitations. Due to restrictions by the limited lab facilities, the isolation and culture of *Brucella* from abortus samples were not carried out. Moreover, a tracing-back survey of the source of sheep (goats) offal is lacking. Animal offal samples have been collected from the local slaughterhouse for further bacteriological experiments, and genetic phylogenetic analysis will provide the available information to reveal the complete transmission chain of events.

CONCLUSION

In the present study, we combined RBPT, SAT, and AMOS-PCR to investigate the cause of an abortion outbreak event in a blue fox farm in Heilongjiang province. Our experiments showed that blue foxes ingesting sheep offal infected with *B. melitensis* was the main cause of the outbreak. These data indirectly verified the severe animal brucellosis epidemic trend in this region, where *B. melitensis* infection was a spillover from the main host to the blue fox. These events pose a public health risk to people in the fur and catering industries and to workers in other breeding industries that provide animal feed. It is thus time to launch an animal brucellosis prevention program against the spread of *Brucella*.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its **Supplementary Information** files.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the Heilongjiang Bayi Agricultural University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

YZ, YM, and YR collected the samples and performed the serology and AMOS-PCR amplifications. ZLiu performed data analysis and drafted the manuscript. YZ and ZLiu conducted epidemiological investigations. YZ and ZLi participated in the design of the study, critically reviewed the manuscript, and managed the project. All authors have read and approved the final version of the manuscript.

FUNDING

This study was supported by Heilongjiang Bayi Agricultural University Funds (No. XDB201820) and Natural Science Talent Support Program (No. ZRCPY201807), the Natural Science Foundation Project of Heilongjiang Province (LH2020C083), the National Natural Science Foundation of China (No. 81703426), and the China Postdoctoral Science Foundation (No. 2019M651312), and National Key R&D Program of China, Grant Numbers 2019YFC1200700, 2019YFC1200601-6; and the National Natural Science Foundation of China (No. 82073624). The funding agencies had no role in the study design, data collection, analysis, decision to publish, or manuscript preparation.

ACKNOWLEDGMENTS

We are grateful to the staff of the blue fox breeding farm for assistance with the sample collection and for their cooperation with the epidemiological investigation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.666254/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 24 June 2021
doi: 10.3389/fvets.2021.597630

Estimation of the Basic Reproduction Numbers of the Subtypes H5N1, H5N8, and H5N6 During the Highly Pathogenic Avian Influenza Epidemic Spread Between Farms

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Veterinary Epidemiology and Economics, a section of the journal Frontiers in Veterinary Science

Received: *21 August 2020*
Accepted: *27 May 2021*
Published: *24 June 2021*

Citation:
Kim W-H and Cho S (2021) Estimation of the Basic Reproduction Numbers of the Subtypes H5N1, H5N8, and H5N6 During the Highly Pathogenic Avian Influenza Epidemic Spread Between Farms. Front. Vet. Sci. 8:597630. doi: 10.3389/fvets.2021.597630

It is important to understand pathogen transmissibility in a population to establish an effective disease prevention policy. The basic reproduction number (R_0) is an epidemiologic parameter for understanding the characterization of disease and its dynamics in a population. We aimed to estimate the R_0 of the highly pathogenic avian influenza (HPAI) subtypes H5N1, H5N8, and H5N6, which were associated with nine outbreaks in Korea between 2003 and 2018, to understand the epidemic transmission of each subtype. According to HPAI outbreak reports of the Animal and Plant Quarantine Agency, we estimated the generation time by calculating the time of infection between confirmed HPAI-positive farms. We constructed exponential growth and maximum likelihood (ML) models to estimate the basic reproduction number, which assumes the number of secondary cases infected by the index case. The Kruskal-Wallis test was used to analyze the epidemic statistics between subtypes. The estimated generation time of H5N1, H5N8, and H5N6 were 4.80 days [95% confidence interval (CI) 4.23–5.38] days, 7.58 (95% CI 6.63–8.46), and 5.09 days (95% CI 4.44–5.74), respectively. A pairwise comparison showed that the generation time of H5N8 was significantly longer than that of the subtype H5N1 ($P = 0.04$). Based on the ML model, R_0 was estimated as 1.69 (95% CI 1.48–2.39) for subtype H5N1, 1.60 (95%CI 0.97–2.23) for subtype H5N8, and 1.49 (95%CI 0.94–2.04) for subtype H5N6. We concluded that R_0 estimates may be associated with the poultry product system, climate, species specificity based on the HPAI virus subtype, and prevention policy. This study provides an insight on the transmission and dynamics patterns of various subtypes of HPAI occurring worldwide. Furthermore, the results are useful as scientific evidence for establishing a disease control policy.

Keywords: avian influenza, basic reproduction number, Korea, H5N1, H5N6, H5N8

INTRODUCTION

Highly pathogenic avian influenza (HPAI) is a highly contagious viral disease that infects domestic poultry and wild birds (1). The HPAI virus can cause an epidemic that may spread rapidly, has a high mortality rate among domestic birds, and devastates the poultry industry (2). Outbreaks of distinct subtypes of HPAI, including H5N1, H5N8, and H5N6, are continually reported worldwide (3–5), and this global HPAI virus dissemination is caused by migratory wild birds (6). The HPAI crisis appears to be a great threat to not only animal health but also public health worldwide. Furthermore, the World Health Organization reported 860 human infection cases of avian influenza A subtype H5N1 (7) after the first human case of HPAI subtype H5N1, which was reported in Hong Kong in 1997 (8).

In South Korea, outbreaks of three different subtypes of HPAI occurred between 2003 and 2018. The first outbreak of H5N1 occurred from December 2003 to February 2004 and had a high mortality rate at poultry farms, especially among chickens (9). Since then, outbreaks of H5N1 have occurred in 2006, 2008, and 2010 (10–12). The novel HPAI subtype, H5N8, was first reported in January 2014 at South Korean poultry farms (13). Genetic analyses of viruses isolated from wild birds and poultry farms showed that migratory birds could be responsible for the first wave of H5N8 outbreaks between January and May 2014 (14). After the first wave, two waves of subtype H5N8 occurred during September 2014 to June 2015 and during September 2015 to November 2015 (15). It was reported that these sporadic outbreaks were caused by viruses reintroduced into Korea by migratory waterfowl (16). In November 2016, a novel genotype of H5N6 that was first detected in wild birds in Korea and HPAI infectious cases was reported at poultry farms (17). Another novel H5N8 virus co-circulated with H5N6 virus during the outbreaks in 2016, from February to June 2017 (18). In November 2017, the novel H5N6 virus was detected at a broiler duck farm and in wild mallards, with infection spreading to poultry farms (19).

The main strategies used to prevent and control HPAI outbreaks are based on the prohibition of movement, preemptive culling, and vaccinations in infected areas (20). Therefore, it is important to understand pathogen transmissibility in a population to establish an effective disease prevention policy. The basic reproduction number (R_0) is one of the important epidemiologic parameters necessary to understand the characterization of disease and the dynamics in a population (21). $R_0$is generally defined as the average number of secondary cases caused by one infectious individual during the entire infectious period in an uninfected population (22). If each infected individual infects more than one other individual, on an average, at any time point, then the epidemic will be sustainable (23). Various methods are used to estimate the reproduction number (24–26), and these have been implemented in the R program (27) and Excel (28) as ready-made procedures.

Reproduction number estimation has been used to understand HPAI epidemic characteristics and to provide insight regarding control measures for epidemics. These farm-to-farm reproduction number estimations were targeted to the HPAI subtype H5N1 and were conducted in Nigeria (29), Romania (30), Thailand (31), Bangladesh (32), India (33), Italy, Canada, and the Netherlands (34). In Korea, there was a mathematical modeling study of the reproduction number for HPAI from 2016 to 2017, but this was limited to the local reproduction number and did not include all epidemics from South Korea (35). We aimed to estimate the serial interval and R_0 of HPAI subtypes H5N1, H5N8, and H5N6, which were associated with nine outbreaks from 2003 to 2018 in Korea, and demonstrate the characterization of each subtype by analyzing HPAI characteristics, including the epidemic days, number of farms, species distribution, serial interval, and R_0. It is expected that the results of the present study will become a foundation for demonstrating the disease dynamics of each HPAI subtype and its characteristics, as well as for establishing effective HPAI control, not only for traditional HPAI subtype H5N1 but also the emerging subtypes H5N8 and H5N6.

MATERIALS AND METHODS

Data Collection

The epidemic data of HPAI outbreaks in Korea were collected by the Animal and Plant Quarantine Agency (APQA) in Gimcheon, Korea (**Table 1**). In Korea, three HPAI subtypes occurred from 2003 to 2017, HPAI subtype H5N1 occurred in a total of 214 poultry farms, H5N8 occurred in 469 farms, and H5N6 occurred in total 362 farms. The livestock owner (including the manager) or veterinarian who found an animal with clinical signs and suspected HPAI was required to report the case to the APQA according to the Prevention of Contagious Animal Disease Act. Cloacal, fecal, and blood samples were collected from sick or dead poultry in reported poultry farms, and HPAI virus was confirmed using reverse-transcriptase polymerase chain reaction at the Avian Influenza Research and Diagnosis Department of the APQA. If the suspected farm was confirmed as HPAI-positive and deemed an infected premise (IP), then depopulation of farms with infected poultry and depopulation of all poultry farms in the protection zone were conducted. If a depopulated farm was found to be positive, then it was defined as a positive premise (PP) (36). Both IP and PP were considered cases in this study. The epidemic curve of these HPAI cases was depicted using the "incidence" package in R (37) to illustrate the weekly reported number of poultry farms in the International Organization for Standardization (ISO) week date system (37) (**Figure 1**). In Korea, there were no poultry farms infected with two HPAI subtypes simultaneously, and each farm only had one subtype in each outbreak.

Based on the APQA epidemiology reports, the HPAI outbreaks were classified as waves when the period between cases was longer than 1 month (38). As a result of this classification, outbreaks of the subtype H5N8, which occurred in 2014 and 2016, were classified as four and two waves, respectively. Four outbreaks, including the H5N1 outbreak in 2003, outbreak in 2006, the fourth wave of the H5N8 outbreak in 2014, and the second wave of the H5N8 outbreak in 2016, were excluded from the analysis because the samples were too small to calculate R_0.

TABLE 1 | HPAI epidemic in Korea from 2003 to 2018.

Subtype	Year of epidemic	Clade	Date	Days of epidemic	Total number of Farms	Cases per day	No. of chicken farms (%)	No. of duck farms (%)	No. of other poultry farms (%)
H5N1	2003	2.5	10/12/2003–05/02/2004	58	18	0.310	7 (38.9)	11 (61.1)	0 (0.0)
	2006	2.2	25/11/2006–06/03/2007	103	7	0.068	4 (57.1)	2 (28.6)	1 (14.3)
	2008	2.3.2	01/04/2008–24/05/2008	54	98	1.815	80 (81.6)	17 (17.3)	1 (1.0)
	2010	2.3.2	29/12/2010–23/05/2011	146	91	0.623	38 (41.8)	50 (54.9)	3 (3.3)
H5N8	2014 1st	2.3.4.4	16/01/2014–29/07/2014	194	212	1.093	39 (18.4)	166 (78.3)	7 (3.3)
	2014 2nd	2.3.4.4	24/09/2014–10/06/2015	260	162	0.623	39 (24.1)	117 (72.2)	6 (3.7)
	2014 3rd	2.3.4.4	14/09/2015–15/11/2015	63	17	0.270	0 (0.0)	14 (82.4)	3 (17.6)
	2014 4th	2.3.4.4	23/03/2016–05/04/2016	14	2	0.143	0 (0.0)	2 (100.0)	0 (0.0)
	2016 1st	2.3.4.4	06/02/2017–14/04/2017	58	40	0.690	16 (40.0)	23 (57.5)	1 (2.5)
	2016 2nd	2.3.4.4	02/06/2017–19/06/2017	18	36	2.000	30 (83.3)	0 (0.0)	6 (16.7)
H5N6	2016	2.3.4.4	16/11/2016–18/02/2017	95	340	3.579	192 (56.5)	140 (41.2)	8 (2.4)
	2017	2.3.4.4	19/11/2017–18/03/2018	121	22	0.182	8 (36.4)	14 (63.6)	0 (0.0)

Serial Interval and Generation Time

A serial interval is the time between successive cases in a chain of transmission, estimated from the interval between clinical onsets in patients (25). We estimated the serial interval of HPAI as the time between the reported date of the first farm with infected cases and secondary farm with infected cases. This estimation was based on the investigation of the epidemic pathway of HPAI transmission, which shows the epidemiologic relationship between the infector and infectee. According to the APQA investigations, HPAI transmission could be possible through wild migratory birds, wild animals, farm owners, managers, staff, vehicles related to the poultry industry, and airborne transmission from nearby infected farms. The epidemic transmission pathway investigation was conducted by an APQA epidemiologic investigator visiting and interviewing the places suspected to be associated with the infected farms, including animal facilities such as hatcheries, feed factories, and live bird markets. The APQA investigated vehicles, people, livestock, and their products that entered an infected farm from 21 days prior to infection and estimated the disease transmissions.

In addition to investigating via interview, the APQA used geographic information to identify HPAI viral transmission by vehicles. In Korea, vehicles related to the poultry industry transporting poultry, poultry products, medicines, feed, and feces must be registered with the Korea Animal Health Integrated System (KAHIS; http://www.kahis.go.kr). The movements of livestock-related vehicles are reported to the KAHIS, making it possible to track the movement of vehicles, people, livestock, and animal products.

Through these interviews and vehicle information, the disease transmission pathway via transportation and human movement was identified. If a clear epidemiologic link to the infected farm could not be found through interviews and movement tracking, then we hypothesized that the farm might have been infected with HPAI by wild migratory birds or wild animals. We then excluded infection thought to be caused by wild birds or wild animals during the estimation of the serial interval because it is not possible to observe the serial interval of virus transmission from wild birds and animals.

The generation time is the modeling term describing the time duration from the onset of transmittable infection in a primary case to the onset of infection in a secondary case infected from the primary case. We defined the generation time as the difference between suspected infection days of the primary farm and secondary farm, which was measured through epidemiologic investigation (**Figure 2**). The suspected infection day was estimated according to the day reported by the farm owner after clinical symptoms were found in the poultry and the period between the infection and latent period of each HPAI subtype in the poultry species. We estimated the suspected infection date from the day the clinical symptoms were reported by subtracting the periods between infection and clinical symptoms. For H5N1, the periods between infection and clinical symptoms were assumed to be 2 days for chickens (9), 4 days for ducks (39), and 3.8 days for other poultry species (9). For H5N8, the periods were 3.2 days for chickens (40), 8.0 days for ducks (15), and 2.0 days for other species (41). For H5N6, the periods were 2.6 days for chickens (42), 4.6 days for ducks (43), and 3.0 days for other species (43).

Based on the generation time between case farms, we calculated the discretized generation time distribution using a function (est.GT) in the R0 package (27). Discretization is performed on the grid [0, 0.5), [0.5, 1.5), [1.5, 2.5), etc… where the unit is time interval of days (27). Time-to-event data were assumed to follow a parametric distribution with a probability density function (PDF). The distribution of generation time is expressed in the form of parametric distribution such as "gamma," "lognormal," or "Weibull," using maximum likelihood. The mean and standard deviation of generation time is provided in the desired time units. The calculated distribution of the generation time in each subtype and outbreaks is depicted in **Figure 3**.

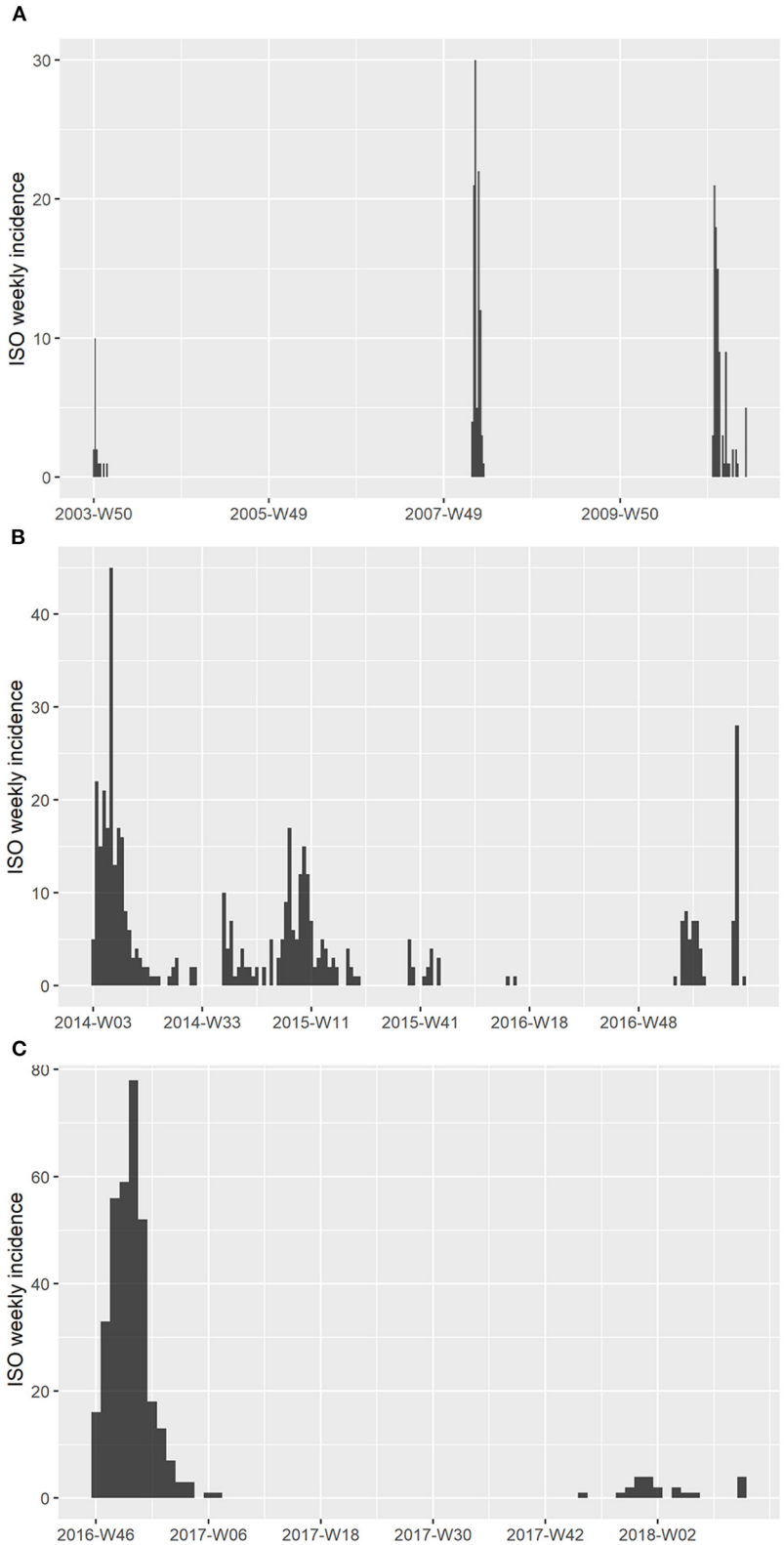

FIGURE 1 | Epidemic curve of HPAI outbreaks in Korea between 2003 and 2018. **(A)** Weekly epidemic case number of HPAI subtype H5N1 from 2003 to 2011. **(B)** Weekly epidemic case number of HPAI subtype H5N8 from 2014 to 2017. **(C)** Weekly epidemic case number of HPAI subtype H5N6 from 2016 to 2018. The x-axis represents the week numbers, which were based on the ISO 8601 week date system.

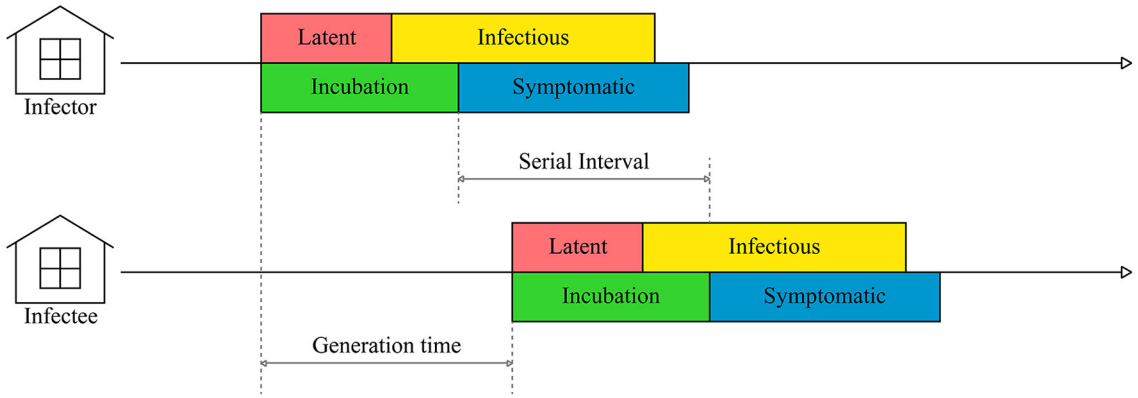

FIGURE 2 | A schematic representation of HPAI transmission between farms.

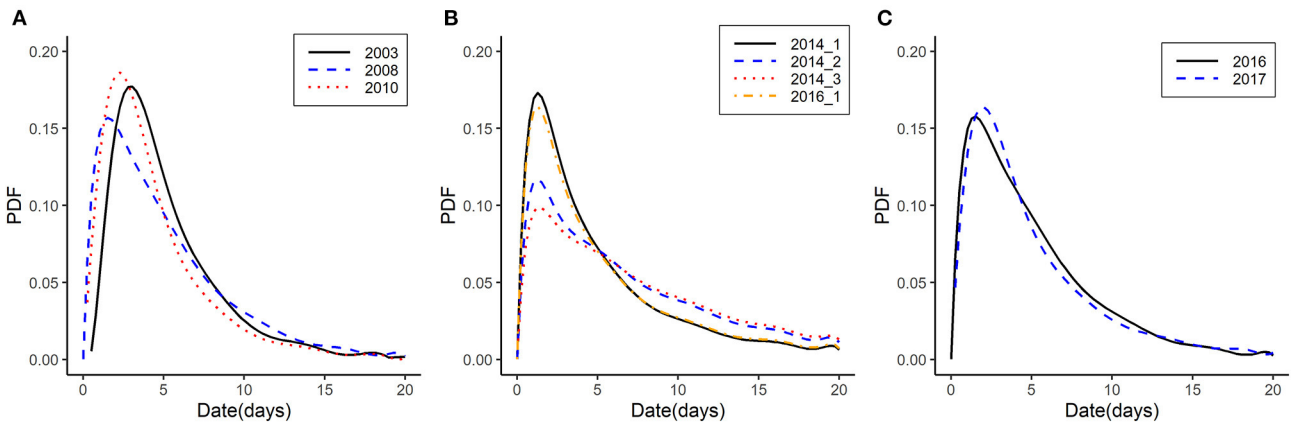

FIGURE 3 | Generation time distribution of HPAI outbreaks from 2003 to 2018 in Korea by HPAI subtype. **(A)** Generation time distribution of HPAI subtype H5N1 in 2003, 2008, and 2010. **(B)** Generation time distribution of HPAI subtype H5N8 during the 2014 first wave, second wave, and third wave and during the 2016 first wave. **(C)** Generation time distribution of HPAI subtype H5N6 in 2016 and 2017. The x-axis represents the days for generation time. The y-axis represents the probability density function (PDF).

Model Assumption and Data Analysis

The study model is based on the susceptible-infected-removed (SIR) compartmental model (44), which divides poultry farms into compartment. A susceptible farm (S) becomes infectious (I) through contact with the possible disease and is then removed (R) by depopulation. The dynamics of an epidemic can be described as the equation given below when N is the sum of S, I, and R.

$$\begin{aligned}\frac{dS}{dt} &= -\frac{\beta IS}{N}\\ \frac{dI}{dt} &= \frac{\beta IS}{N} - \gamma I\\ \frac{dR}{dt} &= \gamma I\end{aligned}$$

In this model, β is a parameter, which controls how much the disease can be transmitted through the exposure of HPAI virus, and γ is a parameter, which expresses how many poultry farms can be removed in a specific period. In this model, the average number of secondary infections caused by an infected host, R_0, equals β/ γ (45).

We constructed exponential growth (EG) and maximum likelihood (ML) models to estimate early reproduction numbers using the R0 package (27) in R (version 3.3.0). The EG model assumes that the initial reproduction ratio can be associated with the EG rate during the early epidemic phase (24). The formula is $R_0 = 1/\mathrm{M}(-r)$, where r denotes the initial EG rate and M stands for the moment generating function of generation time distribution. In the initial EG model, a period from day 1 to day 14 of the epidemics was chosen when the outbreak's growth was exponential. The 14-day interval was selected based on Korea's standstill policy (38). When HPAI outbreaks are reported in South Korea, a standstill policy is implemented for the vehicles, to reduce the spread of HPAI. This policy is intended to minimize the contact between vehicles and suppress the HPAI dissemination. We determined that this intervention affects the basic reproduction number of HPAI. Therefore, we specified the exponential growth for the first 14 days of the epidemic wave in each case.

A function (est.R0.EG) in the R0 package was used (27). We used a sensitivity test in EG to select the period during which growth is exponential as optimized time windows. We used the "sensitivity analysis" function to compute the deviance R-squared statistic over a range of periods.

The ML estimation model assumes that the number of secondary cases caused by an index case is Poisson-distributed with the expected value R_0 (25). The log-likelihood (LL) of R_0 was defined as $LL(R_0) = \sum_{t=1}^{T} \log(\frac{e^{-\mu_t}\mu_t^{N_t}}{N_t!})$, where $\mu_t = R_0 \sum_{i=1}^{t} N_{t-i} w_i$. This model assume that the number of new cases at indexing time t as N= $\{N_t\}$, $t = 0,\ldots T$, a generation time distribution w, and μ_t which represent the total number of cases produced by the earlier case N_t. The likelihood must be calculated on a period of exponential, and the deviance R-squared measure may be used to select the best period that maximized the likelihood. In this study, the range was set as 0.01–50, in which the maximum must be searched. A function (est.R0.ML) in the R0 package was used (27). The goodness of fit of each model was calculated using the chi-square goodness of fit test in R.

The Kruskal-Wallis test was used to determine the statistical differences in epidemics between subtypes (46). The epidemic days, number of farms, cases per day, poultry species distribution of farms, generation time, and R_0 estimated by EG and ML of the three subtypes H5N1, H5N8, and H5N6 were analyzed. The significance level was $\alpha = 0.05$. These statistical analyses were performed using SPSS 22.0 (IBM, Armonk, NY, USA).

RESULTS

HPAI Epidemic in Korea

We investigated 12 HPAI outbreaks of three subtypes, H5N1, H5N8, and H5N6, that occurred from 2003 to 2018 in Korea. **Table 1** presents a summary of the epidemic data, including the period of outbreaks and the number of infected farms that were investigated. The weekly epidemic curves of HPAI outbreaks are shown in **Figure 1** based on the ISO 8601 week date system. The H5N1 HPAI outbreaks (except for the 2008 outbreaks) began between November and February, when the lowest temperature drops below 0°C (**Figure 1A**). Regarding H5N8 in 2014, the second and third waves recurred in September 2015 and 2016, respectively (**Figure 1B**). However, the second wave of H5N6 in 2016 occurred in June (**Figure 1C**). The longest outbreak was the second wave of H5N8 in 2014, which occurred over 260 days. The shortest outbreak was the fourth wave of H5N8 in 2014, which occurred over 14 days. The outbreaks with the most cases (340 poultry farms) and cases per day (3.579 cases per day) were the H5N6 outbreaks in 2016. Regarding H5N8 in 2014, more than 72% of the occurrences were in ducks; however, there was no apparent species specificity for subtypes H5N1 and H5N6.

Serial Interval and Basic Reproduction Number of HPAI in Korea

We selected nine outbreaks with sufficient number of premises to calculate R_0 and analyzed the generation time and initial R_0 using the EG and ML methods (**Table 2**). Generation time distributions are illustrated by each HPAI subtype as the PDF in **Figure 3**. Generation time of H5N1 were estimated between 4.58 and 5.24 days (**Figure 3A**), generation time of H5N8 were estimated to have 6 days or more (6.01–8.23 days) (**Figure 3B**), and generation time of H5N6 were estimated between 5.02 and 5.91 days (**Figure 3C**). R_0 was estimated as 1.65–2.20 for subtype H5N1, 0.03-1.56 for subtype H5N8, and 1.03–1.24 for subtype H5N6 using EG methods. Using ML methods, $R_0$was estimated as 1.68–1.95 for subtype H5N1, 1.03–1.83 for subtype H5N8, and 1.37–1.60 for subtype H5N6.

Most of the R_0in the EG and ML methods were similar, except for the second and third waves of H5N8 in 2014. The R value obtained by the EG method was <1 for the second and third waves of H5N8 in 2014. To select the optimal time windows, sensitivity results of the time windows and R_0 were used (**Table 2**). Optimized time windows selected by sensitivity tests accounted for 69.14% of the outbreak periods, on an average, and the optimal R_0 values in optimized time windows were <1 for the subtypes H5N1 and H5N6 outbreaks.

Epidemic Statistics Between Subtypes

The average values of the number of epidemic days, infected poultry farms, species distribution, and infected farms per day for the three subtypes of nine selected outbreaks were determined (**Table 3**). The average numbers of epidemic days were 86.0 for H5N1, 108.0 for H5N6, and 143.8 for H5N8. The average numbers of farms were 69.0 for H5N1, 107.8 for H5N8, and 181.0 for H5N6. Regarding the species distribution, subtype H5N8 was more highly distributed among duck farms (74.2%) than other subtypes (37.7% for H5N1 and 42.5% for H5N6).

The Kruskal-Wallis H test showed a statistically significant difference in mean generation time among the different subtypes [χ^2 (2) = 6.444; $p = 0.040$], with mean rank scores of 2.33 for subtype H5N1, 7.50 for H5N8, and 4.00 for H5N6. The pairwise comparison showed that the mean H5N8 generation time (7.58 days) was significantly longer than the H5N1 generation time (4.80 days) ($P = 0.03$) (**Table 3**). There were no significant differences among subtypes in epidemic days, number of farms, cases per day, species distributions, or reproduction number.

DISCUSSION

HPAI outbreaks occur continually worldwide and have become a major threat to animal and human public health. In South Korea, eight outbreaks with multiple waves of infections occurred between 2003 and 2018; these involved three different HPAI subtypes, H5N1, H5N8, and H5N6, and massively damaged the poultry industry. Therefore, it is important to understand the HPAI transmissibility at poultry farms to control outbreaks by establishing an effective prevention policy. An effective tool for understanding disease characteristics is the R_0, which is generally defined as the average number of secondary cases caused by one infected individual (21). Therefore, we investigated the transmission dynamics of the HPAI subtypes H5N1, H5N8, and H5N6 by estimating the generation time and R_0. To the best of our knowledge, no previous study has attempted to estimate R_0 of various HPAI subtypes and perform comparative analyses among them. This could be the first study to investigate the disease transmission dynamics of HPAI subtypes H5N1, H5N8, and H5N6, which are emerging worldwide.

TABLE 2 | Generation time and reproduction number of HPAI by EG and ML method.

Subtype	Year of epidemic	Distribution	Mean generation time (95% CI) (Days)	Initial R_0 by EG Method (95% CI)	χ^2 of EG Method	Initial R_0 by ML Method (95% CI)	χ^2 of ML Method	Optimal time windows (percent in total period)	R_0 by EG Method (optimal) (95% CI)
H5N1	2003	Lognormal	5.24 (3.51–6.97)	2.02 (1.02–3.76)	0.33	1.95 (0.81–3.86)	0.33	9–46 (65.52%)	0.18 (0.01–0.51)
	2008	Gamma	4.98 (4.15–5.81)	1.65 (1.02–2.49)	0.33	1.68 (0.92–2.76)	0.34	9–54 (85.19%)	0.74 (0.65–0.82)
	2010	Gamma	4.58 (3.76–5.40)	2.20 (1.51–3.16)	0.30	1.93 (1.10–3.10)	0.30	9–138 (89.04%)	0.77 (0.72–0.83)
H5N8	2014 1st	Lognormal	7.45 (5.83–9.07)	1.56 (0.95–2.23)	0.31	1.83 (1.11–2.81)	0.31	14–125 (57.73%)	0.72 (0.65–0.79)
	2014 2nd	Weibull	8.23 (6.94–9.52)	0.35 (0.00–1.38)	0.36	1.56 (0.70–2.97)	0.35	10–248 (91.92%)	1.01 (0.99–1.03)
	2014 3rd	Weibull	7.39 (4.39–10.39)	0.03 (0.00–0.98)	0.36	1.03 (0.22–2.88)	0.38	10–50 (65.08%)	2.17 (1.26–3.67)
	2016 1st	Weibull	6.01 (4.57–7.45)	1.23 (0.50–2.31)	0.34	1.70 (0.75–3.22)	0.37	2–45 (75.86%)	1.37 (1.13–1.16)
H5N6	2016	Gamma	5.02 (4.56–5.48)	1.24 (0.87–1.73)	0.36	1.60 (1.09–2.25)	0.36	14–94 (85.26%)	0.71 (0.67–0.74)
	2017	Lognormal	5.91 (3.14–8.68)	1.03 (0.01–2.45)	0.38	1.37 (0.34–3.56)	0.38	14–107 (77.69%)	0.90 (0.78–1.01)

SD, standard deviation; EG, exponential growth; ML, maximum likelihood estimation; CI, confidence interval.

TABLE 3 | Epidemic characteristics, mean generation time, and R_0 in two models by HPAI subtype H5N1, H5N8, and H5N6.

Subtype	Average epidemic days	Average number of farms	Cases per day	Chicken (%)	Duck (%)	Etc. (%)	Mean generation time (days)	R_0 by EG Method (95% CI)	R_0 by ML Method (95% CI)
H5N1	86.0	69.0	0.802	41.7 (60.4)	26.0 (37.7)	2.0 (2.9)	4.80 (4.23–5.38)	1.96 (1.48–2.39	1.69 (1.10–2.28)
H5N8	143.8	107.8	0.750	23.5 (21.8)	80.0 (74.2)	4.3 (3.9)	7.58*(6.63–8.46)	1.49 (1.19–1.79)	1.60 (0.97–2.23)
H5N6	108.0	181.0	1.676	100.0 (55.2)	77.0 (42.5)	4.0 (2.2)	5.09 (4.44–5.74)	1.14 (0.76–1.51)	1.49 (0.94–2.04)

EG, exponential growth; ML, maximum likelihood estimation; CI, confidence interval.
**Mean generation time of subtype H5N8 is significantly longer than subtype H5N1 (P = 0.03).*

The R_0 of HPAI H5N1 in Korea estimated in this study was between 1.68 and 1.95, according to the ML method (**Table 1**). The R_0 of subtype H5N1 has previously been estimated in countries such as Italy (1.2–2.7), Canada (1.4–2.7), the Netherlands (1.0–3.0) (34), Romania (1.95–2.68) (30), Bangladesh (0.85–0.96) (32), and Thailand (1.27–1.60) (47). Despite being the same subtype of HPAI, the estimated R_0 subtype H5N1 varied across countries. We assumed that several factors, such as geographic distribution of poultry farms, mixed farming systems, poultry product supply system, and climate, were associated with this difference.

We believe that unique characteristics of the poultry industry in Korea and climatic differences are the major causes for these observed differences. We speculate that the estimated *R0* may be related to characteristics of the Korean poultry industry, such as the coexistence of large-scale commercial farms and small family farms. Among the Organization for Economic Cooperation and Development (OECD) countries, Korea has the lowest availability of arable land per capita (0.03 hectare in 2016) (48). This land scarcity is an important factor leading to high stocking densities (49). A previous study suggested that farms with large flocks and the presence of a neighboring farm within 500 m were risk factors of HPAI at Korean broiler duck farms (50). This high stocking and local density of large-scale poultry farms could increase the likelihood of massive infections when HPAI outbreaks occur in Korea.

Small family poultry farms also represent a biosecurity risk during HPAI outbreaks. Most of these small farms sell live poultry to local markets without going through slaughterhouses; this could be a pathway for the spread of HPAI viruses. Additionally, there was an obvious lack of information regarding the official statistics of poultry farms too small to be defined as agricultural holders in Korea (51). This includes establishments with <0.1 hectares of land or with sales of agricultural products per year or value of agricultural animals less than KRW 1.2 million (USD 1,090).

Secondly, we hypothesize that climate factors during the epidemic period may affect R_0 in these countries. Climate factors could affect HPAI transmission and persistence by altering bird migration, virus shedding between hosts, and virus survival outside the host (52). Climate change is considered to influence the wild bird species composition and their migration cycle, and these changes will affect the transmission intensity of disease (53). Furthermore, temperature and humidity could be related to

viral persistence in the host and environment. An influenza virus transmission experiment using a guinea pig model suggested that relative low humidity and cold temperature were favorable for spreading influenza (54). Liu et al. (55) showed that the environmental temperature decreased shortly before HPAI H5N1 outbreaks in domestic poultry in Eurasia between 2005 and 2006. Additionally, AI viral infectivity remained at lower temperatures (<17°C) during an *in vivo* test (56). Therefore, it is assumed that our estimated R_0 in Korea is higher than the R_0 in Thailand and Bangladesh, where the average annual temperatures and humidity are higher. Based on these results, we assumed that the climate factors were closely related to the R_0estimated in several countries in terms of virus transmission and survivability.

In 2016, two novel HPAI subtypes, H5N6 and H5N8, occurred simultaneously. HPAI H5N6 occurred from November 2016 to February 2017, whereas subtype H5N8 occurred from February to April 2016; the first wave and second wave occurred in June. Although these two subtypes occurred simultaneously, both were novel viruses. The genetic clade analysis suggested that Korean H5N6 viruses are novel reassortments of multiple virus subtypes, and it is difficult for H5N6 virus reassortment to occur during outbreaks that could increase the possibility of viral subtype mutation (5). Additionally, an infection experiment involving wild mandarin ducks demonstrated a difference in viral shedding and viral tropism in H5N8 and H5N6 viruses within the same clade of 2.3.4.4 H5 HPAI viruses (57). Based on these findings, both subtypes were independent of each other, and the virus infectivity could also be different; therefore, different R_0 was expected.

However, our estimated initial R_0 value in 2016 suggested a similarity between the reproduction number represented in subtypes H5N8 (1.70) and H5N6 (1.60) (**Table 2**). Apart from the difference in transmissibility of each virus subtype, the level of transmission between farms in the field may be similar between the two subtypes. However, this presumes that the values of R_0 of the two subtypes were similarly calculated because the biosecurity policy implemented during the outbreaks was identical. The basic reproductive number is affected by the rate of contacts in the host population, the probability of infection being transmitted during contact, and the duration of infectiousness (58). Therefore, it can be estimated that the R_0 of two different subtypes were similar due to the reduction of the poultry population through preemptive culling and the reduction of contact between farms because of the standstill (59).

The quarantine against HPAI in Korea has changed over 14 years after the first HPAI epidemic in 2003. The HPAI prevention policy changed dramatically, especially before and after H5N8 epidemics in 2014. Before the outbreaks, Korea Animal Health Integrated System (KAHIS) was established in 2013 to monitor livestock vehicle movement. In this system, all poultry-related vehicles must be registered with KAHIS and equipped with a global positioning system mandatorily (60). Also during the epidemics, the preemptive depopulation of the protective zone was changed from a radius of 500 m−3 km, and inspections were conducted more than once before releasing poultry and poultry products (36), The influence of these quarantine policy can also be seen in the changes in the R_0 values of each wave of subtype H5N8 that occurred between 2014 and 2016. For H5N8 in 2014, the initial R_0 of each wave showed a tendency to decrease as the outbreak progressed gradually (**Table 2**). This would indicate that the effectiveness of control measures for HPAI were increasing while the waves were passing.

In the Kruskal-Wallis model, H5N1 and H5N8 subtypes showed statistically significant differences in generation time ($P = 0.03$) (**Table 3**). However, there were no significant differences in the epidemic characteristics of the subtypes. There was also no statistical significance in the R_0 obtained through the EG and ML models. This generation time difference in the two subtypes might be associated with subtype pathogenicity in the poultry species. The spread of H5N1 viruses in the field was quickly controlled as a result of the rapid diagnosis of the infections due to the high pathogenicity of these viruses in poultry. In contrast, subtypes H5N6 and H5N8 clustered as clade 2.3.4. H5NX viruses are usually mild in ducks, leading to delayed diagnosis of infections and persistent spread in the wild (61). Therefore, the H5N8 subtype could possibly spread the HPAI virus over a relatively longer period than the H5N1 subtype which could be driven by sub-clinical spread in ducks.

In conclusion, this study showed the characterization of each subtype by analyzing the HPAI characteristics, including the epidemics, number of farms, species distribution, generation time, and R_0 of HPAI subtypes H5N1, H5N8, and H5N6, which were associated with nine outbreaks in Korea between 2003 and 2018. R_0, which is estimated by the generation time, index case, and secondary cases, is essential for identifying the characteristics of HPAI. In particular, our findings suggest that the estimated R_0 might be influenced by the HPAI subtype and might be associated with the seasonal aspects during the early stage, species specificity by virus subtype, and prevention policy. We believe that the results of the present study are helpful for demonstrating the disease dynamics of each HPAI subtype and its characteristics and, thus greatly assist in better disease control strategies. It could be possible to establish systematic quarantine policies to reduce the socio-economic losses caused by HPAI, Especially differences observed between countries with different poultry raising systems and climatic conditions. This study provided insight regarding HPAI transmission of the traditional subtype H5N1 and newly emerging subtypes H5N8 and H5N6. Further research on the basic reproduction numbers of the HPAI subtypes occurring worldwide is required to understand the global dynamics of HPAI transmission.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

W-HK designed the study, investigated data collection, reviewed the data, performed data analysis, and participated in manuscript preparation. SC supervised the project, administrated the project, acquired funds, and participated in the manuscript review.

FUNDING

This research was supported by grants from the National Research Foundation of Korea funded by the Korean Government (NRF-2018R1A2B6002396).

ACKNOWLEDGMENTS

The authors would like to express deep gratitude to Dr. Jeff Bender, professor of the School of Public Health, University of Minnesota, for his comments on the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

BRIEF RESEARCH REPORT
published: 09 July 2021
doi: 10.3389/fvets.2021.673624

Transport of Moving Duck Flocks in Indonesia and Vietnam: Management Practices That Potentially Impact Avian Pathogen Dissemination

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Highly pathogenic avian influenza (HPAI) virus is endemic in Indonesia and Vietnam, where "moving" duck production is commonly practiced. Questionnaire surveys were conducted with transporters of "moving" duck flocks in Indonesia ($N = 55$) and Vietnam ($N = 43$). The main purpose of transportation was to transport duck flocks between rice paddies used for scavenging. Trucks were commonly utilized for transport in both countries (Indonesia: 98.2%, 54/55; Vietnam: 37.2%, 16/43), while boats were only used in Vietnam (62.8%, 27/43). Transporters in Vietnam moved larger flocks and traveled over longer distances. Deaths of ducks due to diseases were reported in both countries (Indonesia: 16.4%, 9/55; Vietnam: 4.7%, 2/43; $p = 0.11$). Throwing away of carcasses was the primary method of disposal of dead birds in Indonesia (60.0%, 33/55), but was not practiced in Vietnam ($p < 0.001$), while more transporters in Vietnam (34.9%, 15/43) buried carcasses compared to Indonesia (6.8%, 4/55; $p = 0.001$). Consumption of carcasses (20.9%, 9/43), sale of dead ducks (14.0%, 6/43) and processing of ducks for fish feed (9.3%, 4/43) was conducted in Vietnam, but not in Indonesia. Vehicles were predominantly cleaned in rivers and stored outside in Vietnam, while cleaning and storage was usually conducted in houses/garages in Indonesia. In conclusion, we identified management practices that potentially impact transmission of avian pathogens, such as HPAI virus. In Indonesia, unsafe management practices were related to multipurpose usage of transport vehicles and disposal of birds in the environment, while in Vietnam, they were related to the mixing of birds during transport, the processing of dead carcasses and the storage and cleaning of transport vehicles.

Keywords: avian influenza, transport, moving ducks, biosecurity, virus transmission, Indonesia, Vietnam

OPEN ACCESS

Edited by:
Anuwat Wiratsudakul, Mahidol University, Thailand

Reviewed by:
Olivia Bowen Faulkner, University of Arkansas, United States
Faten A. Okda, St. Jude Children's Research Hospital, United States

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *28 February 2021*
Accepted: *10 May 2021*
Published: *09 July 2021*

Citation:
Khaw SWS, Vu LT, Yulianto D, Meers J and Henning J (2021) Transport of Moving Duck Flocks in Indonesia and Vietnam: Management Practices That Potentially Impact Avian Pathogen Dissemination. Front. Vet. Sci. 8:673624. doi: 10.3389/fvets.2021.673624

INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) H5N1 virus is endemic in Vietnam and Indonesia and has caused substantial human and poultry losses (1, 2). From 2003 to September 2018, 454 human fatalities were reported out of 860 HPAI human cases worldwide, representing a case-fatality rate of 52.8% (3). At 84.0% (168/200), Indonesia has the highest human case fatality rate globally (3). Vietnam has experienced 64 deaths out of 127 human cases, representing a case fatality rate of 50.4% (3).

From 2004 to 2019, 22.5 and 20.0% of the global HPAI outbreaks in poultry occurred in Indonesia and Vietnam, respectively (4). Since 2003, more than 150 million domestic birds died or were culled as a result of H5N1 outbreaks in Indonesia (5, 6), while in Vietnam more than 52 million poultry losses occurred since 2003, with 86.5% of the domestic poultry population being culled in 2003-04 alone at an estimated cost of about US$205 million (7).

Duck farming is an important sector of the poultry industry in Indonesia and Vietnam. Duck management is classified into "stationary" and "moving" duck production, with stationary flocks allowed to graze around the village vicinity and secured at night near village houses, while moving flocks are moved between areas of recent rice harvests and kept in confinement overnight close to the daytime scavenging locations (8–10). Moving duck flocks are suspected to contribute toward the maintenance and circulation of HPAI viruses (11–14) and research had highlighted that road characteristics (e.g., road density; road length), human and poultry densities and long distance movement might facilitate the spread of HPAI viruses (15–19). Meyer et al. (20) described the actors involved in duck production, providing an overarching description of the poultry value chain system of Vietnam, while Henning et al. (9) described the structure of the moving duck flock network in Indonesia. However, specific transport practices that influence the dissemination of H5N1 virus have yet to be identified.

The objectives of this study were to (1) identify management factors during transport of moving duck flocks in Indonesia and Vietnam that could potentially be associated with an increased risk of avian pathogen dissemination (e.g., HPAI virus), and to (2) compare and contrast differences in movement, management and biosecurity practices implemented by duck flock transporters in Indonesia and Vietnam.

MATERIALS AND METHODS

Study Design

Previous research conducted with moving duck flock owners in Indonesia and Vietnam in 2008 and 2009 described the HPAI H5N1 infection status of ducks and the movements elected by owners for their duck flocks (8, 9). During this research, information about the transport provider used by moving duck farmers was collected. These data comprised the initial dataset of transporters to be contacted. As no register of transporters existed in either country, we used snowball sampling by asking identified transport providers about contact details of additional potential transporters working in the same region. Transporters were identified in six districts of Central Java (Pemalang, Batang, Klaten, Purworejo, Brebes, and Kendal) in Indonesia and in four provinces of the Mekong Delta in Vietnam (Ben Tre, Dong Thap, Tien Gian, and Vinh Long). The aim was to interview about 10 transporters per district or province.

Data collection was conducted in Indonesia by veterinarians from the Disease Investigation Centre (DIC) in Wates, Yogjakarta and in Vietnam by veterinarians from The Regional Animal Health Centre VI, Ho Chi Minh City using an interview process using local languages. All interviewers were trained in data collection.

The study design for this research was reviewed and approved in Indonesia by the Disease Investigation Centre (DIC) in Wates, Yogjakarta; and in Vietnam by the Regional Animal Health Centre VI, Ho Chi Minh City. Data collection for this study was conducted in accordance with the accepted survey guidelines for surveillance activities of both organizations.

Questionnaire

Questionnaires were developed in English and later translated into the national languages (Bahasa, Vietnamese) in order to capture potential associated risk factors associated with spread of avian pathogens such as HPAI virus during the transport of duck flocks: type of transport utilized; number of flocks (and ducks) transported per time period; age of ducks transported; number of duck flocks combined in a transport load; other poultry species transported; number of farms visited to obtain one transport load; cleaning and disinfection before and after transport; location where the transport vehicles were stored, cleaned and disinfected; distance and duration of transport; management of ducks before departure, during transport and after arrival in scavenging area; contact of transported ducks with other poultry and other animals; frequency of transporting ducks; raising of ducks at home by people loading and transporting poultry; transport of items (e.g., chickens, other animals, feed, and eggs) together with ducks; experiences of sickness or deaths of ducks during transport; disposal of dead ducks; and occurrence of health problem in people loading and transporting ducks. Thus, the questionnaires included a mixture of closed and open-ended questions. Copies of the questionnaires are provided in the **Supplementary Material (Data Sheets 1, 2)**.

Pilot testing of the questionnaires was performed with two transporters in both countries before conducting the main survey to identify any problems, misunderstandings or to discover additional risk factors of interest that should be surveyed. The questionnaires were updated accordingly. The total number of questions in the questionnaire was 39, with identical questions being used in Indonesia and Vietnam.

Data analysis was conducted in SPSS (IBM Corp, Release 2019, IBM SPSS Statistics for Windows, Version 26.0) and STATA (StataCorp, College Station, TX, 2019, Stata Statistical Software: Release 16). Descriptive analysis included the calculation of frequencies, means, medians and range values. The command –*tabplot*- in Stata was used to visualize the frequency of responses provided on a 4-point Likert scale. The total number of survey responses for each response category were compared between Indonesia and Vietnam using the Fisher's exact test. To facilitate the utilization of the Fisher's Exact Test for data analysis, Likert scale groups "very important" and "important" were combined into a category "importantly" and Likert scale groups "not important" and "not conducted" were combined into a category "not importantly." Similarly, Likert scale groups "common" and "sometimes" were combined into a category "commonly" and Likert scale groups "seldom" and "not conducted" were combined to a category "infrequently." The non-parametric Mann–Whitney *U*-test was used to compare ordinal and not

normally distributed continuous variables between Indonesia and Vietnam.

RESULTS

A total of 114 transporters of moving duck flocks were interviewed, with 16 transporters being excluded from the analysis as they provided incomplete information in the questionnaire. Thus, 98 transporters provided completed responses to all questions and were analyzed in detail, consisting of 55 transporters from Indonesia and 43 from Vietnam (**Supplementary Table 1 - Data Sheet 3**).

Importance of Transport Activities for Income Generation

As expected, the transport of ducks to scavenging locations was the main activity for transporters (**Figure 1A**) in both countries (Indonesia: 98.2%, 54/55, Vietnam: 95.3%, 41/43; $p = 0.58$). Although not statistically significant, transporting of ducks to markets was less common in Indonesia (Indonesia: 9.3%, 4/43; Vietnam: 16.4%, 9/55; $p = 0.38$), while transport of ducklings to and from hatcheries was more common in Indonesia. Items transported together with ducks include chickens, other birds, feed, and eggs (**Figure 1B**). About 45.5% of transporters from Indonesia indicated that they "commonly" transport duck feed together with ducks compared to only 28.0% of transporters from Vietnam ($p = 0.09$). Additionally, 40.0% of transporters from Indonesia indicated that they "commonly" transport eggs together with ducks, but this was either seldom or not practiced in Vietnam ($p < 0.001$) (**Figure 1B**).

In general, transporters from Indonesia also used their transport vehicles more frequently to transport other items for income generation. This included the transport of feed (Indonesia: 25.5%, 14/55; Vietnam: 2.3%, 1/43; $p = 0.0014$), and transport of non-animal related items (Indonesia: 49.1%, 27/55; Vietnam: 9.5%, 4/42; $p < 0.001$), with the most common items transported being building materials (**Figure 1A**). More Indonesian transporters relied on additional income sources not related to transport (Indonesia: 58.2% 32/55; Vietnam: 23.3%, 10/43; $p < 0.001$), with farming being the most common activity in both countries (**Figure 1A**).

Transport Types Used, Volume of Transport and Distance Traveled

Trucks, boats and motorbikes were used for transportation (**Supplementary Figure 1 - Data Sheet 3**). Trucks were the most common transport type in Indonesia (Indonesia: 98.2%, 54/55; Vietnam: 37.2%, 16/43; $p < 0.001$), while boats were the most common mode of transport in Vietnam (62.8%, 27/43), but were not utilized in Indonesia ($p < 0.001$). Motorbikes were used by one transporter in each country. Almost all transporters (99.0%) utilized only one type of transport.

Of the 70 transporters, who provided data on truck designs, 95.7% (67/70) of trucks were not covered or closed. Trucks had between 1 and 4 levels, with the majority of them (58.6%, 41/70) having three levels. Boats were generally open (82.1%, 23/28) and had between 1 and 3 levels, with 85.7% (24/28) of them having three levels. Neither of the two motorbikes was covered.

Respondents from Vietnam were more likely to store their transport vehicles outside (60.5%, 26/43) compared to Indonesia (16.4%, 9/55; $p < 0.001$). Vietnamese transporters indicated more frequent exposure of their vehicles to wild birds compared to Indonesian transporters [23.3% (10/43) vs. 5.5% (3/55); $p = 0.015$].

The volume of transport and distance traveled in Indonesia and Vietnam are shown in **Table 1**. In Vietnam, transporters using trucks transported more duck flocks per year, over larger distances per year and with a larger number of ducks per flock compared to Indonesia. Individual journeys were ∼25% longer in Vietnam compared to Indonesia, although this was not significant ($p = 0.13$).

Transport of Moving Duck Flocks to and From Scavenging Locations

The locations from where ducks were collected from and transported to by transporters are shown in **Supplementary Figure 2 (Data Sheet 3)**. As expected, the majority of transporters from both countries collected ducks from rice paddies as these represent the main scavenging locations. However, 78.2% (43/55) of transporters in Indonesia collected ducks and 63.0% (34/54) delivered ducks to farms, compared to only 23.3% (10/43) and 20.9% (9/43) from Vietnam ($p < 0.001$ and $p < 0.001$, respectively). About 34.5% (19/55) of transporters in Indonesia collected and 53.7% (29/54) delivered moving ducks to village areas compared to 58.1% (25/43) and 65.1% (28/43) in Vietnam ($p = 0.025$ and $p = 0.30$, respectively), highlighting that within-village scavenging areas are more common in Vietnam.

Collection and delivery of ducks to markets was uncommon for both Indonesia and Vietnam, with about 11% and <5% of transporters in Indonesia and Vietnam, respectively, "commonly" conducting this practice.

Characteristics of return journeys are outlined in **Supplementary Figure 3 (Data Sheet 3)**. Although usually transporters from both countries returned "empty" after delivering ducks, 34.9% (15/43) of transporters in Vietnam and 20.4% (11/54) of transporters from Indonesia ($p = 0.17$) did "commonly" return with other ducks.

A higher proportion of transporters in Vietnam compared to Indonesia provided care to birds during transport. In Indonesia, water and feed was provided to ducks by 13.0% (7/54) and 7.4% (4/54) of transporters, respectively, compared to 88.4% (38/43) and 65.1% (28/43) of transporters, respectively, in Vietnam. Spraying of birds with water was conducted by 5.6% (4/54) of transporters in Indonesia and 32.6% (14/43) of transporters in Vietnam, while rest stops for ducks were provided by 11.1% (6/54) of transporters in Indonesia and 18.6% (28/43) of transporters in Vietnam.

Duck Deaths and Disposal of Carcasses

Out of 41 transporters providing information on the number of duck deaths per truck load in Indonesia, the mean number (median, range) of duck deaths per load was 2.4 (2, 0.5–10), while

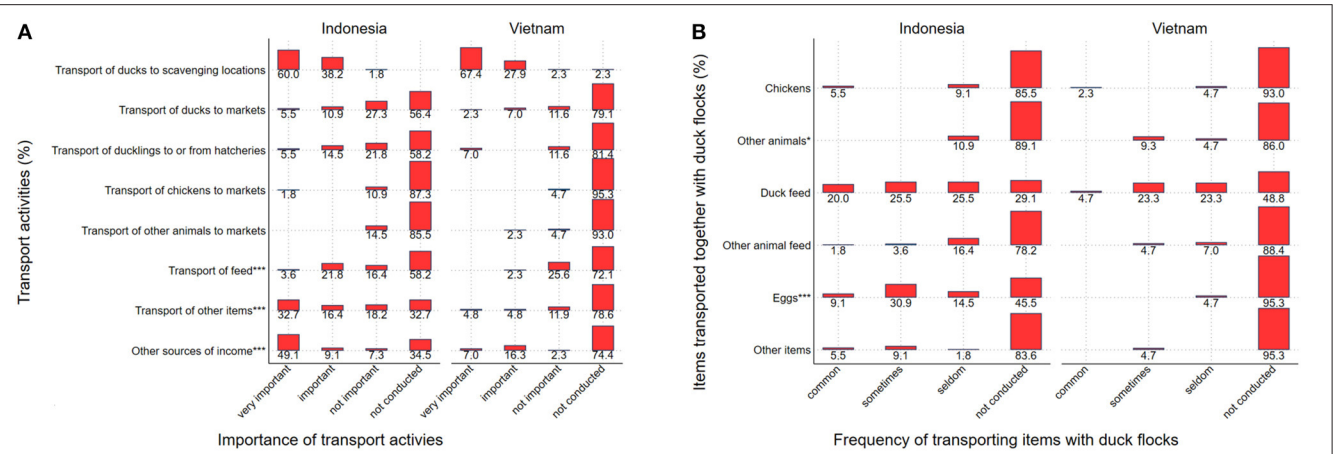


FIGURE 1 | (A) Importance of transport activities for transporters of moving duck flocks in Indonesia ($N = 55$) and Vietnam ($N = 43$), with percent of respondents by importance category. **(B)** Items transported together with ducks to scavenging locations for Indonesia ($N = 55$) and Vietnam ($N = 43$), with percent of respondents by frequency category. *P*-values for comparisons between countries for each category are presented as follows: * for $P \leq 0.05$, *** for $P \leq 0.001$.

TABLE 1 | Volume of transport and distance traveled by transporters of moving duck flocks in Indonesia.

	Transport by truck			Transport by boat
	Indonesia	**Vietnam**	***p*-value**	**Vietnam**
Number of scavenging duck flocks transported per year	44 (48; 2–270) ($N = 45$)	123 (110; 50–220) ($N = 14$)	<0.001	113 (120; 40–250) ($N = 28$)
Number of ducks transported in a single load	431 (450; 100–800) ($N = 51$)	4,792 (750; 300–25,000) ($N = 13$)	<0.001	18,527 (12,000; 800–80,000) ($N = 26$)
Distance traveled per year (in km) to scavenging locations	1,831 (800; 80–20,000) ($N = 37$)	9,483 (10,000; 900–35,000) ($N = 15$)	<0.001	10,450 (6,000; 50–55,000) ($N = 26$)
Distance traveled per journey (in km) to scavenging locations	68 (60; 10–200) ($N = 45$)	94 (80; 25–200) ($N = 16$)	0.13	97 (80; 10–300) ($N = 26$)

Mean (median; minimum - maximum) is shown with number of respondents in brackets. P-value refers to the comparison between Indonesia and Vietnam.

for Vietnam, out of 16 transporters providing information, the mean number (median, range) of duck deaths per truck load was 3.4 (3, 1–10). For 26 transporters with boats from Vietnam who provided data, the mean number (median, range) of duck deaths per load was 2.6 (2, 1–5).

Transporters reported the causes of death as "disease," "dehydration," "injury," "other," and "unknown." Deaths of ducks during transport due to diseases were observed by transporters in both countries at similar frequencies (**Figure 2A**, $p = 0.11$). Injuries occurred in similar frequencies during transport in Indonesia and Vietnam ($p = 0.84$). In contrast, transporters from Vietnam more frequently experienced death of ducks due to dehydration (Vietnam: 72.1%, 31/43; Indonesia: 30.9%, 17/55; $p < 0.001$).

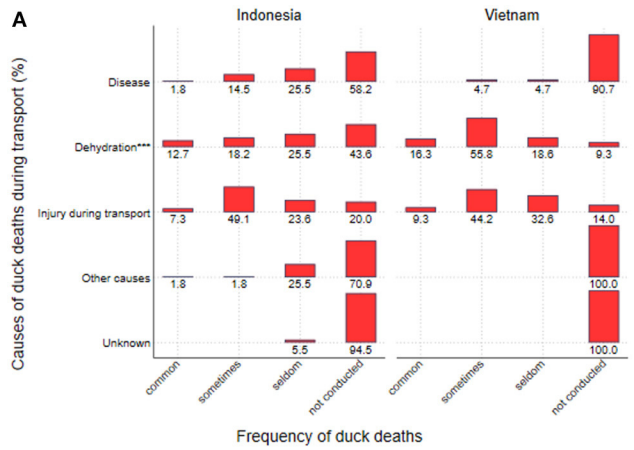
There were considerable differences in how transporters disposed of ducks that died during transport (**Figure 2B**). Throwing away carcasses in the environment was most common in Indonesia (60.0%, 33/55), but not practiced in Vietnam ($p < 0.001$). Sale of dead ducks ($p = 0.006$), processing of ducks for fish feed ($p = 0.034$), giving ducks to neighbors ($p < 0.001$), and household consumption of ducks ($p < 0.001$) were all practiced in Vietnam, but not in Indonesia (**Figure 2B**).

In Indonesia, two transporters specified as "other" management practice that duck carcasses were returned to duck owners (3.6%, 2/55). For Vietnam, "other" management practices also included return of carcasses to duck owners (20.9%, 9/43), while 9.3% (4/43) of transporters provided carcasses to para-veterinarians.

Cleaning and Disinfection of Transport Vehicles

Cleaning and disinfection of transport vehicles in the water of nearby rivers was common in both countries (Vietnam: 67.4%, 29/43; Indonesia: 49.1%, 27/55; $p = 0.10$). However, the most common location for cleaning transport vehicles in Indonesia was inside houses or garages (67.3%, 37/55), but this was less common in Vietnam (25.6%, 11/43; $p < 0.001$). Car washes were more utilized by Indonesian transporters compared to Vietnamese transporters (27.3%, 15/55 vs. 9.3%, 4/43; $p = 0.038$).

The most common cleaning practices, i.e., removing feces from loading surfaces and washing loading surfaces with water



B

Management Strategies	Indonesia	Vietnam	p-value
	% (N)	% (N)	
Burned	0.0 (0)	25.6 (11)	<0.001
Sold	0.0 (0)	14.0 (6)	0.006
Processed for fish feed	0.0 (0)	9.3 (4)	0.034
Given to neighbours	0.0 (0)	25.6 (11)	<0.001
Buried	6.8 (4)	34.9 (15)	0.001
Household consumption	0.0 (0)	20.9 (9)	<0.001
Thrown away	60.0 (33)	0.0 (0)	<0.001
Others	3.6 (2)	30.2 (13)	<0.001

FIGURE 2 | (A) Causes of death ducks during transport in Indonesia ($N = 55$) and Vietnam ($N = 43$) with percent of respondents by frequency category. *P*-values for comparisons between countries for each category are presented as follows: *** for $P \leq 0.001$. **(B)** Disposal of ducks that died during transport in Indonesia and Vietnam.

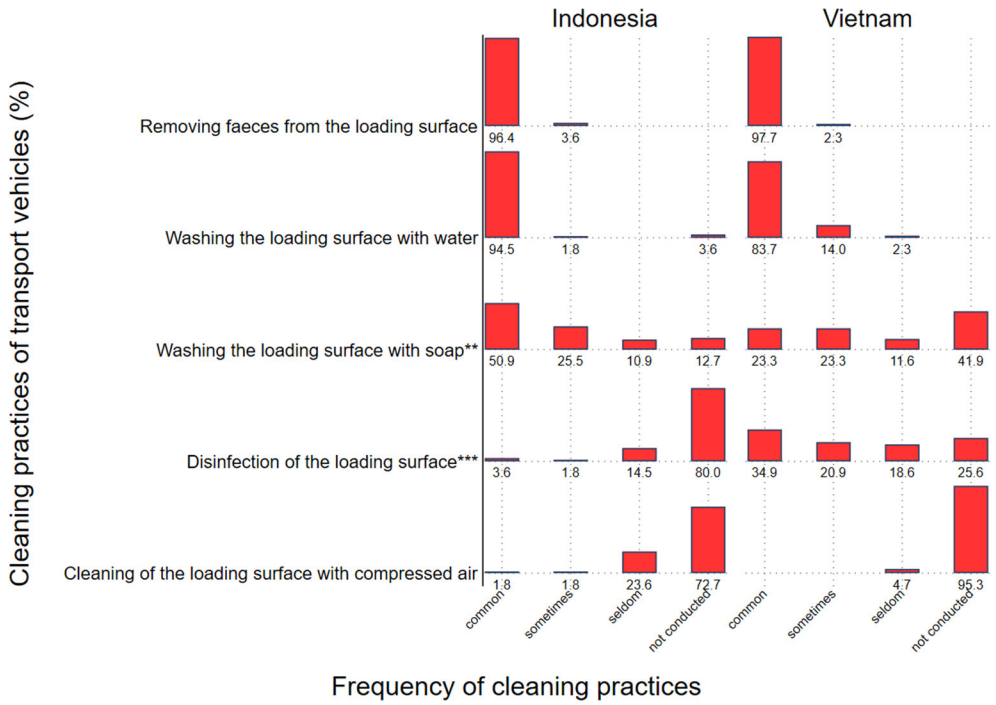


FIGURE 3 | Cleaning practices conducted on transport vehicles in Indonesia ($N = 55$) and Vietnam ($N = 43$) with percent of respondents by frequency category. *P*-values for comparisons between countries for each category are presented as follows: ** for $P \leq 0.01$, *** for $P \leq 0.001$.

were similar for both countries ($p = 1$; and $p = 0.10$, respectively) (**Figure 3**). However, the use of soap to wash loading surfaces was more common in Indonesia compared to Vietnam ($p = 0.0067$), while transporters from Vietnam more "commonly" used disinfectant on loading surfaces compared to Indonesia ($p < 0.001$).

Health of Transporters

Information was also obtained about whether transporters had experienced health problems while loading and transporting ducks. Almost all of transporter respondents from Indonesia (98.2%; $N = 54$) and Vietnam (95.3%; $N = 41$) indicated that they did not experience any health problems ($p = 0.58$).

DISCUSSION

This study investigated factors involved in the transportation of moving duck flocks in Vietnam and Indonesia that might facilitate the transmission of avian pathogens such as HPAI virus, and compared the magnitude of these factors between the two countries. While many practices were common to both countries, there were significant differences between the occurrence of some factors, which might help to identify strategies to reduce HPAI transmission during the transport of ducks in each country.

Compared to Vietnamese transporters, Indonesian transporters had more varied sources of income that contributed to a substantial portion of their financial needs and they were more likely to travel to many different locations, such as markets and hatcheries. The more frequent use of transport vehicles for different purposes may have implications for direct (if ducks are transported at the same time with ducks from other farms or other animal species) or indirect (if cleaning is not conducted properly between transports session) spread of HPAI virus (16, 17, 20). Also, considerably more transporters from Indonesia engaged in buying/selling of ducks in markets as an additional income source. This is a concern because wet markets facilitate interspecies transmission of HPAI virus and are considered to be the most likely source for HPAI outbreaks (16, 18, 21, 22). In contrast, transporters in Vietnam mainly specialized in transporting ducks between scavenging locations and did not generally use transport vehicles for other purposes. Although distances traveled per journey with trucks were similar between both countries, the total distance traveled per year was six times larger in Vietnam and more duck flocks and more ducks per load were transported in Vietnam. Compared to Indonesian transporters, Vietnamese transporters more frequently transported other duck flocks on their return journeys (although not significant at $p < 0.05$), thus proving potential opportunities for dissemination of avian pathogens if surface areas of transport vehicles were not properly cleaned and disinfected (16, 17, 23). Adding to this is the fact that transport vehicles were more commonly stored and left open during transport in Vietnam which suggests a higher likelihood for transport vehicles and duck flocks to be exposed to wild birds which could be harboring or excreting avian pathogens such as HPAI virus (15, 23, 24).

In general, collection and delivery of ducks to markets was not very common for transporters in either Indonesia or Vietnam. This indicates that the collection of ducks and delivery to markets is not in their domain, and is more likely conducted by middle men or traders, who have their own vehicles and collect moving ducks from scavenging areas or from farms. Similar observations were made by Meyer et al. (10) and Meyer et al. (20), who surveyed duck farmers in Vietnam.

Differing from the findings of Henning et al. (9) and Meyer et al. (10), our study found that an overwhelming majority of transporters from Indonesia and Vietnam clean their vehicles regularly after each journey, although both previous studies focussed on duck farmers and not directly on transporters of moving duck flocks. Almost all transporter respondents from both Indonesia and Vietnam removed feces and washed the vehicle loading surfaces, with transporters in Vietnam also commonly using disinfectant. This indicates that transporters recognize to a certain extent, the importance of biosecurity practices to prevent the spread of avian pathogens such as HPAI virus. However, further education to increase the biosecurity awareness among actors within the poultry industry is needed, in particular in HPAI endemically infected countries (25–28).

Transporters from Indonesia more frequently experienced deaths of ducks due to disease during the journeys, while transporters from Vietnam more frequently experience deaths of ducks due to dehydration. This difference may be explained by the fact that Indonesian transporters had more opportunities of direct and indirect contact between ducks as birds were sourced and delivered to a wider range of locations; while transporters from Vietnam generally traveled longer distances resulting in potentially longer stressful periods for ducks.

With regards to the disposal of duck carcasses, guidelines from international organizations recommend burial, composting, incineration, rendering or landfill disposal as they are effective in mitigating virus spread and minimizes public health and environmental effects (25, 29, 30). However, despite education campaigns conducted in Indonesia (25), the preferred method of carcass disposal by Indonesian transporters, was to throw carcasses into the environment (in particular into rivers), which has been described previously (31). This increases the likelihood of direct contact of other birds with the carcasses (31) or that scavengers such as roaming dogs open up carcasses and potentially increase virus exposure in the environment (when carcasses are infectious).

Additionally, it also presents a public health risk if untreated river water is consumed by people, especially given that around 4% of households in Indonesia rely on rivers as their main water supply (32–34). Interestingly, a substantial percentage of transporters from Vietnam indicated appropriate methods of disposal including incineration and burial. However, a sizeable proportion of respondents indicated that they prefer to give away the carcasses that died during transport to neighbors (the carcasses may eventually be consumed by the neighbors) or keep them for consumption within their own household. Corroborating with Manabe et al. (28), this suggests that despite a reasonably high awareness of H5N1 infection, Vietnamese transporters adhered to traditional habits. This may be due to insufficient knowledge about the risks of HPAI virus infection, compounded by financial hardships.

None of the transporters involved in this study was using Personal Protective Equipment (PPE), during handling of ducks or cleaning of transport vehicles. Previous research highlighted that lack of awareness and training, but also that low income influences the under-use of protective equipment in developing countries (35).

Data Limitations

It is difficult to determine if the cohort of transporters recruited into the study is representative of the spatio-temporal distribution of transporters in both countries. This is due to limited literature describing nationwide spatio-temporal distribution of moving duck flocks in both Indonesia

and Vietnam and the non-existence of a sampling frame of transporters for both countries. Government lists of moving duck farmers, let alone transporters of moving duck flocks do not exist in either country. The snowball sampling strategy used in this study was the only methodology that allowed us to overcome this problem. It has been previously noted that duck farming is widespread in West and Central Java in Indonesia; and highly concentrated around the Mekong Delta region in Vietnam (10, 17, 36, 37). Therefore, the use of Central Java and the Mekong Delta regions provided a good representation in terms of concentration of duck farming activities in those countries.

The use of survey questionnaires introduced biasness of various forms: recall bias, as the journeys may have been conducted many months earlier resulting in errors in recording journey characteristics and parameters; and social desirability bias, such as that transporters may be unwilling to disclose past experiences of ill-health after handling ducks; and that they may erroneously reported a greater frequency of disinfecting their transport vehicles than in reality. However, we used well-trained interviewers in this study and we are confident that the information we collected is reliable.

Finally, the data summarized here were collected in 2009. Some might argue that there could be changes to the duck industry since then. However, based on the value chain analysis elucidated by Meyer et al. (20) and further described by Kasim et al. (38), our description of the relationship between duck farmers and transporters appears to be a system that exists till this day.

CONCLUSION

In conclusion, while HPAI is endemic in both Indonesia and Vietnam, known risk factors that perpetuate HPAI in duck farming differ in importance between the two countries. This is due to dissimilarities in the duck farming industries between the two countries that impact the mode of transport used, movement patterns, disposal methods, and cleaning and disinfection approaches. While practices associated with higher biosecurity risk in Indonesia are related to the multipurpose usage of transport vehicles and the disposal of birds in the environment, unsafe practices in Vietnam relate to the potential mixing of birds during transport, the processing of dead carcasses and the storage and cleaning of transport vehicles.

DATA AVAILABILITY STATEMENT

The original data presented in this study are included in the article's **Supplementary Material (Data Sheets 4, 5)**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical approval for interviews with human participants in this study was not provided because the study design for this research was reviewed and approved in accordance with local legislation and institutional requirements in Indonesia and Vietnam. In Indonesia, this was conducted by the Disease Investigation Centre (DIC) in Wates, Yogjakarta; and in Vietnam by the Regional Animal Health Centre VI, Ho Chi Minh City. Data collection for this study was conducted in accordance with the accepted survey guidelines for surveillance activities of both organizations. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JH, JM, LTV, and DY: design of the research study. JH, LTV, and DY: data collection. SWSK and JH: data analysis. SWSK, JH, and JM: development of manuscript. All authors contributed to the manuscript and approved the submitted version.

FUNDING

Funding for this project was provided by the Australian Centre for International Agricultural Research (project no. AH/2004/040). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

We thank all transporters who were involved in this research project.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.673624/full#supplementary-material

Data Sheet 1 | Questionnaire Indonesia.

Data Sheet 2 | Questionnaire Vietnam.

Data Sheet 3 | Supplementary Figures and Tables.

Data Sheet 4 | Raw data Indonesia.

Data Sheet 5 | Raw data Vietnam.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 12 November 2021
doi: 10.3389/fvets.2021.648267

Control and Prevention of Epizootic Lymphangitis in Mules: An Integrated Community-Based Intervention, Bahir Dar, Ethiopia

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From 2010 to 2017, as part of a wider animal welfare program, The Donkey Sanctuary piloted an integrated, community-based model for the control and prevention of epizootic lymphangitis (EZL) in cart mules in Bahir Dar, Ethiopia. Stakeholders included muleteers, service providers, and transport and animal health regulatory authorities. Interventions included muleteer education, wound prevention, harness improvement, animal health professional training, treatment of early EZL cases, euthanasia for advanced cases, and review of transport services and traffic guidelines. The project followed a participatory project management cycle and used participatory learning and action tools to facilitate stakeholder engagement and ownership. Participatory and classical epidemiology tools were employed to raise and align stakeholder understanding about EZL for effective control and prevention and to evaluate the progress impact of the model through annual prevalence surveys. During the intervention, the annual prevalence of EZL reduced from 23.9% (102/430) (95%CI: 19.8%−27.0%) in 2010 to 5.9% (58/981) (95% CI: 4.4%−7.4%) in 2017, and wound prevalence from 44.3% in 2011 to 22.2% in 2017; trends in the reduction of the prevalence maintained in the face of a mule population that increased from 430 in 2010 to ∼1,500 in 2017. While non-governmental organization (NGO)-led interventions can facilitate change by trialing new approaches and accessing new skills and resources, sustainable change requires community ownership and strengthening of service provision systems. To this effect, the project raised muleteer competence in mule husbandry and EZL prevention strategies; strengthened veterinary competence; facilitated more mule-friendly traffic, transport, and waste disposal guidelines and practices; supported mule-community bylaws to control EZL; and established a supportive network between stakeholders including trusting relationships between muleteers and veterinary services. To advance the intervention model in other endemic areas, we recommend elucidation of local epidemiological factors with other stakeholders prior to the intervention, early engagement with veterinary and transport service regulatory authorities, early development of bylaws, exploration of compensation or insurance mechanisms to support euthanasia of advanced cases,

OPEN ACCESS

Edited by:
Satoshi Sekiguchi, University of Miyazaki, Japan

Reviewed by:
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Getahun E. Agga, United States Department of Agriculture (USDA), United States

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Specialty section:
This article was submitted to Veterinary Infectious Diseases, a section of the journal Frontiers in Veterinary Science

Received: *31 December 2020*
Accepted: *05 October 2021*
Published: *12 November 2021*

Citation:
Duguma BE, Tesfaye T, Kassaye A, Kassa A and Blakeway SJ (2021) Control and Prevention of Epizootic Lymphangitis in Mules: An Integrated Community-Based Intervention, Bahir Dar, Ethiopia. Front. Vet. Sci. 8:648267. doi: 10.3389/fvets.2021.648267

and additional social, economic, and epidemiological investigations. In line with the OIE Working Equid Welfare Standards, we suggest that integrated community-based interventions are useful approaches to the control and prevention of infectious diseases.

Keywords: epizootic lymphangitis, Bahir Dar Ethiopia, EZL prevention and control, mule, community-based animal health care

INTRODUCTION

This article reports on a working mule welfare project (the project) that ran from 2010 to 2017 and focused on the control and prevention of wounds and the disease epizootic lymphangitis (EZL) in Bahir Dar, Amhara Region, as an indicator of the success of a more extensive participatory, community-based donkey and mule welfare program in Ethiopia facilitated by The Donkey Sanctuary (TDS), a United Kingdom-based donkey and mule welfare charity.

Around 13.3 million working equids provide essential services in Ethiopia (1). Their contribution to the economy remains generally under-recognized, and services available to the sector are therefore generally poor (2–4).

Around 380,000 of these working equids are mules, with ∼140,000 living in the Amhara Region where ∼56,000 power carts (1). The Amhara Region also has one of Ethiopia's largest populations of working donkeys. Mule numbers in Bahir Dar, capital of the Amhara Region, are increasing, from 430 in 2010 to around 1,500 in 2017, alongside increasing development and a human population in Ethiopia that doubled between 1995 and 2019 (5).

All mules in Bahir Dar power carts. They transport building materials (timber, stone, and cement) and agricultural goods (seeds, fertilizers, pesticides, and harvested produce) to and from markets, grain and flour to and from grinding mills, water where there is no piped supply, solid waste to municipal dumps, and people (6, 7).

Mules represent a significant investment, make a substantial (sometimes the only) financial contribution to muleteer household economies (6, 8), and provide valuable low-carbon community services, thereby serving the Sustainable Development Goals (9). Yet lack of knowledge; poor husbandry; harness wounds; lameness; colic; infectious diseases such as EZL, African horse sickness, tetanus, strangles, and parasites; and a lack of relevant support services all compromise welfare and productivity (7, 8, 10–14). On average, a mule affected with EZL in Gondar, Amhara, is estimated to cost its owner around ETB 6,000 per year (∼GBP 100, July 2021) in lost production (15).

EZL is the most visible and prevalent of the infectious diseases affecting equids in Ethiopia (16–21). In endemic areas, emaciated, abandoned horses and mules with the running sores of advanced EZL can be seen standing in the middle of busy roads where the breezes from passing vehicles provide some respite from flies. Welfare assessment, using the Hand (22–25), identified mule–muleteer–societal relationships, wounds, and EZL as the main welfare challenges to be addressed in this project. Lameness and nutrition would also be addressed tangentially.

EZL is primarily a chronic contagious disease of equids (17, 18, 20, 26), with horses being most susceptible, donkeys least susceptible, and mules in between. The causal agent is *Histoplasma capsulatum* var. *farciminosum* (HCF), a dimorphic fungus (one that can exist in both unicellular (yeast) and filamentous (mycelial or mold) states) which can live independently in soil, making eradication difficult. It is endemic to Ethiopia (27), particularly in the hot, humid upland areas between 1,500 and 2,800 m above sea level (20).

Control of EZL is challenging, with no completely satisfactory treatment (26). Spread between equids is thought to be facilitated by the presence of open wounds, close contact, flies, and poor work, hygiene, or husbandry practices (28). Treatment requires continuing owner compliance. Early identification and intensive follow-up are critical for successful therapy (17, 29). The more advanced the disease, the more guarded the outcome (30, 31). Tincture of iodine (2%) can be used topically, and sodium or potassium iodide can be parenterally administered via drinking water or feed, although lengthy treatment can lead to iodine toxicity. All are available in Ethiopia. The antifungal drug amphotericin B is generally impractical for working equids because of the specialized treatment protocols, potential side effects, and requirement for close monitoring (32). There is no readily available commercial vaccine for prevention, although an attenuated vaccine and a killed formalized vaccine are reported to have been used for its control in some endemic areas of west Asia (27, 33). Currently, therefore, the only viable means of prevention and control of EZL involves close collaboration between mule-using communities, veterinary services, and regulatory authorities, with cases caught early and intractable cases humanely euthanized.

The TDS program in the Amhara Region followed a strategic review with a move from direct veterinary intervention services to community-based approaches measured against welfare outcomes that could better deal with the technical, social, and economic complexities of donkey and mule welfare including the multifactorial nature of a disease such as EZL (34). It drew on lessons from community-based animal healthcare (35, 36) and participatory epidemiology (37, 38) including the global eradication of rinderpest in cattle (39–41).

In summary, the project trialed a community-based approach to understanding and improving cart mule welfare, in a location where wounds and EZL were the most visible welfare challenges with the explicit intention of exploring sustainability.

TABLE 1 | Number of mules sampled for annual cross-sectional survey of EZL and wounds, 2010–2017 (baseline and implementation surveys).

Survey year	Number of mules sampled	Survey method
2010[a]	430	Census survey
2011	623	Census survey
2012	1,128	Census survey
2013	1,266	Census survey
2014	NA[b]	No survey conducted
2015	394	Sample survey
2016	1,436	Census survey
2017	981	Sample survey

[a]*The project baseline survey.* [b]*NA—not applicable.*

METHODS

Materials

Project Study Area

The study was conducted in cart-pulling mules of Bahir Dar city, located next to Lake Tana, source of the Blue Nile, in the Amhara Region, northwestern Ethiopia, at an altitude of 1,820 m above sea level. The average annual rainfall is 1,416 mm. The short rainy season is in March and April, and the long rainy season is from June to September. Bahir Dar has a borderline tropical savannah climate with an average low temperature of 11.7°C and an average high temperature of 26.7°C. The mean relative humidity of Bahir Dar is 58.4% (42).

Project Study Population

The Bahir Dar City Administration provided a mule population estimate of around 500 mules for 2010 at the start of the project. The mule cart sector was informal, mules were not registered nor licensed, and there was little formal engagement between the municipality and sector, so the estimate was based on little data. The baseline survey which aimed for a full census found 430 mules. This number had risen to nearly 1,500 by the end of the project. During the project, the municipality based its annual mule population estimates on project census surveys, rather than its usual general annual estimate of animals in the city. Official full census surveys are conducted every 10 years in Ethiopia. Annual mule numbers are presented in **Table 1**, and the survey methodology is explained in Section Annual cross-sectional surveys: prevalence of EZL and wounds.

Cart mule demographics were fluid. Almost every mule had a dedicated handler (muleteer) who chased work across the city independently, and there appeared to be no stable groupings of mules. Owners with multiple mules generally rent them out in long-term arrangements with the muleteer responsible for almost all aspects of husbandry; and cart mule associations appeared to be loose affiliations. As mule population increased alongside diversification of work opportunities, competition also increased with larger numbers of mules accumulating at collection sites.

Nevertheless, the muleteer community was self-aware. Apart from business risks, EZL was its greatest threat, and many muleteers were aware of the disease and its status across the population. Muleteers were initially wary of the project, but once they understood the project's approach and intentions, most became more trusting. Mules are only kept to work, and working mules are visible, so key informants from within the muleteer community played an essential role in the project's success, helping project staff to find muleteers with mules affected by EZL including in the outer reaches of the city, both during the intervention and the annual surveys.

Project Study Framework

TDS identified the cart mules of Bahir Dar as a target project for two strategic reasons. First, the welfare of cart mules with wounds and EZL is visibly compromised. Second, the visibility of these mules in a regional capital helped raise awareness about working animals more generally and so fed into the awareness-raising and advocacy strand of TDS's broader strategy.

The project followed a participatory project management cycle (PPMC) which allows flexibility, accumulation of learning through review points, and the ability to amend plans and activities in agreement with other stakeholders (43, 44). The cycle can be described as stakeholders identifying and defining the problem together, then planning together, then implementing together, and then monitoring and reviewing together, before starting the cycle again by planning the next stage together, taking into consideration lessons learnt in the previous cycles. Awareness of the need for an exit strategy is explicit from the start, so additional cycles aim increasingly to hand over aspects of the work, while focusing on specific areas of challenge. Also explicit is the need to reduce external contributions to the project over time. This is shown schematically in **Figure 1**.

While TDS identified EZL in mules in Bahir Dar as a social, economic, and animal health and welfare problem, it then needed to involve other stakeholders for the project to work.

Stakeholder Analysis

Stakeholder analysis started by talking with obvious stakeholders such as mule owners and users (muleteers), veterinary service providers, and regulatory officials. TDS then used interviews and stakeholder analysis (45) with these individuals and emerging key informants to understand the wider range of stakeholders, their areas of interest and influence, and the roles they might play in making the project a success.

Epidemiology

The application of epidemiology and epidemiological tools served two main roles during this EZL control and prevention project: to engage all stakeholders with an agreed understanding about EZL prevention and control based on both local knowledge and published articles; and to provide a reliable methodology for annual EZL prevalence surveys for project monitoring and evaluation.

Case Definition and Diagnosis of EZL

The case definition for EZL in this study was a working mule in Bahir Dar with a diagnosis of EZL based on clinical examination with confirmation through direct microscopy.

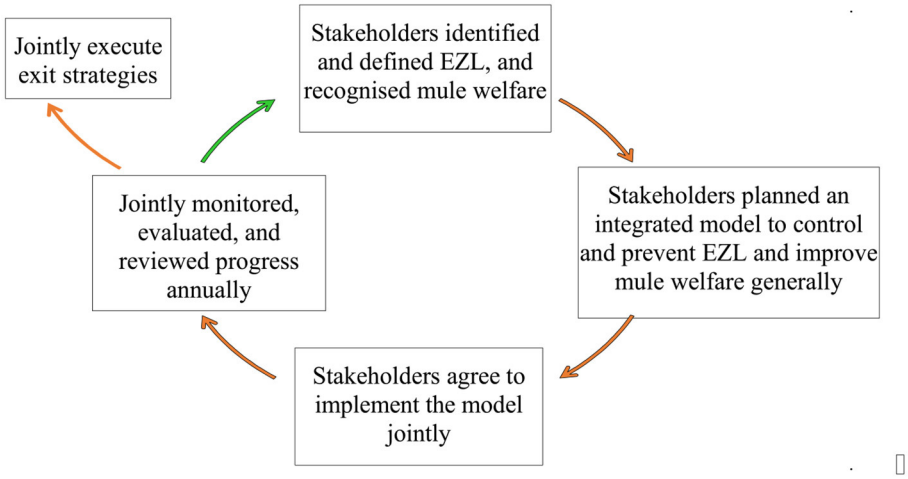

FIGURE 1 | EZL participatory project management cycle (PPMC). Review has been a continuing process within PPMC before implementing the exit strategies. Exit strategies can happen at different times for different components of the project.

The clinical examination protocol for EZL involved inspection of the entire skin surface for ulcerative wounds or suppurative spreading dermatitis and lymphangitis and palpation along the lymphatic vessels and lymph nodes for the presence of nodules. Cases were characterized as advanced by the presence of button-type ulcers or nodules, cording or thickening of lymphatic vessels, or functional abnormalities such as lameness, dyspnea, or visual impairment.

Swab samples from any suspect lesions, including ulcerating harness-related lesions, bit sores, or ocular discharge, and aspirates of nodules along the lymphatic vessels or lymph nodes were collected for direct microscopy. Direct microscopic examination of samples was conducted in collaboration with the Bahir Dar Regional Veterinary Laboratory. Both Gram's and Giemsa stains were applied to examine for pleomorphic yeast-like cells with a halo appearance under the oil immersions lens (29).

Annual Cross-Sectional Surveys: Prevalence of EZL and Wounds

A baseline survey to assess general welfare, including prevalence of EZL and wounds, was conducted by the TDS staff in 2010 before making a final commitment to implement the project. This was a full census survey aiming to reach every mule and involved clinical examination of mules for EZL, open wounds, harness fit and quality, lameness, sex, age [estimate based on dentition (46)], and body condition score [BCS, using a five-level scoring methodology (47)] as potential risk factors for EZL. General observations were also made regarding commodities being transported, working practices, and mule–muleteer relationships. The project monitoring and evaluation strategy was to resurvey mules each year to assess the effectiveness of its protocol by measuring annual change in EZL and wound prevalence. In view of the growth and demographic complexity of the mule sector and because the project was about general welfare as well as EZL, the preferred strategy was to continue with full census surveys, to reach all mules, sample each mule for EZL, and check for open wounds, collect accurate population data, and check general welfare across the growing population of working mules. Resampling of mules was avoided by registering muleteer names during the exercise and marking each mule on the forehead with permanent red ink.

Surveys were conducted by a team composed of veterinarians from Bahir Dar Clinic and Bahir Dar Regional Veterinary Laboratory and TDS staff. To meet the primary objective of controlling EZL, every effort was made to ensure inclusion of each and every mule working, at rest, grazing, left at home, or abandoned for the success and progress impact of the intervention model. Muleteers increasingly assisted in the process as they became more aware that every case is a potential source of infection and needs to be reported as soon as discovered. Other key informants, including the Kebele administration, were also involved in the organization of the census survey. Surveys took place during the month of May when EZL cases are higher and were completed over 2 to 3 weeks.

For reasons outside project control, there was no survey in 2014, and full census surveys were not possible in 2015 and 2017. In 2015, the sample size was calculated based on the 21% prevalence published in a previous Ethiopian study of EZL prevalence in cart mules (17). Assuming 21% prevalence with 95% confidence level, 80% power of test, 5% precision and an estimated population size of 1,400 mules required a minimum sample number of 380 (48), and the project sampled 394 mules. In 2017, because prevalence had risen in 2016, the project reduced the margin of error, assumed prevalence of 50%, precision level of 3% and, with an estimated population of 1,500 mules, calculated a minimum sample number of 889, and 981 mules were sampled (49). Mule selection in 2015 and 2017 used systematic random sampling where every other mule was sampled, and planning together with key informants from

the mule community ensured sampling of mules across the full geographic and demographic range in and around Bahir Dar.

Stakeholder Contributions and Ownership of the Interventions

For project success, all stakeholders needed to reach a common understanding about EZL and what they could do to minimize or prevent it.

With the use of two participatory epidemiology approaches, key informant interviews, and focused group discussions (FGDs), factors associated with EZL were explored using a range of participatory exercises. The purpose of these exercises was to build engagement; to respect and understand local perceptions; and to check that these aligned generally with published epidemiology on EZL. These exercises aimed to allow stakeholders, whatever their level of education or literacy, to contribute their knowledge and experience, share in the analysis, and take ownership of the prevention and control strategy.

The key informant interviews and FGDs were conducted primarily by the TDS program staff. Themes and results were shared with the wider community in subsequent meetings and training sessions through narrative, reporting of ranking results, and map building (37, 50, 51).

Key Informant Interviews

Individual, semi-structured, face-to-face interviews were conducted with 12 key informants. These included five experienced muleteers, one municipality officer, two animal health professionals, two traffic police officers, one senior livestock officer, and one manager of a private waste collection business. Each interview involved a set of questions to open wider conversations that allowed interviewees to add ideas that might not have occurred to the interviewer.

Questions explored the socioeconomic impact of EZL on cart mules in Bahir Dar; factors predisposing to EZL; challenges to controlling and preventing EZL; the roles of the different institutions and associations; and what should be done about EZL.

FGDs

Five FGDs were held to explore muleteer perceptions about EZL and its epidemiology. Participation criteria were agreed with stakeholders such that all participants were experienced muleteers, were members of cart mule associations, and had some form of social responsibility in the community as well as passion and willingness to participate. Muleteers new to the carting business in Bahir Dar who lacked background about EZL and its impact on the city and children driving carts for their older relatives were therefore excluded.

Around 30 mule-related associations were registered with the Bahir Dar municipality, but some were short term, for example, as part of a small microfinance scheme, and were no longer functional. Participants in the FGDs came from the eight most active associations based on the large size of membership, longer years of establishment, integrity of their bylaws, and diverse work type and geographic locations. Despite being members of an association, muleteers work independently, and FGDs were mixed depending on when different muleteers had the time to join a session. Location and timing of FGDs were dictated by the muleteers and their daily schedules.

Topics were explored in the FGDs with the help of participatory learning and action (PLA) tools (50, 51), which allow participants to represent their worlds, their knowledge, and their experience, in a variety of ways including diagrams, pictures, and maps. The discussions between participants as they create the representations, the representations themselves, and the detailed discussion afterwards are all equally important parts of the process. PLA tools help ensure that all participants contribute, even the quietest ones. When facilitated well, the process holds attention beyond that of a general discussion, allowing every point to be fully examined.

The topics explored (and the PLA tools used) were as follows:

- *Socioeconomic impacts of EZL* (brainstorming and discussion);
- *Predisposing factors to EZL*: identify (brainstorming) and rank and discuss (simple ranking);
- *Pairwise ranking*: using the top risk factors identified in the previous exercise, compare and rank these risk factors in pairs;
- *Seasonal calendars*: draw a line that represents your working year; mark the four main seasons—Tseday, Bega, Belg, and Kiremt; add in other major events in the year, e.g., types of work that may vary during the year, and times of the year when EZL cases are lowest and highest; and explain and discuss; and
- *Mapping Bahir Dar from a mule perspective*: participants worked on a map of the city showing collection sites including feeding and watering points, parking, markets, construction sites, and grain mills, adding other meeting sites of importance and marking routes used to move through the city while discussing the challenges they faced (participatory mapping).

Planning and Implementation

Once the various stakeholders had established a common understanding about the problem, TDS facilitated a series of consultative workshops to agree on a collective implementation plan.

Workshops started with a summary of project progress to date (as in Sections Project study framework–Epidemiology) and then used a problem tree exercise (52) to explore root causes and intervention points.

These meetings helped identify key intervention activities, reach an agreement on how the work should be monitored and evaluated, assign roles and responsibilities, and reach an agreement on other implementation modalities and a timeline. The idea of an exit strategy for TDS involvement was introduced from the start to provide focus.

Facilitated learning including practical training—for all stakeholders (including TDS) but most actively for muleteers, harness makers, and animal health professionals—was a central theme, as was ensuring availability of treatment for treatable cases and euthanasia for incurable cases. Other activities aimed at changing attitudes, behaviors, and practice regarding mule transport within the municipality transport officials and traffic police.

The project started with an official public launch. Stakeholders needed to commit to being part of the "project team" before the official launch to help build trust, to reinforce that the project could only succeed as a joint effort, and to mitigate against groups dropping out.

Education of Muleteers

Muleteer education was practical and participatory. It targeted all muleteers and responded to points raised by all stakeholders including the muleteers themselves. TDS was involved with the local veterinary staff in improving muleteer understanding and practical knowledge of mule behavior and handling; improving general mule care and management; and reducing wounds and improving hygiene and segregation practices around EZL. Transport officials and traffic police worked together with muleteers on traffic-related education.

Specifics included herd health and EZL prevention (where herd can mean all the mules in Bahir Dar or a subset, such as those belonging to one muleteer, or all members of a cart mule association, if they can be managed in any way separate from other mules); use of improved harness (saddle, bits, and straps made of natural materials) and implications of sharing harness; daily mule checks for early identification of all health problems including wounds and EZL; early treatment including how to engage with veterinary services; principles and practice of wound management (including application of tincture of iodine); case segregation while housing, feeding, and being at collection sites; understanding the concept of euthanasia and how it supports control and prevention of EZL; and safe road use including knowledge of road safety regulations and good communication while driving.

Mule welfare training modules were initially developed and delivered monthly by TDS staff and then through training delegates selected by their cart mule associations who provided training to other muleteers, including new muleteers outside their own association. The treatment protocol for active EZL cases required muleteers to clean wounds and apply tincture of iodine initially under the direct supervision of an animal health professional. This training support was later taken over by local animal health professionals. Harness training was facilitated by TDS harness specialists (animal health assistants trained by TDS international harness specialists). Road safety training was facilitated by local traffic police officers.

Training of Harness Makers

Almost all cart mule wounds were related to poor harnessing practice, and the project therefore needed to address this. It developed, tested, and piloted improved saddle prototypes, humane bits, and canvas straps. Training was provided to 12 harness makers stationed at two locations in Bahir Dar on the making, fitting, repair, and maintenance of this harness. TDS initially supplied the raw materials, e.g., wood, canvas, and the bit-making tools, during the prototype and testing phase.

Training of Animal Health Professionals

Local animal health professional staff included veterinary surgeons (6-year university training), BVScs (Bachelors of Veterinary Science, 3-year university training), and animal health assistants (3-year technical college training) at the public veterinary clinics in central Bahir Dar, Meshanti, Zanzelima, Gonbat, and Tis-Abay. Except central Bahir Dar with six to eight veterinary staff, other public veterinary clinics were health posts with one to two veterinary staff. There were also around seven or eight private animal health practitioners across Bahir Dar. Turnover among veterinary staff in public clinics, particularly the small health posts, was high, although some interested individuals remained engaged with the project even after promotion.

Before the project, muleteers were not taking mules with EZL to veterinary clinics for two reasons: because the animal health staff lacked competence in EZL diagnostics, treatment, and euthanasia (and equid medicine generally) and because clinics lacked the necessary drugs.

TDS therefore trained the animal health professionals and equipped the public veterinary clinics with tincture of iodine (2%) and potassium iodide.

Animal health professional training was prepared in four modules: mule behavior and handling; EZL diagnosis, treatment, and euthanasia; EZL epidemiology and herd health; and community facilitation skills and equine husbandry. Each module was delivered as a Continuing Professional Development (CPD) course and followed up with more informal practical hands-on training in the field at veterinary clinics.

Once the project was established, veterinarians with competence to train others (see Section Monitoring and evaluation of the project), including in communication skills, were selected for Training of Trainer (ToT) training so that they could continue to train muleteers and deliver further CPD training to other animal health professionals as necessary. Trainees were followed monthly by the TDS team up until 2015 and semiannually thereafter. The project targeted all animal health professionals for training.

Treatment of Early EZL Cases and Euthanasia of Advanced Cases

Treatment and euthanasia were critical elements of the intervention to contain spread of EZL because untreated or abandoned EZL mule cases remain a source of infection for other mules in Bahir Dar.

Cases were classified as early, established, advanced, and untreatable (recommended for euthanasia). Treatable cases were treated by incision of nodules when present, application of tincture of iodine, and administration of parenteral iodides (potassium iodide, Ubiche) in drinking water (30). Length of treatment increased the more severe the case classification. For the detailed procedure and outcome, please refer to Supplementary Section of the manuscript.

Euthanasia was performed using intravenous injection of barbiturates (Pentoject® 200 mg/ml solution, pentobarbital sodium 20% w/v, XVD132, Animalcare Limited, UK).

TABLE 2 | The Donkey Sanctuary general competence framework for trainees.

Competence level	Description and assessment process
Starter	All trainees start at this level to acknowledge and encourage interest. No real knowledge or experience but with active interest.
Becoming independent	Acknowledgement of starting actively along training journey. An active trainee, a reflective learner, with good attitude and regular attendance.
Independent	A trainee who has continued to show competence in what was taught and to demonstrate reflective practice, for at least 6 months after completing the formal training. Assessed by the TDS staff through practical follow-up field work.
Trainer	Independent practitioner in a primary area of competence, with additional independent competence following Training of Trainers (ToT) training. Identified for ToT training by demonstrating good communication skills, an interest in helping others to learn, strong reflective practice, and an interest and enthusiasm for becoming a trainer, and who has then reached independent level in training practice (assessed by the same competence framework).

Monitoring and Evaluation of the Project

Progress indicators for each aspect of the project were identified and agreed on by all stakeholders. Annually repeated cross-sectional surveys were conducted to assess prevalence of EZL and wounds as key indicators for the success of the project (see Sections Project study population and Annual cross-sectional surveys: prevalence of EZL and wounds).

Training was assessed using the TDS four-level general competence framework for trainees: starter, becoming independent, independent, and trainer (see **Table 2**).

Field reports were prepared monthly, project progress reports were prepared quarterly, and project evaluation reports were prepared annually. All stakeholders took part in consultative review workshops. External evaluations were conducted by Amhara Region regulatory signatories (Bureau of Livestock Agency and Bureau of Finance and Economics) midterm and at the end of the 5-year project agreement between TDS and the Government.

Project Exit Strategy

The project started with a 5-year agreement between TDS and the regional bureaus. The project aimed to find an approach to improving mule welfare, including reducing prevalence of EZL, that could be owned and sustained locally with a minimum of external input. Reflective learning among all stakeholder groups, including TDS, would be a central part of the work. TDS envisaged gradually reducing its involvement over further project cycles, while continuing to help refine the approach and institutionalize key components such as cart mule business, training, and animal welfare standards.

This would involve empowering communities, mainstreaming best practices into the relevant sectors, recognizing EZL as a notifiable disease by the Bahir Dar municipality, transforming the local veterinary clinics to handle EZL cases and euthanize advanced cases by the regional livestock agency, formalizing and regulating mule-powered transport by the transport sector, supplying improved harness by local harness makers, formulating local community bylaws among the cart muleteers, and ideally encouraging institutions and organizations such as veterinary schools, the Ethiopian Veterinary Association, or the Ethiopian Animal Health Assistant Association to review their curricula, strengthen communication and community-engagement practices, and offer equine CPD training.

Gradually refining and reducing involvement over time was the project's exit strategy—a tailing out rather than an abrupt end. However, this was not possible for reasons outside project control.

Statistical Analysis

Information obtained from key informants was captured using facilitator notes and summarized into thematic areas and presented as a narrative. Results of FGDs obtained using different tools were presented in a map and a table.

Statistical analysis for annual prevalence study was carried out with STATA software version 11, using the chi-square statistical test, with the significance test set at a p-value < 0.05. Mule demographics and prevalence studies were presented using proportions.

To examine the statistical significance for the persistence of the prevalence reduction, one-way ANOVA trend analysis was executed for prevalence reduction of both EZL and wounds across the years.

RESULTS

Demography—Survey of Mules and Muleteers

Owners sourced mules from local livestock markets around Bahir Dar including Yigodi, Merawi, and Bahir Dar central market. These mules are likely to have come through more distant markets in Este, Debre Tabor, and Adet, which are part of an equine trade network originating from South Wollo that extends across Central, Southern, and Western Ethiopia.

Mule transport is currently considered an informal economic activity and is unlicensed. Training and support services are limited, with no training available in mule handling, husbandry, or business management.

The major items transported by cart mules in Bahir Dar included construction supplies (56.6%) such as wood, stone, cement, gravel, and sacks of sand to meet the demand for Bahir City expansion; agricultural produce from nearby farms to markets (27.3%); and other commodities for sale and household use (16.1%) including water and flour.

TABLE 3 | Age structure of working mules in Bahir Dar in 2010 (number (N) and %), from baseline data.

Mules < 5 years *N* (%)	Mules 5–10 years *N* (%)	Mules > 10 years *N* (%)
47 (11)	241 (56)	142 (33)

Table 3 presents the age structure of the mule population of Bahir Dar in 2010 (extracted from 2010 baseline survey data), showing that 52% of mules were male and 48% of mules were female.

As part of the baseline, the TDS team assessed the working condition of mules to be generally poor. Almost all carts and harnesses and harnessing systems were poor. All drivers use a stick or whip to drive mules. Mules work throughout the day; there was no shifting practice unlike cart horses in other areas of Ethiopia. They stay loaded without feed and water at collection sites for an extended period particularly on non-market days. Cart owners work with an EZL case until the disease advances to affect the mule's ability to work, for example, by its impact on locomotion or its respiratory system, and finally, they abandon the mule.

Study Framework: Results of the PPMC Approach

Results of the PPMC approach with its collaborative working and flexible review points showed themselves in various ways.

Muleteers at the start mistrusted TDS and the veterinary services, used traditional treatments, and avoided outside interference. However, when they understood that the project was genuine about working with them and saw the potential benefits, they engaged with the veterinary service, with muleteers starting to accept euthanasia. Stakeholders took their own initiatives within the project structure: some cart mule associations introduced EZL bylaws; transport officers and traffic police in consultation with the cart mule associations developed mule-friendly improvements in traffic regulations supported with information bulletins put out through municipality media channels; and traffic police reported reduced numbers of road traffic accidents involving cart mules as the project progressed.

Stakeholder Analysis

Active involvement of diverse stakeholders is a central aspect of a community-based approach. The full list of stakeholders engaged with this project are listed in **Box 1**, grouped by affiliation.

BOX 1 | Project stakeholders by affiliation.

Project stakeholders
Muleteers
Muleteers in general
Delegates from eight of the most active of the 30 cart mule associations
Private sector
Cart and harness makers
Private animal health professionals/clinics
Licensed solid waste management cooperatives
Bahir Dar city administration
Animal health professionals from Bahir Dar clinics and subclinics
Bahir Dar city municipality
Transport officers (make regulations)
Traffic police (enforce regulations)
Solid waste management staff
Amhara regional bureaus
Livestock Agency
Regional Livestock Officer
Lab technicians from Bahir Dar Regional Laboratory
Bureau of Transport
Bureau of Finance and Economics
Regional NGO desk
The Donkey Sanctuary employees
Animal health professionals: veterinarians and allied professionals including harness specialists
Social science staff
Education specialists

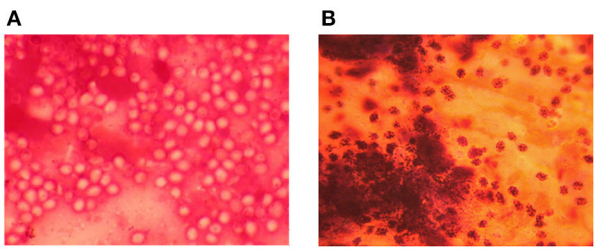

FIGURE 2 | Photomicrographs of *Histoplasma capsulatum* var. *farciminosum* yeast cells showing intact nonstaining yeast cells sampled from an early case **(A)** and disintegrated yeast cells with deep-staining dotted granules from an advanced case **(B)**.

Epidemiology

Case Definition and Diagnosis of EZL

The case definition for EZL provided a common understanding of the disease for diagnosis and training. It was observed consistently during the study that intact and non-staining yeast cells of HCF were common in early cases of EZL, and disintegrated yeast cells with deep-staining dotted granules were more common in relatively advanced cases (see **Figures 2A,B**). These observations have not been reported in other studies and are therefore of note.

The case definition provided the basis for the annual prevalence surveys. The results from the prevalence survey are presented in **Table 4**.

Epidemiology: Annual Change in Prevalence of EZL in Bahir Dar

As shown in **Table 4**, the prevalence of EZL reduced from 23.9 to 5.9% during the course of the project. The prevalence of wounds reduced from 44.3 to 22.2%. The greatest changes came in the first 2 years of the project, with the prevalence of EZL reducing faster than that of wounds. After year 3, the improvements plateaued, with rises in 2015 for wounds and in 2016 for EZL as the

TABLE 4 | Prevalence of EZL in cart mules in Bahir Dar from baseline and annual prevalence surveys.

Year	Test positive	Prevalence (%)	95% CI (%)	Total tested sample
2010	102	23.9%	19.8%–28.0%	430
Total (baseline)	102			430
2011	79	12.7%	10.0%–15.3%	622
2012	54	4.8%	3.5%–6.0%	1,128
2013	75	5.9%	4.6%–7.2%	1,266
2014	NA[a]	NA	NA	NA
2015	23	5.8%	3.5%–8.2%	394
2016	144	10.0%	8.5%–11.6%	1,436
2017	58	5.9%	4.4%–7.4%	981
Total (project)	433			5,827

[a]*NA—Data not available.*

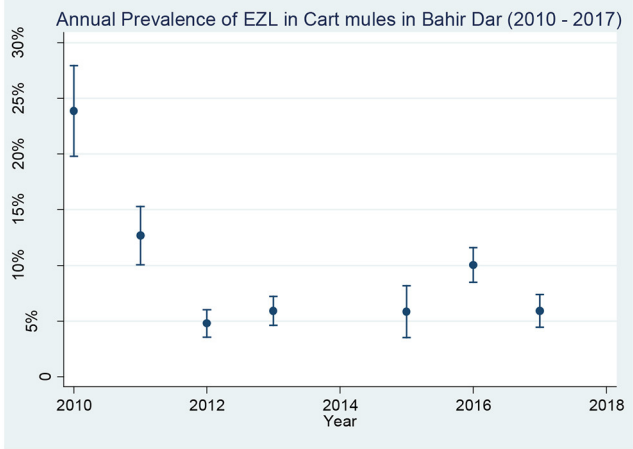

FIGURE 3 | Annual prevalence of EZL in cart mules in Bahir Dar from 2010 to 2017 [NB: Error bars represent 95% confidence interval around EZL prevalence value].

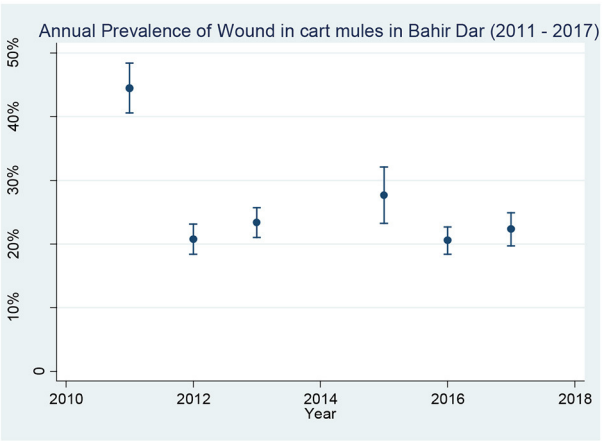

FIGURE 4 | Annual prevalence of open wounds in cart mules in Bahir Dar from 2010 to 2017 [NB: Error bars represent 95% confidence interval around wound prevalence value].

project tested handing more responsibility to private veterinary clinics. Despite this, trend analysis showed that the prevalence reductions in both EZL and wounds over the whole duration of the project were statistically significant ($\chi^2 = 26.57$; $p < 0.001$). Results from the baseline survey in 2010 and the subsequent annual surveys for the prevalence of EZL and wounds during the intervention period from 2011 to 2017 are presented in **Figures 3**, **4**. No data were available for 2014 for reasons external to the project.

The prevalence reduction difference from 2010 to 2017 was 18.0% for EZL and 22.1% for wounds. The prevalence reduction trend analysis for both EZL and wound was statistically significant ($\chi^2 = 39.02$; $p < 0.001$). Wound data for 2010 were excluded because open wounds and healed wounds had not been put into different categories. An observation during the annual surveys was that while almost all the open wounds continued to originate from poor harnessing, the severity of wounds was reducing with fewer extensive or infected wounds or discharging abscesses, compared to the baseline stage.

Stakeholder Contributions

Key Informant Interviews

Individual key informant interviews gave insight about how informants understood EZL and related matters, with agreement about the following general themes.

All key informants believed that mules arrive from external markets free of EZL and only become infected in Bahir Dar. Informants agreed that EZL has a devastating socioeconomic effect, and follow-up discussions explored the cost of the mule (∼ETB 10,000 = ∼GBP 165) compared to the cost of treatment (∼ETB 1,000 for early cases). Besides its socioeconomic impact on cart mule business, abandoned mules were a public concern contributing to a poor image of the city. Traffic police complained that abandoned EZL cases were sources of car accidents at various occasions, and an abandoned mule could be a cause of more than one accident over time, particularly at night. Waste collectors believed that terminally sick mules could easily be transported to disposal sites and killed there as it was tiresome to deal with a dead body elsewhere in the city. They suggested for a coordinated effort of stakeholders. Animal health professionals complained about the lack of treatment options and required updated skill sets in all equine practice, not just EZL as a single disease entity. Cart mule association delegates explained how EZL is a real worry to their business. They complained that new owners are a risk because they do not know how to identify, manage, and prevent EZL. They requested that regulatory authorities formalize and develop the sector. There was general agreement that the predisposing factors were wounds mainly from ill-fitting harnessing, poor hygiene, and lack of segregation practices; the main challenges for control are lack of an agreed-upon sound treatment protocol, poor harnessing practice, lack of awareness, lack of mule movement

TABLE 5 | Pairwise ranking of the predisposing factors identified by experienced muleteers during focused group discussions.

Predisposing factors of EZL	Proximity to EZL case (PE)	Owner's lack of experience (OE)	Fly season (FS)	Open wound (OW)	Poor harness (PH)	Poor hoof care (HC)	Working condition (WC)
Proximity to EZL case (PE)[a]		**PE**	**PE**	**OW**	**PE**	**PE**	**PE**
Owner's lack of experience (OE)			OE	OW	OE	OE	OE
Fly season (FS)				OW	FS	FS	FS
Open wound (OW)					OW	OW	OW
Poor harness (PH)						PH	PH
Poor hoof care (HC)							HC
Working condition (WC)[b]							
Total	OW	PE	OE	FS	PH	HC	WC
Rank (score[c])	1st (6)	2nd (5)	3rd (4)	3rd (4)	4th (2)	5th (1)	6th (0)

[a]Proximity at work, feeding, water, grazing, and housing. [b]Types of work engaged such as transporting construction, logs, and flour. [c]Score = number of other factors considered more important than during the individual pairwise comparisons.

control, and the business being informal and owned mainly by a resource-poor class of community and illiterate people; and EZL could be prevented easily through collaboration of relevant sectors including prevention of wounds, segregation of cases, and removal of abandoned EZL mule. Transport officers believed that in the long term, EZL will not be a problem in Bahir Dar because the cart mule work will soon be replaced by motorized vehicles.

FGDs

The results from the individual exercises used in the FGDs are presented below.

Socio-economic impacts of EZL (brainstorming and discussion). Muleteers compared the impact of EZL with the impact of other key endemic diseases that affect their cart mules, specifically colic, African horse sickness, and EZL. They described the negative socioeconomic impact of each on the business: colic occurs rarely and kills only one animal at a time, and it can be prevented; African horse sickness is a risk to other mules but comes once in several years and yet kills some mules while other mules recover; EZL is also a risk to other mules, occurs throughout the year, and has no reliable treatment option, and most EZL cases eventually die.

Predisposing Factors to EZL (Brainstorming, Card Ranking, and Pairwise Ranking). Muleteers identified the main predisposing factors for EZL as open wounds, proximity to another mule affected by EZL, owners' lack of knowledge and experience to prevent EZL, fly season, poor harnessing practice, hoof care, and working conditions.

These factors were then debated in more detail using pairwise ranking in which each factor is compared individually to all the others in turn. A summary of the result is presented in **Table 5**.

Participating muleteers ranked open wounds as the most important factor affecting spread of EZL, followed by proximity to other mules affected by EZL; next were owner's lack of experience and fly season, which were of equal importance; then poor harness was more important than poor hoof care practice; and work type was the least important factor.

Seasonal Calendar. The owners concluded that mules get the disease throughout the year; however, new cases were observed more after the long rainy season during the months of September and October, remain low during the dry seasons, and rise again following the small rainy season in April and May of the year.

Participatory Mapping. Key informants identified cart mule entry points to Bahir Dar, as well as movement and distribution. They located mule pathways and sites where cart mules come together such as markets, construction sites, flour mills, water points, grazing fields, healthcare units, and large group housing sites. The owners worked together to map these using referring points, which were translated onto a Google Map (see **Figure 5**).

The key informants summarized that cart mules were free to work anywhere in the city, be it markets, construction, or other working sites. There was no restriction of movement for an EZL case until it is abandoned. An abandoned EZL case still had access to socialize with apparently normal mules at feeding and water points, other collection sites, or grazing areas. Abandoned cases stay alive for months, and that is when they remain the source of infection for other mules.

Results of Implementation

Education of Muleteers

Table 6 shows the numbers of muleteers who attended structured, subject-based workshops which aimed to raise animal welfare awareness and change behaviors. The subject areas were mule behavior, handling, and care; mule harnessing practice and road traffic rules; wound management, including application of tincture of iodine; and EZL, i.e., detection and reporting, prevention measures, and euthanasia.

Examples of how education activities improved the mule-health-related practices of muleteers included increased number of equine cases visiting the public vet clinic; adoption of project EZL treatment protocols with wound management using application of tincture of iodine and gradual acceptance of euthanasia; segregation of infected and non-affected mules at collection and grazing sites; disinfection of harness materials; greater vigilance in reporting suspected cases of EZL as a

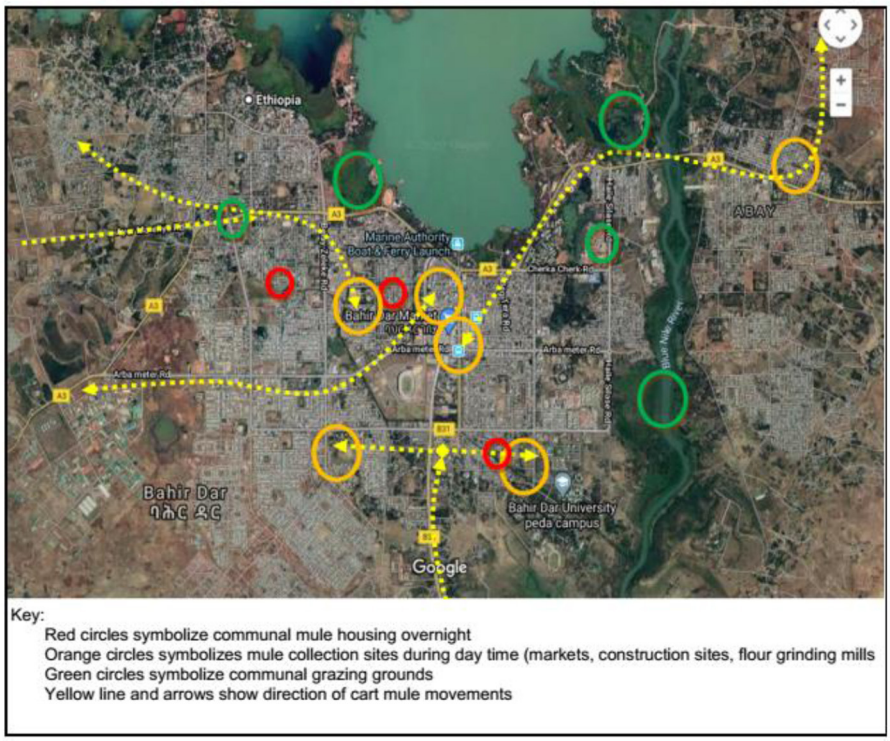

FIGURE 5 | Cart mule movement dynamics and gathering sites in Bahir Dar.

TABLE 6 | Numbers of muleteers attending different training sessions.

Subject/Year	Mule behavior, handling, and care	Mule harnessing practice; road traffic rules	Wound management, including application of tincture of iodine	EZL: detection and reporting, prevention measures, and euthanasia
2011	870	300	169	370
2012	627	624	124	227
2013	580	552	116	380
2014	600	178	73	410
2015	420	164	107	320
2016	280	388	45	187

notifiable disease; and increased use of cart and animal fitted reflectors when driving at night. In general, the muleteers understood that the success and benefit of the intervention relied on shared responsibility, with each taking responsibility not only for their own mule but also for the herd.

Competence levels of individual muleteers are not reported because individual follow-up was difficult, and competence assessment was therefore based on feedback at meetings, field observations at working sites, and observation of changes in practice. Based on these observations, the majority of muleteers were in the "becoming independent" and "independent" category, apart from a few exceptional muleteers who took it upon themselves to mentor others.

Owners with EZL cases were more immediately attentive and engaged than those who had apparently EZL-free mules, but as the intervention progressed, some owners with advanced EZL cases became reluctant to attend the training or treatment clinics because they feared pressure for euthanasia.

The results of the awareness-raising training sessions with traffic police, transport officers, waste collectors, and other municipality development agents came through their changing, more constructive relationships with the muleteers.

Other events, such as World Animal Day, which was observed every year during the first week of October and promoted animal welfare more widely, demonstrated the growth in confidence of project stakeholders who were able

TABLE 7 | Trainings provided for animal healthcare professionals and number of attendees.

Subject/year	Mule behavior, handling, and care	EZL diagnosis, treatment, and euthanasia	EZL epidemiology and principles of herd health	Community facilitation skills and equine husbandry
2011	24	24	24	18
2012	21	18	18	20
2013	21	13	15	18
2014	16	16	14	16
2015	6	6 (4 ToT[a])	8 (4 ToT[a])	
2016			11	11

[a] *ToT—trainers' training was provided at a later stage so that new staff can be trained by the local veterinarians.*

to present themselves in a positive light and to show themselves united.

Training of Harness Makers

Of the 12 harness makers, eight successfully completed the training and were producing improved prototypes of saddle and humane bits and straps. During the intervention period, the harness makers produced 584 improved cart saddles, 430 humane bits, and 893 canvas straps and collars, which were exchanged with poor traditional types.

Training of Animal Health Professionals

Attendance at training sessions for local animal health professionals is presented in **Table 7**. The training was intensive in terms of time and tasks. Mule behavior training was particularly challenging for trainees, most of whom had no experience in working professionally with mules. For various reasons, some veterinarians were irregular in attendance. Most veterinarians demanded incentives for the follow-up treatments. The owners' pressure to get his mule healed and TDS commitment to invest in the treatment were boosters for the success of the training and treatment. Despite the challenges mentioned above, all clinic veterinary staff achieved independent competence during the course of the project, assessed through long-term clinic follow-up. Two veterinarians from Bahir Dar clinic and two from animal health posts excelled in their competence as trainees and then trainers and played an important role in ongoing project success.

Treatment of Early Cases and Euthanasia of Advanced Cases

Treatment and euthanasia were critical components of the project to remove cases from the herd. For the euthanasia protocol, please see the **Supplementary Materials** section. Before the project, euthanasia was rarely done and had not been considered as a disease control practice in the area. Constraints were lack of drugs and a system for the disposal of carcasses as well as difficulty to get owners' consent without any compensation.

In piloting this model, TDS made drugs available, trained professionals, and raised public awareness in the perceptions and value of euthanasia. The Bahir Dar City municipality cooperated in the provision of trailers, disposal sites, and organizing associations working on city sanitation for disposal of euthanized mules. Getting owners' consent to euthanize mules with poor EZL prognosis was the inherent challenge throughout the intervention. Nevertheless, a total of 123 mules were euthanized during the period of the project.

Development of cart mule association bylaws helped reinforce good practice among members and influenced non-members through peer association. The Bahir Dar municipality also, for the first time, developed a range of bylaws relating to working equids; however, this came toward the end of the project period, and no mechanism for effective enforcement was developed before the project ended.

Evaluation of the Project by Signatories

The project was originally signed for 5 years with the intention of piloting a community-based system to control and prevent EZL. The Amhara Regional Livestock Agency, the Bahir Dar City Administration, and Regional Bureau of Transport, all regulated and coordinated by the NGO desk of the Regional Bureau of Finance and Economics, jointly conducted midterm and final evaluations.

The midterm evaluation focused on stakeholder commitment, coordination mechanisms, and testing the impact on the prevalence of EZL. Finding a dramatic reduction in the prevalence of EZL opened the eyes of many stakeholders, particularly the muleteers, animal health professionals, and traffic police.

The final evaluation focused greater emphasis on drawing conclusions about best practice and sustainability. The evaluating team reflected that the project impact was significant and obvious to the general public in Bahir Dar. The most publicly noticeable change was avoidance of abandoned EZL-affected mules in the middle of roads in Bahir Dar city, a prime tourist destination in Ethiopia. The practice of abandoning a mule at the end of its working life to continue to suffer in pain and die of deprivation of food and water is visibly cruel, is a public worry, and portrays a bad image for the city. All parties witnessed and shared success stories: the livestock agency witnessed the treatment success and training modalities for its animal health professionals; the municipality witnessed and appreciated the value of coordination in transforming livelihoods of muleteers; and the traffic police

officers witnessed a persistent reduction of cart-mule-associated road traffic accidents.

A learning point was the time needed to establish a community-based project and engage stakeholders. One 2-year project extension was completed to strengthen the intervention, but for reasons beyond its control, the project then ended.

Outcome of Project Exit Strategy

The objective of an exit strategy is for the project as an entity to end its input, leaving behind a self-sustaining positive impact.

The benefits of the euthanasia program to mule herd health and the mechanism of operation have been recognized in Bahir Dar. A bylaw was established to sustain the change and control and prevent EZL. The articles contained in the bylaw require notification of an EZL case to the nearest traffic police or public veterinary clinic within 48 h, segregation of an EZL case, euthanasia of a terminally sick EZL case, prevention of wound, and registration of new muleteers as they join the business. The Bahir Dar city administration mainstreamed the disposal mechanism of dead mules alongside solid waste.

The traffic regulations mainstreamed animal-powered transport systems into the Bahir Dar traffic public awareness-raising program to minimalize road traffic accidents. Subjects promoted include use of reflectors; improved harness and mule health and welfare; a minimum muleteer age; and skill and knowledge of driving to prevent road traffic accidents.

Trust has been established between Bahir Dar public veterinary clinic service providers and muleteers for the treatment of early cases and euthanasia of terminally sick cases.

However, the long-term sustainability of the EZL project relies on many factors including further formalization of animal-powered transport into the city/regional development program alongside recognition that it is not about to be replaced by mechanized transport and transformation of animal healthcare delivery to include equine medicine and welfare. Also the low status of the sector, the low level of formal education among muleteers with limited alternative livelihood options, the current informal nature of the business, and the influx of new mules into the city remain challenges to sustainability.

DISCUSSION

Overview

The TDS tested a community-based model for the control and prevention of EZL and wounds in cart mules in Bahir Dar as part of a wider donkey and mule welfare program in the Amhara Region of Ethiopia. The focus of the project was identified following a welfare assessment using the 'Hand' tool. Previous studies in Bahir Dar (13, 53) and across multiple countries (54) reported lameness as the most common problem facing working equids. This assessment identified mule–muleteer–societal relationships, wounds, and EZL as the most serious challenges facing mules in Bahir Dar with lameness, nutrition, and abandonment at end of life as additional problems.

The lack of a reliable treatment or a commercially available vaccine limits options for EZL control and prevention. As a result, EZL poses a threat to mule cart businesses and to mule welfare and is the commonest cause of mule abandonment in Bahir Dar. Mules that can no longer pull carts for whatever reason are left to die of thirst and starvation, are often associated with traffic accidents (18, 27, 31), and present a poor image of the city.

TDS recognized that most working equine welfare problems are a result of poor management practices, lack of an affordable healthcare model, and underlying socioeconomic factors and that an effective and lasting solution to EZL control and prevention required involvement of all the relevant stakeholders, including muleteers, service providers, and policy makers, and the interplay of participatory and classical epidemiology. The project built on lessons from previous unsuccessful attempts to control EZL through treatment-only interventions in Bahir Dar and elsewhere (30, 33). TDS identified EZL as a visible indicator of poor welfare for cart mules in Bahir Dar and therefore also of wider program success.

With the ratification of the OIE Working Equid Welfare Standards, the project saw itself as modeling an approach to how these standards might be implemented and therefore increase interest in the outcomes of the work.

Impact

With a drop in prevalence of EZL from 23.9% (102/430) in 2010 to 5.9% (58/981) in 2017 and of open wounds from 44.3% in 2011 to 22.2% in 2017, the project suggests that prevalence of wounds and EZL can be significantly reduced in working mules through a community-based intervention as described. These drops in prevalence were associated with improved owner husbandry and handling of mules, improved cart and harness design, more mule-friendly municipal traffic practices, more trusting relationships between mule owners and local veterinary staff, improved equine medical competence among local veterinary staff, treatments provided for EZL, reduced abandonment of sick animals, mule community bylaws relating to EZL control put in place, and a means for animals with advanced incurable EZL to be humanely euthanized.

EZL prevalence in 2010 was within the range reported across Ethiopia in cart horses (16, 55), which varied from 39.1% in Mojo to 21.1% in Nazret, and was comparable to reports from Ejaji and Bako where prevalence was 21% in cart mules (17). Prevalence of wounds in 2010 was also comparable to previous reports in Bahir Dar and the adjacent town of Adet (8, 56), where most wounds were also associated with poor harnessing practice (10). Prevalence of EZL in 2017 in Bahir Dar was below any previously published reports of EZL prevalence in working equids in an EZL endemic area of Ethiopia. This reduction in EZL prevalence over the course of the project was significant. The authors are not aware of any other published reports of projects intended to control and prevent EZL in similar or indeed any other circumstances.

The drop in EZL and wound prevalence took place within the first 2 years and over the first 3 years of the project, respectively. These reduced prevalence rates were maintained in the face of year-on-year rises in the population of working mules. It is possible that part of the reduction in prevalence was due

to a dilution effect from newly arrived mules, but this factor would not explain the consistent sustained reduction. Equally, the arrival of new mules occurred alongside an increase in mule use and busier collection points, factors that could facilitate an increased rate of EZL spread. During World War II, it was the collection and mixing of horses that challenged the control of EZL (27). With the annually increasing population, we believe that if it were not for the control imposed by the project, the prevalence could have worsened. The rise in the prevalence of EZL in 2016 after a trial in 2015 to hand treatment responsibility to private veterinarians showed how quickly prevalence can rise and highlighted the lack of an economic model for effective private veterinary services in Bahir Dar.

The move in Ethiopia to delineate public and private roles in veterinary services is still in its infancy and beyond the project's scope, but this holds out hope for the future (57). Future projects might help facilitate herd health veterinary service models, possibly with well-established cart mule associations as the herd, whereby instead of being paid for individual treatments, veterinarians are paid collectively against reduction in incidence and prevalence for all association mules.

Possible factors constraining further reductions in prevalence of EZL include the dynamic and unregulated nature of the cart mule business in Bahir Dar with changing demographics; new muleteers with limited experience of wound management and EZL control; mixing of mules during the course of their work, feeding, watering, and general husbandry' unregulated movement around the city of known EZL cases; treatment factors including the investment in time needed; the financial investment in the mule making it difficult for muleteers to accept euthanasia; and the lack of an enabling regulatory framework. Further demographic research on the mule population would have provided rich data but was not possible within the resources of the project. From a welfare point of view, the failure to reduce wound prevalence below 20% was disturbing; however, there was an observed reduction in wound severity during the annual surveys.

In EZL endemic areas, dealing rapidly with EZL cases which act as potential sources of infection, through treatment of treatable cases or euthanasia of untreatable cases, is vital for effective control and prevention. In the project, TDS supplied medicines, equipment, and euthanasia drugs and used each treatment as an opportunity to build competence of animal health professionals. Treatment outcomes were comparable to previously published reports (30, 31, 33, 58, 59).

Epidemiology

The application of epidemiology and epidemiological tools served two roles in this project. First was to corroborate existing knowledge, take forward understanding about EZL, and inform the work. Second was to engage stakeholders so that they understood and took ownership of the steps needed to control and prevent the disease. The project used both participatory and classical epidemiology methods.

Although elucidating causal relationships between EZL and wounds or other associated factors was not an objective of the study, it was important to corroborate potential risk factors associated with EZL as mentioned in the literature including open wounds, hygiene, and collection site practice (17, 27, 60). Using participatory epidemiology tools, we found that muleteers already recognized proximity to an EZL case in a population (including abandoned mules), open wounds, poor harnessing, new owners with limited experience, poor hoof care, poor working conditions, and fly season as predisposing factors to EZL (**Table 5**).

Key informants claimed that mules get EZL throughout the year, with new cases being more common following a rainy season (30). However, the project observed the highest numbers of new EZL cases just before the long rainy season, after which untreatable cases were abandoned and die, and the cycle continues. Possible reasons for this include that during the rainy season, economic activities like construction will be reduced, which affects muleteers' income, affecting their expenditure on the care of mules.

The project was not able to explore potential clustering effects of mules temporarily gathered at a given working station. Mules mix fairly flexibly at daytime collection sites, grazing areas, and communal housing sites (see map in **Figure 4**); EZL-affected individuals are not quarantined; most mules in Bahir Dar work all day with no shift system; owners with more than one mule are generally renting them out. So clustering was considered unlikely to affect results significantly. Nevertheless, ideally, the project would have explored mule demographics in greater detail epidemiologically, socially, and economically.

Community-Based Approach and Sustainability

The project provides an example of how NGO-led interventions can test new models for change through better access to targeted funding, skill sets, and resources. However, ownership by the local community is needed to make the change last.

In this project, the community-based approach and participatory methods were essential to success by empowering stakeholders, increasing engagement, ensuring local ownership, and building bridges and common understanding between different stakeholder groups. The results of the project are similar to the successes of other community-based animal healthcare initiatives (35, 36, 61).

Encouraging signs of growing stakeholder ownership included development of bylaws and guidelines and use of media to transmit information. The public launch of the project and yearly follow-up events such as World Animal Day proved beneficial in creating a sense of teamwork among the stakeholders and also worked as awareness-raising and advocacy tools.

The successes of the project were achieved despite working animals having no mention in Ethiopia's federal 5-year Growth and Transformation Plans and there being no animal welfare legislation, poor equine health and welfare practices, and little recognition of the valuable social services provided by mules and

muleteers. The achievements in shifting viewpoints, particularly among the regulatory authorities, were therefore significant.

Sustaining project impact may require policy changes such as more flexible approaches to pharmaceuticals and equipment procurement by the veterinary regulatory authorities to allow government clinics to respond better to local healthcare priorities or more effective regulation of public and private veterinary roles to allow development of more effective service models.

The sustainability challenges facing the project are common to all donor veterinary projects, and not unique to community-based projects nor to NGO-led projects. The global campaign to eradicate rinderpest took decades and many different approaches, and the current campaign to eradicate peste des petits ruminants is also a long-term campaign. Empowering local veterinary services and involving communities are common to stories of success.

The project responds to a recently published work by Gizaw et al. (62), which assessed veterinary service delivery in Ethiopia, by demonstrating a model for development of services that can work for marginalized mule–muleteer communities in urban settings. The work by Gizaw et al. and this project highlight the need for a study of appropriate veterinary service design and community facilitation skills within the curricula of veterinary training institutes to ensure animal health and welfare professionals' awareness of the different approaches that can be used to achieve improved health and welfare for animals, particularly in resource-poor or otherwise marginalized communities. While holistic socioeconomic transformation of the mule sector will take time, the project has shown what is possible and can act as a seed for change.

Cost-Effectiveness and Carbon Benefits

The ∼ETB 10,000 (∼GBP 165) cost of a mule in Bahir Dar reported by muleteers compared to the ∼ETB 1,000 cost of EZL treatment if caught early gives a sense of scale to the recent (2021) estimates by Molla et al. (15), in their study of the economic costs of EZL in cart mules and horses in two urban locations in the Amhara Region, with an average annual animal level loss of ETB 6,587 per cart animal per year averaged out between EZL-affected and EZL-unaffected animals.

Using the above figures with mule numbers in Bahir Dar and reduction in EZL prevalence, together with project costs, allows estimation of the cost-benefit. Figures are not presented for this project because they are broad approximations, but they do suggest that there should be a cost-benefit for repeating this work where there are working mules in EZL endemic areas, that the approach could be institutionalized sustainably within public/private veterinary services in Ethiopia (63, 64), and that as the confidence of muleteers and other stakeholders grew, they might realize the benefits of investing in the improvements, including veterinary treatments. Future projects would do well to include economic analysis into their work from the start through collaboration with socioeconomists, ideally from local institutes.

It is also worth noting that Molla et al. (15) did not specifically include additional potential benefits that might accrue to the project from reduced wounds and increased work efficiency from improved communication between mules, muleteers, and municipality. Nor do they include potential carbon/climate/ecosphere-related benefits.

High-welfare mules, working safely and efficiently, are an effective low carbon form or transport, particularly for short distances (65). Throughout the project, transport officials in Bahir Dar held to the idea that mule transport will be replaced by motorized, currently fossil-fuel powered, vehicles, even in the face of year-on-year increases in mule numbers. Nevertheless, they also, for the first time, introduced municipal bylaws to facilitate more efficient, high-welfare mule transport. This demonstrates that community-based projects might play a part in establishing an environment conducive to low-carbon transportation.

Study Limitations and Challenges

In this study, the socioeconomic conditions and demographic volatility of working mules made follow-up of individual new cases problematic and unaffordable, particularly as EZL and wounds are generally protracted conditions requiring complex interventions. Elucidation of causal relationships between associated risk factors was not pursued as a project priority. To do this within the constraints of such a project would face ethical challenges.

The challenge of exploring clustering effects has been discussed above alongside the desirability for future projects to explore other epidemiological, social, and economic aspects of the cart mule sector, possibly through using a cross-disciplinary team with involvement from local institutes.

Sampling was challenging because of the dynamic nature of the mule population. The plan was to do a full census every year; however, this was not possible in some years. For sample surveys in 2015 and 2017, to reduce the duration of work disruption for muleteers and to ensure representation, mules were mostly examined at their working stations. At times, this posed challenges to the practicality of our systematic random sampling strategy of sampling every other mule (e.g., in a work station where we found only one mule). While there is a possibility that this might introduce sampling bias, we went beyond calculated sample size to minimize this possibility. The risk that vets drawn toward EZL cases despite the sampling methodology might overestimate EZL prevalence was also a possibility.

Unlike most towns, in Bahir Dar, equine power transport is limited to cart mules (6). With increasing urbanization and a high unemployment rate, rapidly growing cart mule numbers presented a challenge to the project as mostly inexperienced new muleteers with new mules arrived. Nevertheless, established muleteers, trained animal health professionals, and traffic police officers took responsibility to engage with new arrivals, and EZL and wound prevalence reductions remained comparatively low.

While muleteers in Bahir Dar are all male, this is not the case elsewhere in Ethiopia. There appears to be a more even balance of female and male equine owners in rift valley towns near Ziway where EZL is also present. In future projects, it would be useful to explore similarities and differences between male and female muleteers.

While case definitions for the different stages of EZL may seem clear to animal health professionals, all treatment protocols

require the compliance of muleteers, each of whose social and economic circumstances are different. Some with advanced case of EZL chose to move to the periphery of the town and work outside normal hours to continue making money from their infected mule in the face of social disapproval. Future projects might want to consider compensation or insurance schemes to improve compliance and help reduce prevalence still further; however, these need to be carefully designed and run, both with stakeholder involvement, if they are to be effective. If the status and economic security of the cart mule sector were to rise, these options might become more feasible and acceptable.

The continuing belief by planners and regulators that cart mules will be replaced by motor transport has held back the development of animal-powered transport despite its social value. The project has helped break down this prejudice slightly, but ideally, animal-powered transport should be included in government development plans. Similarly, a lack of understanding about animal welfare presents a challenge to improving the efficiency and effectiveness of the sector.

Applicability and Replicability

The project is applicable not only to other cities in Ethiopia with endemic EZL but also to animal healthcare challenges in other circumstances, including other countries with EZL (62). Successful replication requires recognition of the time needed to build the project on a secure foundation of stakeholder engagement from community to regulatory level. This includes establishing a common understanding of the epidemiology prior to the intervention and close engagement with veterinary and transport service regulatory bodies. Specifically for EZL, early development of community-level and municipality bylaws would be helpful and a compensation or insurance mechanism to facilitate euthanasia of advanced cases would be worth exploring.

CONCLUSIONS

The project achieved its aim of demonstrating an affordable sustainable approach to improving mule welfare with a reduction in EZL and wounds in cart mules in Bahir Dar between 2010 and 2017 despite rapidly changing mule demographics.

Every step was manageable within existing local institutions, with locally available resources, and economic considerations suggest it could be affordable. Although, for reasons outside its control, the project could not go far enough in embedding the processes, much of the routine work was already being handed over.

To replicate the intervention in other endemic areas, we recommend engaging with stakeholders and establishing the epidemiology at the start, developing bylaws, and exploring an insurance or compensation mechanism for euthanasia cases.

Participatory methodologies were essential for engaging stakeholders and empowering communities, and the lessons learnt show the value of a community-based approach to infectious disease control alongside wider human and animal welfare benefits, particularly in resource-poor or otherwise marginalized communities.

With the OIE Working Equid Welfare Standards now adopted internationally, the authors suggest that integrated community-based interventions are a useful approach to EZL control and prevention in endemic areas within wider working equid welfare improvement programs.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The project was signed by government authorities following a critical review of the respective experts. For the animal studies, including mule sampling and the overall clinical trial, we obtained ethical approval from Gondar University. Informed consent was also obtained from owners for the participation of their animals in this study. For muleteers and key informants we obtained oral consent from individual participants at each stage of the study. Written informed consent for participation was not required according to the national legislation or the institutional requirements.

AUTHOR CONTRIBUTIONS

BD and SB designed and supervised the study. TT, AKassaye, and AKassa implemented the programme on the ground. BD, SB, and AKassaye wrote up the study. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by The Donkey Sanctuary credited.

ACKNOWLEDGMENTS

We would like to thank all the project stakeholders who showed what is possible when people work in collaboration; The Donkey Sanctuary field team in Ethiopia who delivered the program, of which this project was a part; Sally Price, who trained the local team on community-based participatory approaches; Chris Garrett, who did the same in developing practical harness competence; Joe Anzuino, for supporting the veterinary aspect; Angie Garner, for facilitating the availability of supplies; and The Donkey Sanctuary who funded the work through the generosity of its supporters. We would also like to acknowledge the enormous contribution made by the reviewers who gave generously of their time to help strengthen and refine this article.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.648267/full#supplementary-material

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