VETERINARY BACTERIAL ZOONOSES

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PUBLISHED IN: Frontiers in Veterinary Science

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88945-592-8
DOI 10.3389/978-2-88945-592-8

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VETERINARY BACTERIAL ZOONOSES

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Pets are our passionate companions and friends. Watercolor by Menachem Banai. Photographer: Arik Shilo, Israel.

Humans, animals and microorganisms all share the same planet, the last playing critical roles in the cycling of nitrogen and sulfur in nature and the degradation of organic materials. Unfortunately, micro-organismal populations also include infectious *bacteria* and viruses that cause diseases, with a few that have fatal consequences. We chose veterinary bacterial zoonoses as our Research Topic with the aim of delivering up-to-date scientific knowledge on the subject, addressing the topics of detection approaches, vaccine development and host immune response.

Our Research Topic alludes to the One Health approach in addressing three important bacterial diseases, Brucella, Mycobacteria and Chlamydia. A short chapter also elaborates on a highly pathogenic field isolate of Mycobacterium avium spp. Avium and an atypical enteropathogenic Escherichia coli O98 as evolving zoonotic risks. The cover illustration is intended to raise our awareness of the fact that pets play a role in our life as passionate and compassionate friends, but that they also pose a health risk due to carrying a bacterial or a viral zoonotic agent. We hope our Research Topic will further the pursuit of these topics and spark research in other important diseases.

Citation: Ding, J., Banai, M., Yu, S., Ting, X., eds. (2018). Veterinary Bacterial Zoonoses. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-592-8

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frontiers in Veterinary Science

EDITORIAL
published: 06 August 2018
doi: 10.3389/fvets.2018.00162

Editorial: Veterinary Bacterial Zoonoses

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Keywords: bacterial zoonoses, bacterial bioinformatics, brucellosis, tuberculosis, paratuberculosis, chlamydia

Editorial on the Research Topic

Veterinary Bacterial Zoonoses

Zoonotic diseases provide a unique angle of looking at the One Health attitude toward infectious diseases. One Health is a recent approach taken by the community in addressing different environmental risks, depending on the pathogenicity (disease manifestation by a bacterium in a host) and the way the disease is disseminated in the human environment. Here, zoonotic diseases add unique perspectives to the pathogens harnessing their animal hosts to spread in nature via their milk, placenta, and visceral organs, respectively, adding zoonotic risks to food borne diseases involving unpasteurized or uncooked materials.

In this Research Topic we chose *Brucella*, *Mycobacterium,* and *Chlamydia* as disease types which represent facultative vs. obligate intracellular parasites of eukaryotic cells, respectively. The facultative bacteria, although replicating as intracellular parasites, spread due to their ability to exist outside their hosts. *Chlamydia*, on the other hand, survive in the eukaryotic cells. *Brucella* exploit throphoblasts as a resource of cell replication until a critical cell mass can burst by induction of abortion, spreading the bacteria in the closest environment and sustaining the infection in naïve populations, respectively. *Mycobacterium* exploit a similar mechanism of developing a massive bacterial inoculum as an infective dose that spreads by aerosols. *Chlamydia,* as obligate intracellular parasites, can only establish a successful passage following direct contact between their hosts. Understanding these principles in disease dissemination thus provide different leads to controlling the disease by developing relevant scientific tools.

The zoonotic impact of a disease could be determined by studying the correlation between their common existence between animals and humans. High throughput serological tests have been established to conduct rapid and accurate mass testing of a population but these only provide estimates of the herd status without informing on the Brucella agent that was involved in the infection. For the latter, strain isolation and typing is needed. Similarly, serological tests cannot reveal the persistence of a pathogen in the animal population in comparison to its spread in the human population.

Critically, vaccines are used as an effective prophylactic means against diseases. However, should vaccines be developed to protect the animals or humans, even if doing so may have a negative economic effect on farmers? In cases where a vaccine has been successfully developed, how can we use it in the field without hampering serological diagnosis? Some of these questions are addressed in our Research Topic.

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: *12 June 2018*
Accepted: *27 June 2018*
Published: *06 August 2018*

Citation:
Ding J, Banai M, Yu S and Ting X (2018) Editorial: Veterinary Bacterial Zoonoses. Front. Vet. Sci. 5:162. doi: 10.3389/fvets.2018.00162

Dongri et al. used Multiple Locus-Variable Number Tandem Repeat Analysis (MLVA) as a means of analyzing the common existence of *B. canis* between canine and humans, proven to be highly specific in determining the epidemiology of the disease in the area.

Unlike *B. canis*, *B. melitensis* is considered the severest human pathogen associated with small ruminants. Similar to the case in Israel, *B. melitensis* is also endemic to China, and vaccination is included in both countries as a prophylactic measure aimed at reducing the spread of the disease. The Israeli laboratory is serving as a national and international reference laboratory for the disease. Because Rev. 1 vaccination is enforced in the country, here, Banai et al. have exploited their national position in developing DIVA tests (distinguishing infected from vaccinated animals). Therefore, a combined approach of screening the population using highly sensitive tests, such as Fluorescence polarization assay (FPA), and confirming the infection status, using the Complement Fixation Test (CFT), has shown promise in successfully determining the brucellosis cases in the small ruminants.

Sun et al. have characterized *B. melitensis* isolates in China using a combined strain typing approach that included both Multilocus Variable-number tandem-repeat Analysis (MLVA), Single Nucleotide Polymorphism (SNP), and Multilocus Sequence BruMLS. This showed that Chinese isolates are more closely related to isolates from East Mediterranean and Middle Eastern countries.

Zhu et al. reported on the isolation of a novel, highly pathogenic field isolate of *M. avium* spp. Avium. This highlights the importance of bacteriological culturing of infected materials in showing possible existence of zoonotic variants within *M. avium*. Once an isolate has been obtained, data can be added to its genome. Recruiting strain isolation was also explored by Qi et al. as a method of studying antibiotic susceptibilities by an atypical enteropathogenic *E. coli* (EPEC) strain which added important information to control the disease in endangered Golden Snub-Nosed monkeys.

Due to a shortage in commercially available ELISA based detection kits, Xu et al. exploited the usage of mAbs targeting Ag85A from *M. tuberculosis* in surveying bovine tuberculosis. Developing monoclonal antibodies is a well-known approach in targeting specific epitopes. From five hybridomas two were selectively effective in distinguishing this antigen from Ag85B and At85C, allowing the pursuit of ELISA based detection of bovine tuberculosis.

Chlamydia psittaci causes chlamydiosis among birds which might then lead to avian influenza-like diseases in humans. Liu et al. compared the expression of PmpD-N of *C. psittaci* in the host, based upon two different promoters which contribute to the development of DNA vaccines against *C. psittaci*.

Research and development establish key components in advancing control and diagnosis of livestock diseases. As an example, Xin et al. explored means of detection of bovine tuberculosis based upon measurement of expression of IFN-ϒ and other cytokine mRNAs or proteins following induction of peripheral blood mononuclear cells from naturally or experimentally infected cattle by purified protein derivative (PPD-6) and mycobacterial early secretory ESAT-6 and culture filtrate CFP-10 proteins. Although showing signals that could be correlated with infection they concluded the test is still premature for field diagnosis.

A similar approach was undertaken by Zhang et al. in pursuit of the activation of the mitogen activated protein kinase (MAPK) signal by *B. abortus* outer membrane protein 25. For this, signals of the MAPK pathways were detected, such as p38 phosphorylation, and the extracellular-regulated protein kinase (ERK) and Jun-N-terminal kinase (JNK), were activated respectively. From this, the authors concluded that Omp25 played a role in *Brucella* activation of the MAPK signal pathway.

microRNAs (miRNAs) are well studied factors which function at the posttranscriptional step as regulators of biological pathways. Intriguingly, these molecules could be considered valuable in the diagnosis of infectious diseases, or as therapeutic agents, respectively. Dong et al. took the task of reviewing this subject in focusing on the possible exploitation of using miRNAs as biomarkers of infectious diseases among livestock.

To summarize, this Research Topic focuses on three important zoonotic diseases which explore bacterial diagnosis, epidemiology, laboratory works with clinical samples and research, and the development involved in advancing diagnostics as well as developing novel vaccines. We hope our Research Topic contributes to furthering the One Health approach.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

We thank Victor Kouassi, Journal Development Specialist, for his invitation to write the Editorial chapter in the eBook.

Conflict of Interest Statement: 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 reviewer CS and handling Editor declared their shared affiliation.

frontiers in Veterinary Science

PERSPECTIVE
published: 05 January 2018
doi: 10.3389/fvets.2017.00234

Perspectives and Outcomes of the Activity of a Reference Laboratory for Brucellosis

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One health is an emerging conceptual approach geared to harmonize the activities of the public health, veterinary services, and extension services within a single operative structure. Brucellosis is an important zoonosis worldwide, mostly involving nomadic populations but may often affect transboundary animal management and exotic domesticated animal farming such as camels and buffalo. Here, we provide contemporary knowledge on the disease and its causative agent, a Gram-negative bacteria belonging to the genus *Brucella*. Further, because of the zoonotic importance, we emphasize the need to assign a national reference laboratory for the disease and discuss how this would integrate into a "One Health" system. *Brucella* vaccines are live attenuated strains possessing the smooth phenotype, and vaccination, therefore, hampers the ability to maintain a national surveillance program due to concerns regarding the false positive vaccine-induced responses. In order to overcome these failings, we developed a combined approach based on rapid screening of mass numbers of serum samples by the fluorescence polarization assay, a cost-effective and accurate method, and confirmation of the true positive reactors by the complement fixation test, a highly specific method that is less sensitive to vaccine-induced antibodies. We demonstrate how, despite the high vaccination coverage of the small ruminant population in Israel, our results proved to be effective in discriminating between vaccinated and infected animals. The speed and accuracy of the method further justified immediate declaration of 37% of flocks as cleansed from brucellosis, thus reducing the burden of repeated tests among this population.

Keywords: ***Brucella*, bacteriology, serology, reference laboratory, lipopolysaccharide**

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: *16 October 2017*
Accepted: *14 December 2017*
Published: *05 January 2018*

Citation:
Banai M, Itin R and Bardenstein S (2018) Perspectives and Outcomes of the Activity of a Reference Laboratory for Brucellosis. Front. Vet. Sci. 4:234. doi: 10.3389/fvets.2017.00234

INTRODUCTION

Brucellosis is one of the few severe zoonoses with worldwide distribution (1). The disease is associated with domestic animals, which play important roles in the dairy and meat industries (2) and is disseminated to the human population as a family or tribe cluster infection (3). Awareness of the association of the disease with domestic animals has raised in 1887 when Sir Bruce first identified the organism in pathological human samples, followed by implementation of serological diagnosis and *Brucella* isolation diagnostic approaches, respectively (4). To date, despite a century of learning, the disease still prevails at high rates in countries of the Latin America, South East Asia, the Middle East, and Persian Gulf (1).

Until the late 1960s, the international community recognized only six *Brucella* species based upon their unique association with a natural host in which they cause abortion in the

last trimester of pregnancy (5). Four of these were considered zoonotic: *Brucella abortus* infecting cattle, but found associated with camelids and other bovids, *Brucella melitensis* infecting small ruminants but posing risk to bovids and camelids, and *Brucella suis* and *Brucella canis* infecting suids and canids, respectively. Two others have been recognized as non-zoonotic *Brucella* species: *Brucella ovis*, associated with orchitis and epididymis in rams and *Brucella neotomae,* infecting wood rats. Interestingly, recent studies have identified two potentially zoonotic sea mammalian species: *Brucella ceti* and *Brucella pinnipedialis*, associated with cetacean and pinniped brucellosis, respectively (6, 7). Molecular studies further showed that the genomes of the six-species, later conceived "Classical *Brucella*," were highly homologous, justifying their unification into one single species, *B. melitensis*, upon which their sub-taxon being determined according to morphological and biochemical characteristics (8).

The discovery of two more classical *Brucella* species isolated from vole and red fox or soil, e.g., *Brucella microti*, endowed with higher metabolic activity (9, 10) and the *Brucella papionis* baboon-associated strain (11), further extended with the addition of the atypical strains belonging to the more basal lineages, e.g., *Brucella inopinata* BO1 (12) and BO2 human pathogens, and the red fox *Brucella vulpis* (13) or the motile frog isolates, has opened the door to widening the genus structure.

Most *Brucella* species have two chromosomes, of roughly 2.1 and 1.2 Mbp, ranging at 57.8% GC content (14). *Brucella* species lack common virulence factors such as motility, plasmids, and exotoxins. In contrast, they are equipped with a complete *virB* operon that endows them with an active type IV secretion mechanism (15) and a sheathed flagellum (16) found active in the basal frog lineages (17). The *Brucella* cell envelope contains lipopolysaccharide (LPS), a molecule shared by all Gram-negative bacteria. Due to a non-canonical structure, its lipid-A conveys a stealthy infection following the development of a poor innate immune response (18). The role *Brucella* LPS plays in the process of *Brucella* trafficking into the replicative niche of *Brucella*-containing vacuoles is currently debated (19). In contrast, its role as a major humoral stimulating antigen has laid out the structure for the serological diagnosis of brucellosis.

The epidemiology of brucellosis in Israel underwent significant changes throughout the years, affected mainly by the socioeconomic and geopolitical restrains in the region. During the 1950s and 1960s, *B. abortus* prevailed in the country. The disease was eradicated in 1985 following implementation of a "test and cull" policy combined with a full dose, sub-cutaneous S19 vaccination of replacement females. Later on, the increase in the small ruminant population and market demands eventually led to the emergence of *B. melitensis* in the country. A control program was then instigated in the 1990s based upon Complement Fixation Test (CFT) serological surveys of the adult population and implementation of a combined "test and cull" and live Rev. 1 vaccination program (20). Due to the lack of sustainable financing, the program was ceased in 1997 and the disease re-emerged in the beginning of the 21st century, leading to a record number of new human cases as well as the infection of a large dairy cattle farm in the southern region of Israel (Negev) (21).

THE CONCEPT OF A REFERENCE LABORATORY FOR BRUCELLOSIS

The "One health" approach addresses the multi-disciplinary facets of disease complexity involving livestock biosecurity, environmental conditions, veterinary, and medical extension services, as well as farm to fork aspects. Setting up a national reference laboratory complements the "One Health" concept in providing a centralization center with expertise on the standardization of methods corresponding with epidemiology, diagnosis, and human treatment, as well as implementation of prophylaxis and control programs in the livestock population. The laboratory should then focus its activity on strain isolation and typing (gold standard test) and characterization of serological tests that confirm exposure and/or infection among livestock populations and humans, respectively.

Brucella TYPING

In recent years, a return to a nomen-species structure of genus *Brucella* has gained support (22). In the laboratory, new isolates are first characterized according to their susceptibility to Fuchsin and Thionin dyes, as well as growth dependence on an enhanced CO_2 atmosphere, and H_2S and urease production. Staining by the modified Ziehl–Neelsen method is a rapid supplementary technique applied to confirm the disease at point of care sites based upon characteristic cell morphology and staining of *Brucella* species (23). In past years, *Brucella* metabolism was analyzed by the old oxygen consumption test, but this test has recently been replaced by a robust microplate metabolome method (24). Use of phage typing has remained supplementary to the methods, but the availability of different sources of phage variants complicate standardization of such analyses (25). Unfortunately, access to brucellaphage seed stocks has become rare and this situation has been worsened by the lack of knowhow on brucellaphage propagation and preparation of master routine test dilutions of phage suspensions emphasizing the need for harmonization and unification of a worldwide *Brucella* typing and classification system.

Brucella VACCINES

Only a few *Brucella* vaccines have been approved for use in the field, all based upon live attenuated strains (26). Live vaccines are superior to killed or acellular vaccines as they survive in the host for a sufficient period of time required to induce a strong immune response by continuously challenging the host immune system. It was estimated that a good vaccine strain must survive for a minimum of 7.9 ± 1.2 weeks before clearance of the organisms by the immune system takes place. In contrast, virulent vaccine strains survive longer in the host, ruling out their use as suitable vaccines. The vaccine proficiency of the strain is established as inducing host cellular immune response active in clearance of a challenge strain (27) in comparison to an un-vaccinated control animal (28, 29). In contrast, the humoral response plays mainly as a secondary function in conveying protective immunity during early dissemination of the pathogen in the blood (30).

Unfortunately, antibodies elicited by the vaccine interfere with surveillance and monitoring programs, due to the lack of effective DIVA methods that could distinguish between vaccine and field strain serology. Reference laboratories must play a role in determining vaccine qualities, establishing standard seed stocks and confirming vaccine lot qualities prior to vaccination/usage (20).

Brucella abortus S19/B19 and *B. melitensis* Rev. 1 have been established as official live attenuated reference vaccine strains for cattle and small ruminants, respectively. In recent years, *B. abortus* RB51 has been endorsed as a compromised vaccine fulfilling the protective activity against *B. abortus* in cattle without eliciting conflicting smooth antibodies that hamper serological testing (31). Readers are referred to a comprehensive review on the evolutionary adaptation of *Brucella* species to their hosts and how vaccination may intervene with the emergence of novel *Brucella* (4). Further reading is recommended regarding vaccine efficacy and risks associated with Rev.1 and S19 implementation at different conditions (26, 32).

MOLECULAR TYPING

Brucella typing and classification can now be achieved by molecular approaches. DNA amplification of a specific sequence by PCR is a basic approach in bacteriology aimed at gene cloning, on the one hand, and gene characterization on the other and is currently widely used in diagnosis (33). AMOS PCR (standing for *B. abortus*, *B. melitensis*, *B. ovis*, and *B. suis*) was established in the 1990s as a primary method of classification of these species. The method is based on the IS*711* sequence inserted at different allelic sites in the chromosomes of the different species, producing characteristic amplified fragments of the target sites. Among *B. abortus*, AMOS PCR is limited to bvs. 1, 2, and 4, inferring insufficient sensitivity of this method to other *B. abortus* biovars (34, 35). Several other PCR-based molecular methods have been described in the literature culminating to the approval of the Bruce-ladder PCR by the OIE as a recommended approach (36).

Variable number tandem repeats (VNTR) that is also referred to as multiple loci VNTR analysis, is a molecular amplification method of micro- and macro-satellite DNA fragments that depict specific molecular fingerprints associated with an epidemiological strain. The method targets 16 molecular sites on the *Brucella* chromosome, divided into eight Panel A markers that specifically distinguish between *Brucella* species and 5 and 3 Panel B hypervariable epidemiological genotypes, respectively (37). Although effective in defining epidemiological linkages among isolates, this method suffers a major flaw by including multiple *Brucella* biovars within a single genotype.

HIGH-THROUGHPUT MOLECULAR TYPING

Whole genome sequencing has been instigated in recent years in genomic characterization of bacteria. At the chromosomal level, single-nucleotide polymorphism plays an important tool in identifying phylogenetic linkages. This method has been widely implemented in describing *Brucella* phylogenetic linkages as well as associating novel strains into genus *Brucella*. Development of a national bank of a strain collection helps in showing global trends of *Brucella* spread or clonal evolution for which multi-locus-sequence analysis is considered a favored approach (36).

SEROLOGICAL TESTS

Brucella species cross-react among each other as well as with *Yersinia enterocolitica* O9 and other Gram-negative bacteria (38). Furthermore, disease first progresses with the development of IgM antibodies and further succeeds by the development of IgG antibodies during the chronic state or alongside pathogen persistence in the animal (39). Finally, antibody isotypes might vary upon the challenge dose, the site of infection, and the capacity to establish bacteremia or develop a limited local infection in a specific organ or tissue, as well as antibiotic treatment. Therefore, these factors must be taken into account when designing a serological approach in the laboratory.

A reciprocal correlation exists between a test's sensitivity and its specificity. Sensitivity is increased at the expense of increased background noise, therefore, reducing specificity. In contrast, specificity is increased by reducing background noise and cross reactivity with heterologous antibodies at the expense of reduced sensitivity. IgM antibodies are pentamers, which promote the development of a net between antibodies and cells, thus causing agglutination in the tube [serum agglutination test (SAT)]. In contrast, IgG antibodies interact with the host's complement fixing system. Such a function forms the basis of the CFT, a lytic readout response against targeted antigens. Because IgM and IgG antibodies rise at different stages of the disease, agglutination and CFT complement each other in diagnosis, the first highlighting on acute infection whereas the latter indicates a chronic persisting infection, respectively. Thus, CFT provides a higher predictive value than SAT and, as such, has been affirmed by OIE as the prescribed method for animal trade between countries.

The enzyme-linked immunosorbent assay (ELISA) and fluorescence polarization assay (FPA) are two high-throughput serological techniques aimed at detecting serum antibodies (analyte) in a given sample. Because the two systems employ different underlying principles, their performance by sensitivity and specificity may vary. Two ELISA methods have been validated, indirect ELISA that measures binding of secondary antibodies to a primary antibody isotype bound onto the *Brucella* LPS antigen, and competitive ELISA, which measures the competitive binding between anti-*Brucella* LPS monoclonal antibodies and host's antibody onto the same reactive site. Both ELISA methods suffer from having non-specific binding of conjugate to background substrates and as such, their reading signal is increased. To avoid background noise, blocking and washing steps are introduced as intermediated steps in the method, thus reducing analyte concentration in the system and reducing the test's sensitivity.

Unlike ELISA, FPA measures antibody (analyte) binding to a soluble antigen by a homogenous measurement method without interventional steps such as blocking and washings. Because of the principle of homogeneity, this method measures the interaction of all participating antibody isotypes in the sample with

the soluble antigen, establishing the highest final reading. It is considered, therefore, highly sensitive. Test specificity has been similarly achieved due to using the soluble *Brucella* O-PS antigen, which omits Lipid A and core LPS epitopes in the reaction mixture. The method thus achieves a maximum performance value of close to 200%, surpassing ELISA as the best performing technique (40).

Plate agglutination methods, such as Rose Bengal Test (RBT), resemble FPA as homogenous methods. Unlike FPA, RBT is performed manually, limiting the number of samples a laboratory can process per day. RBT is considered, therefore, a screening approach surpassed by the high-throughput ELISA and FPA methods, however, because of its low price, some laboratories rather use this test as an alternative test to CFT.

The establishment of an international standard serum, which aimed to internationally harmonize test results, was considered by the OIE, leading to development of the second International standard anti-*Brucella abortus* Serum (second ISABS) arbitrarily assigned with 1,000 IU for SAT and CFT tests, respectively (23). Local serum standards could thus be established in different countries, allowing for the first time, the comparison of serological tests among different countries. A similar serum standard against *B. melitensis* infection in the small ruminant population has recently been described (41). Nonetheless, neither standard sera directly correlate with the diagnosis of human brucellosis.

DECISION-MAKING

Small ruminant population in Israel includes intensively managed flocks grown in rural places for dairy and meat production and extensively managed open flocks often pasteurizing on agricultural lands at southern Israel (Negev), and less at the northern part of the country. Human brucellosis is mostly associated with the nomadic animals due to close contacts with the animals. Following the 1990s' campaign (20), implementation of a full dose, ocular Rev. 1 vaccination of the young replacement females was enforced in the country. In spite of the vaccination program, due to cessation of the national control program, a new burst of human cases rose, most occurring in the Negev, leading to a public outcry to instigate a new national campaign.

The new program targeted the Negev, covering about 250 pasteurizing flocks (some 15,000 animals) in close proximity to dairy cattle herds, and others of risk to humans, including more than 1,000 flocks (over 250,000 heads) respectively.

In order to overcome DIVA concerns, we chose FPA as a screening method (being rapid and highly sensitive), and CFT [omitting most vaccine reacting individuals from the population (42)] as a confirmative method.

TABLE 1 | Calculation of the agreement between fluorescence polarization assay (FPA) and Complement Fixation Test (CFT) observations by the Cohen's Kappa test.

	FPA positive	FPA susceptible	Total
CFT positive	97	10	107
CFT negative	269	725	994
Total	366	735	1,101

Number of observed agreements: 822 (74.66% of the observations).
Number of agreements expected by chance: 699.1 (63.50% of the observations).
Kappa = 0.306.
SE of kappa = 0.027.
95% confidence interval: from 0.253 to 0.358.
The strength of agreement is considered to be "fair."

TABLE 2 | Comparison of numbers of responders between fluorescence polarization assay (FPA) and Complement Fixation Test (CFT) among serum samples randomly taken at early stages of the 2016 campaign.

	FPA positive	FPA suspect	FPAn
	3,066	3,179	37,511
CFTp	2,255 (36.1%)		
CFT	3,990 (63.9%)		

P, positive; N, negative; S, suspected.

RESULTS AND CONCLUSION

For statistical purposes, we chose sampling data that were available from the second cycle of the brucellosis campaign. At this stage, we expected most of the population to be tested negative by FPA as most infected animals have already been excluded from the population. Other FPA results could have been distributed between FPA positive (FPAp) and FPA suspect (FPAs) reactors, respectively. Among these reactors, we anticipated that FPAs reactors would belong to the vaccinated population expected to be found CFT negative (CFTn). We chose a free "QuickCalcs" calculator, which compares agreement between two methods in terms of a Kappa value, based upon the number of selected categories in the system. Comparison was conducted upon the observations obtained from the two population categories, FPAp:CFTp and FPAs:CFTn, respectively. As shown in **Table 1**, a total of 4,684 animals were tested by the FPA method revealing 1,101 (23.5%) of FPAp and FPAs responders in total that further were distributed within groups as follows: FPAp:CFTp (97, 8.8%), FPAs:CFTp (10, 0.9%), FPAp:CFTn (269, 24.4%), and FPAs:CFTn (725, 65.8%), respectively. As shown in **Table 1**, the inter-rater qualitative agreement by Cohen's Kapp test indicated fair agreement between FPA and CFT tests, with Kappa coefficient of 0.306 (95% CI 0.253–0.358).

As seen in **Table 2**, we tested 43,756 animals representing 760 flocks during the first cycle of the campaign. FPAs (3,179) and FPAp (3,066) comprised approximately 14.3% of the population. At the flock category, a total of 482 (63.2%) flocks were suspect/positive by FPA, 203 (26.7%) comprised true CFTp and 279 (36.7%) were CFTn, respectively, allowing omission of the latter group from future monitoring and thus reducing the burden on the laboratory. This highlights on the advantage of using FPA on a wide scale surveillance campaign in a population with a high vaccine coverage, first as a screening approach, using CFT as a confirmation method, and then,

as a sole method during the finalization of the eradication campaign. In conclusion, our work confirms the feasibility of using FPA in screening and eradication of vaccinated flocks in other places worldwide.

ETHICS STATEMENT

The study was exempt from one or more of the above requirements because it was conducted for the purpose of the control/eradication of brucellosis during a national campaign.

AUTHOR CONTRIBUTIONS

MB conceived the project and wrote the manuscript. SB produced the data and RI assisted in performing the tests and data organization.

FUNDING

Research was supported by Grant No. 845-0289-13 from Chief scientist, Ministry of Agriculture. We thank our Israeli colleagues at the Veterinary District Office, Beer Sheva, and support provided by the CVO and Israeli veterinary services.

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Conflict of Interest Statement: 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 is currently co-organizing a research topic with one of the authors MB and confirms the absence of any other collaboration.

frontiers in Veterinary Science

REVIEW
published: 01 November 2017
doi: 10.3389/fvets.2017.00186

Circulating MicroRNAs As Potential Biomarkers for Veterinary Infectious Diseases

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MicroRNAs (miRNAs) are a kind of small non-coding RNA molecules that could regulate multiple biological pathways at posttranscriptional level. Over 2,000 miRNAs have so far been discovered in humans, and many of them are found to be linked to various kinds of diseases. Thus, miRNAs are being considered as clinical diagnostic and therapeutic targets. With the discovery of high stability of circulating miRNAs in various kinds of mammalian body fluids, the potential of circulating miRNAs as diagnostic/prognostic biomarkers of infectious diseases aroused great interest among researchers. As far as human diseases are concerned, some biomarkers based on circulating miRNAs have been progressed to clinical application. In veterinary fields, however, this concept is only beginning to come into view. In this review, we summarize an update of preclinical studies on using circulating miRNAs as diagnostic biomarkers to combat infectious diseases that affect domestic animals.

Keywords: circulating microRNA, biomarkers, infectious diseases, veterinary, diagnosis

OPEN ACCESS

Edited by:
Jiabo Ding,
China Institute of Veterinary Drug Control, China

Reviewed by:
Yuehua Ke,
Institute of Disease Control and Prevention, China
Wen-juan Liu,
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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 August 2017*
Accepted: *17 October 2017*
Published: *01 November 2017*

Citation:
Dong H, Gao Q, Peng X, Sun Y, Han T, Zhao B, Liu Y, Wang C, Song X, Wu J and Yang L (2017) Circulating MicroRNAs As Potential Biomarkers for Veterinary Infectious Diseases. Front. Vet. Sci. 4:186. doi: 10.3389/fvets.2017.00186

INTRODUCTION

MicroRNAs (miRNAs) are small, endogenously expressed, non-coding RNA transcripts, about 22 nucleotides in length, with unique sequences targeting mRNAs for posttranscriptional regulation. Various researches have showed that miRNAs play important roles in various physiological and pathological processes, such as immune response, inflammatory response, and tumor occurrence (1, 2). The dysregulations of miRNAs have also been implicated in various kind of diseases, including diabetes, kidney disease, cancer, and many infectious diseases. Since 2008, the stable existence of miRNAs was identified in different type of body fluids (such as urine, serum, and plasma) (3–5). The application prospect of circulating miRNAs as non-invasive diagnostic biomarkers of disease status attracts the interest of researchers (5–7). At present, compared to the studies of circulating miRNAs in human diseases, only very limited studies are focused on miRNAs in veterinary infectious diseases and most are related to cellular miRNAs, instead of circulating miRNAs. Recently, the significance of functions of miRNAs in the host response to several veterinary virus diseases has been comprehensively reviewed (8, 9). In this review, we mainly focus on the research progress of using circulating miRNAs as diagnostic biomarkers for infectious diseases of domestic animals.

FUNCTION OF miRNAs

Over 30 years ago, the first miRNA was identified in the nematode *Caenorhabditis elegans* with the identification of the developmental regulator *lin-4* (10). In the year of 2000, another miRNA, let-7, regulating the timing of *C. elegans* development, was found to be conserved in a wide range

of animal species, including human, and this result indicates this kind of small regulatory RNAs has a more general biological function (11, 12). In 2002, Calin and colleagues found that miR-15 and miR-16 were tumor suppressors for chronic lymphocyte leukemia, which demonstrated for the first time the relationship between dysregulation of intracellular miRNAs and disease (13). Shortly afterward, researchers found that the expression levels of let-7 were closely related to prognosis for lung cancer survival (14). More and more research literatures have highlighted that alterations in miRNA expression profile were related to a wide range of human diseases, especially in the studies of various cancers (15–17).

In terms of biological function of miRNAs, it was first reported in the researches about mouse models deficient for Dicer and DGCR8. DGCR8 plays an important role in miRNA biogenesis, while Drosha participates in other RNA metabolic pathways. Therefore, knockout of the two genes resulted in loss of most miRNAs and embryonic lethality (18, 19). However, individual miRNAs are seems to be not required for specification of individual tissues. In addition, miRNAs are usually required for maintaining tissue homeostasis, since the expression levels of many tissue-restricted miRNAs are usually downregulated in the process of illness (12).

Currently, over 2,000 miRNAs have been reported according to results of human transcriptome, and it is believed that over 60% of coding genes are regulated by miRNAs in the genome (20). These findings indicate miRNAs may participate in the vast majority of physiological pathways. Recently, the immunoregulatory roles of miRNAs during infection have been widely studied. For example, various miRNAs have been demonstrated to regulate the toll-like receptor 4 pathway in the host innate immune response (21, 22).

CIRCULATING miRNAs AS DIAGNOSTIC BIOMARKERS

A large number of studies have demonstrated that dysregulation of circulating miRNAs are associated with a wide variety of diseases progression and syndromes. At present, the relationships between profile changes of circulating miRNAs and human diseases (such as virus infections, cancers, and liver injury) have been widely reported (5, 23, 24). In the respect of veterinary infectious diseases, many studies have also demonstrated the correlation between expression profiles of circulating miRNAs and various bacteriosis (Paratuberculosis), virus disease [foot-and-mouse disease, bovine viral diarrhea (BVD)], and parasitosis (Echinococcosis).

Immune and non-immune cells could actively release miRNAs into extracellular environments (25, 26). In 2008, researchers found for the first time that circulating miRNAs were present in the samples of serum and plasma. Subsequently, circulating miRNAs were also found in other body fluids, such as saliva, urine as well as semen (27, 28). For a long time period, invasive tissue biopsies served as the main diagnostic methods for cancer; however, the existence of circulating miRNAs in blood samples of cancer parents undoubtedly significantly promotes the development of diagnostic techniques for cancer (4, 5, 29, 30). For some infectious diseases that lack of blood-based diagnostic methods, circulating miRNA is likely a good choice in term of establishing new diagnostic methods. For instance, human tuberculosis is an infectious disease that a number of researches have established new diagnostics methods using circulating miRNAs, and currently, dozens of circulating miRNAs have been identified related to this disease. However, the results obtained by different researchers exhibited somewhat inconsistency, which may be due to the different test samples (serum and sputum), different test technologies (miRNA-seq, microarray, and RT-qPCR), and data normalization methods (31).

In addition, circulating miRNAs are remarkably stable under harsh conditions, such as extended storage, boiling, low or high pH, as well as multiple freeze–thaw cycles (3, 5, 23). A study of detecting bovine serum samples from experimental infections with *Mycobacterium avium* subspecies paratuberculosis (MAP) demonstrated that the circulating miRNA profile of samples, which were stored at −20°C for 10–15 years, was remarkably similar to that of fresh serum samples (stored at −80°C for less than one year) (7). In another study, researchers evaluated the stability of miRNA levels in plasma and serum from healthy dogs after storage at room temperature for different time points, and it was found that miRNAs were sufficiently stable in serum or plasma stored at room temperature for 1 h but not for 24 h (32). The remarkable stability of circulating miRNAs in body fluids is considered to be mainly attributed to two mechanisms: (1) circulating miRNAs could form a protein–miRNA complex with argonaute proteins or high-density lipo-proteins and (2) they could incorporate into exosomes (33).

Based on all these features of circulating miRNAs mentioned above, they have attracted great attention of researchers as a kind of potential diagnostic biomarker for various diseases.

ANALYSIS OF CIRCULATING miRNA

As mentioned previously, circulating miRNAs can be detected in many kind of biofluids, while majority of current studies about circulating miRNAs are based on serum and plasma. In addition, the abundance of circulating miRNAs varies in different type of body fluids (27, 34). Due to this reason, here we only focus on discussion of the main points of detecting circulating miRNAs in plasma or serum samples.

Previous studies about analysis of serum and plasma samples demonstrate that different sample treatment methods can affect the quality of extracted miRNAs (34, 35). For example, as heparin could inhibit downstream PCR assay, collection of peripheral blood samples should use EDTA-coated tubes (36, 37). In addition, while separation of serum or plasma, hemolysis should be strictly avoided, as intracellular miRNAs from platelets and erythrocytes can introduce significant bias in the expression profiles of circulating miRNAs (34, 35, 38). To determine whether the serum and plasma samples are contaminated by hemolysis, the relative expression levels of the specific miR-451 of erythrocyte and the stable miR-23a could be used as an indicator (35).

Currently, several miRNA extraction kits are available to extract circulating miRNAs from blood. In a recent study, Guo and colleagues compared the performance of five kits (ThermoFisher Scientific Ambion TRIzol LS Reagent, Qiagen Circulating Nucleic Acid Kit, QiaSymphony RNA extraction kit, Qiagen miRNEasy, and the Exiqon MiRCURY RNA Isolation Kit) for isolating circulating extracellular sRNAs, including miRNAs, and their variant isoforms (isomiRs), and transfer RNA-derived small RNAs (tDRs), and other miscellaneous sRNAs. In the respect of isolation of miRNAs, Ambion TRIzol LS produced the most reads for miRNAs, the repeatability of QiaSymphony RNA extraction kit was the highest, and Circulating Nucleic Acid Kit detected the greatest number of miRNAs. In addition, Circulating Nucleic Acid Kit also detected the most singleton miRNAs, which could explain why this kit was able to detect the most miRNAs (39).

As miRNAs are different from mRNAs in the respects of biochemistry and molecular structure, the techniques for miRNAs detection are not completely the same as that of mRNAs detection (40–42). Here, we mainly describe three frequently used techniques of miRNAs detection.

Reverse transcription quantitative real-time PCR is the most widely used technology for detecting miRNA expression level. As the sequences of miRNAs are very short, the experimental flow is different from the conventional reverse transcription PCR of mRNAs. At present, there are two common strategies used to detect the expression of circulating miRNAs: (1) using stem-loop reverse transcription followed by TaqMan PCR analysis (43) and (2) polyadenylated and reverse transcribed by a poly(T) adapter for quantitative RT-PCR using the miRNA-specific forward primer and sequence complementary to the poly(T) adapter as the reverse primer (44). It is worth noting that RT-qPCR-based technologies are unable to identify new miRNAs and special attention is needed for the design of standardized internal controls (40, 45, 46).

Hybridization-based technologies usually depend on DNA capture probes that are immobilized on a microarray so that the fluorescent signal intensities could be quantified to evaluate expression levels of different miRNAs. At present, many commercial miRNA array products are available, such as GeneChip® miRNA Arrays (Affymetrix), miRCURY LNA™ microRNA Arrays (Exiqon), and SurePrint miRNA Microarrays (Agilent) (47). Compared to other methods, the specificity and dynamic range of hybridization-based technologies are relatively lower. Thus, the results of microarray methods are usually needed to verify by RT-qPCR (48). Recently, the NanoString Technologies, Inc. established a new hybridization-based method (nCounter® miRNA Expression Assay) that does not require a PCR amplification step or direct labeling of target miRNAs (49). This approach has several advantages: it is as sensitive as RT-PCR; it has high throughput; and it could test up to 800 distinct miRNA variant targets in the same assay (31). However, similar to RT-qPCR, these hybridization-based methods are unable to identify novel miRNAs.

Unlike the methods mentioned above, miRNA-seq methods are able to discover global expression profiling of the whole miRNA transcriptome from certain biological sample (50). Compared to microarrays, the most considerable advantage of miRNA-seq is that it does not limit to detection of known miRNAs. miRNA-seq starts with constructing a cDNA sequence library reversely transcribed from short sRNAs selected by different methods, for example, size-selected gel electrophoresis. The prepared, indexed, and pooled cDNA library is subsequently sequenced using different sequencing platforms. However, special attention should be paid that technical biases inherent to different sequencing technologies may generate unreal reads (51, 52).

DIFFERENTIAL EXPRESSION OF CIRCULATING miRNAs IN VETERINARY INFECTIOUS DISEASES

Foot-and-Mouth Disease (FMD)

Foot-and-mouth disease, caused by foot-and-mouth disease virus, is a highly contagious infectious disease with significant economic impact. FMD can affect livestock and wild cloven-hoofed animals worldwide. This disease is characterized by fever and blister-like sores in the mouth, on the teats, on the tongue and lips, and between the hooves.

Using miRNA PCR array plates, Stenfeldt and colleagues detected the alterations of miRNA levels in bovine serum samples collected from three different phases of FMDV infection (acute, persistent, and convalescent phases) compared to uninfected animals. A total of 169 abundant miRNAs were detected in serum samples, and 3 differentially expressed miRNAs were identified: the expression of miR-1281 was significantly reduced at both acute and persistent infection stages; bta-miR-17-5p was expressed in the highest level at acute infection stage, whereas the expression level of bta-miR-31 was the highest during FMDV persistence. In addition, as cattle that cleared infection resembled the baseline profile, the authors considered that serum miRNA profiling could be used for identification of subclinically infected FMDV carriers (53).

Bovine Viral Diarrhea

Bovine viral diarrhea virus (BVDV) is an important pathogen of cattle with a global distribution and causes major economic losses (54). Animals infected with BVDV could exhibit a variety of clinical symptoms including diarrhea, depression, and pyrexia ranging from clinically mild to severe. However, in many cases, the symptoms of BVDV infections are subclinical and difficult to detect (55–57).

To identify circulating miRNAs that could be used for biomarkers indicating the timing of BVDV exposure to cattle, Taxis and colleagues investigated miRNA profiles of BVDV infected colostrum deprived male Holstein calves at four different time points: prior to infection (day 0) and at 4, 9, and 16 days postinfection. In accordance with short duration of fever and lymphopenia, the expression levels of two circulating miRNAs (Bta-miR-423-5p and Bta-miR-151-3p) were different between BVDV challenged group and control group across time examined. However, only the expression level of Bta-miR-151-3p was significantly increased at the time point of 9 days postinfection compared to the control animals. Thus, these two miRNAs could not be considered as appropriate diagnostic biomarkers (58).

It is widely known that persistently infected (PI) animals are the primary source of BVDV infection, and eliminating PI animals is the main measure of prevention and control of BVD. Thus, identification of different level of expression of circulating miRNAs in serums of PI cattle could be an idea approach for seeking diagnostic biomarkers of this disease.

Ebola

The disease of Ebola caused by Ebola virus (EBOV) is a hemorrhagic infectious disease. This disease usually causes a severe hemorrhagic fever in humans and non-human primates. Early diagnosis of EBOV is of significant importance for implementation of effective interventions and prevention of the spread of infection. However, EBOV-infected patients usually do not exhibit typical symptom at the early stage for diagnosis; therefore, current diagnostic methods, such as detecting viral RNA or antigen in suspected patients, are only effective at the late stage (59).

Chen and colleagues identified a miRNA-like fragment (miR-VP-3p) generated by EBOV in the serum of Ebola patients, which was detectable before EBOV genomic RNA in the serum. Thus, this miRNA-like fragment may sever as a biomarker for early diagnosis of this disease (59). In another study, Duy and colleagues tested the circulating miRNA profiles in both rhesus macaques and humans. In total, eight circulating miRNAs (hsa-miR-146a-5p, hsa-miR-18b-5p, hsa-miR-21-3p, hsa-miR-22-3p, hsa-miR-29a-3p, hsa-miR-432-5p, hsa-miR-511-5p, and hsa-miR-596) were selected as an EBOV classifier. Further studies indicated that this classifier could correctly categorized infection status in 86% human and rhesus macaque samples (64/74). More importantly, 50% (6/12) presymptomatic rhesus macaques could also be diagnosed using this classifier (60). Using circulating miRNAs (or miRNA-like fragment), the two early diagnosis methods for Ebola will significantly promote controlling of fulminating infectious disease in the world. In addition, the finding of miR-VP-3p was a powerful evidence that RNA viruses were able to express miRNA-like small RNAs.

Paratuberculosis

Paratuberculosis (also called Johne's disease) is a chronic disease caused by MAP. This disease can cause substantial economic losses to cattle industry due to increased premature culling, replacement costs, decreased milk yield, reduced feed conversion efficiency, fertility problems, reduced slaughter values, and increased susceptibility to other disease or conditions. However, the lack of accurate and reliable diagnostic tests is the main challenge faced in the control of paratuberculosis in cattle (61).

To identify novel diagnostic and prognostic miRNA biomarkers, Farrell and colleagues applied RNA-seq approaches to detect the circulating miRNA profiles in serum from an experimental JD infection model (six experimental MAP-challenged calves and six age-matched controls). However, the miRNA expression levels were almost the same when comparing serum collected from MAP-challenged animals to the control group at 6 months postinfection (62). In another study, the circulating miRNA expression profiles of seropositive cattle ($n = 5$) and seronegative cattle ($n = 7$) were also detected at both early and late stages of infection, while no significant differentially expressed circulating miRNAs were detected (7). Recently, Malvisi and colleagues investigated the miRNAs related to MAP infection, and seven miRNAs (bta-mir-19b, bta-mir-19b-2, bta-mir-1271, bta-mir-100, bta-mir-301a, bta-mir-32, and Novel:14_7917) were reduced and two (bta-mir-6517 and bta-mir-7857) increased in positive animals vs. unexposed animals (63). However, it is worth noting that Malvisi and colleagues studied the miRNA profiles of whole blood, rather than circulating miRNAs of serum or plasma. As is known to all, many components in the whole blood are rich in miRNA; thus, there is no comparison between this study and the two previous ones.

Staphylococcus aureus Infection

Staphylococcus aureus is one of the most prevalent pathogens causing chronic intramammary infection in cattle, responsible for substantial milk, quality, and economic loss in dairy farming worldwide. Sun and colleagues investigated the miRNA expression profiles of milk exosomes from four Holstein cows, whose mammary gland was infected with *S. aureus*, during mid-lactation prior to and after infection (48 h). Two miRNAs (bta-miR-142-5p and bta-miR-223) were identified as the potential indicators for early diagnosis of bacterial infection of the mammary gland (64).

Echinococcosis

Echinococcosis, caused by *Echinococcus granulosus* sensu lato (cystic echinococcosis) or *Echinococcus multilocularis* (alveolar echinococcosis), has become a major population health problem and economic importance worldwide. About 466 million people living in Asian region, over half exposed inhabitants (in particular herdsman and farmers), are at high risk of infection with *E. granulosus* and *E. multilocularis* (65).

As antibodies of this pathogen are unable to be detected at the early stages of infection (66), effective biomarkers for early and specific diagnosis of *E. multilocularis* are urgently needed. To find potential diagnostic targets for echinococcosis, Guo and colleagues compared the circulating miRNA expression profiles in the serum collected from the *E. multilocularis*-infected and uninfected mice at 4 weeks post-infection, using RNA sequencing method. In total, the expression levels of 58 circulating miRNAs were different, of which 21 were upregulated and 37 were significantly reduced.

In addition, the expression levels of six circulating miRNAs, which were verified to be differently expressed at 4 weeks postinfection, were further analyzed at different time points postinfection. The expression levels of mmu-miR-107-3p, mmu-miR-103-3p, mmu-miR-146a-5p, and mmu-miR-21a-3p were found to be significantly increased compared with the early stage of infection. The expression level of mmu-miR-339-5p was significantly increased at the early stage of infection, while no difference was observed after 4-week infection. On the contrary, mmu-miR-222-3p was significantly down regulated in the course of *E. multilocularis* infection (67).

Porcine Whipworm

The *porcine whipworm*, *Trichuris suis*, which is epidemic all over the world, lives in the large intestine of pigs. The pigs infected with this pathogen are usually subclinical, but worm loads are usually related to reduced feed efficiency, reduced growth rates, hemorrhagic diarrhea, and death (68).

To find circulating miRNAs of importance in *T. suis* infections in pigs, Hansen and colleagues investigated the expression levels of 16 preselected circulating miRNAs in serum samples collected from infected and uninfected pigs at different time points postinfection. The expression level of one circulating miRNA, ssc-let-7d-3p, was significantly increased in infected pigs at 8 weeks postinfection. As the pre-patent period of *T. suis* is 6–7 weeks, this miRNA operating at 8 weeks cannot be detected pre-patent infection with *T. suis*. Thus, its usefulness as a biomarker for early diagnosis may be limited (69).

CIRCULATING PARASITE-DERIVED miRNAs

Currently, more and more miRNAs have been identified in parasitic helminths, and many of them can be detected in serum and plasma samples. Thus, these worm-derived miRNAs could be used as potential biomarkers for the early detection of particular helminth infections (70).

Guo and colleagues compared the expression levels of circulating miRNAs in the serum from the *E. multilocularis*-infected and uninfected mice, and 2 *E. multilocularis*-derived circulating miRNAs (emu-miR-10 and emu-miR-277) were identified in all 15 *E. multilocularis*-infected serum (67).

TABLE 1 | Circulating microRNAs (miRNAs) profiled in selected studies of veterinary infectious diseases.

Name of disease	Biological fluid	Notable miRNAs detected	Reference
Foot-and-mouth disease	Serum	bta-miR-17-5p, bta-miR-1281, bta-miR-31	Stenfeldt et al. (53)
Bovine viral diarrhea	Serum	bta-miR-423-5p, bta-miR-151-3p	Taxis et al. (58)
Ebola virus disease	Serum/ plasma	miR-VP-3p, hsa-miR-146a-5p, hsa-miR-18b-5p, hsa-miR-21-3p, hsa-miR-22-3p, hsa-miR-29a-3p, hsa-miR-432-5p, hsa-miR-511-5p, hsa-miR-596	Chen et al. (59), Duy et al. (60)
Staphylococcus aureus infection	Milk exosomes	bta-miR-142-5p, bta-miR-223	Sun et al. (64)
Echinococcosis	Serum	mmu-miR-107-3p, mmu-miR-103-3p, mmu-miR-146a-5p, mmu-miR-21a-3p, mmu-miR-339-5p, mmu-miR-222-3p, emu-miR-10, emu-miR-277	Guo and Zheng (67)
Porcine whipworm	Serum	ssc-let-7d-3p	Hansen et al. (69)
Schistosomiasis	Plasma	Bantam, miR-3479, miR-10, miR-3096, and sja-miR-8185	Cheng et al. (71)

To find *schistosome*-specific miRNAs as potential biomarkers for the diagnosis of *Schistosomiasis*, Cheng and colleagues detected the miRNAs expression profiles in the plasma from rabbits infected with *Schistosoma japonicum* by a deep sequencing method. They identified five *schistosome*-specific miRNAs (Bantam, miR-3479, miR-10, miR-3096, and sja-miR-8185) in the plasma of rabbits infected with *S. japonicum*, and four of the five *schistosome*-specific miRNAs were also detected in the plasma of *S. japonicum*-infected mice. Among these *schistosome*-specific miRNAs mentioned above, miR-10 showed diagnostic potential for *S. japonicum* infection, while this result is still needed to be further confirmed in other animal models (71).

Table 1 provides summary information on circulating miRNA biomarker studies for diagnosis of veterinary infectious diseases.

CONCLUSION

In this review, recent works of circulating miRNAs involved in diagnosis of veterinary infectious diseases were introduced. Compared to thousands of published articles about utilization of circulating miRNAs as diagnostic biomarkers in human diseases, researches focusing on circulating miRNAs in animals of veterinary relevance are still deficient. This may be mainly due to that the cost of circulating miRNAs detection is much more expensive than traditional diagnostic methods in domestic animal diseases. However, the greatest advantage of circulating miRNAs is that it could be used as markers for the early detection. Upon outbreak of infection diseases, this method could quickly identify infected animals so as to prevent the spread of the epidemic and reduce economic losses, particularly for animals of high economic value, such as breeding stocks and dairy animals. In the future, with the advances in molecular biology, it is possible that the circulating miRNAs detection method will be moved from the molecular biology lab to the clinic.

In addition, in term of human disease studies, most miRNA biomarkers reported in the literature have failed to enter clinical practice because of inconsistent and irreproducible findings. One of the important reasons is that the characteristics of clinical cases (such as age, gender, ethnicity, and medical history) are usually uncontrollable. Unlike human studies, the use of experimental animals in veterinary studies can be standardized according to the needs of experimental design, which might make the veterinary related research results more repeatable and more accurate. Thus, circulating miRNAs will have a broad application prospects in the field of veterinary diseases diagnosis.

AUTHOR CONTRIBUTIONS

HD drafted the initial manuscript. All authors have read and approved the version to be published.

FUNDING

This work was supported by the National Natural Science Foundation of China (No. 31602055 and No. 31602017).

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Conflict of Interest Statement: 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 January 2018
doi: 10.3389/fvets.2017.00243

Isolation, Identification, and Characterization of a New Highly Pathogenic Field Isolate of *Mycobacterium avium* spp. *avium*

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Avian tuberculosis is a chronic, contagious zoonotic disease affecting birds, mammals, and humans. The disease is most often caused by *Mycobacterium avium* spp. *avium* (*MAA*). Strain resources are important for research on avian tuberculosis and vaccine development. However, there has been little reported about the newly identified *MAA* strain in recent years in China. In this study, a new strain was isolated from a fowl with symptoms of avian tuberculosis by bacterial culture. The isolated strain was identified to be *MAA* by culture, staining, and biochemical and genetic analysis, except for different colony morphology. The isolated strain was Ziehl-Zeelsen staining positive, resistant to p-nitrobenzoic acid, and negative for niacin production, Tween-80 hydrolysis, heat stable catalase and nitrate production. The strain had the DnaJ gene, IS*1245*, and IS*901*, as well. Serum agglutination indicated that the *MAA* strain was of serotype 1. The *MAA* strain showed strong virulence *via* mortality in rabbits and chickens. The prepared tuberculin of the *MAA* strain had similar potency compared to the *MAA* reference strain and standard tuberculin *via* a tuberculin skin test. Our studies suggested that this *MAA* strain tends to be a novel subtype, which might enrich the strain resource of avian tuberculosis.

Keywords: avian tuberculosis, *Mycobacterium avium* spp. *avium*, isolation, identification, characterization

OPEN ACCESS

Edited by:
Joy Scaria, South Dakota State University, United States

Reviewed by:
Kuldeep Dhama, Indian Veterinary Research Institute (IVRI), India
Shankar Thangamani, Midwestern University, 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: *13 September 2017*
Accepted: *20 December 2017*
Published: *15 January 2018*

Citation:
Zhu L, Peng Y, Ye J, Wang T, Bian Z, Qin Y, Zhang H and Ding J (2018) Isolation, Identification, and Characterization of a New Highly Pathogenic Field Isolate of Mycobacterium avium spp. avium. Front. Vet. Sci. 4:243. doi: 10.3389/fvets.2017.00243

INTRODUCTION

Avian tuberculosis is a serious, chronic infectious zoonotic disease in poultry, pet, or captive birds, animals, and humans that is caused by *Mycobacterium avium* spp. *avium* (*MAA*), a subspecies of the *M. avium* complex (MAC). Avian tuberculosis was classified as a List B disease by the World Organization for Animal Health and as a third-class animal disease in China. Clinical manifestations in birds include emaciation, depression, and diarrhea, and the prominent feature is the formation of caseous tubercular nodules and granulomas with calcification in the liver, spleen, intestine, and bone marrow (1–3). Humans exposed to infected birds may acquire a zoonotic infection, particularly in immunocompromised people such as those with HIV (3, 4). Once fowls in poultry farms are infected, avian tuberculosis can persist for a long time and is difficult to eradicate, due to the chronic carrier state and shedding of bacteria by infected fowls (5, 6). This disease can have a serious economic impact associated with mortality, morbidity, and a reduction of egg production (1, 6). The best way is to quarantine and slaughter the infected ones (6, 7), as well as antibiotic treatment with the infected individuals (8, 9).

The MAC is a group of closely related non-tuberculous mycobacterial species and subspecies which includes both veterinary and opportunistic human pathogens. In addition to birds, MAC may also infect different animal species, such as swine, cattle, deer, sheep, goat, horses, cats, dogs, and exotic species. The species within MAC are divided into the following subspecies according to taxonomical classification: *MAA*, the etiological agent of avian tuberculosis; *M. avium* spp. *paratuberculosis*, the etiological agent of Johne's disease; *M. avium* spp. *silvaticum*, previously called "wood pigeon *Mycobacterium*"; *M. avium* spp. *hominissuis*, frequently isolated from pig and human and *M. intracellulare*, a closely related pathogen of birds with a lower prevalence (2, 6, 10). Although the subspecies of MAC differ greatly in their host range and growth potential, it has been reported that tuberculous lesions caused by *MAA*, *M. avium* spp. *paratuberculosis*, *M. avium* spp. *hominissuis*, and *M. avium* spp. *silvaticum* are indistinguishable (11–15). In addition, coinfection with different members of M. tuberculosis complex and *M. avium* subspecies or with other mycobacterial species combinations is not rare in animal and human hosts (16, 17). Accurate identification and discrimination of mycobacterial species and subspecies is essential to determine their significance, pathogenicity, diagnosis, epidemiology, and most beneficial control program (18–20).

The identification and preservation of microbial strains is an important strategic resource for disease epidemiology research and disease prevention. Since the first report of avian tuberculosis in 1978 in China, there have been additional reports about the disease (7). As known, avian tuberculosis is sporadic and has a low incidence. Recent years, there were reports about incidence of avian disease in the poultry farm, but not large scale occurrences of disease (7, 21, 22). Isolation and identification of *MAA* strains from pathological materials could provide abundant resources for avian tuberculosis research. Although more attention has recently been paid to avian tuberculosis research, fewer *MAA* strains have been isolated and identified in recent years because of the high biological safety risks and low economic value of *MAA* strains in China. There were almost no reports regarding the isolation of *MAA* strains from diseased species, and no systematic studies were carried out on the characteristics of *MAA* strains.

In this work, we first isolated an *MAA* strain from the typical tubercular nodules of a fowl with possible avian tuberculosis symptoms. We then systematically identified the isolated strain *via* a series of assays and further investigated the virulence and potency of the identified strain.

MATERIALS AND METHODS

Ethics Statement

The present study was approved by the Laboratory Animal Ethics Committee of China Institute of Veterinary Drug Control and was also approved by the Ministry of Agriculture and the Bureau of Animal Husbandry. The experiments were performed in compliance with the "Regulations of the People's Republic of China on the Administration of Experimental Animals" and the "Guidelines for the ethical review of experimental animal welfare in Beijing."

Mycobacterium avium spp. *avium* strains were cultured in an air-conditioned, air filtered, biosafety level III facility. The experimental use of serum samples, including sample collection, handling, testing, and personal protection, complied with the General Requirements for Laboratory Biological Safety of China, GB19489 (2008).

MAA Strains

Mycobacterium avium spp. *avium* strains CVCC275, CVCC276, and CVCC277 were the virulent reference strains for serotype 1, serotype 2, and serotype 3, respectively. *MAA* strain CVCC68201 was the virulent strain for avian tuberculin purified protein derivative (PPD) production and inspection (23).

Tissue Origin and Examination

One fowl showing the clinical signs (swollen joints, lameness, emaciation, tubercle formation under the skin, and granulomas in the conjunctival sac) of avian tuberculosis and poor health was transferred to the avian diseases section, Shandong Agricultural University, College of Animal Science and Veterinary Medicine. The fowl was euthanized and underwent a necropsy examination, and tissue containing typical tubercles was collected in 50 mL screw cap containers, packed in dry ice chambers, and then sent to the China Institute of Veterinary Drug Control.

After thawing the tissue samples, portions were formalin fixed, embedded in paraffin blocks, and stained by the Ziehl-Neelsen (Z-N) technique to detect *Mycobacteria* (24). An additional tissue sample was fixed in neutral-buffered formalin and embedded in paraffin wax. Thereafter, the sample was sectioned, stained and transported to perform histopathological examination.

Mycobacterial Isolation

Approximately 1 g of tissues were pooled into a test tube containing 1 mL of sterile PBS and homogenized for 1 min. Two hundred microliters of the mixture was inoculated onto two slopes of Petragnani medium (25). The inoculated slopes were incubated at 37°C for 4 weeks. Colony morphology was visualized with the naked eye.

Grown Colony Staining

The grown colony on the slope was checked by Gram staining and Z-N acid-fast staining (24, 26). The studies were carried out using a Gram-staining kit and Z-N staining kit (Land Bridge Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions. The colony, confirmed to contain Gram-positive bacteria and acid-fast bacteria (AFB), was streaked out for further study.

Biochemical Identification of the AFB Isolates

Biochemical identification of the AFB isolates was based on multiple tests, which included the p-nitrobenzoic acid (PNB) growth assay, niacin production, Tween-80 hydrolysis, heat stable catalase activity, and nitrate reductase activity (27, 28).

PNB Growth Assay

The AFB isolates were screened by a growth test on a slope of Petragnani medium containing 500 μg/mL PNB (29). The inoculated slope was incubated at 37°C for 4 weeks, and then growth conditions were visualized.

Niacin Production Assay

The AFB isolates were washed off from the slope of Petragnani medium. Next, 0.9 mL of bacterial suspension was added to 0.2 mL of 3% benzidine ethanol, followed by the addition of 0.2 mL of 10% hydrogen bromide. The color reaction and precipitation reaction were observed.

Tween-80 Hydrolysis Assay

The Neutral Red/Tween-80 solution was prepared by adding 0.5 mL of 0.8% Neutral Red to 100 mL of 0.5% Tween-80/PBS solution (pH 7.0), followed by sterilization at 116°C for 20 min. The AFB isolates were washed off from the slope and resuspended to a final concentration of 10 mg/mL with PBS. Then, 0.5 mL of bacterial suspension was added to 2 mL Neutral Red/Tween-80 solution and incubated at 37°C for 10 days. The color change of the Neutral Red/Tween-80 solution was observed daily.

Heat Stable Catalase Assay

First, 0.5 mL of the bacterial suspension prepared above (10 mg/mL) was added to a test tube and incubated at 68°C for 20 min. Then, 0.5 mL of 30% H_2O_2 in 10% Tween-80 (pH 7.0) was added to the bacterial culture. The evolution of O_2 gas caused frothing indicative of catalase activity, whereas the absence of O_2 gas bubbles demonstrated a loss of enzymatic activity.

Nitrate Reductase Assay

Nitrate reduction was performed by the classical procedure with liquid reagent. First, 10 mM $NaNO_3$/PBS solution (pH 7) was prepared and sterilized at 116°C for 20 min. Next, 0.5 mL of the bacterial suspension prepared above (10 mg/mL) was added to 2 mL of $NaNO_3$/PBS solution. The mixture was incubated at 37°C for 2 h, followed by the addition of 100 μL of 18% HCl and 100 μL of 0.2% sulfanilamide solution and later by 100 μL of 0.1% naphthylethylenediamine solution. The system was stored at 2–8°C for 2 weeks, and the color reaction was visualized.

Genetic Identification of the AFB Isolates

The AFB isolates were examined by PCR for the detection of DnaJ, IS1245, and IS901 gene fragments (30). The colony on Petragnani medium was washed off from the slope and washed with PBS. After heat inactivation at 60°C for 2 h, genomic DNA was extracted using a genomic DNA purification kit (Promega, Madison, WI, USA). Primers were designed for amplification of DnaJ, IS*1245*, and IS*901* fragments (**Table 1**) (30). Multiplex PCR was programmed as 1 cycle at 96°C for 2 min, 35 cycles at 96°C for 10 s, 58°C for 10 s, 72°C for 1 min, and 1 cycle at 72°C for 2 min. The PCR products were visualized by DNA gel electrophoresis.

TABLE 1 | The PCR primers used to identify the acid-fast bacteria isolates.

Gene	Sequence of primer	Length of PCR product (bp)
DnaJ	5′-GACTTCTACAAGGAGCTGGG-3′ 5′-GAGACCGCCTTGAATCGTTC-3′	140
IS*1245*	5′-GAGTTGACCGCGTTCATCG-3′ 5′-CGTCGAGGAAGACATACGG-3′	385
IS*901*	5′-GGATTGCTAACCACGTGGTG-3′ 5′-GCGAGTTGCTTGATGAGCG-3′	577

Serological Identification of the AFB Isolates

The serotype of the AFB isolates was identified by the serum agglutination method as described below (31). The antigens of serotype 1, 2, and 3 were prepared from *MAA* reference strains CVCC275, CVCC276, and CVCC277, respectively. The AFB isolates, as well as the three strains, were cultured on Lowenstein-Jensen solid medium at 37°C for 18 days. The bacteria were washed off using 200 mL of sterile PBS containing sterile glass beads and inactivated at 37°C for 7 days with shaking up to four times a day. The mixture was centrifuged to remove the supernatant. After washing three times, the bacterial pellet was suspended in 0.4% sodium citrate/PBS to a concentration of approximately 10^{10} CFU/mL. The suspended bacteria were used as antigen for the serum agglutination assay.

The AFB isolates were cultured on Petragnani medium at 37°C for 4 weeks. The bacterial culture was washed off using sterile PBS containing sterile glass beads and centrifuged at 10,000 rpm for 5 min to remove the supernatant. The bacterial pellet was weighed and resuspended in sterile PBS to a concentration of 0.1 mg/mL. Three 8-week-old SPF chickens were inoculated intravenously with 0.2 mL of the above bacterial suspension, with the same amount of PBS as a control. After 60 days, sera were collected from the wing vein for the serum agglutination assay.

For the serum agglutination assay, one drop of serum from the wing vein and one drop of bacterial suspension were mixed on a slide. After 1 min, the results were graded as negative (−), weakly positive (+), positive (++), and strongly positive (+++), according to agglutination degree as no agglutination (−), less than 25% agglutination (+), approximately 50% agglutination (++), and more than 75% agglutination (+++), respectively.

Virulence

The AFB isolates were cultured on Petragnani medium at 37°C for 4 weeks. The bacterial culture was washed off using sterile PBS and centrifuged at 10,000 rpm for 5 min to remove the supernatant. The bacterial pellet was weighed. The virulence of the AFB isolates in rabbits and chickens was studied according to the "The People's Republic of China veterinary biological product regulation" (25).

For virulence in rabbits, the above bacterial pellet was resuspended in sterile PBS to a final concentration of 0.5, 1, 2, or 4 mg/mL. Six healthy rabbits at 2 kg of body weight (Albino, Vital River) were inoculated intravenously in the ear with 1 mL of the bacterial suspension, while the same amount of PBS was used as a control. The inoculated rabbits were observed for 30 days, and survival was recorded.

For virulence in chickens, the above bacterial pellet was resuspended in sterile PBS to a final concentration of 0.5, 1.25, 2.5, or 5 mg/mL. Five 8-week-old SPF chickens (Vital River) were inoculated intravenously with 0.2 mL of the bacterial suspension, while the same amount of PBS was used as a control. The inoculated rabbits were observed for 60 days, and survival was recorded.

Potency and Specificity

Preparation of PPD of Avian Tuberculin

Avian tuberculin was prepared according to the rules for tuberculin production and inspection (25), as described briefly below. The AFB isolates were streaked out from the Petragnani slope medium to 200 mL of Sauton liquid medium and grown at 37°C for 40–45 days to prepare the seed strain. The seed strain was inoculated into 1,000 mL of Sauton liquid medium and grown at 37°C for 90 days. The culture was inactivated at 121°C for 30 min and filtered through a hollow fiber membrane to remove the bacteria. The filtrate was mixed with 40% (w/v) TCA solution to a final concentration of 4%. The mixture was incubated overnight at 2–8°C. The pellets were collected and washed three times with 1% TCA solution. The pellets were centrifuged at 4°C, 5,000 rpm for 30 min, and the pellet was dried and weighed. The pellet was dissolved in 1 M NaOH, adjusted to pH 7.4 using 1 M HCl, and filtered through sterilizing Chua's filter. The final product was the avian tuberculin PPD for further testing, referred to as PPD-AFB. The avian tuberculin PPD from CVCC68201 (PPD-CVCC68201) was prepared using the same procedure.

Preparation of Allergen and Sensitization of Guinea Pigs

Mycobacterium avium spp. *avium* strain CVCC68201 was cultured at 37°C for 20 days. The bacteria were scraped from the slope, weighed, dissolved in sterile PBS, and inactivated at 121°C for 30 min. The bacterial suspension was mixed with Fruend's incomplete adjuvant to prepare an emulsion at a concentration of 40 mg/mL bacteria. The emulsion was subpackaged and inactivated at 80°C water bath for 2 h, and the emulsion was used as the allergen. Sixteen guinea pigs at 400 g of body weight (Hartly, Vital River) were sensitized with 0.5 mL of allergen by deep intramuscular injection in the inner thigh and maintained for 35 days under specific pathogen-free conditions. After the sensitization period, guinea pigs were shaved in a 3 cm^2 area on the hip, at the opposite side of allergen injection. The standard avian tuberculin PPD (PPD-S, IVDC) was diluted 100-fold, and 0.1 mL diluted standard avian tuberculin PPD was injected into the shaved area of the guinea pigs. The diameter of reactions on the skin was measured after 24 h, and the guinea pig was positive for sensitization if the area of skin thickness was over 1 cm^2.

Potency Calibration and Specificity Test of Avian Tuberculin PPD

The potency calibration of PPD was carried out according to the relevant rules (24–26, 32). For the potency calibration, the PPD-AFB was diluted to two concentrations of 0.1 and 0.25 dose/mL. Additionally, the PPD-S and PPD-CVCC68201 were diluted to two concentrations of 100 and 250 IU/mL. The shaved area of the sensitized guinea pigs was divided into two equal blocks. Each block contained three randomized injection sites resulting in six total injections, with a replicated injection of each PPD concentration in each guinea pig. Guinea pigs were injected intradermally with a volume of 0.1 mL of designated PPDs. A total of 12 guinea pigs (6 guinea pigs for each PPD concentration) were used for PPD inoculation. An additional 4 guinea pigs were used as negative controls. No adverse effect resulted from the procedures used, and the animals were monitored daily by the animal care staff. After 24 h, the skin thickness was measured using Vernier calipers.

The specificity of PPD testing was performed as described below (25, 33). Six guinea pigs at 400 g of body weight (Hartly, Vital River) were shaved in an area (3 cm × 9 cm) on both sides of the chest. PPD-AFB, PPD-S, and PPD-CVCC68201 were diluted to 2×10^4 IU/mL. Similar to the potency test, the guinea pigs were injected intradermally with one volume of 0.1 mL of designed PPDs with replicated injections of each PPD. After 24 h, the skin inflammatory reaction was observed.

Statistical Analysis

The statistical package SPSS and one-way ANOVA method were used for data analyses. The results were expressed as the mean ± SD. The *p*-values were considered significant at ($*p<0.05$).

RESULTS

Identification of the Pathological Tissue

The pathological tubercle contained a yellow-white cheese-like substance with a coated envelope after cutting. After smearing and Z-N staining, a large number of mycobacteria were observed (data not shown). With further histopathological examination of the tubercle, caseous necrosis appeared in the center of the tubercle with a large number of lymphoid cells, epithelioid cells, and Langerhans multinucleated giant cells (**Figure 1A**). This was a typical avian tuberculosis symptom.

On the slopes of the Petragnani medium inoculated with homogenized tissue, transparent, round, thick, drip-like colonies were observed (**Figure 1B**, arrow) after incubation at 37°C for 4 weeks. The colonies did not have a yellowish-white, cream like appearance (24, 25, 34). While streaking, the colonies had a threadlike filament (data not shown). The colonies were further tested by Gram staining and Z-N staining. As shown in **Figure 1C**, the colonies were Gram-positive, bluish-purple staining, and contained straight or curved rod-shaped bacteria. Additionally, the grown colonies were Z-N staining positive with red staining and a length of 1–3 μm (**Figure 1D**). The above results indicated that the colonies were AFB, i.e., mycobacteria.

Biochemical Characterization of AFB Isolates

Five different biochemical tests, including resistance to PNB, niacin production, Tween-80 hydrolysis, heat stable catalase and nitrate production, were applied to the AFB isolates (**Table 2**). The AFB isolates showed positive results for resistance to PNB. For the niacin production test, a clear pellet, not a peach red pellet

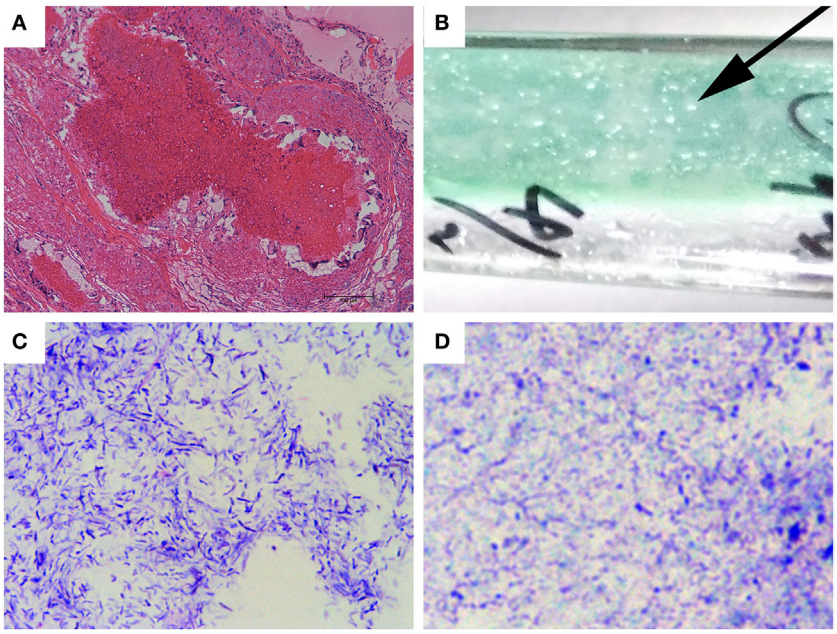

FIGURE 1 | Tubercle staining and mycobacterial isolation. **(A)** Histopathological staining of the pathological tubercle from the diseased fowl. Bar, 100 μm. **(B)** Growth of isolated mycobacteria colonies from the tissue homogenate on a slope of Petragnani medium. The arrow indicates a typical clone. **(C,D)** Photographies of the mycobacterial colonies after Gram staining **(C)** and Z-N acid-fast staining [**(D)**, 1,000×].

TABLE 2 | Distinguishing characteristics of the acid-fast bacteria (AFB) isolates.

Characteristics	Resistant to p-nitrobenzoic acid	Niacin production	Tween-80 hydrolysis	Catalase at 68°C	Nitrate reduction
AFB isolates	+	–	–	–	–

was observed, indicating a negative result. The test for Tween-80 hydrolysis was negative, indicated by no color change of from neutral red. The heat stable catalase activity was negative, as no bubble was generated in the reaction solution. The reaction system was very light pink, suggesting the absence of nitrate reductase in the AFB isolates. From the above biochemical tests, the AFB isolates most closely resemble*MAA* in their enzymatic activities.

Genetic Characterization of AFB Isolates

Using multiplex PCR, the AFB isolates and the CVCC6801 strain produced three bands (140, 385, and 577 bp) corresponding to the DnaJ gene, IS*1245*, and IS*901* (**Figure 2**). As the isolates were *IS901*+, *IS1245*+, and dnaJ+ (30, 35), the isolates were of the *MAA* strain.

Serological Characterization of AFB Isolates

The *MAA* strain has three serotypes: serotype 1, serotype 2, and serotype 3 (2). Using the serum agglutination method, the antigen prepared from AFB isolates showed a strong agglutination reaction to the serum from chickens infected with the CVCC275 strain but no agglutination reaction to the serum from chickens infected with the CVCC276 and CVCC277 strains (**Table 3**). The *MAA* strains CVCC275, CVCC276, and CVCC277 were the reference strains for serotype 1, serotype 2, and serotype 3, respectively. The results suggested that the AFB isolates were an *MAA* strain of serotype 1.

Virulence of the AFB Isolates

To evaluate the virulence of the AFB isolates, mortality in rabbits and chickens was recorded during the observation period post-injection of the prepared bacteria. As shown in **Figure 3A**, the six uninfected rabbits remained healthy and alive until the 30th day of the observation period. Among the six rabbits injected with 0.5 mg of bacteria, three of the rabbits died, and three remained alive until the end of the observation period. For the remaining three groups of rabbits inoculated with 1, 2, and 4 mg of bacteria, all rabbits died during the observation period. Moreover, the rabbits died at earlier dates when inoculated with higher doses of bacteria.

As shown in **Figure 3B**, the five uninfected chickens remained healthy and alive until the 60th day of the observation period. All of the chickens in the groups injected with different doses of bacteria, from 0.1 to 1.0 mg, died during the 60 days observation period. Additionally, the chickens died earlier when given a higher inoculation dose of bacteria.

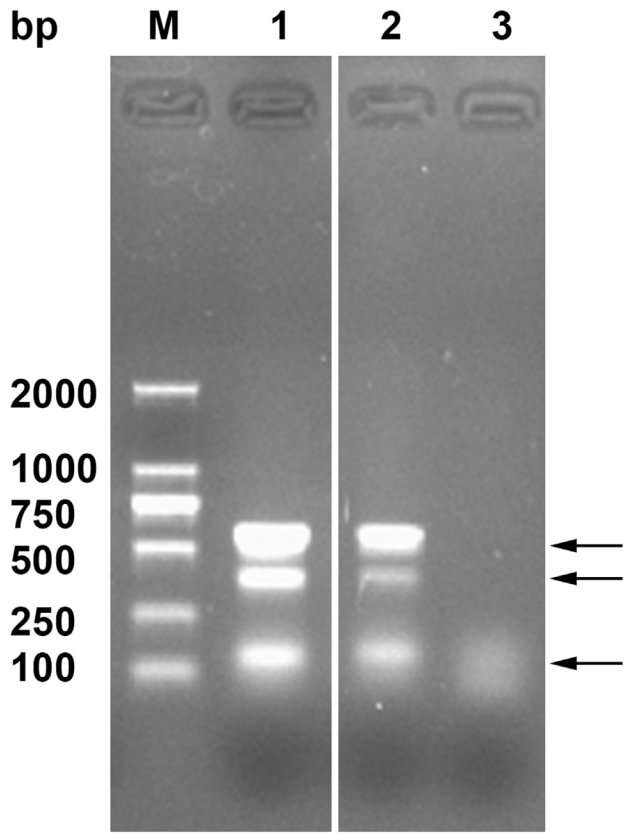

FIGURE 2 | Genetic identification of the acid-fast bacteria (AFB) isolates by Multiplex PCR. Lane M, DNA ladder, Lane 1, AFB isolates, Lane 2, CVCC68201, and Lane 3, PBS. The arrows indicate the three typical amplified bands of DnaJ gene, *IS1245*, and *IS901*.

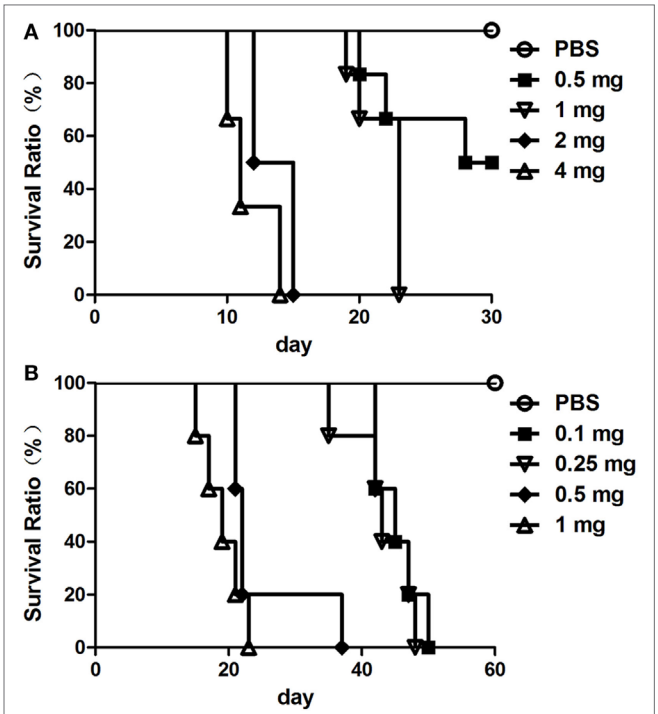

FIGURE 3 | The virulence of the acid-fast bacteria (AFB) isolates. Five groups of healthy rabbit **(A)** and chicken **(B)** was inoculated with different amounts of *Mycobacterium avium* spp. *avium* bacteria. The virulence of the AFB isolates is represented by the survival ratio of rabbits **(A)** and chickens **(B)**.

TABLE 3 | Serotype identification of the acid-fast bacteria (AFB) isolates.

	Antigen prepared from		
	CVCC275	**CVCC276**	**CVCC277**
Sera[a] from the AFB isolates inoculated chicken	+++	–	–

[a]*The sera from three inoculated chicken was all tested and showed same result.*

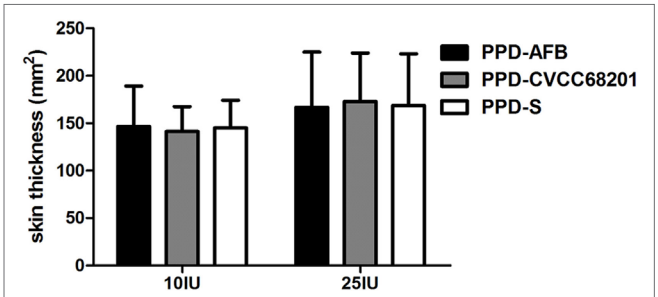

FIGURE 4 | The potency of the acid-fast bacteria (AFB) isolates. The purified protein derivative (PPD) was prepared from the AFB isolates and was calibrated by traditional PPD skin test. The potency is represented by the area of skin thickness after PPD injection on sensitized guinea pigs.

According to the definition of *MAA* virulence (25), the results indicated that the AFB isolates were *MAA* strain with strong virulence.

Potency and Specificity of the PPD Prepared from AFB Isolates

The potency of the AFB isolates was evaluated by calibrating the prepared avian tuberculin PPD from the AFB isolates. As shown in **Figure 4**, PPD-AFB, PPD-S and PPD-CVCC68201 showed no significant difference when analyzed for indurations (skin thickness) in sensitized guinea pigs. Thus, the PPD prepared from the AFB isolates had a similar potency to the standard PPD and the PPD from the reference *MAA* strain, and the AFB isolates had a similar potency to the reference *MAA* strain.

TABLE 4 | Specificity of the p-nitrobenzoic acid (PPD) prepared from the acid-fast bacteria isolates.

PPD	**Testing-PPD**	**PPD-S**	**PPD-CVCC68201**
Skin inflammatory reaction	–	–	–

The specificity of the AFB isolates was analyzed by the skin test in unsensitized guinea pigs with PPD-AFB, PPD-CVCC68201, and PPD-S. Similar to PPD-CVCC68201 and PPD-S, PPD-AFB could not induce a skin inflammatory reaction (**Table 4**). Thus,

the PPD prepared from the AFB isolates was specific for PPD prepared from the *MAA* strains.

DISCUSSION

In this work, we isolated an *MAA* strain from caseous tubercular nodules of a fowl with avian tuberculosis. The isolated strain was a typical *MAA* strain of serotype 1, genotype IS*901*+ and IS*1245*+ according to biochemical, serological and genetic identification. The identified *MAA* strain was a virulent strain and showed similar PPD potency as the reference *MAA* strain. Interestingly, our study revealed that this isolated *MAA* strain showed a novel colony appearance not previously reported.

Diagnosis of avian tuberculosis is frequently based on clinical symptoms, postmortem gross lesions and Z-N staining of the smear. However, Z-N staining for demonstrating acid-fast bacilli is limited because of its low sensitivity and detection rate (36). Serological diagnosis method such as hemagglutination, complement fixation, ELISA, and Western-blot analysis might be most useful in late-stage infections based on the process of mycobacteria evading the host immune system. Also the request of specific antigen might limit the board application of these methods (37). The PCR approaches, including PCR, multiplex PCR, PCR restriction fragment length polymorphism (RFLP), and mycobacterial interspersed repetitive units and variable number tandem repeats (MIRU-VNTR) typing are also capable of specifically detecting DNA fragments, thus acting as a diagnostic procedure (30, 35, 38, 39). However, PCR products can have false positive results because of contamination (40). The PPD skin test is the only method recommended by OIE, but the result reading is affected by the dose and injection manner (24). Comparing everything, the gold standard technique for avian tuberculosis is culturing from diseased tissue for isolation, genotypic and phenotypic characterization of the suspect pathogen (2). This method is reliable, but it requires more time and high quality culture medium.

For the conventional culturing technique of the *MAA* strain, it is important to use the proper selective medium (41). It was reported that *M. avium* grows best on media such as Lowenstein-Jensen medium, Herrold's medium and Middlebrook medium (6), which were also recommended by the World Organization for Animal Health as the culturing medium of *MAA* (24). In this study, we used Petragnani medium to isolate the *MAA* strain from the tubercle (25, 26). The Petragnani medium was reported to have similar colony yielding ratio as Lowenstein-Jensen medium for Mycobacterium tuberculosis (42). As known, culturing of mycobacteria is time-consuming to complete, as it takes at least 4–8 weeks for visible colonies to appear (5, 37). For *MAA* strain, the culture should be incubated for at least 12 weeks using Lowenstein-Jensen medium, Herrold's medium and Middlebrook medium (43). It took only 4 weeks to observe transparent, round, thick, drip-like colonies on Petragnani medium (**Figure 1B**). Thus, culturing Petragnani medium for isolation of *MAA* strain was time-saving and offered greater efficiency for avian diagnosis.

The traditional method for species and subspecies typing of *Mycobacterium* was according to growth on distinct medium and morphology of the grown colony. The *MAA* strain usually had yellowish-white, round, and cream-like colonies (25) (Insights into the mycobacterium infection). In our study, the isolated *MAA* strain had a novel colony appearance, as round, thick, drip-like colonies (**Figure 1B**). *MAA* forms colonies of different morphologies, including smooth transparent, smooth opaque, and rough, which might influence pathogenicity, virulence, drug susceptibility and macrophage survival (44–46). Here, this isolated *MAA* strain had similar virulence and PPD potency compared to the *MAA* reference strain CVCC68201 (**Figures 3** and **4**). This finding might indicate that this isolated *MAA* strain might be of a novel subtype and have differences in some unknown phenotype. However, the traditional method was limited in use because of the high biosafety requirement, and it was more time-consuming and required more complicated techniques. Genetic approaches would applied for further subtyping of this newly isolated *MAA* strain, as RFLP and DNA sequencing of distinct gene cluster for subtyping of diverse *MAA* isolates (47, 48), short sequence repeats sequencing and MIRU-VNTR typing for subtyping of *M. avium* spp. *paratuberculosis* (49–51). For example, IS901 RFLP typing was used to discriminate *MAA* field isolates (52, 53). As reported, a cluster of genes were proved to be related to colony morphology, such as MAP1156 (diacyglycerol O-acyltransferase), MAP 1152 (PPE protein), and Lsr2 etc. in *M. avium* spp. *paratuberculosis* (54), MAV_4334 (nitroreductase family protein), MAV_5106 (phosphoenolpyruvate carboxykinase), MAV_1778 (GTP-binding protein LepA), etc. in *M. avium* spp. *hominissuis* (55). In addition, glycopeptidolipids and its biosynthesis were reported to be related to the surface properties and colony morphology of mycobacteria (56–58). DNA sequencing of these gene clusters might give a clue for further subtyping of this isolated *MAA* strain with a round, thick, drip like colony appearance (**Figure 1B**).

Virulence is a reflection of pathogenicity of the bacterial pathogen. The virulence of *MAA* is affected by various factors and could change with different hosts and environments (59). The extent of intracellular replication in cell cultures, guinea pigs and mice is widely used as a measure for mycobacterial virulence (60, 61). Additionally, the mortality of animals inoculated with mycobacteria is also used as an indicator of virulence (25, 62). The definition of the *MAA* virulent strain is that inoculation of 0.1–1.0 mg bacteria would lead to the death of chickens or that 1 mg of bacteria would lead to the death of rabbits (23, 25). The isolated *MAA* strain here was proven to have strong virulence (**Figure 3**). Compared to intracellular bacterial replication or macrophage infection (59, 63), it was more intuitive to use mortality as an indicator of virulence, especially for a bacterial pathogen of strong virulence.

In conclusion, in this study, we isolated and identified a strain of *M. avium* spp. *avium*. The virulence and the derivative PPD of the strain were also characterized. Our work provided a novel reference strain for the intensive study of avian tuberculosis. This work not only enriched strain resources for *M. avium* spp. *avium* but also provided a realistic candidate strain for further development of *M. avium* spp. *avium* vaccines.

ETHICS STATEMENT

The present study was approved by the Laboratory Animal Ethics Committee of China Institute of Veterinary Drug

Control and was also approved by the Ministry of Agriculture and the Bureau of Animal Husbandry. The experiments were performed in compliance with the "Regulations of the People's Republic of China on the Administration of Experimental Animals" and the "Guidelines for the ethical review of experimental animal welfare in Beijing." *MAA* strains were cultured in an air-conditioned, air filtered, biosafety level III facility. The experimental use of serum samples, including sample collection, handling, testing, and personal protection, complied with the General Requirements for Laboratory Biological Safety of China, GB19489 (2008).

AUTHOR CONTRIBUTIONS

LZ conceived and designed the experiments and wrote the whole manuscript. YP, JY, TW, ZB, and HZ provided assistance of the experiments. YQ revised the manuscript. JD supervised the experiments and revised the manuscript.

FUNDING

This work was supported by the National Key R&D Program of China (2016YFD0500902).

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Conflict of Interest Statement: 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: 13 December 2017
doi: 10.3389/fvets.2017.00217

Identification of Atypical Enteropathogenic *Escherichia coli* O98 from Golden Snub-Nosed Monkeys with Diarrhea in China

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Fecal samples (n = 76) were collected from 38 snub-nosed monkeys (*Rhinopithecus roxellana*) in Shennongjia National Nature Reserve (China) and examined for the presence of enteropathogenic *Escherichia coli* (EPEC). The 56 samples originated from 30 free-ranging monkeys on the reserve and 20 samples from 8 captive monkeys that were previously rescued and kept at the research center. Eight diarrhea samples were collected from four of the eight captive monkeys (two samples from each monkey), and two EPEC strains (2.6%) (95% confidence interval 0.3–9.2%) were isolated from two fecal samples from two diarrheic monkeys. Both strains belonged to serotype O98 and phylogenetic group D (*TspE4C2*$^+$, *ChuA*$^+$). The virulence gene detection identified these strains as an atypical EPEC (aEPEC) (*bfpB*$^-$, *stx1*$^-$, and *stx2*$^-$) with the subtype *eae*$^+$, *escV*$^+$, and *intimin*β$^+$. These strains were highly sensitive to all the antibiotics tested. The lethal dose 50% of the two isolates in Kunming mice was 7.40×10^8 CFU/0.2 mL and 2.40×10^8 CFU/0.2 mL, respectively, indicating low virulence. Based on the report that this serotype had been isolated from some other non-human animals and humans with diarrhea, the first identification of aEPEC O98 strains and their drug resistance profile in *R. roxellana* is of ecological significance for disease control in this endangered species.

Keywords: enteropathogenic *Escherichia coli*, *Rhinopithecus roxellana*, diarrhea, virulence, non-human primates

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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 September 2017*
Accepted: *30 November 2017*
Published: *13 December 2017*

Citation:
Qi M, Wang Q, Tong S, Zhao G, Hu C, Chen Y, Li X, Yang W, Zhao Y, Platto S, Duncan RI, Chen J, Chen H and Guo A (2017) Identification of Atypical Enteropathogenic Escherichia coli O98 from Golden Snub-Nosed Monkeys with Diarrhea in China. Front. Vet. Sci. 4:217. doi: 10.3389/fvets.2017.00217

INTRODUCTION

The golden snub-nosed monkey (*Rhinopithecus roxellana*) is a listed class A protected and endangered (EN) species in the International Union for Conservation of Nature (IUCN) Red List.[1] It is distributed in the Sichuan, Gansu, Shanxi, and Hubei provinces in China (1, 2). Diseases represent one of the major threats to the survival of these monkeys (1), and therefore, it is important

[1] http://www.iucnredlist.org.

to identify pathogens of these animals, which may affect their health. However, there has been little research published on potential pathogens of golden snub-nosed monkeys (3–5). The main obstacles to conducting research in this species are the difficulties in accessing the monkeys and in collecting suitable samples in a non-invasive way.

Feces is the most readily available type of sample for many animals. Enteropathogenic *Escherichia coli* (EPEC) is a major pathogen identified in feces that causes infantile diarrhea in humans in both developing and developed countries and results in thousands of deaths worldwide each year (6, 7). The pathogenesis of EPEC depends on the chromosomal pathogenicity island locus of enterocyte effacement (LEE). The LEE contains a number of genes, including *eae*, which is essential for inducing the formation of characteristic attaching and effacing lesions in the intestinal epithelium (8). EPEC strains are classified as typical EPEC and atypical EPEC (aEPEC) on the basis of the presence of *E. coli* adherence factor plasmid, which carries the *bfp* gene, encoding the bundle-forming pili. Typical EPEC strains have mainly been isolated from humans, but they have been identified in other animals such as monkeys, dogs, cats, birds, and deer (9–12). They are considered to be a leading cause of infantile diarrhea in developing countries (13). The most important epidemiological feature of EPEC infection is its significantly high prevalence in children aged 0–5 years (14, 15).

In addition, aEPEC strains have been isolated from a wide range of hosts including calves, dogs, cats, cervids, sheep, goats, pigs, non-human primates, and humans (9, 16–21). In addition, the aEPEC strains isolated from non-human primates and humans have a very high degree of similarity (9, 22), underlying their zoonotic nature.

The purpose of this study was to identify EPEC strains in the feces of golden snub-nosed monkeys in the Shennongjia National Nature Reserve, PR China, and to provide information to aid in disease control and protection of this EN species.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of Hubei Regulations for the Administration of Affairs Concerning Experimental Animals (2005), Ethical Committee for Experimental Animals of Huazhong Agricultural University. The protocol was approved by the Ethical Committee for Experimental Animals of Huazhong Agricultural University (Permit Number: SYX–K(ER)2010-0029).

Sample Collection

The Shennongjia National Nature Reserve hosts approximate 1,280 monkeys distributed in 8 areas, with 80 wild monkeys in Dalongtan area (**Figure 1A**) and 8 captive monkeys in Xiaolongtan area. The accessible monkeys included a community of 30 free-ranging monkeys in Dalongtan and 8 captive monkeys in Xiaolongtan. These two areas are connected with the valley in the Hubei Conservation and Research Center for the Golden Monkey at Shennongjia National Nature Reserve, Hubei Province, China.

FIGURE 1 | Monkeys sampled in this study. **(A)** The monkeys from a one-male and multi-female unit waiting for food in the tree before feeding time; **(B)** monkeys fed with different food, which varies with the seasons; **(C)** the monkeys kept in the cage which is located in an open area.

The community was established at Dalongtan area in 2006 and is composed of five families: four one-male and multi-female units and one all-male unit. The monkeys gather at a feeding station (**Figure 1A**) three times a day (10:00–11:00, 14:00–15:00, and 18:00–19:00 h) where one of the staff from the observation station feeds them with sweet potatoes, oranges, peaches, apples, or peanuts. The food provided by the observation station to the monkeys varies with the season (**Figure 1B**). During the intervals between feeding periods, the monkeys return to the forest where they eat forage like other wild monkeys. The eight captive monkeys (BB2, QQ1, QQ2, HH3, YY2, TT, JJ, and LL2) kept at Xiaolongtan (**Figure 1C**) were rescued from different areas of Shennongjia Reserve (**Table 1**). These animals had physical injuries caused by fighting in the adults (BB2, QQ1, QQ2, HH3, YY2, and TT) and by falling from tree branches in the sub-adults (JJ and LL2) when they were saved. After recovery, they were kept at Xiaolongtan for research. Their enclosures are located in an open place (**Figure 1C**); the floors of the enclosures are washed daily with running water from a hose by the staff of the center. The water from the floor cleaning disperses into the surrounding environment.

TABLE 1 | Information on golden snub-nosed monkeys with fecal samples.

Sampling time	Location	Monkey ID	Sex	Age	Health status	Number of samples collected
July–September 2013	Dalongtan	BT	M	Adult	H	2
		DD1	F	Adult	H	2
		HH1	F	Adult	H	2
		YY1	F	Adult	H	2
		TJ	F	Sub-adult	H	1
		DWB	F	Adult	H	2
		NN	M	Adult	H	2
		XB1	F	Adult	H	2
		XH	F	Adult	H	2
		HH2	F	Adult	H	2
		ME	F	Sub-adult	H	2
		XY	F	Sub-adult	H	2
		XB2	M	Adult	H	2
		LN	F	Adult	H	2
		GG	F	Adult	H	2
		FF	M	Sub-adult	H	1
		YZ	F	Sub-adult	H	1
		YE	F	Sub-adult	H	1
		XXL	M	Sub-adult	H	1
		YJ	M	Sub-adult	H	2
	Xiaolongtan	BB2	M	Adult	D	2
		QQ1	F	Adult	H	3
		QQ2	M	Adult	H	3
		HH3	F	Adult	D	2
		YY2	F	Adult	D	2
		TT	M	Adult	H	3
		JJ	F	Sub-adult	D	2
		LL2	F	Sub-adult	H	3
March 2014	Dalongtan	XX	M	Adult	H	2
		BB1	F	Adult	H	2
		HHE	F	Adult	H	2
		LL1	F	Adult	H	2
		SB	F	Sub-adult	H	2
		DW	M	Sub-adult	H	2
		XHT	M	Adult	H	3
		WY	M	Adult	H	2
		YH	M	Adult	H	2
		JW	M	Sub-adult	H	2
Total						76

adult, more than 5 years; D, diarrhea; H, healthy; sub-adult, 2–4 years.

Each monkey was individually identified by giving a unique name and code based on its hair color, body shape, and other morphological features. Fecal samples were collected during three field trips of 1 week's duration each in August and October 2013 and in March 2014.

Each time, the samples were collected by two researchers who were familiar with the monkeys before the morning (10:00–11:00 h) and afternoon (14:00–15:00 h) feeding periods, when the monkeys were waiting for their food (**Figure 1A**). Each researcher was responsible for one family at a time. As soon as the monkeys defecated, the feces were collected immediately. The fresh feces were immediately placed in sterile disposable plastic bags and stored in a portable refrigerator at 2°C–8°C. Within 2 h after collection, the samples were transported to the laboratory and kept at −20°C until further analysis.

Detection of Fecal Pathogens

The fecal samples were sent to the Testing Department, XISHAN Biotechnology Inc. (Vanton Research Laboratory, Suzhou, China) for the detection of common bacterial pathogens (Catalogue# PA21) including *E. coli*, *Salmonella*, and *Shigella*, following the protocol from China national standard GB/T14926.11-2001 (23), GB/T 14926.1-2001 (24), GB/T 14926.47-2008 (25), and for *Campylobacter* spp. and *Yersinia* with real-time PCR (qPCR).

For *E. coli* isolation, fecal samples were inoculated on MacConkey agar plates (Oxoid, England) and incubated at 37°C for 16–24 h (23). The morphological and cultural characteristics and biochemical properties were analyzed according to previous protocols (26).

For *Salmonella* isolation, the fecal samples were cultured in selenite enrichment broth at 37°C overnight, and thereafter the culture was transferred on *Shigella* and *Salmonella* agar plates for 18–24 h at 37°C (24). The suspicious colonies were subjected to PCR amplification with primers specific to the *ITS* gene (**Table 2**).

For *Shigella* isolation, the fecal samples were cultured on MacConkey agar plates at 37°C overnight (25), following which suspicious colonies were subjected to PCR amplification with primers specific to the *virF* and *ipaH* genes (**Table 2**).

For *Campylobacter* spp. and *Yersinia* detection, the fecal samples were mixed with phosphate-buffered saline and centrifuged at 500 *g* for 10 min, and the supernatant was used to extract the DNA using a TianGen DNA extraction kit (TianGen, Beijing, China) according to the manufacturer's instructions. The DNA was subjected to qPCR with the primers and reaction conditions described in previous reports (34, 35).

In addition, the presence of diarrhea-related parasitic helminths (Catalogue# PA31) was investigated by microscopic observation of worm eggs, following the protocol from China national standard GB/T 18448.6-2001 (36). Briefly, fecal samples were mixed with saturated saline in a 5-mL tube and allowed to rest for 5 min. After discarding large floating objects, the tubes were filled with saturated saline. A cover glass was pressed gently

TABLE 2 | Primer sequences, annealing temperatures, and sizes of amplified fragments from selected genes of the target pathogens.

Primers	Sequence(5′–3′)	Product size (bp)	Anneal temperature (°C)	Pathovar	Reference
TspE4C2-F	GAGTAATGTCGGGGCATTCA	152	55	*E. coli* phylogenetic groups	Clermont et al. (27)
TspE4C2-R	CGCGCCAACAAAGTATTACG				
ChuA-F	GACGAACCAACGGTCAGGAT	279	55		Clermont et al. (27)
ChuA-R	TGCCGCCAGTACCAAAGACA				
YjaA-F	TGAAGTGTCAGGAGACGCTG	211	55		Clermont et al. (27)
YjaA-R	ATGGAGAATGCGTTCCTCAAC				
eae-F	TCAATGCAGTTCCGTTATCAGTT	482	58		Vidal et al. (28)
eae-R	GTAAAGTCCGTTACCCCAACCTG				
escV-F	ATTCTGGCTCTCTTCTTCTTTATGGCTG	544	62		Antikainen et al. (29)
escV-R	CGTCCCCTTTTACAAACTTCATCGC				
bfpB-F	GACACCTCATTGCTGAAGTCG	910	60		Antikainen et al. (29)
bfpB-R	CCAGAACACCTCCGTTATGC				
Intimiα	CCTTAGGTAAGTTAAGT	558	52	EPEC	Adu-Bobie et al. (30)
Intimiβ	TAAGGATTTTGGGACCC	562	50		
Intimiγ	ACAAACTTTGGGATGTTC	562	58		
Intimiδ	TACGGATTTTGGGGCAT	563	52		
Reverse	TTTATGTGCAGCCCCCCAT				
Intimiε-F	CCCGAATTCGGCACAAGCATAAGC	2,608	68		Oswald et al. (31)
Intimi ε-R	AGCTCACTCGTAGATGACGGCAAGCG				
stx1A-F	CGATGTTACGGTTTGTTACTGTGACAGC	244	62		Müller et al. (32)
stx1A-R	AATGCCACGCTTCCCAGAATTG			EHEC	
stx2A-F	GTTTTGACCATCTTCGTCTGATTATTGAG	324	61		Müller et al. (32)
stx2A-R	AGCGTAAGGCTTCTGCTGTGAC				
elt-F	GAACAGGAGGTTTCTGCGTTAGGTG	655	60		Müller et al. (32)
elt-R	CTTTCAATGGCTTTTTTTTGGGAGTC				
estIa-F	CCTCTTTTAGYCAGACARCTGAATCASTTG	157	62	ETEC	Müller et al. (32)
estIa-R	CAGGCAGGATTACAACAAAGTTCACAG				
estIb-F	TGTCTTTTTCACCTTTCGCTC	171	58		Müller et al. (32)
estIb-R	CGGTACAAGCAGGATTACAACAC				
invE-F	CGATCAAGAATCCCTAACAGAAGAATCAC	766	62		Müller et al. (32)
invE-R	CGATAGATGGCGAGAAATTATATCCCG			EIEC	
astA-F	TGCCATCAACACAGTATATCCG	102	58		Antikainen et al. (29)
astA-R	ACGGCTTTGTAGTCCTTCCAT				
aggR-F	ACGCAGAGTTGCCTGATAAAG	400	58	EAEC	Antikainen et al. (29)
aggR-R	AATACAGAATCGTCAGCATCAGC				
pic-F	AAATGTCAGTGAACCGACGATTGG	1,111	60		Antikainen et al. (29)
pic-R	AGCCGTTTCCGCAGAAGCC				
ITS-F	TATGCCCCATCGTGTAGTCAGAAC	312	58	*Salmonella* spp.	Park et al. (33)
ITS-R	TGCGGCTGGATCACCTCCTT				
virF-F	AGCTCAGGCAATGAAACTTTGAC	618	58	*Shigella* spp.	Vidal et al. (28)
virF-F	TGGGCTTGATATTCCGATAAGTC				
IpaH-F	CTCGGCACGTTTTAATAGTCTGG	933	58	*Shigella* spp.	Vidal et al. (28)
IpaH-R	GTGGAGAGCTGAAGTTTCTCTGC				

EAEC, enteroaggregative Escherichia coli; EHEC, enterohemorrhagic Escherichia coli; EIEC, enteroinvasive Escherichia coli; EPEC, enteropathogenic Escherichia coli; ETEC, enterotoxigenic Escherichia coli.

on top of the liquid level and allowed to sit for 20 min, and the sample was then observed under a microscope for worm egg examination.

PCR-Based Genotyping of *E. coli* Isolates

Genotyping of *E. coli* was performed using a triplex PCR assay with a combination of three gene markers (*ChuA*, *yjaA*, and *tspE4C2*) (27, 37). The resulting PCR products allowed classification of the strains into one of the four major phylogenetic lineages: A1, B1, B2, and D. The primer sequences for the PCR and the sizes of the amplified products are listed in **Table 2**. The primers were synthesized by Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China).

Each PCR was performed in a final volume of 25 μL, which included 12.5 μL of 2× PCR Mix (TransGen, Beijing, China), 0.5 μL of 10 nmoL of each primer, 11 μL of ddH_2O, and 1 μL of bacterial culture. All PCRs were performed with an initial denaturation step at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final single extension step at 72°C for 5 min. PCR products were detected with electrophoresis in 1% agarose gels, along with a DL2000 DNA ladder (TaKaRa, Dalian, China), and visualized under UV illumination after staining with ethidium bromide.

Analysis of Virulence Factors with PCR

Diarrheagenic *E. coli* falls into six categories based on the virulence markers: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffuse-adhering

E. coli (28, 29, 32, 38). Twelve virulence-associated genes (*eae*, *escV*, *bfpB*, *stx1A*, *stx2A*, *elt*, *estIa*, *estIb*, *invE*, *astA*, *aggR*, and *pic*) were selected to differentiate these categories of diarrheagenic *E. coli* with PCR, with the specific primers listed in **Table 2**. The *eae*-positive *E. coli* isolates were analyzed further to determine the *eae* subtypes of α, β, γ, δ, and ε (30, 31). All the primers were synthesized by Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China).

Serotyping of Isolates

Serotyping was performed by employing a standard slide agglutination test with standard antisera purchased from the China Institute of Veterinary Drug Control (26, 39). The *E. coli* strains were grown overnight in tryptic soy broth (BD, New Jersey, USA) and then autoclaved at 1.05 kgf/cm^2 for 2 h. The strains were serotyped with standard antisera against all antigens of EPEC, ETEC, EHEC, STEC, EAEC, and EIEC of O1–O163, according to the manufacturer's instructions.

Lethal Dose 50% (LD_{50}) Test of the Isolates

The LD_{50} in mice was determined using a protocol described previously (40, 41). Briefly, female-specific pathogen free Kunming mice aged 4–6 weeks were purchased from China Hubei Provincial Center for Disease Control and Prevention. Each group included six mice that were injected intraperitoneally with the bacterial culture. The LD_{50} dose was calculated on the basis of Karber's formula (40).

Antimicrobial Sensitivity Testing

Bacterial antimicrobial susceptibility was determined using VITEK 2 (BioMérieux, Hazelwood, MO, USA) for all 16 drugs in 6 different categories: aminoglycosides: amikacin, gentamicin, and tobramycin; cephalosporins: cefepime, cefotetan, ceftazidime, ceftriaxone, and cephazolin; β-lactam: ampicillin, aztreonam, and meropenem; fluoroquinolones: ciprofloxacin and levofloxacin; penicillins: imipenem and piperacillin; sulfonamide: cotrimoxazol. Each antibiotic was twofold serially diluted from 128 to 0.125 μg/mL. *E. coli* strain ATCC 25922 was used as the quality control strain. The final results were interpreted as sensitive (S), intermediate (I), or resistant (R) on the basis of the Clinical and Laboratory Standards Institute (42) Guidelines.

Statistical Analysis

The positive rate (%) of the samples for pathogen isolation was defined as number of positive specimens/number of specimens tested. The 95% confidence interval (CI) for the positive rate was calculated with online epitools, which were based on the method described previously (43).[2]

RESULTS

Sample Collection

A total of 76 fecal samples were collected, which included 56 samples from 30 free-ranging monkeys in the Dalongtan area and 20 samples from 8 captive monkeys in the Xiaolongtan area, with an average of 1–3 samples from each monkey. Among all the samples collected, eight were diarrheic feces from four captive monkeys with diarrhea, whereas all the other fecal samples were normal in shape, dark, and odorless (**Table 1**).

Pathogen Detection

Two *E. coli* strains were isolated from the 76 samples, with a positive rate in feces of 2.6% (2/76) (95% CI, 0.3–9.2%). The morphological and cultural characteristics and biochemical properties of the isolates determined them to be typical *E. coli*. In addition, both strains were from two of the four captive monkeys with diarrhea, who were kept in different enclosures. The positive rate of the strains in the diarrheic monkeys was 50.0% (2/4) (95% CI, 6.8–93.2%), while a positive rate of 0% (0/34) (95% CI, 0.0%, 10.3%) was found in the other 34 monkeys without diarrhea.

The morphological and cultural characteristics and biochemical properties were in agreement with the standards of typical *E. coli*. The isolates produced smooth, circular and bright pink or red colonies on MacConkey agar plates. The culture smear was identified as Gram negative by observation under the light microscope. Biochemical tests demonstrated that the isolates could ferment glucose, sorbitol, and xylose, and they were positive in the indole and methyl red tests. Moreover, the isolates could utilize ornithine and lysine, but they could not produce hydrogen sulfide and were negative in the Voges–Proskauer reaction.

In addition, all fecal samples were negative for other pathogens commonly associated with diarrhea such as *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, and helminths.

Genotyping and Serotyping of *E. coli* Isolates

The PCR-based genotyping was performed with a triplex PCR. The *TspE4C2* (152 bp) and *ChuA* (279 bp) amplicons were found to co-exist, confirming that both strains belong to group D (**Figure 2A**).

The analysis of the virulence genes showed that both strains possess the *eae* (482 bp) and *escV* (544 bp) gene amplicons, but lacked *bfpB*, *stx1*, and *stx2*. These findings confirmed that the isolates are aEPEC strains (**Figure 3A**). Further subtyping of the virulence genes with regard to the *eae* type showed the presence of only the amplicon of the *intimin* β gene (562 bp), which indicates that both strains belong to the β subtype (**Figure 3B**).

In addition, serotyping with the standard antiserum to O antigens demonstrated that both strains are of serotype O98 (**Figure 2B**).

LD_{50} Testing of *E. coli* Isolates

The LD_{50} of the two *E. coli* strains was tested in mice to determine their virulence. The mice were injected intraperitoneally with the bacteria and observed for 36 h. The mice who challenged with the EPEC strains began to die at 24 h post-challenge and the mortality being positively correlated to the bacterial concentrations in the next 12 h, while the mice in the control group were healthy. The strains had LD_{50} of 7.40×10^8 CFU/0.2 mL and 2.40×10^8 CFU/0.2 mL, respectively (Table S1 in Supplementary Material), which demonstrates that they belong to the class of low virulence.

[2] http://epitools.ausvet.com.au/content.php?page=home.

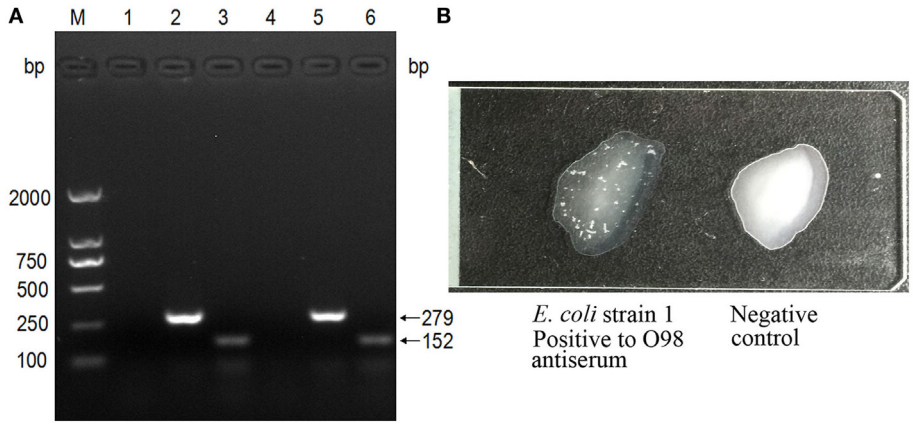

FIGURE 2 | Genotyping and serotyping of *Escherichia coli* isolates. **(A)** The triplex PCR specific for *E. coli* phylogenetic groups. (M) DNA ladder (DL2000); lanes 1–3: PCR products with primers specific to the genes *yjaA*, *ChuA*, and *TspEC2*, using the template of strain 1; lanes 4–6: PCR products with primers specific to the genes *yjaA*, *ChuA*, and *TspEC2*, using the template of strain 2. **(B)** The strains were typed by the slide agglutination test with standard antisera against all antigens of enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffuse-adhering *E. coli* (DAEC) of O1–O163. The left was O98 positive, shown by the apparent white agglutinating clusters, while the right was negative.

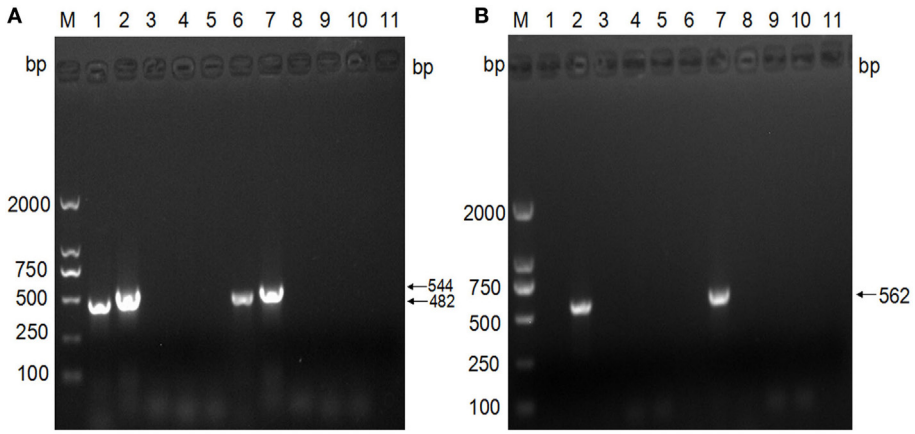

FIGURE 3 | Subgenotyping of *Escherichia coli* isolates based on the virulence genes. **(A)** PCR analyses of virulence factors. M: DNA ladder (DL2000); lanes 1–5: PCR products with primers specific to the genes *eae*, *escV*, *bfpB*, *stx1*, and *stx2*, using the template of strain 1; lanes 6–10: PCR products with primers specific to the genes *eae*, *escV*, *bfpB*, *stx1*, and *stx2*, using the template of strain 2; lane 11: negative. **(B)** PCR analysis of intimin subtypes. M: DNA ladder (DL2000); lanes 1–5: PCR products with primers specific to *intimin* α, *intimin* β, *intimin* γ, *intimin* δ, and *intimin* ε, using the template of strain 1; lanes 6–10: PCR products with primers specific to *intimin* α, *intimin* β, *intimin* γ, *intimin* δ, and *intimin* ε, using the template of strain 2; lane 11: negative control.

Antimicrobial Susceptibility of *E. coli* Isolates

Escherichia coli strain ATCC 25922 and the two isolates were cultured overnight, and then 0.1 mL fresh culture of each strain was streaked on Luria-Bertani agar plates and incubated for 12 h, and the antimicrobial susceptibility of each strain was tested by VITEK 2. As expected, the quality control strain was sensitive to all 16 antibiotics that were tested. Meanwhile, both aEPEC isolates were discovered to be similarly sensitive to all 16 antibiotics (**Table 3**).

DISCUSSION

The aEPEC is a well-known zoonotic pathogen that causes diarrhea in a wide range of hosts including humans and different species of non-human animals (9, 16–21). In this study, both strains were identified as group D serotype O98. This type of aEPEC serotype is not among those usually affecting humans, as defined by the World Health Organization (44), which include O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 and is therefore less studied. Nevertheless, this aEPEC

TABLE 3 | Antibiotic susceptibility testing of atypical EPEC isolates.

Category	Antibiotics (128 μg/mL)	MIC (μg/mL)/ interpretation	
		Strain 1	Strain 2
Aminoglycosides	Amikacin	≤2/S	≤2/S
	Gentamicin	≤1/S	≤1/S
	Tobramycin	≤1/S	≤1/S
Cephalosporins	Cefepime	≤1/S	≤1/S
	Cefotetan	≤4/S	≤4/S
	Ceftazidime	≤1/S	≤1/S
	Ceftriaxone	≤1/S	≤1/S
	Cephazolin	≤2/S	≤2/S
β-lactams	Ampicillin	≤2/S	≤2/S
	Aztreonam	≤1/S	≤1/S
	Meropenem	≤1/S	≤1/S
Fluoroquinolones	Ciprofloxacin	≤0.25/S	≤0.25/S
	Levofloxacin	≤0.25/S	≤0.25/S
Penicillins	Imipenem	≤1/S	≤1/S
	Piperacillin	≤4/S	≤4/S
Sulfonamide	Cotrimoxazol	≤20/S	≤20/S

EPEC, enteropathogenic Escherichia coli; S, sensitive.

serotype has lately been recognized as an important emerging pathogen more frequently isolated from human patients with diarrhea than the typical EPEC (45). Furthermore, aEPEC serotypes have previously been reported to be common in animals such as cattle (46) and dogs (47). In a survey aimed at isolating EPEC from cattle farms and abattoirs in Ireland, 140 strains were isolated from 2,700 samples, including feces from cattle on farms, carcasses, hides, and soil. All the strains belonged to aEPEC, covering nine serotypes: O145, O2, O26, O25, O29, O98, O103, O15, and O108. Among them, O98 strains comprised 6.4% and originated mainly from fecal samples (46). In a similar survey in dogs in Brazil, the EPEC and aEPEC strains were divided into 23 serotypes (21 aEPEC and 2 EPEC serotypes) that were isolated from 13% of dogs with diarrhea and 8.3% without diarrhea. The O98 strain was also one of the aEPEC strains identified (47). In addition, O98 was reported to have been isolated from pigs with gastrointestinal disease (48) and has been isolated from humans with diarrhea (49).

In our study, given that the samples of diarrhea were negative for other common diarrhea-associated pathogens such as *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*, and helminths, it is likely that the aEPEC strains were the cause of diarrhea in these animals. The lack of aEPEC isolates from the samples from the other two monkeys with diarrhea could be related to reduced bacterial shedding in fecal samples or to the low temperature, which has been previously shown to decrease the number of *E. coli* when stored at −20°C for a few days (50). However, the true reason is unknown. In addition, it is necessary to identify the virus and nutrition as the cause of diarrhea in the future to access the true pathogenic factor.

Fortunately, both the EPEC isolates were sensitive to the 16 antibiotics tested. It indicates that these drugs might be used when the monkeys require some medication. This drug susceptibility may be associated with the fact that the monkeys have lived in the mountains for generations, do not receive antibiotic treatment, and are segregated from domestic animal species that may have been administered antibiotics when they are sick. Meanwhile, antibiotic-resistant genes cannot be spread from the water, soil, or other contaminants in the environment to the monkeys.

Regarding the possible source of the aEPEC strains, it is possible that they were carried by clinically healthy monkeys and later caused diarrhea as an opportunistic pathogen, as reported previously by other investigators (51). In fact, aEPEC strains could be isolated from 3.9% of normal feces from cattle (46). In this study, the overall positive rate in feces was 2.6% (2/76) (95% CI, 0.3–9.2%), which is close to the cattle carrier rate.

Despite the very limited number of samples with O98 in this study, and the fact that the pathogenicity in this monkey species remains to be determined, our finding is of importance because it is the first report of aEPEC O98 in this species of monkey. Additional studies should be performed by increasing the number of sampled monkeys and extending the sampling time frame to assess whether aEPEC O98 is a common pathogen in this species of monkey, and even other species in the wild, to evaluate fully the ecological risk of aEPEC O98.

In conclusion, this is the first study to report the presence of aEPEC O98 strains in golden snub-nosed monkeys with diarrhea. The results could be of significance in protecting this rare primate species, owing to the potential pathogenicity of these aEPEC O98 strains.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Hubei Regulations for the Administration of Affairs Concerning Experimental Animals (2005), Ethical Committee for Experimental Animals of Huazhong Agricultural University. The protocol was approved by the Ethical Committee for Experimental Animals of Huazhong Agricultural University (Permit Number: SYX–K(ER)2010-0029).

AUTHOR CONTRIBUTIONS

MQ and QW performed sample collection, performed tests, and drafted the manuscript. ST and GZ contributed to parts of the sample collection. AG and CH conceived and designed the study and corrected the manuscript. WY, YZ, XL, and JC coordinated the field work. HC and YC performed some of the experiments. RD analyzed the data. SP revised the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by the National Science and Technology Infrastructure Program of China (#2013BAD03B01-01) and Special Fund for Agro-scientific Research in the Public Interest

(#201403054). We greatly appreciate the Administrative Bureau of Shennongjia National Nature Reserve, the reserve staff and other researchers, Yuncai Xiao, Jingyuan Yang, Hui Yao, Huiliang Yu, Jie Zhou, Penglai Fan, Surong Zhou, Shangfei Li, and Lijuan Yao, for their support in the sample collection.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fvets.2017.00217/full#supplementary-material.

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Conflict of Interest Statement: 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 reviewer VN and handling Editor declared their shared affiliation.

frontiers in Veterinary Science

ORIGINAL RESEARCH
published: 18 December 2017
doi: 10.3389/fvets.2017.00223

MLVA and LPS Characteristics of *Brucella canis* Isolated from Humans and Dogs in Zhejiang, China

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Background: *Brucella canis* is a pathogenic bacterium that causes brucellosis in dogs, and its zoonotic potential has been increasing in recent years. *B. canis* is a rare source of human brucellosis in China, where *Brucella melitensis* has been the major pathogen associated with human brucellosis outbreaks. In late 2011, a case of a *B. canis* infection was detected in a human patient in Zhejiang Province, China. To compare the genotypes between strains of *B. canis* isolated from the patient and from dogs, a multiple-locus variable-number tandem-repeat analysis (MLVA-16) was performed. In addition, the lipopolysaccharide-synthesis-related genes were analyzed with the *B. canis* reference strain RM6/66.

Results: 32 *B. canis* strains were divided into 26 genotypes using MLVA-16 [Hunter-Gaston Diversity Index (HGDI) = 0.976]. The HGDI indexes for various loci ranged between 0.000 and 0.865. All four Hangzhou isolates were indistinguishable using panel 1 (genotype 3) and panel 2A (genotype 28). However, these strains were distinctly different from other isolates from Beijing, Jiangsu, Liaoning, and Inner Mongolia at Bruce 09. The emergence of a human *B. canis* infection was limited to an area. Comparative analysis indicated *B. canis* from canines and humans have no differences in lipopolysaccharide-synthesis locus.

Conclusion: The comprehensive approaches have been used to analyze human and canine *B. canis* isolates, including molecular epidemiological and LPS genetic characteristics. Further detailed analysis of the whole genomic sequencing will contribute to understanding of the pathogenicity of *B. canis* in humans.

Keywords: MLVA, LPS biosynthesis, *Brucella canis*, humans and dogs, epidemiology

OPEN ACCESS

Edited by:
Jiabo Ding,
China Institute of Veterinary Drug Control, China

Reviewed by:
Yuanzhi Wang,
Shihezi University Medical School, China
Dongbo Sun,
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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 October 2017*
Accepted: *05 December 2017*
Published: *18 December 2017*

Citation:
Piao D, Wang H, Di D, Tian G, Luo J, Gao W, Zhao H, Xu W, Fan W and Jiang H (2017) MLVA and LPS Characteristics of Brucella canis Isolated from Humans and Dogs in Zhejiang, China. Front. Vet. Sci. 4:223. doi: 10.3389/fvets.2017.00223

INTRODUCTION

Brucellosis is one of the most common zoonotic diseases worldwide. In north of China, brucellosis is a serious endemic disease mainly caused by *Brucella melitensis* infection (biovars 1 and 3). Currently, there were 95 human brucellosis surveillance counties nationwide in order to grasp the epidemiological features. In recent years, a few papers about canine brucellosis have published (1–5). The new available data of canine brucellosis showed the outbreak trends in Beijing and other provinces (6–9).

Human brucellosis infection from *Brucella canis* is not common, probably because *B. canis* is rough lipopolysaccharide devoid of the O-side. Indeed, in *Brucella* spp., the smooth lipopolysaccharide, is highly correlated with pathogenesis (10). Some classical *Brucella* strains, as well as the recently isolated new species, express a smooth phenotype (11, 12). It has been shown that the smooth LPS might play an important role in the invasion and intracellular multiplication of *Brucella* spp. (13). Several LPS biosynthesis genes have been recognized, most of them clustered in the *wbk* and *wbo* genetic regions (14). However, *B. ovis* is naturally devoid of the *wbo* region (15). On the other hand, the *wbk* cluster is present in both the *B. ovis* and the *B. canis* (16).

Brucella melitensis was the predominant species associated with humans and animals brucellosis outbreaks in China (9). However, *B. canis* infection cannot be ignored. The epidemiological data of human canine brucellosis and etiology study have been limited in China. *B. canis* is known to infect humans, yet only a few cases have ever been reported. On November 10, 2011, a 45-year-old woman who developed fever, back pain, and fatigue was diagnosed with pleurisy at the Jiaxing People's Hospital, Zhejiang, China. The rough rose bengal plate agglutination test (RRBT) and rough standard tube agglutination test (RSAT) assays were positive. *B. canis* was also isolated from the blood culture. Because of scarce *B. canis* infection cases in humans, any potential genetic diversity of the *B. canis* strain isolated from different hosts should be investigated. Therefore, the aim of this study was to evaluate the epidemiological relatedness among Chinese *B. canis* strains isolated from dogs and humans. First, variable number of tandem repeats assay was performed to assess the diversity from the different regions, especially in the same geographical area, Zhejiang province. Second, 21 LPS-synthesis related genes were amplified and sequenced, in order to detect genetic mutations between human and canine *B. canis* strains. This comparative analysis will contribute to knowledge of the *Brucella* virulence and microevolution.

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

This study is a retrospective investigation of historical collections strains using modern typing methods and study protocol was approved by the Ethics Committees of National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. Informed consent was obtained from the patient before diagnosis in the study. We isolated *Brucella* spp. from patient' blood sample with his agreement.

Human *B. canis* Infection Detection

For detection of antibodies against rough *Brucella* spp., the RRBT and RSAT were performed as previously described (17). Blood culture was performed by biphasic blood bottle (BioMérieux Industry, France) by following the manufacturer's protocol. 7 days later, some colonies were found in solid phase and inoculated into *Brucella* Agar Medium.

B. canis Identification and DNA Preparation

Species identification was determined on the basis of phenotype identification procedure (18). 32 *B. canis* strains isolated from Zhejiang, Beijing, Jiangsu, Liaoning, and Inner Mongolia between 1986 and 2011 were included. The genomic DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen China Ltd., China) by following the manufacturer's protocol. Species-level identification was performed using Suis-ladder PCR and real-time PCR assay (19, 20). The procedure of *Brucella* isolates were carried out in biosafety level 3.

MLVA Typing and Data Analysis

The multiple-locus variable-number tandem-repeat analysis (MLVA-16) was performed as previously described (21–23). Sizes of PCR products were evaluated by capillary electrophoresis on an ABI Prism 3130 automated fluorescent capillary DNA sequencer (Applied Biosystems). Genomic DNA from reference strain *B. canis* RM6/66 (NC_010103.1 and NC_010104.1) was used as positive control. The cluster analysis was performed using the UPGMA algorithm (Unweighted Pair Group Method Algorithm) with Euclidean distance matrices. Hunter and Gaston diversity indexes were calculated[1]. Web-based MLVA database[2] was utilized to compare different strains.

LPS-Synthesis Characterization of *Brucella* Strains: PCR Assay and Sequence Analysis

21 LPS-synthesis related genes were analyzed. Primers and amplification were previously described (13). The resultant PCR products were purified and sequenced at Qingke Biosciences Company (Beijing, China). The sequence data were compared by web-based BLAST.[3] Reference strain *B. melitensis* 16M sequences was available in GenBank (AE008917 and AE008918).

LPS Extraction and SDS-Polyacrylamide Gel Electrophoresis (PAGE)

The LPS was extracted using Lipopolysaccharide Extraction Kit (iNtRON Biotechnology Ltd., Korea) by following the manufacturer's protocol. SDS-PAGE was performed as previously described (24).

RESULTS

Identification of Human Brucellosis by Serological Tests

The serum of the patient was tested by RRBT and RSAT tests for anti-R antibodies, and yielded positive results for antibodies against rough *Brucella* spp. Blood culture was performed by biphasic blood bottle. A *B. canis* strain was isolated and showed the same phenotypic characteristics with *B. canis* RM6/66. The location of *B. canis* isolated from human and animals between 2006 and 2011 was shown in **Figure 1**.

[1] http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl.
[2] http://mlva.u-psud.fr/.
[3] http://blast.ncbi.nlm.nih.gov/Blast.cgi/.

MLVA Analysis of Human and Canine *B. canis* Strains

32 *B. canis* strains were grouped into 26 genotypes using MLVA-16 [Hunter-Gaston Diversity Index (HGDI) = 0.976]. The HGDI indexes ranged between 0.000 and 0.865 (**Table 1**; **Figure 2**). 11 loci were of low discriminatory power (HGDI < 0.2). Bruce 09 and Bruce 16 displayed high discriminatory power (HGDI > 0.8), which contained 10 and 7 alleles, respectively. Comparative genetic profiles by MLVA-16 indicated that MLVA-8 and MLVA-11 genotypes of human *B. canis* strain were identical to *B. canis* isolates from dogs. Exception of bruce30 loci, four loci (bruce04, bruce07, bruce09, and bruce16) of panel 2B showed diversity (**Table 2**).

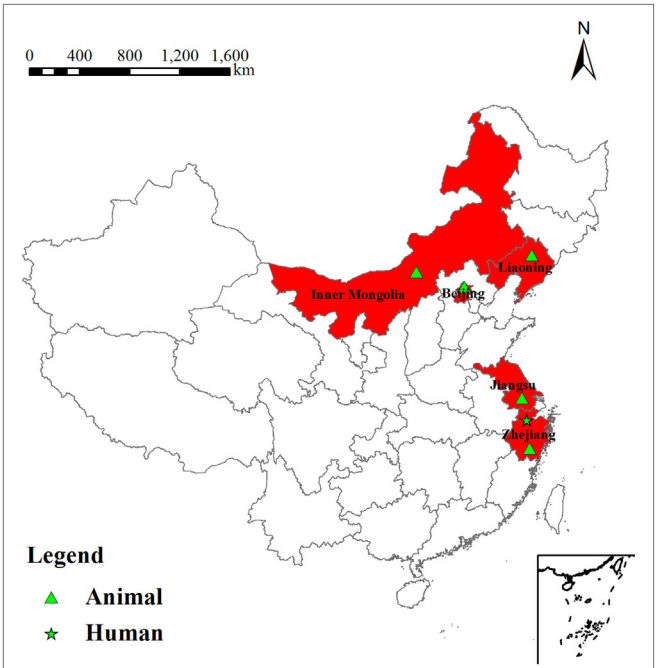


FIGURE 1 | Location of *Brucella canis* isolated from human and animals between 2006 and 2011.

TABLE 1 | Hunter-Gaston Diversity Index (HGDI) for the 32 Chinese *Brucella canis* isolates.

Locus	No. of alleles	HGDI[a]	CI 95%[b]
MLVA-16	26	0.976	0.945–1.000
MLVA-8	3	0.284	0.092–0.476
Bruce06	1	0.000	0.000–0.194
Bruce08	1	0.000	0.000–0.194
Bruce11	2	0.175	0.011–0.340
Bruce12	1	0.000	0.000–0.194
Bruce42	1	0.000	0.000–0.194
Bruce43	1	0.000	0.000–0.194
Bruce45	1	0.000	0.000–0.194
Bruce55	2	0.121	0.000–0.268
Panel 2A	3	0.603	0.506–0.699
Bruce18	3	0.603	0.506–0.699
Bruce19	1	0.000	0.000–0.194
Bruce21	1	0.000	0.000–0.194
Panel 2B	26	0.976	0.945–1.000
Bruce04	6	0.607	0.451–0.763
Bruce07	7	0.758	0.649–0.867
Bruce09	10	0.865	0.803–0.927
Bruce16	7	0.821	0.747–0.894
Bruce30	1	0.000	0.000–0.194

[a]*Hunter and Gaston index.*
[b]*Precision of the diversity index, expressed as 95% upper and lower boundaries.*

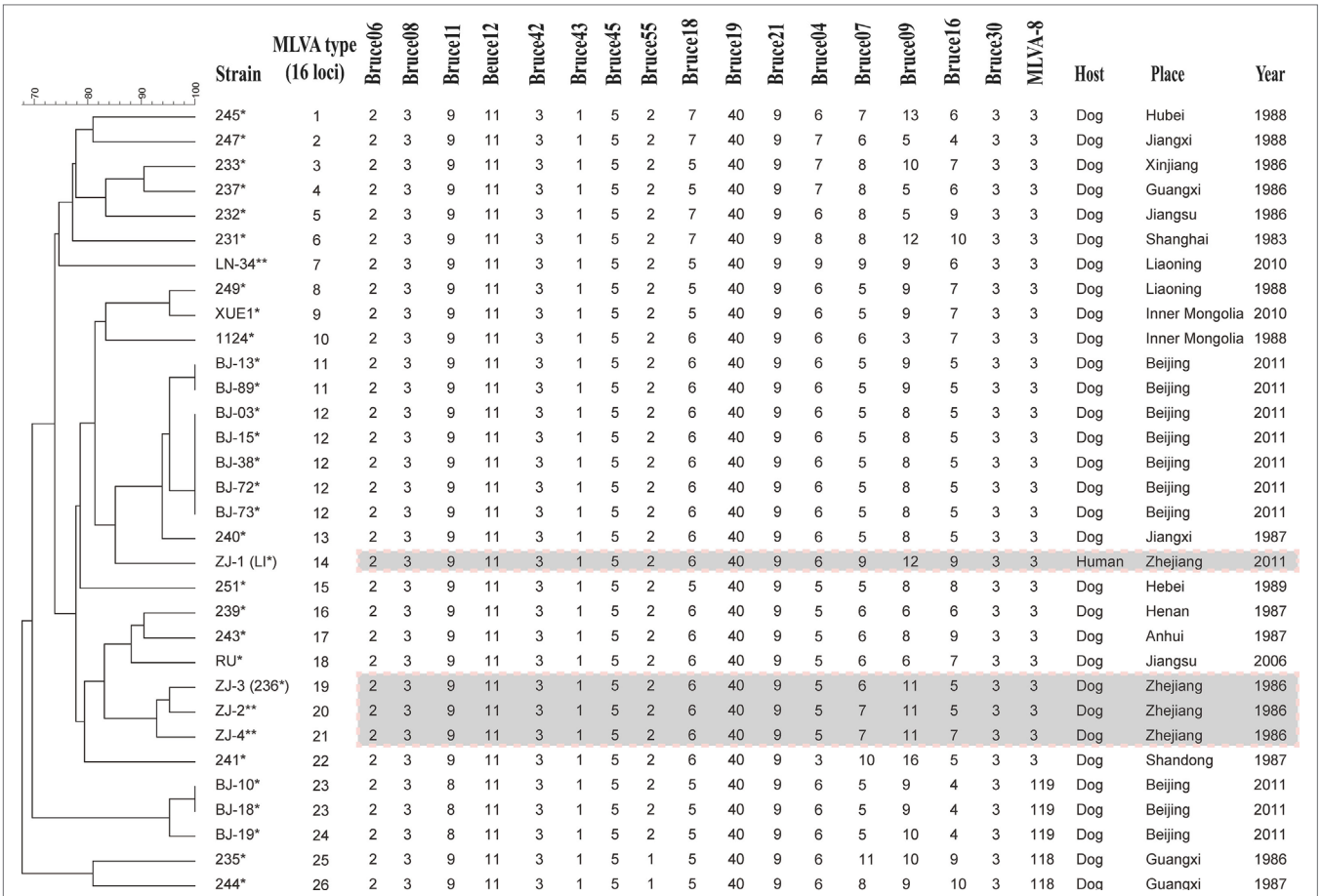
Genetic Characteristics of Human and Canine *B. canis* LPS

21 LPS-synthesis genes were analyzed with the reference strain *B. canis* RM6/66. Comparative analysis indicated *B. canis* from canines and humans had no differences in LPS-synthesis locus. We next sought to extend the LPS-synthesis genetic characterization between *B. canis* isolated from human and canines. We also extracted the LPS and performed SDS-PAGE analysis. The results revealed no difference was found between *B. canis* isolated from human and canines (**Figure 3**).

DISCUSSION

Brucella canis infection was first reported in China in 1984 (6). A nationwide survey on the *B. canis* infection was conducted in 25 provinces. Epidemic regions were primarily located in south and southeast China during the 1970s and 1980s (6). In 2011, a *B. canis* outbreak was investigated in a beagle dog breeding farm in Beijing. 48.75% (39/80) was positive by RSAT (7). On November 10, 2011, a 45-year-old woman who developed fever, back pain, and fatigue was diagnosed with pleurisy at the Jiaxing People's Hospital, Zhejiang, China. Although the epidemiological contact history, brucellosis symptoms were atypical. The RRBT and RSAT tests for anti-R antibodies yielded positive results, considered to be evidence of *B. canis* infection. On December 12, the patient blood was sent to the Zhejiang Center for Disease Prevention and Control. After several days, an isolate was collected from the blood culture. This isolate displayed rough colony using acridine orange staining. It also showed strong agglutination with anti-R monospecific sera and weak agglutination with anti-*Brucella* monospecific sera. Surveillance data showed most human brucellosis cases were caused by *B. melitensis* species (8, 9). There were few reported human brucellosis cases caused by *B. canis* species. A detailed understanding of genetic diversity of circulating *B. canis* strains in animals and humans should be achieved to provide appropriate prevention measures for subsequent eradication programs.

Hangzhou city is low prevalence of brucellosis in domestic animals and human populations. We discovered that four *B. canis* strain were classified into genotype 3 by MLVA-8 analysis. As subset panel 2A loci showed no diversity, the genotypic polymorphism was solely due to the panel 2B loci (**Table 2**). The lack of genetic diversity in panel 1 and panel 2A suggests that these *B. canis* strains are highly homogenous. Further analysis showed the three dog MLVA genotypes were only a single-locus variant or double-locus variants in bruce07 (panel 2B) and bruce21 (panel 2A), respectively. However, the human isolate shared only bruce30 (panel 2B) from the dog isolates, which originated from



Strain	MLVA type (16 loci)	Bruce06	Bruce08	Bruce11	Beuce12	Bruce42	Bruce43	Bruce45	Bruce55	Bruce18	Bruce19	Bruce21	Bruce04	Bruce07	Bruce09	Bruce16	Bruce30	MLVA-8	Host	Place	Year
245*	1	2	3	9	11	3	1	5	2	7	40	9	6	7	13	6	3	3	Dog	Hubei	1988
247*	2	2	3	9	11	3	1	5	2	7	40	9	7	6	5	4	3	3	Dog	Jiangxi	1988
233*	3	2	3	9	11	3	1	5	2	5	40	9	7	8	10	7	3	3	Dog	Xinjiang	1986
237*	4	2	3	9	11	3	1	5	2	5	40	9	7	8	5	6	3	3	Dog	Guangxi	1986
232*	5	2	3	9	11	3	1	5	2	7	40	9	6	8	5	9	3	3	Dog	Jiangsu	1986
231*	6	2	3	9	11	3	1	5	2	7	40	9	8	8	12	10	3	3	Dog	Shanghai	1983
LN-34**	7	2	3	9	11	3	1	5	2	5	40	9	9	9	9	6	3	3	Dog	Liaoning	2010
249*	8	2	3	9	11	3	1	5	2	5	40	9	6	5	9	7	3	3	Dog	Liaoning	1988
XUE1*	9	2	3	9	11	3	1	5	2	5	40	9	6	5	9	7	3	3	Dog	Inner Mongolia	2010
1124*	10	2	3	9	11	3	1	5	2	6	40	9	6	6	3	7	3	3	Dog	Inner Mongolia	1988
BJ-13*	11	2	3	9	11	3	1	5	2	6	40	9	6	5	9	5	3	3	Dog	Beijing	2011
BJ-89*	11	2	3	9	11	3	1	5	2	6	40	9	6	5	9	5	3	3	Dog	Beijing	2011
BJ-03*	12	2	3	9	11	3	1	5	2	6	40	9	6	5	8	5	3	3	Dog	Beijing	2011
BJ-15*	12	2	3	9	11	3	1	5	2	6	40	9	6	5	8	5	3	3	Dog	Beijing	2011
BJ-38*	12	2	3	9	11	3	1	5	2	6	40	9	6	5	8	5	3	3	Dog	Beijing	2011
BJ-72*	12	2	3	9	11	3	1	5	2	6	40	9	6	5	8	5	3	3	Dog	Beijing	2011
BJ-73*	12	2	3	9	11	3	1	5	2	6	40	9	6	5	8	5	3	3	Dog	Beijing	2011
240*	13	2	3	9	11	3	1	5	2	6	40	9	6	5	8	5	3	3	Dog	Jiangxi	1987
ZJ-1 (LI*)	14	2	3	9	11	3	1	5	2	6	40	9	6	9	12	9	3	3	Human	Zhejiang	2011
251*	15	2	3	9	11	3	1	5	2	5	40	9	5	5	8	8	3	3	Dog	Hebei	1989
239*	16	2	3	9	11	3	1	5	2	6	40	9	5	6	6	6	3	3	Dog	Henan	1987
243*	17	2	3	9	11	3	1	5	2	6	40	9	5	6	8	9	3	3	Dog	Anhui	1987
RU*	18	2	3	9	11	3	1	5	2	6	40	9	5	6	6	7	3	3	Dog	Jiangsu	2006
ZJ-3 (236*)	19	2	3	9	11	3	1	5	2	6	40	9	5	6	11	5	3	3	Dog	Zhejiang	1986
ZJ-2**	20	2	3	9	11	3	1	5	2	6	40	9	5	7	11	5	3	3	Dog	Zhejiang	1986
ZJ-4**	21	2	3	9	11	3	1	5	2	6	40	9	5	7	11	7	3	3	Dog	Zhejiang	1986
241*	22	2	3	9	11	3	1	5	2	6	40	9	3	10	16	5	3	3	Dog	Shandong	1987
BJ-10*	23	2	3	8	11	3	1	5	2	5	40	9	6	5	9	4	3	119	Dog	Beijing	2011
BJ-18*	23	2	3	8	11	3	1	5	2	5	40	9	6	5	9	4	3	119	Dog	Beijing	2011
BJ-19*	24	2	3	8	11	3	1	5	2	5	40	9	6	5	10	4	3	119	Dog	Beijing	2011
235*	25	2	3	9	11	3	1	5	1	5	40	9	6	11	10	9	3	118	Dog	Guangxi	1986
244*	26	2	3	9	11	3	1	5	1	5	40	9	6	8	9	10	3	118	Dog	Guangxi	1987

FIGURE 2 | Dendrogram based on the MLVA-16 genotyping assay showing relationships of the four *Brucella canis* isolates from Hangzhou in this study to other *B. canis* strains. Strain: strain name in the laboratory in which the DNA extraction was done; year/place: year and country of isolation (when known); * indicates these strains were previously analyzed in Di et al. (23). ** indicates these strains have been analyzed in this study.

TABLE 2 | MLVA-8 and MLVA-11 genotypes and numbers of tandem-repeat units for panel 2B loci in *Brucella canis* isolates from Zhejiang province and other regions by MLVA-16.

Strain	Host	Year	MLVA-8 genotype	MLVA-11 genotype	Panel 2B				
					Bruce04	Bruce07	Bruce09	Bruce16	Bruce30
ZJ-1 (LI*)	Human	2011	3[a]	28	6	9	12	9	3
ZJ-2**	Dog	1986	3[a]	28	5	7	11	5	3
ZJ-3 (236*)	Dog	1986	3[a]	28	5	6	11	5	3
ZJ-4**	Dog	1986	3[a]	28	5	7	11	7	3
RU*	Dog	2006	3[a]	28	5	6	6	7	3
LN-34**	Dog	2010	3[a]	26	9	9	9	6	3
XUE1*	Dog	2010	3[a]	26	6	5	9	7	3
BJ-03*, BJ-15*, BJ-38*, BJ-72*, BJ-73*	Dog	2011	3[a]	28	6	5	8	5	3
BJ-13*, BJ-89*	Dog	2011	3[a]	28	6	5	9	5	3
BJ-10*, BJ-18*	Dog	2011	New[b]	26	6	5	9	4	3
BJ-19*	Dog	2011	New[b]	26	6	5	10	4	3

[a]*Genotype 3 (2-3-9-11-3-1-5-2).*

[b]*New genotype (2-3-8-11-3-1-5-2).*

** indicates these strains have been analyzed in Di et al. (23).*

*** indicates these strains have been analyzed in this study.*

the same breeding farm. These minor mutants probably reflect adaptation or microevolution (21). Although the three dog isolates analyzed in this study were from 25 years earlier, with no reported intermediate infection, it is of interest that the time-distant isolates showed greater homology than geographically distant isolates, consistent with local persistent transmission.

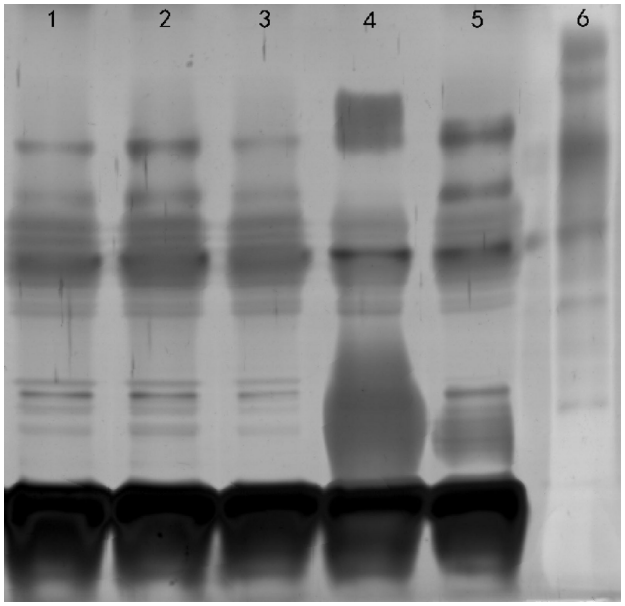


FIGURE 3 | SDS-PAGE analysis of LPS. Lane 1, *Brucella canis* ZJ-1 (human); lane 2, *B. canis* ZJ-2 (dog); lane 3, *B. canis* RM6/66; lane 4, *Brucella melitensis* 16M; lane 5, *B. suis* 1330S; lane 6, molecular weight marker.

It may suggest a recent evolution from a common ancestor in Hangzhou.

Additionally, these four *B. canis* isolates were compared with some dog isolates from other China provinces: Beijing, Jiangsu, Liaoning, and Inner Mongolia, between 2006 and 2011. Diversities were found with MLVA-16. Previously we observed that *B. canis* isolates from dogs either separate into a known genotype 3 or a new genotype with MLVA-8 (7). Interestingly, the Hangzhou strains were distinctly different from the above-mentioned strains because of the bruce09. It could be deduced that the Hangzhou strains may have originated from different restricted geographic region compared with the other strains. The differences may be explained by the bacterial adaptation to host and environment that produce genetic changes or polymorphisms. Zhejiang province was an epidemic region where *B. melitensis* was the dominant epidemic species and *B. abortus* was an accessory species (unpublished). However, *B. canis* infection has complicated the current epidemic situation. Nationwide, the true incidence of *B. canis* in humans remains unknown; therefore, sero-prevalence survey data are needed to determine the risk of *B. canis* infections. The human canine brucellosis was limited to one area, which is useful in monitoring the source of infection when outbreaks occur in other areas free canine brucellosis.

For LPS-synthesis-related genes, no mutations were detected among these strains, demonstrating the presence of the same LPS genetic loci responsible for rough morphology. We also extracted LPS and performed SDS-PAGE analysis. But, no difference was found between *B. canis* isolated from human and canine. Importantly, the human *B. canis* strain is a precious resource for studying *Brucella* pathogenicity. The whole genome sequencing of the human *B. canis* strain is ongoing and could be useful for the development of diagnostic tools, in order to reduce health complications of this infection in dogs and humans.

CONCLUSION

For the first time, comprehensive approaches have been used to analyze human and animal *B. canis* isolates with molecular epidemiological and genetic characteristics. Further detailed analysis of the whole genomic sequencing will contribute to understanding of the pathogenicity of *B. canis* in human.

ETHICS STATEMENT

After approval by the ethics committee, we included patient data in this study (Ethics Committee, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention).

AUTHOR CONTRIBUTIONS

HJ and HW designed this study and did most of the typing work. HW, JL, WX, and WG were in charge of the epidemiological investigation and collection of Hangzhou strains. GT, HZ, and DP prepared the DNA samples. HW and DP wrote the report. All authors read and approved the final manuscript. HJ and WF are guarantors of the paper.

FUNDING

This study was funded by the National Nature Science Foundation (81271900), National Key Program for Infectious Diseases of China (2013ZX10004221, 2013ZX10004805 and 2014ZX10004003), the Science and Technology Basic Work Program (2012FY111000). The funders contributed to the study design and data collection.

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Conflict of Interest Statement: 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: 14 December 2017
doi: 10.3389/fvets.2017.00215

Multiple Locus Variable-Number Tandem-Repeat and Single-Nucleotide Polymorphism-Based *Brucella* Typing Reveals Multiple Lineages in *Brucella melitensis* Currently Endemic in China

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OPEN ACCESS

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Reviewed by:
Hai Jiang,
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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 September 2017
Accepted: 29 November 2017
Published: 14 December 2017

Citation:
Sun M, Jing Z, Di D, Yan H, Zhang Z, Xu Q, Zhang X, Wang X, Ni B, Sun X, Yan C, Yang Z, Tian L, Li J and Fan W (2017) Multiple Locus Variable-Number Tandem-Repeat and Single-Nucleotide Polymorphism-Based Brucella Typing Reveals Multiple Lineages in Brucella melitensis Currently Endemic in China. Front. Vet. Sci. 4:215. doi: 10.3389/fvets.2017.00215

Brucellosis is a worldwide zoonotic disease caused by *Brucella* spp. In China, brucellosis is recognized as a reemerging disease mainly caused by *Brucella melitensis* specie. To better understand the currently endemic *B. melitensis* strains in China, three *Brucella* genotyping methods were applied to 110 *B. melitensis* strains obtained in past several years. By MLVA genotyping, five MLVA-8 genotypes were identified, among which genotypes 42 (1-5-3-13-2-2-3-2) was recognized as the predominant genotype, while genotype 63 (1-5-3-13-2-3-3-2) and a novel genotype of 1-5-3-13-2-4-3-2 were second frequently observed. MLVA-16 discerned a total of 57 MLVA-16 genotypes among these *Brucella* strains, with 41 genotypes being firstly detected and the other 16 genotypes being previously reported. By BruMLSA21 typing, six sequence types (STs) were identified, among them ST8 is the most frequently seen in China while the other five STs were firstly detected and designated as ST137, ST138, ST139, ST140, and ST141 by international multilocus sequence typing database. Whole-genome sequence (WGS)-single-nucleotide polymorphism (SNP)-based typing and phylogenetic analysis resolved Chinese *B. melitensis* strains into five clusters, reflecting the existence of multiple lineages among these Chinese *B. melitensis* strains. In phylogeny, Chinese lineages are more closely related to strains collected from East Mediterranean and Middle East countries, such as Turkey, Kuwait, and Iraq. In the next few years, MLVA typing will certainly remain an important epidemiological tool for *Brucella* infection analysis, as it displays a high discriminatory ability and achieves result largely in agreement with WGS-SNP-based typing. However, WGS-SNP-based typing is found to be the most powerful and reliable method in discerning *Brucella* strains and will be popular used in the future.

Keywords: *Brucella melitensis*, MLVA, multilocus sequence typing, whole-genome sequence, single-nucleotide polymorphism, phylogeny

INTRODUCTION

Brucellosis is one of the world's most important zoonotic diseases causing great damage to husbandry industry and public health (1). While some developed countries have successfully eradicated this disease, it remains the major concern for many developing countries in Africa, South America, and much of Asia including China (2–9). Brucellosis is caused by intracellular Gram-negative bacteria belonging to *Brucella* genus. So far six species have been officially identified based on differences in pathogenicity, host preferences, and conventional phenotyping methods: *Brucella abortus* (cattle), *Brucella melitensis* (goat and sheep), *Brucella suis* (pigs), *Brucella canis* (dogs), *Brucella ovis* (sheep), and *Brucella neotomae* (rodents). More recently, additional species have been suggested originating from a wide range of hosts, *Brucella ceti* (cetaceans), *Brucella pinnipedialis* (seals), *Brucella microti* (voles), *Brucella papionis* (baboons), and *Brucella vulpis* (foxes) (10–12).

In China, brucellosis is regarded as a remerging disease with previous high incidence occurred in 1950s–1980s (13, 14). In 1960s, a comprehensive control measures including animal vaccination, test-slaughter, and movement restriction were introduced to control this disease, thereafter a low-incidence period was observed from 1980s to 1990s. During that period, brucellosis incidence in domestic animals was kept at below 0.3%, while human brucellosis rate ranged around 0.05–0.10 cases per 100,000 residents. However, during the past decades, outbreaks of brucellosis in domestic animals and human have been increasingly reported, especially in north of China where brucellosis was highly endemic in history, such as Inner Mongolia, Xinjiang, Qinghai, and Ningxia. In recent years, brucellosis is rapidly spreading across the mainland with the trend from northern area to southern provinces where brucellosis was not historically serious. Over a long period of time, test-slaughter as major measure has been implemented to control brucellosis in domestic animals, now this strategy is proven to be less effective. To alleviate the worsening *Brucella* infection, vaccination on domestic animals was reintroduced to Inner Mongolia in 2012. Until 2015, the overall outbreaks of brucellosis in cattle and sheep had been significantly decreased (from 4,741 in 2012 to 1,534 in 2015), although it remained as the most severe brucellosis-endemic region according to data shown in Chinese Official Veterinary Bulletin.[1] Contemporarily, other provinces with no vaccination implemented saw an increased number of brucellosis outbreaks in domestic animals, among them Xinjiang was mostly affected (from 413 in 2012 to 927 in 2015). According to the lately issued National Brucellosis Control Plan of China (2016–2020), a comprehensive of measures are going to be applied to control animal brucellosis. From 2016, all the provinces in northern China will carry out vaccination program against brucellosis in bovine and small ruminants, meanwhile more stringent rules will come into effect to restrict animal movement from brucellosis high-incidence region to low-incidence region. In support of the plan, bacteriological surveillance in domestic animal reservoir is specifically emphasized.

The knowledge on current major *Brucella* species, biovar and genotype, and their geographic distribution is of great valuable, especially for selecting an appropriate vaccine, tracking-back infections sources, and monitoring transmission routes. In China, a range of *Brucella* species and biovars has been reported in animals, including *B. melitensis* (biovar 1, 2, and 3), *B. abortus* (biovar 1–7, 9), *B. suis* (biovar 1 and 3), *B. canis*, and *B. ovis*. However, human brucellosis was mainly caused by *B. melitensis*, implying a major source from infected small ruminants. National sentinel surveillance has been established to monitor the seroprevalence of brucellosis in animals, but systematic bacteriological survey was seldom carried out. From 2009, authorized by Ministry of Agriculture of China (MoA), Chinese Animal Health and Epidemiology Center carried out etiological investigation on cattle and small ruminant brucellosis in Northern China where animal vaccination has not been conducted, such as Xinjiang, Shanxi, and Hebei provinces. So far, a quite number of *Brucella* isolates has been obtained and most of them were *B. melitensis*.

Here, in this study, for improving our understanding of the currently endemic *B. melitensis* strains in China, a popularly used *Brucella* typing method of MLVA-16 [multiple locus variable-number tandem-repeat (VNTR) assay] was applied to these *B. melitensis* strains collected by our lab. This *Brucella* typing scheme, utilizing 16 VNTRs, has been proven to have the ability to differentiate *Brucella* species, biovar, and even the isolates (15, 16). More importantly, there is an online database of MLVA-16 profiles available to the all researchers allowing comparison of *Brucella* strains in the global scope.[2] Another *Brucella* typing method based on whole-genome sequence (WGS) extracted single-nucleotide polymorphisms (SNPs) was also applied to these Chinese *B. melitensis* strains. It provides unprecedented resolution in deciphering phylogenetic relationships among different *Brucella* species (17, 18), as well as a great power to distinguish closely related isolates within a species (15, 19–24). As NGS technique becomes more affordable to many laboratories, full genome sequences of *Brucella* isolates with a diverse of geographical backgrounds can be available on publically accessible database. It provides a substantial foundation for widespread use of this reliable method in *Brucella* isolate typing and phylogenetic relationship analysis. In addition, the previously used multilocus sequence typing (MLST) based on smaller number of SNPs was also evaluated in this paper.

MATERIALS AND METHODS

Strains Background

From 2010 to 2016, systematic bacteriological isolations were conducted on tissue and milk samples collected from cattle and small ruminants serologically positive to *Brucella* infection. A total of 110 *B. melitensis* strains were obtained from a multiple locations involving 7 Northern provinces and 50 counties. By further biotype characterization based on conventional serotyping, most of these *B. melitensis* strains were identified as biovar 3

[1] http://www.moa.gov.cn/zwllm/tzgg/gb/sygb/.

[2] http://microbesgenotyping.i2bc.paris-saclay.fr.

($n = 99$, 90%), and only a small part of strains were found to be biovar 1 ($n = 9$, 8%) and biovar 2 ($n = 2$, 2%).

MLVA Genotyping

MLVA including eight minisatellite loci (panel 1: Bruce06, 08, 11, 12, 42, 43, 45, and 55) and eight microsatellite loci (panel 2, subdivided into panel 2A: Bruce18, 19, 21; and panel 2B: Bruce04, 07, 09, 16, and 30) was performed as previously described (15, 24). For each locus, Hunter and Gaston diversity index (HGDI) were calculated by online software.[3] Cluster analysis on strains was conducted using BioNumerics software (version 7.6, Applied Maths, Belgium) and based on the categorical coefficient and UPGMA. Web-based MLVA database (see text footnote 2) was utilized for the convenience of comparing strains from different countries.

MLST Typing

Multilocus sequence typing genotyping was performed with the method described by Whatmore et al. (25). To increase the discriminatory ability on *Brucella* strains, the more informative scheme including 21 loci (BruMLSA21) was used. Each new allele of the 21 loci was given a distinct numerical designation following up the PubMLST databases.[4] Each unique allelic profile for these 21 loci was identified as a sequence type (ST). The assembled sequences of the 21 loci were then concatenated, and phylogenetic analyses on all identified STs were conducted using MEGA software (version 5.1) as described earlier.

Whole-Genome Sequencing, Assembling, and Annotation

Whole-genome sequencing was performed on the Illumina HiSeq2000 platform at Novogene (Novogene, Beijing, China). The generated reads were assembled into contiges using *Brucella* 16M as the reference (GeneBank: NC003317 and GenBank: NC003318). GeneMarkS was used to retrieve the related coding gene. Transfer RNA (tRNA) genes were predicted by the tRNAscan-SE. Putative tRNA and rRNA genes were analyzed by the tRNAscan-SE and rRNAmmer, respectively. Small nuclear RNAs were predicted by BLAST against the Rfam database. Six databases were routinely used to predict gene functions including GO, KEGG, COG, NR, Swiss-Prot, and TrEMBL.

WGS-SNP Discovery and Phylogenetic Tree Construction

We introduced Prokka to fully annotate draft bacteria genomes. All annotated assemblies in GFF3 format was used as input files for to conduct core-pan analysis (26). SNP sites were extracted from core gene alignment file generated by Roary (27). The missing and ambiguous data and gap were excluded. A matrix data containing the orthologous SNPs were generated. The filtered dataset was applied to conduct evolutionary analyses using the MEGA software (version 5.1). Neighbor-joining tree was constructed using Jukes–Cantor model and the percentage bootstrap confidence levels of internal branches were calculated from 1,000 resamplings of the original data. The *B. abortus* 2308 strain was selected as outgroup.

[3]http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl.

[4]http://pubmlst.org/brucella/.

RESULTS

MLVA Typing and Analysis

MLVA-8 Analysis

Among 110 *B. melitensis* strains, MLVA-8 typing comprising eight panel 1 loci generated five genotypes, with VNTR patterns described as 1-5-3-13-2-2-3-2 (82 strains, 75%), 1-5-3-13-2-3-3-2 (13 strains, 12%), 1-5-3-13-2-4-3-2 (11 strains, 10%), 1-5-3-13-3-2-3-2 (3 strains, 3%), and 1-5-3-14-2-2-3-2 (1 strain) (**Table 1**). Of them, three genotypes, previously numbered as genotype 42 (1-5-3-13-2-2-3-2), 63 (1-5-3-13-2-3-3-2), and 43 (1-5-3-13-3-2-3-2), are typical East Mediterranean lineage and have been reported in China (2, 16, 28–30). Genotype 42 is most frequently observed and widely distributed throughout the mainland, while genotype 63 and 43 are less reported and only endemic in local area such as Xinjiang, Inner Mongolia, Qinghai, and Shanxi. The genotype with the profile of 1-5-3-13-2-4-3-2 was the most lately identified and only detected previously in single *B. melitensis* strain collected from Xinjiang (31). Here, in this study, more strains were identified as the same MLVA-8 genotype, and they were exclusively collected from Xinjiang, suggesting the existence of a new lineage in this local area. The remaining MLVA-8 genotype (1-5-3-14-2-2-3-2) was never reported before. This novel profile may represent a highly mutated *B. melitensis* strain, as it is well separated from all others common strains according to MLVA-16 clustering tree (**Figure 1**).

MLVA-16 Analysis

MLVA-16 (including panel 1 and panel 2 loci) displayed higher discriminatory power and generated 57 genotypes within the same collection of *B. melitensis* strains (**Figure 1**). The high diversity revealed by MlVA-16 is mainly contributed by three panel 2B loci (Bruce04, 16, 30) with the HGDI values ranging from 0.743 to 0.777 (**Table 2**). By comparing with the data deposited in MLVA database, 41 genotypes comprising 70 strains were found to be unique to China, while the remaining 16 genotypes have been reported in Turkey and Kazakhstan (4, 8). In China, these 57 MlVA-16 genotypes demonstrate different geographic distribution. 47 genotypes are found to be provincially specific and the other 10 genotypes are shared by two or more provinces. MLVA-16 genotype described as 1-5-3-13-2-2-3-2-4-41-8-7-4-3-6-6 is

TABLE 1 | MLVA-8 genotypes identified in 110 Chinese *Brucella melitensis* strains.

MLVA-8 genotype	Copy no. in each variable-number tandem-repeat locus	No. of strains	Percentage
42	1-5-3-13-2-2-3-2	82	75
63	1-5-3-13-2-3-3-2	13	12
N/A	1-5-3-13-2-4-3-2	11	10
43	1-5-3-13-3-2-3-2	3	2
N/A	1-5-3-14-2-2-3-2	1	
Total		110	

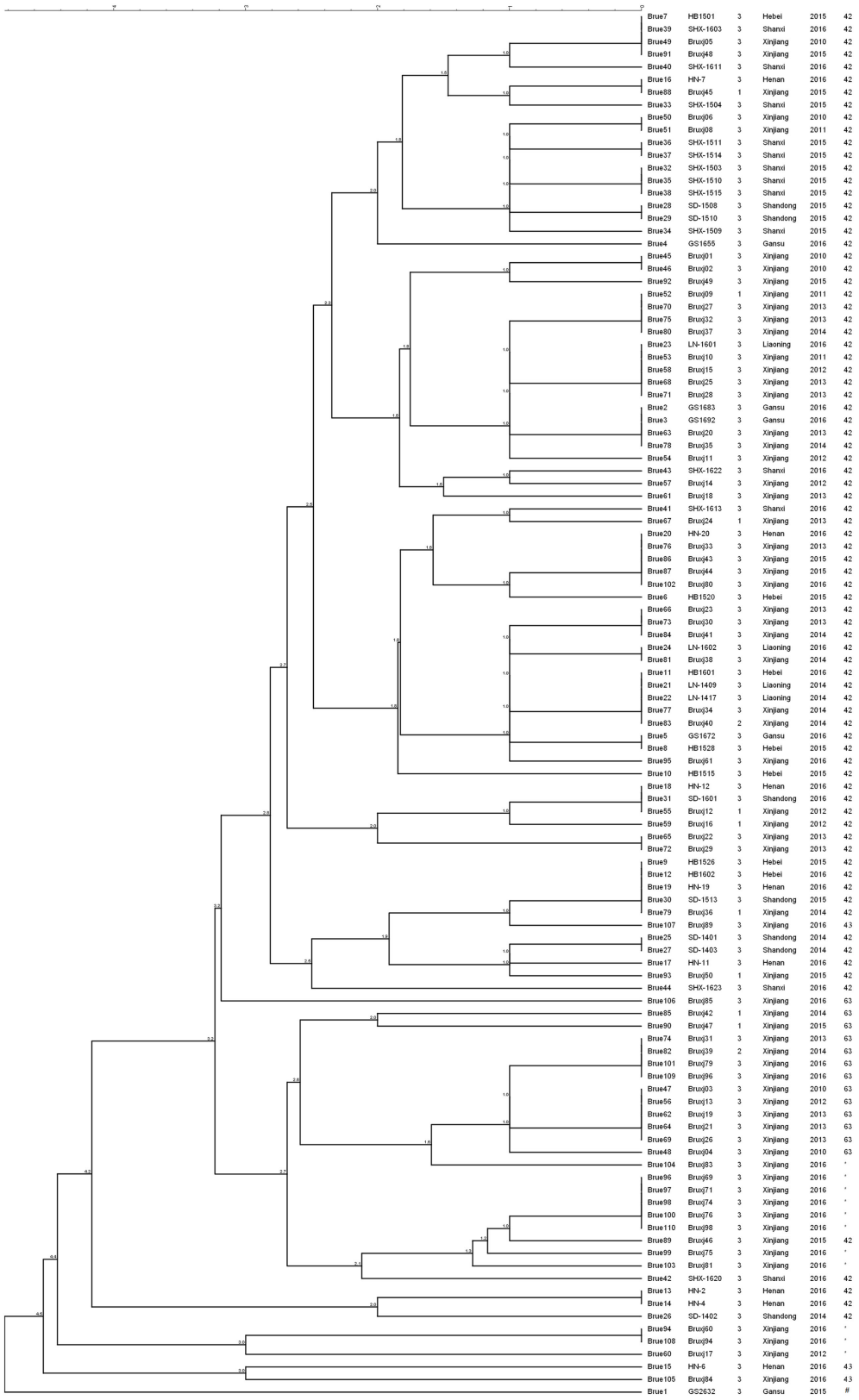

FIGURE 1 | Clustering analysis of 110 *Brucella melitensis* isolated from China based on dataset of 16 variable-number tandem-repeats. In the columns, the following data are indicated: key, strain ID, biovar, isolation location, isolation date, and MLVA-8 genotype ID. * indicates the MLVA-8 genotype with the profile of 1-5-3-13-2-4-3-2; # indicates the newly detected MLVA-8 genotype of 1-5-3-14-2-2-3-2 in this study.

TABLE 2 | Number of alleles and HGDI values of *Brucella melitensis* strains isolated in China.

Variable-number tandem-repeat (VNTR) locus		Alleles number	Copy number of VNTR	HGDI	Confidence interval	Max (Pi)
Panel 1	Bruce06	1	1	0.000	0.000–0.064	1.000
	Bruce08	1	5	0.000	0.000–0.064	1.000
	Bruce11	1	3	0.000	0.000–0.064	1.000
	Bruce12	2	13, 14	0.018	0.000–0.053	0.991
	Bruce42	2	2, 3	0.071	0.006–0.136	0.964
	Bruce43	3	2, 3, 4	0.392	0.290–0.494	0.764
	Bruce45	1	3	0.000	0.000–0.064	1.000
	Bruce55	1	2	0.000	0.000–0.064	1.000
Panel 2A	Bruce18	2	4, 5	0.018	0.000–0.053	0.991
	Bruce19	2	41, 46	0.018	0.000–0.053	0.991
	Bruce21	1	8	0.000	0.000–0.064	1.000
Panel 2B	Bruce04	6	3, 4, 5, 6, 7, 8	0.743	0.702–0.783	0.382
	Bruce07	2	4, 5	0.054	0.000–0.111	0.973
	Bruce09	4	3, 7, 8, 9	0.106	0.027–0.184	0.945
	Bruce16	11	2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13	0.777	0.733–0.822	0.318
	Bruce30	8	4, 5, 6, 7, 8, 9, 10, 11	0.738	0.679–0.797	0.436

most widely distributed genotype and shared by four provinces of Xinjiang, Hebei, Henan, and Shandong. Based on the diverse MLVA-16 genotypes identified in this study, especially those endemic in local regions, a proper tracking-back analysis for infection sources could be achieved.

MLST Typing

By BruMLSA21 typing, 6 STs were identified among these 110 Chinese *B. melitensis* strains (Table S1 in Supplementary Material). One ST, known as ST8, comprises 91 strains and is found to be predominant over all other STs. The other five STs were firstly detected and designated as ST137, ST138, ST139, ST140, and ST141 in MLST database (see Text footnote 4). ST139 consists of 15 strains obtained from three provinces of Shandong, Henan, and Xinjiang, representing the second frequently observed *B. melitensis* lineage in China. ST137, ST138, ST140, and ST141 were identified in individual strain. Phylogenetic tree was constructed using all *B. melitensis* STs deposited in database, including the five newly detected STs in this study (**Figure 2**). 29 STs were separated into three lineages with distinct geographic origins, Americas (Africa), West Mediterranean, and East Mediterranean (25). All novel STs detected in this study together with the predominant ST8 were clustered into East Mediterranean lineage, indicating a close phylogenetic relationship of Chinese *B. melitensis* strains to those from East Mediterranean region. This result is in agreement with the finding based on MLVA analysis. According to database, ST8 is the most common ST identified in *B. melitensis* and shows a wide distribution covering many European and Asian Countries. By phylogeny, ST137, ST140, and ST141 represent stains emerging from the prevailing ST8, while ST138 and ST139 represent another branch that is genetically connected to stains discovered from Egypt, Syria, and Iraq.

General Genomic Features

Nine *B. melitensis* strains representing different MLVA-8 genotypes were subject to whole-genome sequencing. The generated scaffold sequence covered 99.77% of complete genomic sequence when compared to reference strain 16M. G/C content was calculated as 57.24%. An average of 3,291 protein-encoding genes (±5) were predicted among these draft genomes. One copy of 5s, 16s, and 23s subunits in rRNA operon were detected in each strain, meanwhile 50 ± 1 copies of tRNA and 17 ± 2 copies of sRNA were predicted within these genomes (**Table 3**).

WGS-SNP-Based Phylogenetic Analysis

To better understand the evolutionary status of Chinese *B. melitensis* strains in a global context, WGS-SNP-based phylogenetic tree was constructed using 81 *B. melitensis* genomes deposited in NCBI genome bank and the nine genomes of sequenced in this study (Table S2 in Supplementary Material). *B. melitensis* biovar 1 strain 16M (NC_003317 and NC_003318.1) was chosen as reference for SNP calling. By Roary, 8,903 SNPs were extracted from 1,798 core genes. After filtering the sites lying in positions containing gaps and missing data, a total of 6,421 reliable SNPs were obtained and used for phylogenetic analysis.

In phylogenetic tree, 90 *B. melitensis* strains with a diverse geographic backgrounds were clustered into four major clades (labeled as Clade A1, A2, B, and C) corresponding spatially to the potential origins of these strains (**Figure 3**). Clade A1 comprised strains mainly isolated from the Americas, while the clade A2 represented the strains with Africa sources. All strains collected from Italy, Egypt, and Morocco formed clade B, and in many literatures it was termed as Western Mediterranean lineage. The clade C, comprising the largest *B. melitensis* populations with a wide spatial distribution from the Mediterranean region to Asian countries, was recognized as Eastern Mediterranean lineage. 17 Chinese strains including the nine selected in this study were falling into Clade C, and further separated into five clusters (cluster a, b, c, f, and l). The cluster a, b, and c comprise the most strains unexceptionally collected from China (13/17), representing the main *B. melitensis* lineage uniquely endemic in China. These clusters emerge from the same internal node with cluster d comprising the strains collected from Middle East region,

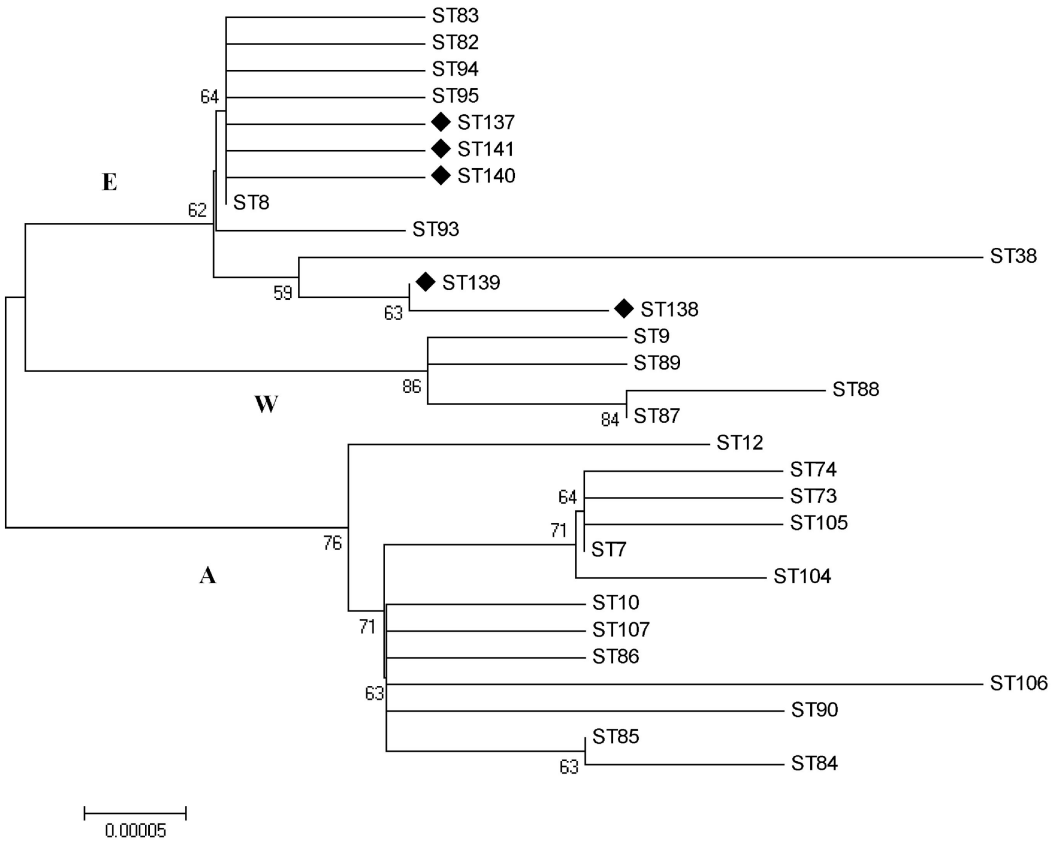

FIGURE 2 | Phylogenetic analysis of newly detected ST137, ST138, ST139, ST140 and ST141 together with 24 sequence types (STs) previously reported in *Brucella melitensis*. In the phylogenetic tree, distinct three clades were observed, with A standing for America/Africa lineage, W and E for West Mediterranean and East Mediterranean lineages.

TABLE 3 | Genomic features of nine *Brucella melitensis* strains isolated from China.

Strain ID	MLVA-8 genotype	Scaffold no.	Genome size (bp)	Gene number	Transfer RNA	sRNA	TRF	T4SS
Bruxj85	63	25	3,288,124	3,294	50	17	80	13
Bruxj20	42	24	3,286,504	3,287	49	16	81	13
HN06	43	24	3,286,987	3,296	51	17	88	13
Bruxj21	63	25	3,287,098	3,289	51	17	78	13
Bruxj71	N/A	25	3,287,527	3,293	51	15	82	13
Bruxj09	42	27	3,286,702	3,291	51	18	83	13
HB1526	42	27	3,287,364	3,285	51	18	78	13
Bruxj08	42	28	3,287,443	3,289	51	19	81	13
Bruxj38	42	27	3,288,458	3,293	51	16	82	13

suggesting a common origin for *B. melitensis* endemic in these two regions (**Figure 3**). Cluster f (comprising NI, Bruxj09 and Bruxj20) represents a minor Chinese lineage and is closely related in phylogeny to the strains collected from Caucasian region, such as Georgia and some Russian Republics of Dagestan, Kalmykia, Sarotov, and Stavropol (cluster g). The strain HN-6 was clustered into cluster m, demonstrating a long distance in phylogenetic tree to other Chinese strains. It represents a rarely observed lineage in China, but frequently detected in East Mediterranean and Middle East countries such as Turkey, Syria, and Kuwait (cluster m). This result support previous findings based on MLVA analysis that Chinese *B. melitensis* lineages are fundamentally East Mediterranean region originated (2, 8, 31), but with a more definitive scope pointing to Middle East and Caucasian countries.

DISCUSSION

For many years, the traditional phenotyping based on host specificity, growth feature, biochemical reaction, serotyping, and bacteriophage typing has been the golden standard for *Brucella* characterization at both species and biovar levels. In China, *B. melitensis* has long been recognized as the main species causing

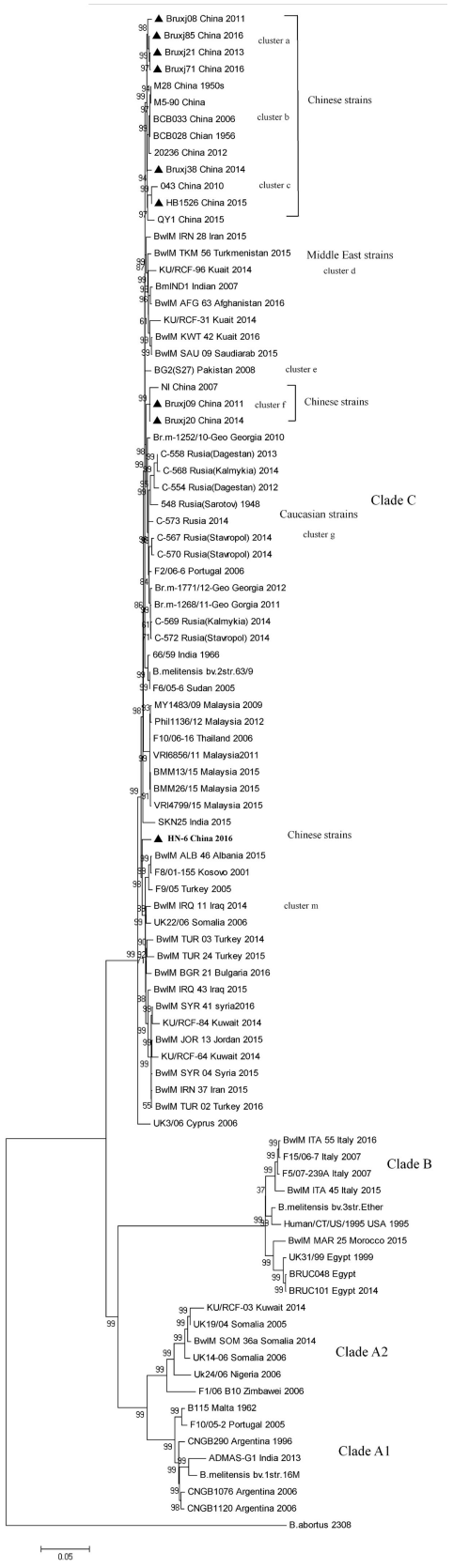

FIGURE 3 | Continued

FIGURE 3 | Continued
Whole-genome sequence-single-nucleotide polymorphism-based phylogenetic analysis of 9 *Brucella melitensis* strains isolated from China with the 81 *B. melitensis* strains selected from NCBI database. Neighbor-joining tree was constructed using Jukes–Cantor model and the setting of bootstraps was 1,000. *Brucella abortus* referential strain 2308 was included as outgroup. Strain information as ID, isolation location, and date are listed.

brucellosis in human and domestic animals. However, at the subspecies level, change of predomiant biovar has been observed in past years. In previous high-incidence stage (1950s–1980s), biovar 1 was the most frequently observed and accounted for around 55% of *B. melitensis* strains collected from human patients and infected animals. While in current reemergence stage, biovar 3 is most often observed and responsible for 90% of small ruminant infections as data shown in this study. Studies on human brucellosis also revealed the similar result (28, 30). Existence of a predominant biotype in a brucellosis-endemic area makes the species and biovar as epidemiological markers much less significant, especially in tracing newly occurred cases to their sources. Thus, other *Brucella* typing methods with higher discriminatory power are particularly needed.

Of the *Brucella* typing methods used so far, MLVA has been proven to be highly powerful in discerning *Brucella* isolates and was popularly used over global laboratories. MLVA-8 genotyping (based on panel 1 loci) may provide a limited discriminatory power, but it is still informative for understanding the relationships between *Brucella* isolates with a diverse geographic sources. Before this paper, a decade MLVA-8 genotypes have been reported in China, of which genotypes 42 and 63 are the mostly frequently detected genotypes with a relatively wider distribution than other genotypes (2, 28–30, 32, 33). The most recently detected genotype (1-5-3-13-2-4-3-2) in this study represents the single-locus variants from already existed genotype 42 or 63, implying the ongoing mutation of *B. melitensis* in a specific niche. Further MLVA-16 genotyping (panel 1 and 2 loci) revealed much more diversity, which is mainly due to three highly variable loci (Bruce04, 16, and 30) and one loci with medium variability (Brue09) (**Table 2**). In these loci, tandem-repeat numbers show successive one-unit difference, implying a stepwise mutation mode taken by *B. melitensis* in a specific environment. It also suggests that the novel MLVA-16 genotypes identified in this study may represent the most recently mutated strains from a few ancestors. Up to now, around 120 MLVA-16 genotypes have been identified among *B. melitensis* strains collected in China, with which proper epidemiological investigations in outbreaks of human and animal brucellosis can be achieved.

As MLVA-16 is comparatively inexpensive and a large database is available, it will certainly remain an important epidemiological tool for *Brucella* infections analysis in the near future. The reliability of this method was partially confirmed by WGS-SNP-based phylogenetic analysis. As data shown in this paper, 90 *B. melitensis* strains with diverse geographic origins were separated into similar clusters in both WGS-SNP phylogenetic and MLVA-16 clustering trees (**Figures 3** and **4**). The Cluster A, B, C1, and C2 discerned by MLVA-16 analysis is perfectly corresponding in their respective to Clade A1, B, A2, and C in phylogenetic tree, making the strains

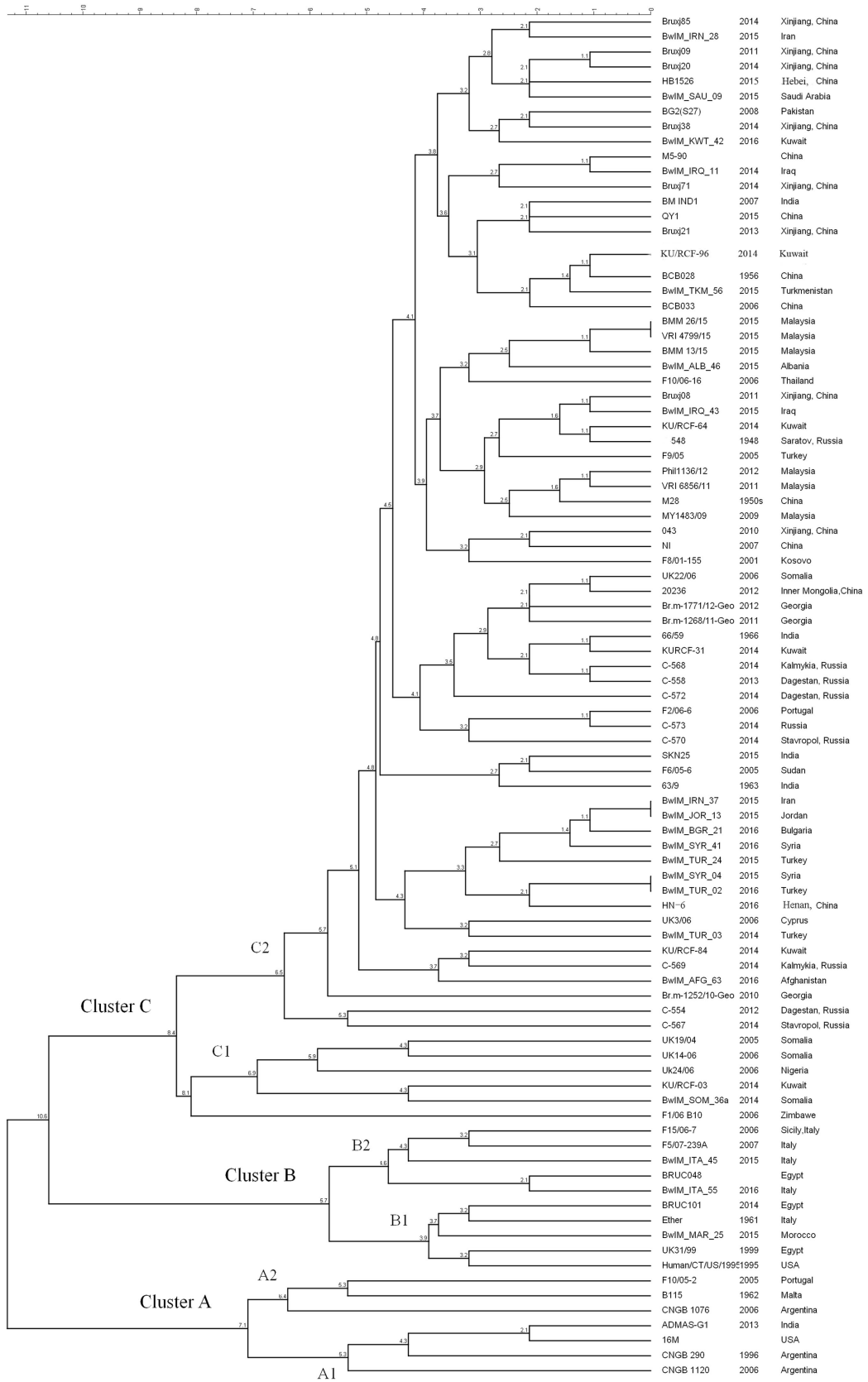

FIGURE 4 | MLVA-16 clustering analysis of 90 *Brucella melitensis* strains including 81 strains selected from NCBI database and 9 strains used in this study.

from America, Africa, Western Mediterranean, and Eastern Mediterranean/Asia backgrounds clearly separated. However, WGS-SNP-based phylogeny demonstrated higher reliability than MLVA-16 clustering, given its ability in resolving strains from a narrower geographic region. For example, the Chinese strains (cluster a, b, and c), which were well grouped together in phylogenetic tree, scattered across MLVA-16 clustering tree and intersected with other strains. SNPs appear to be a better choice for *Brucella* typing and related phylogenetic analysis, as they are evolutionarily stable, and more importantly, thousands of SNPs scattered in genome provide the finest resolution. As NGS becomes more affordable to local laboratories, the widely accepted MLVA-16 will be inevitably replaced by more reliable SNPs typing based on WGSs.

Multilocus sequence typing has been also accepted as a tool for bacterial typing and epidemiological studies, and the resulting data is as well ideal for phylogenetic studies. The previous BruMLSA9 scheme, utilizing 9 discrete genomic loci comprising of 4,396 bp, detected around 30 SNPs from worldwide collected *B. melitensis* isolates and resolved them into 6 STs (from ST7 to ST12) (34). By this scheme, the majority of *B. melitensis* strains endemic in China were identified to be ST8, and a few novel STs were also detected (31, 35). The recently developed BruMLSA21 (comprising 10,244 bp within 21 gene loci) displayed higher discriminatory power and detected a total of 29 STs worldwide, including the 5 novel STs identified in this study. Comparing to MLVA-16 and WGS-SNP-based typing, BruMLSA21 provided a limited ability in discerning highly homogeneous *B. melitensis* isolates. However, with a robust MLST database available to all researchers, it remains useful for strain comparison.

CONCLUSION

Due to the homogeneity of the *B. melitensis* species, especially under the current circumstance that the epidemic *Brucella* agents are overwhelmingly *B. melitensis* biovar 3, the traditional biotyping is of limited epidemiological value. WGS-SNP-based typing is so far the most powerful tool in differentiating *Brucella* isolates, with which multiple lineages were identified among *B. melitensis* strains currently circulating in China. In evolutionary relationship, the Chinese lineages are more closely connected to the strains from East Mediterranean and Middle East countries, such as Turkey, Kuwait, and Iraq. In the near future, MLVA typing will certainly remain an important epidemiological tool for *Brucella* infections analysis, as it displays a high discriminatory ability and achieves result largely in agreement with WGS-SNP-based typing. Based on the large number of MLVA-16 genotypes obtained so far, proper epidemiological investigations could be carried out when outbreaks of human and animal brucellosis occurred in China.

AUTHOR CONTRIBUTIONS

MS designed the study, analyzed the data, and drafted the manuscript. ZJ made WGS-SNP calling. DD, HY, ZZ, QX, and XZ undertook the data analysis work. XW, BN, XS, and CY performed portions of experiment. ZY, LT, and JL were responsible for strain collection and storage. WF oversaw the biosafety issue.

ACKNOWLEDGMENTS

This research was sponsored jointly by National Animal Disease Surveillance Project of Chinese Ministry of Agriculture and Qingdao Postdoctoral Applied Research Program (108756). We acknowledge many colleagues working in local animal disease control organizations who provided great help in collecting strains across China. We thank Fang Xu for her excellent technical support and Dr. Huifang Xu for her comments that greatly improved the original manuscript. Special respect was paid to Miss. Jinping Li for her hard work on *Brucella* research, without her dedication, the current work would be not achieved.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fvets.2017.00215/full#supplementary-material.

TABLE S1 | MLVA-16 dataset of 110 *Brucella melitensis* strains collected from 2010 to 2016 in China.

TABLE S2 | MLVA-16 dataset of 90 *Brucella melitensis* used for whole-genome sequence-single-nucleotide polymorphism-based phylogenetic analysis.

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Conflict of Interest Statement: 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: 13 December 2017
doi: 10.3389/fvets.2017.00197

Outer Membrane Protein 25 of *Brucella* Activates Mitogen-Activated Protein Kinase Signal Pathway in Human Trophoblast Cells

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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 September 2017
Accepted: 01 November 2017
Published: 13 December 2017

Citation:
Zhang J, Zhang Y, Li Z, Liu J, Shao X, Wu C, Wang Y, Wang K, Li T, Liu L, Chen C and Zhang H (2017) Outer Membrane Protein 25 of Brucella Activates Mitogen-Activated Protein Kinase Signal Pathway in Human Trophoblast Cells. Front. Vet. Sci. 4:197. doi: 10.3389/fvets.2017.00197

Outer membrane protein 25 (OMP25), a virulence factor from *Brucella*, plays an important role in maintaining the structural stability of *Brucella*. Mitogen-activated protein kinase (MAPK) signal pathway widely exists in eukaryotic cells. In this study, human trophoblast cell line HPT-8 and BALB/c mice were infected with *Brucella abortus* 2308 strain (S2308) and 2308ΔOmp25 mutant strain. The expression of cytokines and activation of MAPK signal pathway were detected. We found that the expressions of tumor necrosis factor-α, interleukin-1, and interleukin-10 (IL-10) were increased in HPT-8 cells infected with S2308 and 2308ΔOmp25 mutant. S2308 also activated p38 phosphorylation protein, extracellular-regulated protein kinases (ERK), and Jun-N-terminal kinase (JNK) from MAPK signal pathway. 2308ΔOmp25 could not activate p38, ERK, and JNK branches. Immunohistochemistry experiments showed that S2308 was able to activate phosphorylation of p38 and ERK in BABL/c mice. However, 2308ΔOmp25 could weakly activate phosphorylation of p38 and ERK. These results suggest that Omp25 played an important role in the process of *Brucella* activation of the MAPK signal pathway.

Keywords: ***Brucella*, 2308ΔOmp25, mitogen-activated protein kinase, cytokines, HPT-8 cells**

INTRODUCTION

Brucella spp. are Gram-negative facultative intracellular pathogens that can cause diseases of worldwide significance (1, 2). *Brucella* can cause epididymitis, orchitis, or abortion in animals (3). Infection in humans can cause fever or arthritis (4, 5). It resulted in heavy economic losses (6).

There are three groups of major outer membrane proteins (Omps) in *Brucella* (7). Group 1 Omps consist of two major Omps: Omp10 and Omp19. Group 2 Omps consist of two major Omps: Omp2a and Omp2b. Group 3 Omps consist of two major Omps: outer membrane protein 25 (Omp25) and Omp31. Omp25 was a primary protein that was released by *Brucella* when it invaded host cells (8). Omp25 was involved in attachment or invasion to the host cells and intracellular survival or reproduction of *Brucella*, which plays an important role in *Brucella* virulence. Omp25 mutant was attenuated in animals (9, 10). Therefore, Omp25 is an important virulence factor of *Brucella*.

Mitogen-activated protein kinase (MAPK) is one signal transduction pathway in organisms. It is associated with many profiles and processes of the cell, such as auxesis, development, proliferation,

differentiation, and apoptosis (11). MAPK includes four subfamilies: p38, ERK1/2, Jun-N-terminal kinase (JNK), and ERK5 (12). MAPK is implicated in bacterial pathogenesis as demonstrated by the induction of inhibition of ERK1/2 and p38 branches during infection with *Salmonella typhimurium* (13), *Yersinia* (14, 15), *Listeria monocytogens* (16, 17), and *Mycobacterium* (18). In the inflammatory response, MAPK signal pathway can mediate secretion of IL-8, interleukin-10 (IL-10), tumor necrosis factor-α (TNF-α), and other cytokines by epithelial cells (19). In this report, we used S2308 and 2308ΔOmp25 to infect HPT-8 cells and mice, and the expression of cytokines were detected. In addition, we analyzed the effects of Omp25 on the MAPK signal pathway, with the aim to understand the function of Omp25 in the pathogenesis of *Brucella*.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were performed in strict accordance with the Experimental Animal Regulation Ordinances defined by the China National Science and Technology Commission. The study was approved by the Institutional Committee of Post-Graduate Studies and Research at Shihezi University, China (No. 2012-9). Animals are provided with humane care and healthful conditions. All efforts were made to minimize animal suffering.

Bacterial Strains and Cell Line

Brucella abortus 2308 strain was obtained from the Center of Chinese Disease Prevention and Control (Beijing, China). 2308ΔOmp25 mutant was constructed and kept by our laboratory. *Brucella* was cultured in tryptic soy agar or tryptic soy broth (TSB) (Sigma, St. Louis, MO, USA) at 37°C with 5% CO_2 (vol/vol). The human trophoblast cell line HPT-8 (obtained from Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco Life Technologies, Rockville, MD, USA) at 37°C with 5% CO_2 (vol/vol).

Mice

Six-week-old BALB/c female mice were obtained from the Experimental Animal Center of the Academy of Military Medical Science (Beijing, China). Animals were maintained in barrier housing with filtered inflow air in a restricted-access room in pathogen-limited conditions. All experimental procedures and animal care were performed in compliance with institutional animal care regulations. And all experimental procedures and animal care were performed in Biosafety Level 3 Laboratory.

Brucella Cell Infection Assay

HPT-8 cells were infected with S2308 and 2308ΔOmp25, as previously described (20). The bacteria for infection studies were prepared before the experiment was executed. S2308 and 2308ΔOmp25 were cultured in TSB at 37°C with 5% CO_2 (vol/vol) until logarithmic growth phase. Then, 2×10^6 cells/well were cultured in 6-well plates for 24 h at 37°C under 5% CO_2, and then infected with S2308 or 2308ΔOmp25 at a multiplicity of infection (MOI) of 100 bacteria per cell. Culture plates were centrifuged at 350 × *g* for 5 min at room temperature. At 45 min post-infection, the cells were washed thrice with medium without antibiotics and then incubated with 50 μg/mL of gentamicin (Invitrogen, Carlsbad, CA, USA) for 1 h to kill extracellular bacteria. Afterward, the medium was removed and replaced with fresh DMEM with 10% FBS containing 25 μg/mL gentamicin (defined as time 0). Uninfected cells were used as control.

Detection of Cytokines

HPT-8 cells were infected with S2308 and 2308ΔOmp25 according to the above description. At 4, 8, 24, and 48 h post-infection, supernatant was collected and filtered with 0.22 μm filter membrane (Millipore, MA, USA). Then, supernatant was centrifuged at 16,000 × *g* for 15 min at 4°C. The levels of TNF-α, interleukin-1 (IL-1), and IL-10 were measured using an ELISA Quantikine Human Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All assays were performed in triplicate and the concentration of each cytokine in the cell supernatant was calculated using a linear regression equation obtained from the absorbance values of standards, according to the manufacturer's protocol. All assays were performed three times.

Determination of MAPK Branches Associated with the Secretion of TNF-α

To confirm the MAPK signal pathway associated with the secretion of TNF-α, HPT-8 cells were pre-treated with p38 inhibitor (10 μM), or JNK inhibitor (10 μM) at 37°C for 1 h and then infected with S2308 or 2308ΔOmp25 at a 100:1 MOI according to the above description. At 4, 8, 24, and 48 h post-infection, supernatant was collected and measured the levels of TNF-α according to the above description. All assays were performed three times.

Western Blotting Analysis

HPT-8 cells were infected with S2308 and 2308ΔOmp25 according to the above description. The activation of p38, ERK1/2, and JNK was detected in infected cells, as previously described (21). Briefly, at 24 h post-infection, supernatant was discarded and cells were lysed in ice-cold Radio Immunoprecipitation Assay Lysis Buffer (Beyotime Institute of Biotechnology, Shanghai, China) for 30 min, then centrifuged at 16,000 × *g* for 30 min at 4°C. The supernatant was collected and concentration was detected with BCA protein assay kit (Sangon Biotech, Shanghai, China). 500 μg protein samples separated by 12% SDS-PAGE and electro-transferred to a nitrocellulose membrane using a Mini Trans-Blot Cell (Bio-Rad, Hercules, CA, USA) at 200 mA for 1 h. Unbound sites on the membrane were blocked in 5% nonfat milk in Tris-buffered saline Tween-20 (TBST) buffer for 1 h at room temperature. Then, the membrane was washed three times with TBST buffer and incubated with rabbit anti-human anti-p38,

anti-ERK, or anti-JNK polyclonal antibody (pAb; diluted 1:1,000; Bioworld, Minneapolis, MN, USA) at room temperature for 1 h. After being washed three times, the membrane was incubated with peroxidase conjugated goat anti-rabbit IgG for 1 h at room temperature. After a further washing step, bound conjugate was visualized with an ECL Plus Western Blotting Substrate kit (Thermo Fisher Scientific, USA). All assays were performed three times.

Brucella Infection in Mice and Cytokine Measurement

BALB/c mice were infected with *Brucella* as previously described (22). Briefly, 6-week-old BALB/c female mice ($n = 5$ per group) were randomly divided into three groups. Group 1 and 2 were inoculated intraperitoneally (i.p.) with 200 μL phosphate-buffered saline (PBS; Sigma-Aldrich, MO, USA) containing 1×10^6 CFU of S2308 or 2308ΔOmp25, respectively, group 3 was inoculated i.p. with 200 μL PBS as negative control. Serum samples were obtained from peripheral blood of immunized mice 2, 4, 6, 8, and 10 weeks post-immunization (23). Serum samples were diluted with sample diluent buffer (1:5). The level of TNF-α was measured using an ELISA Quantikine Mouse Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. All assays were performed in triplicate and the concentration of each cytokine in the cell supernatant was calculated using a linear regression equation obtained from the absorbance values of standards, according to the manufacturer's protocol. All assays were performed three times.

Tissue Specimens

BALB/c mice ($n = 25$ per group) were inoculated with S2308 and 2308ΔOmp25 according to the above description. At 2, 4, 6, 8, and 10 weeks post-immunization, mice were euthanized and uteruses were removed aseptically. The uteruses were collected, formalin-fixed, and paraffin-embedded, as previously described (24).

Immunohistochemistry

The immunohistochemistry assay was performed as previously described (25). Briefly, the embedded paraffin will be serial sectioned by slicer, with a thickness of 5 μm and mounted on slide glasses coated with poly-L-lysine (Beyotime Institute of Biotechnology, Shanghai, China). Subsequently, the tissue sections were routine deparaffinized and dehydrated with xylene and ethanol. After three times washed with distilled water, the tissue sections were microwaved in 10 mM citrate buffer (pH 6.0; Sigma-Aldrich, MO, USA) at 95°C for 10 min for antigen retrieval and naturally cooled at room temperature. Then, the endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide buffer (Sigma-Aldrich, MO, USA) for 10 min at room temperature. The tissue sections were incubated overnight at 4°C with primary antibody (rabbit anti-mouse anti-p38 or anti-ERK pAb; dilution 1:200; Bioworld, Minneapolis, MN, USA). Subsequently, the sections were washed three times with PBS, each time for 5 min. The sections were incubated at 37°C for 30 min with the secondary antibody (biotinylated goat anti-rabbit IgG; dilution 1:500; ZSGB-BIO, Beijing, China) followed by washing three times with PBS, each time for 5 min. Finally, the tissue sections were dropwise added 100 μL DAB Plus Substrate buffer (Thermo Fisher Scientific, USA) containing 2% (v/v) DAB Plus Chromogen (Thermo Fisher Scientific, USA), and the nuclei were counterstained with hematoxylin. The tissue sections were dehydrated, transparented, and sealed with ethanol, xylene, and Neutral gum.

Statistical Analysis

Cytokine production was expressed as the mean cytokine concentration ± SD. Statistical analysis was performed with Student's unpaired *t*-test. The differences between groups were

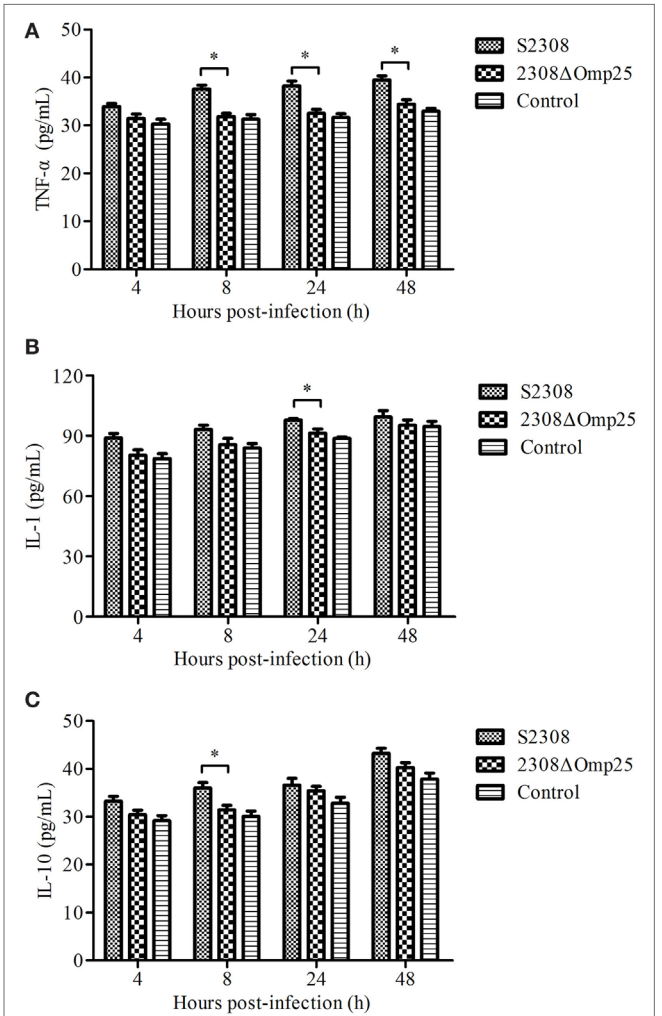

FIGURE 1 | Production of cytokines in S2308 and 2308ΔOmp25-infected HPT-8 cells. HPT-8 cells were infected with S2308 or 2308ΔOmp25 at a 100:1 MOI. Control group was uninfected. At 4, 8, 24, and 48 h post-infection, supernatant samples were collected and TNF-α **(A)**, IL-1 **(B)**, and IL-10 **(C)** levels were assayed by ELISA. Cytokines production is expressed as the mean cytokine concentration ± SD for each group of cells. Significant differences between the S2308 and 2308ΔOmp25 are indicated by * ($P < 0.05$). OMP25, outer membrane protein 25; IL-1, interleukin-1; IL-10, interleukin-10; MOI, multiplicity of infection; TNF-α, tumor necrosis factor-α.

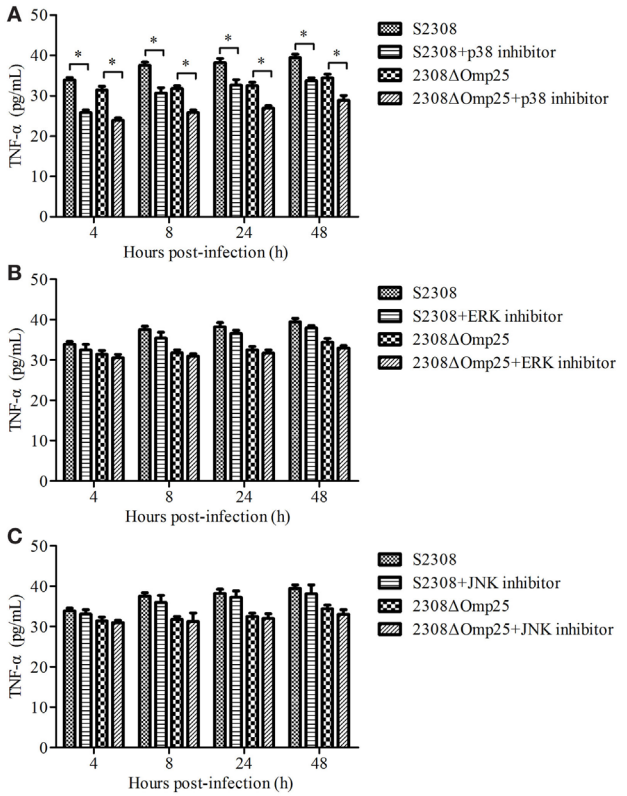

FIGURE 2 | Production of TNF-α in S2308 and 2308ΔOmp25-infected HPT-8 cells. HPT-8 cells were pre-treated with p38 inhibitor (10 μM) **(A)**, ERK inhibitor (10 μM) **(B)**, or JNK inhibitor (10 μM) **(C)** at 37°C for 1 h and then infected with S2308 or 2308ΔOmp25 at a 100:1 MOI. At 4, 8, 24, and 48 h post-infection, supernatant samples were collected and TNF-α levels were assayed by ELISA. TNF-α production is expressed as the mean cytokine concentration ± SD for each group of cells. Significant differences between the S2308 and 2308ΔOmp25 are indicated by * ($P < 0.05$). OMP25, outer membrane protein 25; JNK, Jun-N-terminal kinase; MOI, multiplicity of infection; TNF-α, tumor necrosis factor-α.

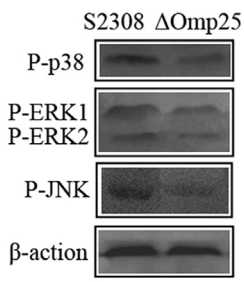

FIGURE 3 | Activation of p38, ERK1/2, and JNK kinases in S2308 and 2308ΔOmp25-infected HPT-8 cells. HPT-8 cells were infected with S2308 or 2308ΔOmp25 at a 100:1 MOI. At 24 h post-infection, supernatant was discarded, and cells were lysed. Then, the activation of p38, ERK1/2, and JNK kinases was detected by Western blotting. Infections with 2308ΔOmp25 induced a markedly weaker stimulation of p38, ERK1/2, and JNK kinases, with S2308 demonstrating a slightly higher capacity of activation than 2308ΔOmp25. OMP25, outer membrane protein 25; JNK, Jun-N-terminal kinase; MOI, multiplicity of infection.

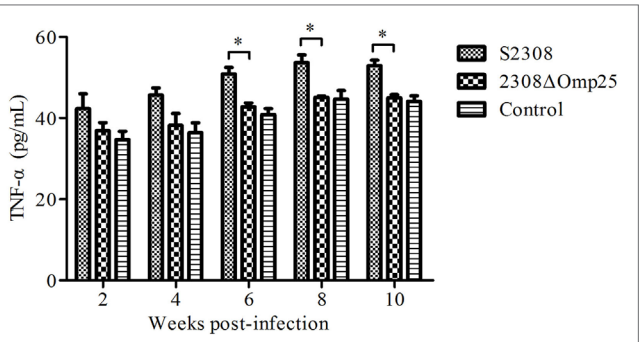

FIGURE 4 | Production of TNF-α in S2308 and 2308ΔOmp25 vaccinated BALB/c mice. BALB/c mice were inoculated with 1×10^6 CFU of S2308 or 2308ΔOmp25. Control groups received PBS. At 2, 4, 6, 8, and 10 weeks post-immunization, serum samples were collected ($n = 5$ per time point) and TNF-α levels were assayed by ELISA. TNF-α production is expressed as the mean cytokine concentration ± SD for each group of mice. Significant differences between the S2308 and 2308ΔOmp25 are indicated by * ($P < 0.05$). OMP25, outer membrane protein 25; TNF-α, tumor necrosis factor-α; PBS, phosphate-buffered saline.

analyzed by analysis of variance (ANOVA) followed by Tukey's honestly significant difference post-test, by comparing all the groups to one another. Results expressed as percentages were analyzed by the Fisher test. The differences between groups were analyzed by ANOVA using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). P values of <0.05 were considered statistically significant.

RESULTS

2308ΔOmp25 Induces Lower Levels of Cytokines

To detect the expression level of cytokines, we collected supernatant from HPT-8 cells infected with S2308 and 2308ΔOmp25 and then monitored expression levels of cytokine TNF-α, IL-1, and IL-10 by ELISA. Supernatant from HPT-8 cells infected with S2308 produced higher amounts of TNF-α (**Figure 1A**), IL-1 (**Figure 1B**), and IL-10 (**Figure 1C**) than did supernatant from uninfected cells ($P < 0.05$) and this difference increased with time. Slightly higher cytokine production levels were observed in 2308ΔOmp25-infected cells than in control cells (**Figure 1**), but there was no significant difference between 2308ΔOmp25 group and control group ($P > 0.05$).

Expression of TNF-α Associated with p38 Branch

We next evaluated which branch associated with secretion of TNF-α. HPT-8 cells were pre-incubated for 1 h with 10 μM p38, 10 μM ERK, or 10 μM JNK inhibitors and then infected with S2308 or 2308ΔOmp25 for 4, 8, 24, and 12 h. The levels of TNF-α were assessed in the supernatants of the S2308 or 2308ΔOmp25-infected p38 inhibitor, ERK inhibitor or JNK inhibitor-treated cells. At 4, 8, 24, and 12 h, the S2308 or 2308ΔOmp25-infected p38 inhibitor-treated cells produced higher levels of TNF-α than S2308 or 2308ΔOmp25-infected

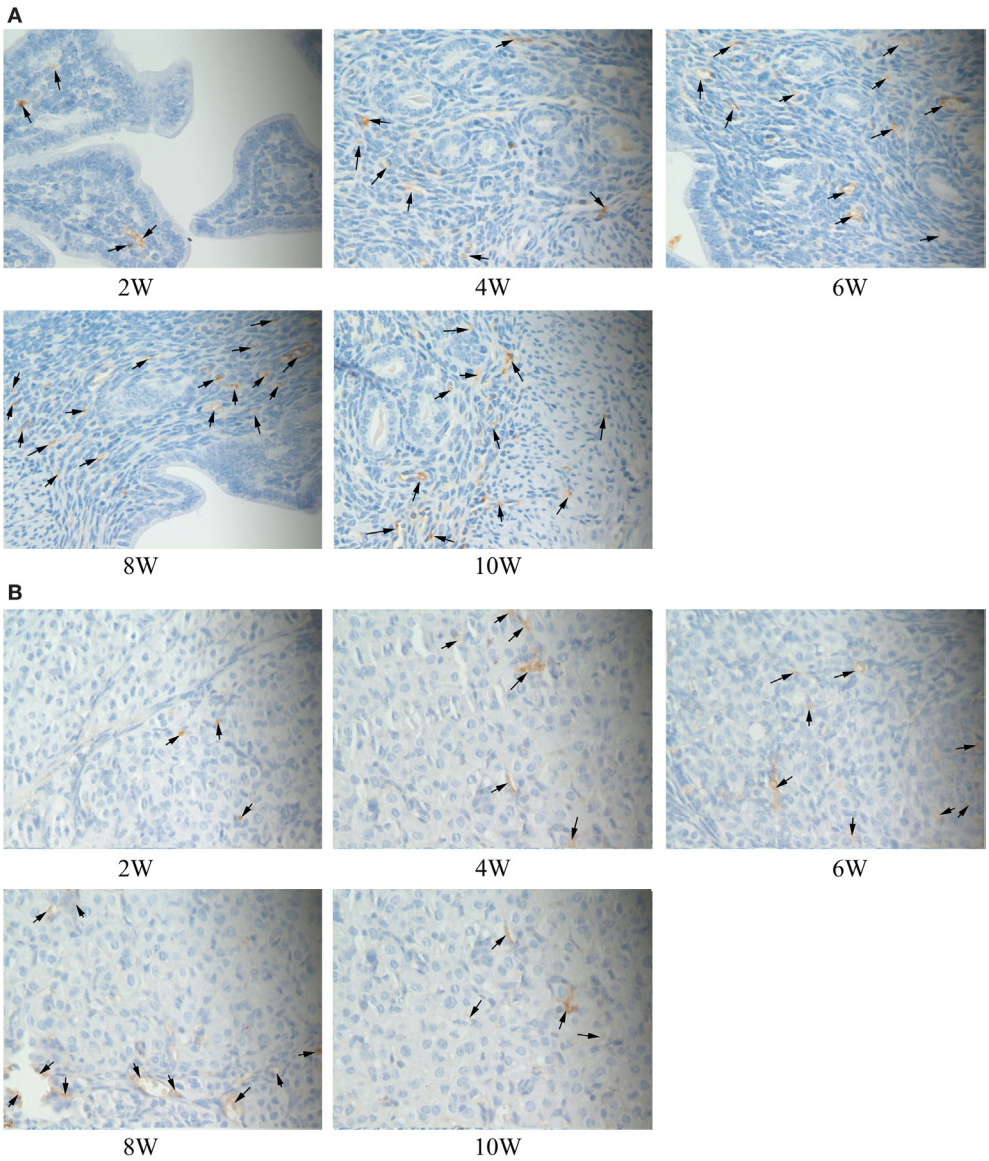

FIGURE 5 | Immunohistochemical detection of phosphorylation proteins in p38 signal pathway branch. BALB/c mice were inoculated with 1×10^6 CFU of S2308 or 2308ΔOmp25. At 2, 4, 6, 8, and 10 weeks post-immunization, uterus tissues were removed and detected by immunohistochemistry. The phosphorylation proteins in p38 signal pathway had been detected and found in the uterus tissues of S2308 immunized mice **(A)**. But the immune complexes of phosphorylation proteins in p38 signal pathway were weakly stained in the uterus tissues of 2308ΔOmp25 immunized mice **(B)**. OMP25, outer membrane protein 25.

cells ($P < 0.05$; **Figure 2A**). However, there was no significant difference between S2308 or 2308ΔOmp25-infected ERK inhibitor and JNK inhibitor-treated cells and S2308 or 2308ΔOmp25-infected cells ($P > 0.05$; **Figures 2B,C**). These results showed that p38 branch could induce secretion of TNF-α in S2308 or 2308ΔOmp25-infected cells.

2308ΔOmp25 Activates Weak MAPK Pathway

To assess activation of p38 and ERK1/2 kinases, HPT-8 cells were infected with the 2308ΔOmp25 mutant and the parental strain S2308 at a MOI of 100. We found that at 24 h post-infection, the activation process triggered by S2308 resulted in a phosphorylation of the p38, ERK1/2, and JNK kinases (**Figure 3**). Infections with 2308ΔOmp25 induced a markedly weaker stimulation of p38, ERK1/2, and JNK kinases, with S2308 demonstrating a slightly higher capacity of activation than 2308ΔOmp25 mutant (**Figure 3**). These results show that OMP25 involved in activating of MAPK pathway.

2308ΔOmp25 Induces Lower Levels of TNF-α in Peripheral Blood of Mice

To detect the expression level of TNF-α in animal, we collected sera from mice inoculated with S2308, 2308ΔOmp25, or PBS

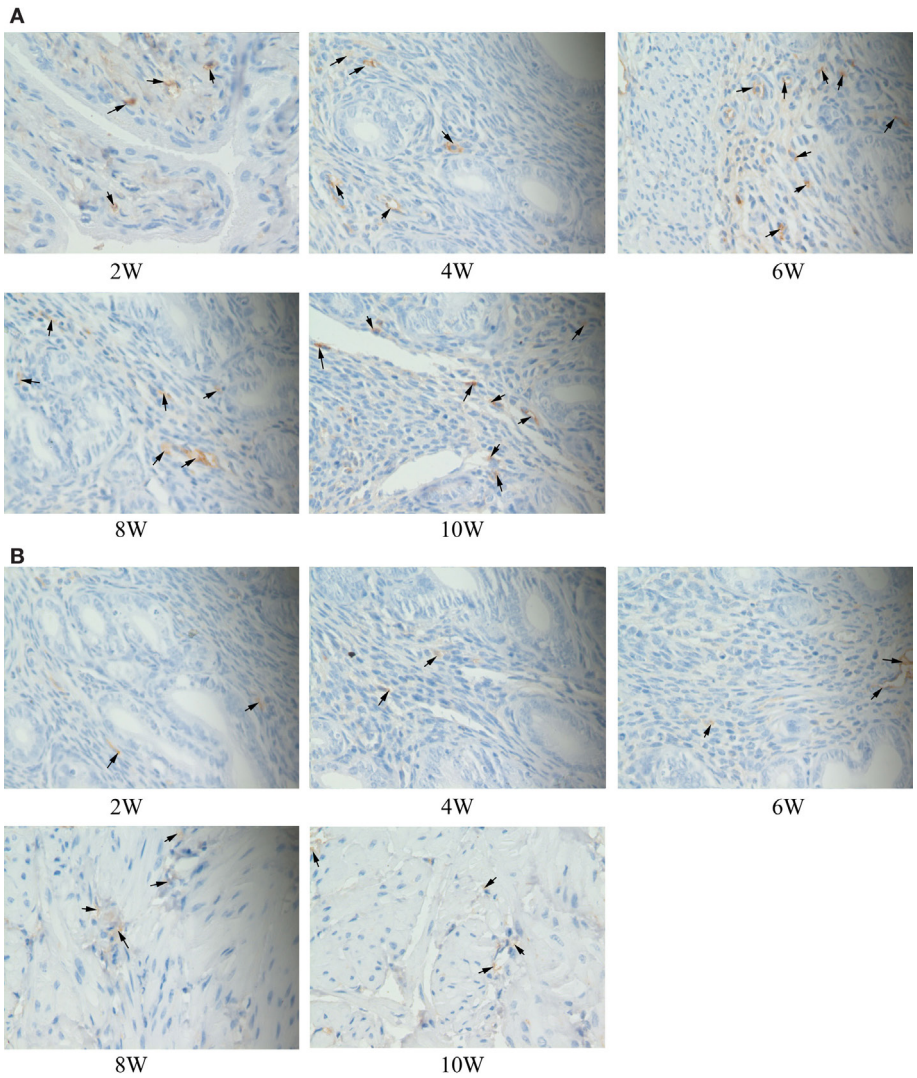

FIGURE 6 | Immunohistochemical detection of phosphorylation proteins in ERK signal pathway branch. BALB/c mice were inoculated with 1×10^6 CFU of S2308 or 2308ΔOmp25. At 2, 4, 6, 8, and 10 weeks post-immunization, uterus tissues were removed and detected by immunohistochemistry. The phosphorylation proteins in ERK signal pathway had been detected and found in the uterus tissues of S2308 immunized mice **(A)**. But the immune complexes of phosphorylation proteins in ERK signal pathway were weakly stained in the uterus tissues of 2308ΔOmp25 immunized mice **(B)**. OMP25, outer membrane protein 25.

and then measured expression levels of TNF-α by ELISA. Serum samples from mice inoculated with S2308 produced higher amounts of TNF-α (**Figure 4**) than did serum samples from mice inoculated with 2308ΔOmp25 or PBS ($P < 0.05$) and this difference increased with time. Slightly higher cytokine production levels were observed in 2308ΔOmp25 immunized mice than in PBS immunized mice ($P > 0.05$) (**Figure 4**). Total TNF-α levels increased with time.

Immunohistochemical Staining

The immune complexes of p38 and ERK phosphorylation proteins were located in the cytoplasm of the uterus tissues, and they were strongly stained as tan or brownish yellow. The phosphorylation proteins in p38 and ERK signal pathways had been detected and found in the uterus tissues of S2308 immunized mice (**Figures 5A** and **6A**). But the immune complexes of phosphorylation proteins in p38 and ERK signal pathways were weakly stained in the uterus tissues of 2308ΔOmp25 immunized mice (**Figures 5B** and **6B**). These results showed that S2308 was able to activate phosphorylation of p38 and ERK in BABL/c mice. However, 2308ΔOmp25 could weakly activate phosphorylation of p38 and ERK.

DISCUSSION

Brucella could infect many kinds of cells, but the main host cells are macrophages and trophoblasts (26). In animals, abortion is associated with a rapid proliferation of *Brucella* within the placenta. Trophoblasts are primary cellular targets for *Brucella* in the natural host. The presence of high bacterial loads within

placental trophoblasts ultimately results in disruption of the placenta and infection of the fetus. Omps of *Brucella* play an important role in the process of pathogen (27). Omp25 an important Omp, it involved in growth, colonization, and proliferation of *Brucella* (28). The Omp25 mutant strain attenuated *Brucella* infection abilities and changed the response of host cells (10). TNF-α is one of important factors that involved in many of the body's immune and inflammatory responses (29). The expression of TNF-α was related with Omp25 and ERK pathway (27). Our results found that there was a significant difference in the expression of TNF-α between S2308 and 2308ΔOmp25. These results suggested that Omp25 may play an important role in the progress of expression of TNF-α when *Brucella* infected cells.

Phosphorylation of MAPK p38 pathway acts as a "switch" role in regulating the production of cytokines. The whole process is through a typical pathway: MAPKKK → MAPKK → MAPK (12). SB208035 is the inhibitor of p38 signal pathway. It has been reported that p38 inhibitors could inhibit the production of IL-1, IL-10, and TNF-α in peripheral blood cells (30). TNF-α could activate p38 pathway (31). Our results showed that 2308ΔOmp25 was weaker to activate p38 pathway in MAPK signal pathway than S2308. The reason may be the low expression of TNF-α in 2308ΔOmp25. It suggested that the production of TNF-α may be related with the activation of p38 pathway. When we used p38 inhibitors to deal with HPT-8 cells, and detected the expression of TNF-α in the culture medium. We found that the expression of TNF-α has been inhibited. It suggested that when S2308 or 2308ΔOmp25-infected HPT-8 cells, the p38 pathway in the MAPK signal pathway was related with the expression of TNF-α.

Brucella can lead many organs happening pathological damages, particularly in chronic infection stage. In the experiment, we found that the expressions of TNF-α significantly increased when S2308 or 2308ΔOmp25-infected mice or HPT-8 cells. It showed that TNF-α was one of cytokines that happened significantly change when *Brucella* infected hosts (organisms and cells), and it may be related to the progress of inflammation. From the result of immunohistochemistry, we found that phosphorylation proteins of p38 and ERK signal pathway in uterine tissues of mice. In addition, we also found that the immune complexes of phosphorylation proteins in p38 and ERK signal pathways were weakly stained in the uterus tissues of 2308ΔOmp25 immunized mice. These results suggested that Omp25 participated in phosphorylation of p38 and ERK signal pathway proteins. Previous studies have reported that the MAPKs are a target for immune intervention by virulent smooth *Brucella* (32). Our results further suggest that Omp25 played an important role in activating MAPK signal pathway in smooth *Brucella*.

In conclusion, we found that *Brucella* can affect the expression of TNF-α by activating MAPK signal pathway, and the expression was higher in S2308 than 2308ΔOmp25. It suggested that Omp25 played an important role in activating MAPK signal pathway when *Brucella* infected hosts. These results established theoretical foundation for further studying pathogenic mechanisms and proinflammatory mechanisms of *Brucella*.

ETHICS STATEMENT

The study was approved by the Institutional Committee of Post-Graduate Studies and Research at Shihezi University, China (No. 2012-9). All efforts were made to minimize animal suffering.

AUTHOR CONTRIBUTIONS

JZ, YZ, LL, and HZ designed the experiments. ZL, JL, YW, TL, and LL performed the experiments and analyzed the data. XS, KW, CW, LL, CC, and HZ contributed reagents/materials/analysis tools. JZ, YZ, ZL, LL, and HZ wrote and revised the paper.

FUNDING

This work was supported by grants from the National key Research and Development Program of China (2017YFD0500304), the National Natural Science Foundation of China (31460650, 31602080, 31360610, and 31402166), the Training Program for Excellent Young Teachers Colleges and Universities of Corps (CZ027202), the Foundation of the Technology Department of Henan Province (172102310335), and the Foundation of the Education Department of Henan Province (16A230013).

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Conflict of Interest Statement: 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: 06 March 2018
doi: 10.3389/fvets.2018.00028

Limitations of Using IL-17A and IFN-γ-Induced Protein 10 to Detect Bovine Tuberculosis

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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: *21 September 2017*
Accepted: *09 February 2018*
Published: *06 March 2018*

Citation:
Xin T, Gao X, Yang H, Li P, Liang Q, Hou S, Sui X, Guo X, Yuan W, Zhu H, Ding J and Jia H (2018) Limitations of Using IL-17A and IFN-γ-Induced Protein 10 to Detect Bovine Tuberculosis. Front. Vet. Sci. 5:28. doi: 10.3389/fvets.2018.00028

Bovine tuberculosis (bTB) is primarily caused by infection with *Mycobacterium bovis*, which belongs to the *Mycobacterium tuberculosis* complex. The airborne route is considered the most common for transmission of *M. bovis*, and more than 15% of cattle with bTB shed the *Mycobacterium*, which can be detect by nested PCR to amplify mycobacterial *mpb*70 from a nasal swab from a cow. To screen for cytokines fostering early and accurate detection of bTB, peripheral blood mononuclear cells were isolated from naturally *M. bovis*-infected, experimentally *M. bovis* 68002-infected, and uninfected cattle, then these cells were stimulated by PPD-B, CFP-10-ESAT-6 (CE), or phosphate-buffered saline (PBS) for 6 h. The levels of interferon gamma (IFN-γ), IFN-γ-induced protein 10 (IP-10), IL-6, IL-12, IL-17A, and tumor necrosis factor alpha mRNA were measured using real-time PCR. To explore the cytokines associated with different periods of *M. bovis* infection, cattle were divided into three groups: PCR-positive, PCR-negative, and uninfected using the tuberculin skin test, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IFN-γ release assay (IGRA), CFP-10/ESAT-6 (CE)-based IGRA, and nested PCR. The expression of IP-10, IL-17A, and IFN-γ proteins induced by PPD-B, CE, or PBS was detected by ELISA. The results showed that levels of PPD-B-stimulated IL-17A and IP-10 (mRNA and protein), and CE-induced IP-10 (mRNA and protein) were significantly higher in cattle naturally or experimentally infected with *M. bovis* than in those that were uninfected. The levels of PPD-B- or CE-induced IL-17A and IP-10 (protein) could be used to differentiate *M. bovis*-infected calves from uninfected ones for 6 to 30 weeks post-infection, whereas PPD-B- and CE-induced IP-10 and IL-17A mRNA expression could be used to differentiate *M. bovis*-infected calves from uninfected ones between 6 and 58 weeks post-infection. However, CE-induced IL-17A (protein) was not a reliable indicator of *M. bovis* infection in cattle that were confirmed positive for infection by nested PCR. Furthermore, the levels of PPD-B- or CE-induced IP-10 and IL-17A protein were lower than IFN-γ in *M. bovis*-infected cattle. Therefore, IL-17A and IP-10 protein are not suitable biomarkers for bTB. Antigen-induced IP-10 mRNA should be analyzed further for their potential to be used in the diagnosis of bTB.

Keywords: bovine tuberculosis, IFN-γ-induced protein 10, IL-17A, interferon gamma, nested PCR

INTRODUCTION

Bovine tuberculosis (bTB) is primarily caused by infection with *Mycobacterium bovis*, which belongs to the *Mycobacterium tuberculosis* complex (MTC) that includes *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. caprae*, *Mycobacterium pinnipedii*, *M. mungii*, and *M. canetti* (1, 2). *M. bovis* could infect wild animals and has a relatively wider host range than *M. tuberculosis* (3). Humans can also be infected with *M. bovis* through the ingestion of raw milk products or inhalation of aerosols (4). The airborne route of infection is considered the most common for transmission of *M. bovis*, and more than 15% of cattle infected with *M. bovis* shed the *Mycobacterium* (5), mainly early in infection (6). Therefore, early and accurate detection of bTB is important to control transmission of *M. bovis* spreading to other animals (7).

The traditional bTB diagnostic methods are the tuberculin skin test (TST) and interferon gamma (IFN-γ) release assay (IGRA). Both TST and IGRA are based on the detection and comparison of cell-mediated responses induced by bovine purified protein derivatives (PPD-B) and avian purified protein derivatives (PPD-A) (8). PPD-B is obtained from a culture of virulent *M. bovis*, but shared antigens with non-pathogenic environmental *mycobacteria* can reduce the specificity of the TST (9). Although PPD-A is used in IGRA to exclude environmental *Mycobacterium* infections, it has failed to detect some *M. bovis*-infected cattle in bTB- and paratuberculosis- coprevalent dairies. Therefore, TST and IGRA based on *M. bovis*-specific antigens such as CFP-10 and ESAT-6 were established to obtain higher specificity in the diagnosis of bTB (8–11). Considering that neither TST nor IGRA can differentiate between stages in the progression of bTB, a nested PCR assay based on the amplification of a fragment of *mpb*70 was established and used to detect mycobacteria in milk, nasal exudates, and bronchoalveolar lavage (BAL) fluid (12).

To develop more effective and accurate methods to diagnose tuberculosis, new candidate biomarkers have emerged recently for the diagnosis of this disease in humans and cattle. These potential biomarkers include tumor necrosis factor alpha (TNF-α), interleukin-2, IL-1β, IL-17A, and IFN-γ-induced protein 10 (IP-10). IP-10 and IL-17A, particularly, have the potential to be used to differentiate between active and latent TB in humans. However, there are only a few studies on screening for bTB-related cytokines.

In this study, we screened for cytokines related to bTB and explored whether cytokines could be used to detect *M. bovis*-infected cattle [including those that were PCR-positive (PCR-P) and those that were PCR-negative (PCR-N)]. First, we isolated peripheral blood mononuclear cells (PBMCs) from naturally *M. bovis*-infected, from experimentally *M. bovis* 68002-infected and from uninfected cattle, and then we stimulated the PBMCs using PPD-B, CFP-10-ESAT-6 (CE), or phosphate-buffered saline (PBS) for 6 h. The levels of IFN-γ, IP-10, IL-6, IL-12, IL-17A, and TNF-α mRNA transcripts were determined using real-time PCR. To explore whether IP-10 and IL-17A can be used to detect all *M. bovis*-infected cattle, cattle were divided into three groups: nested PCR-P, nested PCR-N, and uninfected cattle as determined by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, CE-based IGRA, and nested PCR. The expression of IP-10, IL-17A, and IFN-γ proteins induced by PPD-B or CE was detected by ELISA.

MATERIALS AND METHODS

Ethics Approval

The six Luxi beef calves used in the present study were treated carefully and according to the protocol approved by the Animal Care and Use Committee of the China Institute of Veterinary Drug Control. The animal ethics committee approval number is SYXK (2005-0021).

Bacterial Species and Plasmids

Mycobacterium bovis 68002 was isolated in China and preserved in the China Institute of Veterinary Drug Control, Beijing, China. *M. bovis* 68002 can cause tubercles in the lungs, liver and other organs after intravenous injection into calves. It was defined as highly virulent and used to prepare bovine tuberculin in China. Bovine tuberculin (PPD-B, Harbin Pharmaceutical Group, Heilongjiang Province, China) was used in the TST. The CFP-10/ESAT-6/TB10.4 protein cocktail (0.5 mg/ml, endotoxin less than 10 EU/mg) was prepared in the Institute of Animal Sciences (IAS-CAAS), and used to make the protein cocktail-based skin test. CFP-10-ESAT-6 (20 μg/ml, CE expressed and purified in our lab, with a Trx-His-S tag at the N-terminus, with endotoxin at a concentration less than 10 EU/mg) was used in the CE-based IGRA. PET [20 μg/ml, the tag protein of the pET(32a) + vector was expressed and purified in our lab, with endotoxin at a concentration of less than 10 EU/mg] was used as control for CE. Bovine IFN-γ (500 μg/ml, expressed and purified in our lab, with a 6-His tag at the N-terminus, with endotoxin at a concentration less than 10 EU/mg) was used as a reference standard in the IGRA for the quantitative analysis of IFN-γ in bovine plasma.

Skin Test Procedure

The TST was performed as the Chinese diagnostic standard for bTB (GB/T 18645-2002), and the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test was previously established in our laboratory. As described in an earlier report (9), PPD-B (PPD-B, 2,500 IU/cattle) and the CFP-10/ESAT-6/TB10.4 protein cocktail were intradermally injected (0.1 ml each) into two sites on the same side of a cow's neck. Differences in skin thicknesses (mm) pre- and 72 h post-injection were calculated. With the GB/T 18645-2002, if the difference in skin thicknesses was ≥4 mm, the cattle were considered as *M. bovis*-infected; if the difference in skin thicknesses was <2 mm, the cattle were considered *M. bovis*-uninfected; if the difference in skin thicknesses then were between 2 and 4 mm, the cattle were suspected to be *M. bovis*-infected, and were retested after an interval of 60 days. If the difference in skin thicknesses was ≥2 mm, the cattle were considered *M. bovis*-infected. The cutoff value for the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test was obtained by ROC analysis based on 125 uninfected cattle and 117 *M. bovis*-infected cattle, whose results were confirmed by nested PCR (9). For the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, if the difference in skin thicknesses was ≥1.1 mm, the cattle were considered *M.*

bovis-infected; if the difference in skin thicknesses was <1.1 mm, the cattle were considered free from bTB.

Bovine IFN-γ, IP-10, and IL-17A ELISAs

All cattle tested by skin test were also tested by IGRA (*Mycobacterium bovis* Gamma Interferon Test Kit for cattle, Bovigam, Prionics AG, Schlieren, Switzerland) according to the manufacturer's instructions. Briefly, heparinized blood was collected from each cow and dispensed into a 24-well cell culture plate (1.5 ml/well, five wells for each cow), and stimulated with 100 μl of PPD-A (Avian Tuberculin PPD, 300 μg/ml, Prionics AG, Schlieren, Switzerland), PPD-B (Bovine Tuberculin PPD, 300 μg/ml, Prionics AG, Schlieren, Switzerland), PBS, PET, and CE, respectively. Plasma was collected from each well after incubation for 24 h at 37°C in 5% CO_2.

The IFN-γ in plasma was detected using a *Mycobacterium bovis* Gamma Interferon Test Kit. PPD-B-simulated blood plasma having an OD value more than 0.100 above that of plasma stimulated with PPD-A and PBS, and CE-stimulated blood plasma having an OD value more than 0.100 above that of plasma stimulated with PBS indicated cattle were *M. bovis*-infected. The cutoff value for the CE-based IGRA was obtained in our lab by ROC analysis based on 96 uninfected cattle and 258 *M. bovis*-infected cattle, whose results were confirmed by nested PCR. The IFN-γ in plasma could be quantified using recombinant bovine IFN-γ as the reference standard.

IFN-γ-induced protein 10 and IL-17A in plasma were detected using Bovine IP-10 ELISA VetSet (Kingfisher Biotech Inc., Saint Paul, MN, USA) and Bovine IL-17A ELISA VetSet (Kingfisher Biotech Inc., Saint Paul, MN, USA).

Nested PCR Analysis

Nested PCR was conducted to confirm the *M. bovis* infection as reported before (11). Briefly, nasal swabs of each cow were collected, and immersed immediately into 2 ml of sterile PBS. Then, DNA was extracted and dissolved in 20 μl of Tris buffer, and a nested PCR was performed to amplify a region of the *mpb70* gene of MTC bacteria as described previously.

Analysis of Cytokine Gene Expression by Real-time PCR

Peripheral blood mononuclear cells were isolated from heparinized blood using the density gradient centrifugation method with Ficoll-Hypaque Density Solution (TIAN JIN HAO YANG Biological Manufacture Co., Ltd., Tianjin). Red blood cells (RBCs) were separated from PBMCs by RBC lysis buffer (Invitrogen, USA). After that, PBMCs were washed with PBS three times and resuspended in RPMI1640 medium [with 10% (vol/vol) fetal bovine serum, 25 mM HEPES buffer, 2 mM L-glutamine, 100 U/ml of penicillin, and 0.1 mg/ml streptomycin]. The PBMCs were seeded into a 48-well cell culture plate at 4×10^6 cells in a total volume of 250 μl. Following isolation, PBMCs were subsequently incubated at 37°C in 5% CO_2 with PPD-B, CE, PET, or PBS for 6–8 h. After incubation, the cells were immediately lysed with 750 μl of TRIzol (Invitrogen, USA), and total RNA was isolated using an RNAeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol and eluted from the column with 50 μl of DEPC water. One microliter of RNA inhibitor (RI) was added to the RNA solution to protect against RNAase. The RNA was quantified with a spectrophotometer. One microgram of RNA was pre-mixed with 0.5 μg of Oligo dT (15) (Promega, USA) and DEPC water. A total of 12 μl of the pre-mixture was warmed in a water bath at 70°C for 5 min, and then immediately cooled in an ice bath for 5 min. The full-length cDNA was reversely transcribed in a 25-μl reaction mixture containing a pre-mixture of 5 μl of 5 × MLV Buffer (TaKaRa, Japan), 1.25 μl of 10 mM dNTP (TaKaRa, Japan), 0.5 μl of RI (TaKaRa, JPN), 1 μl of M-MLV (Promega, USA), and 5.25 μl of DEPC water. The mixture was incubated at 30°C for 10 min, followed by 42°C for 60 min, and then at 70°C for 5 min. The cDNA was stored at −80°C.

Real-time PCR was carried out with TaqMan® Real-Time PCR Master Mix (ABI). The primer and probe sequences (**Table 1**) were designed and synthesized by Shanghai GengCore Biotechnologies Co., Ltd. All reactions were performed in a 25-μl volume containing 10 μl of TaqMan Real-Time PCR Master Mix, 0.2 μl of Primer F (10 μM), 0.2 μl of Primer R (10 μM), 0.2 μl of Taqman-Probe (20 μM), and 1 μl of template cDNA. All reactions were run in triplicate and were carried out in 96-well Rxn Plate (ABI, USA) sealed with optical adhesive film (ABI, USA) on an ABI7900HT Fast Real-Time PCR System (ABI, USA). The instrument was programmed to cycle at 95°C for 2 min, followed by 40 cycles of 15 s at 95°C and then 1 min at 60°C. Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method, with *β-actin* as the reference gene, and the PBS-stimulated sample from each cow was used to calibrate PBMCs responses.

Animals and Infection

To preliminarily screen for the cytokines related to *M. bovis* infection, 10 naturally *M. bovis*-infected and 5 uninfected Holstein cows were determined by TST, CFP-10/ESAT-6/TB10.4 protein

TABLE 1 | Primer and probe sequences used in real-time PCR.

Primer and probe	Sequences
β-actin-F	5′-GCCCTGAGGCTCTCTTCCA-3′
β-actin-R	5′-GCGGATGTCGACGTCACA-3′
β-actin-P	5′-FCATGGAATCCTGCGGCATTCACG-BHQ1-3′
IFN-γ-F	5′-GCTGATTCAAATTCCGGTGGA-3′
IFN-γ-R	5′-CAGGCAGGAGGACCATTACG-3′
IFN-γ-P	5′-FTCTGCAGATCCAGCGCAAAGCC-BHQ1-3′
IP-10-F	5′-GTCCTTAGAAAAACTTGAAGTCATTCC-3′
IP-10-R	5′-TTCTTGATGGTCTTAGATTCTGGATTC-3′
IP-10-P	5′-FCCCACGTGTCGAGATTATTGCCACAA-BHQ1-3′
IL-6-F	5′-AAATGGAGGAAAAGGACGGA-3′
IL-6-R	5′-TGATTTCCCTCATACTCGTTCTG-3′
IL-6-P	5′-FCTTCCAATCTGGGTTCAATCAGGCGA-BHQ1-3′
IL-12p40-F	5′-TGCACAAGCTCAAGTATGAAAACTA-3′
IL-12p40-R	5′-ACCTCCACCTGCCGAGAAT-3′
IL-12p40-P	5′-FCAGGGACATCATCAAACCAGACCCAC-BHQ1-3′
IL-17A-F	5′-AGAAGGCCCACCGATTATCA-3′
IL-17A-R	5′-CCACCTTCCCTTCAGCATTGA-3′
IL-17A-P	5′-FACTCTCCACCGCAATGAGGACCCTG-BHQ1-3′
TNF-α-F	5′-AGAAATTAGGGATGTAGGGAAGTGA-3′
TNF-α-R	5′-CTTGTGGACCCCAGGGAGTT-3′
TNF-α-P	5′-FTGGACAACGGGCCACCAACCA-BHQ1-3′

cocktail-based skin test, IGRA, and CE-based IGRA. The PBMCs from each cow were isolated and treated with PPD-B, CFP-10-ESAT-6 (CE), or PBS for 6–8 h. The levels of IFN-γ, IP-10, IL-6, IL-12, IL-17A, and TNF-α mRNA transcripts were measured using real-time PCR.

To verify the cytokines related to *M. bovis* infection, six male Luxi beef calves aged 1–2 months from a bTB-free dairy farm were randomly selected for testing by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, and CE-based IGRA, and maintained in a biosafety level-3 facility. Three calves were intravenously injected with 10^6 C *M. bovis* 68002; another three calves were intravenously injected with PBS to serve as uninfected controls. Animals in each group were monitored for 1 year, and tested using TST and CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test before injection, at 8 and 32 weeks post-infection. These six calves were also tested using IGRA and CE-based IGRA before injection, and at 8, 24, 48, 72, and 96 h, and 2, 4, 6, 8, 12, 16, 20, 26, 30, 34, 38, 45, 50, and 58 weeks post-infection. The levels of IFN-γ, IP-10, and IL-17A mRNA transcripts and protein induced by PPD-B, CE, PET, or PBS were determined before injection and at 6, 26, 30, and 58 weeks post-infection.

To explore whether PPD-B- or CE-induced IP-10, IP-17A, and IFN-γ proteins could be used to determine the stage of *M. bovis* infection, more than 1,000 Holstein cows were detected by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, CE-based IGRA. The nasal swabs from *M. bovis*-infected cows were collected and detected by nested PCR, then the PCR-P swabs were confirmed by *mycobacteria* culture. The *M. bovis*-infected cows could be divided into two groups (**Table 2**): PCR-P (21 cows determined positive for *M. bovis* by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, CE-based IGRA, and nested PCR were selected for the study) and PCR-N (21 cows determined positive for *M. bovis* by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA and CE-based IGRA, but negative by nested PCR were selected for the study). Heparinized whole blood from each cow was collected and stimulated with PPD-B, CE, or PBS for 24 h, and the plasma was collected from each well. The levels of IP-10, IL-17A, and IFN-γ protein expression in plasma were determined by ELISA.

TABLE 2 | Characteristics of PCR-positive (PCR-P), PCR-negative (PCR-N) and NC groups.

Diagnostic methods	*Mycobacterium bovis*-infected cattle		NC
	PCR-P	PCR-N	
TST	+	+	−
CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test	+	+	−
IGRA	+	+	−
CE-based IGRA	+	+	−
Nested PCR	+	−	−

TST, tuberculin skin test; IGRA, interferon gamma (IFN-γ) release assay; CE-based IGRA, CFP-10/ESAT-6 (CE)-based IFN-γ release assay; PCR-P, cattle determined to be infected with M. bovis by nested PCR; PCR-N, cattle determined not to be infected with M. bovis by nested PCR; NC, cattle from a bovine tuberculosis-free dairy farm considered to be uninfected with M. bovis.

The uninfected animals were from a bTB-free dairy farm and determined free from bTB by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, CE-based IGRA, and nested PCR. All animals used in this study were determined free of paratuberculosis by *Mycobacterium paratuberculosis* Antibody Test Kit for Cattle (IDEXX Montpellier SAS, France) and free of brucellosis by Svanova Brucella-Ab C-ELISA (Svanova Biotech AB, Uppsala, Sweden).

Bacteriological Analysis

Three *M. bovis* 68002-infected calves and one naturally *M. bovis*-infected cow were slaughtered after the experiment. Tissue samples from the lung, liver, spleen, bronchial lymph nodes, and kidney were collected and analyzed using nested PCR, *Mycobacterium* culture, and hematoxylin–eosin staining.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA) followed by Kruskal–Wallis test or Spearman correlation using GraphPad Prism 5 software (San Diego, CA, USA). A p value of <0.05 (two-tailed) was considered significant.

RESULTS

Screening of Cytokines Related to *M. bovis* Infection in Naturally *M. bovis*-Infected Cattle

To screen for cytokines related to *M. bovis* infection, 10 naturally *M. bovis*-infected and five uninfected cows were determined by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, and CE-based IGRA. Levels of IFN-γ, IP-10, IL-6, IL-12, IL-17A, and TNF-α mRNA in PBMCs from each cow stimulated with PPD-B, CE, PET, or PBS were measured using real-time PCR. The levels of IFN-γ, IP-10, IL-6, IL-17A, and TNF-α mRNA were significantly higher in both PPD-B- and CE-stimulated PBMCs from *M. bovis*-infected cows than from uninfected cows (**Figures 1A,B**), whereas the levels of IL-12 mRNA were significantly higher only in PPD-B-stimulated PBMCs from *M. bovis*-infected cows relative to those in uninfected cows (**Figure 1A**). The transcript levels of these six cytokines induced by PET were similar to those exposed to PBS, and there was no difference in these levels between *M. bovis*-infected and -uninfected cows (**Figure 1C**). The transcript levels of these six cytokines induced by PPD-B in PBMCs from both *M. bovis*-infected and -uninfected cows were higher than those induced by CE (**Figures 1A,B**). The levels of IFN-γ, IP-10, and IL-17A transcripts induced by PPD-B or CE in PBMCs from *M. bovis*-infected cows were higher than those of IL-6, IL-12, and TNF-α.

Correlations among the levels of transcripts of the six cytokines were determined, and IFN-γ, IP-10, IL-17A, and TNF-α induced by PPD-B or CE showed good correlation with each other (**Table 3**). IL-6 mRNA induced by PPD-B or CE showed good correlation with IL-17A, IFN-γ, and TNF-α mRNA. The levels of PPD-B-induced IL-12 significantly correlated with those of IFN-γ, IP-10, IL-17A, and TNF-α, whereas the levels of CE-induced IL-12 only correlated with those of IL-6. TNF-α levels showed

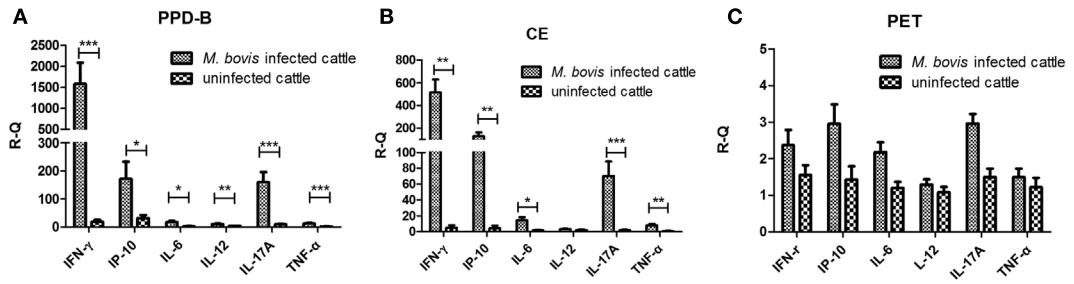

FIGURE 1 | Levels of cytokine mRNA in *Mycobacterium bovis*-infected cattle and bovine tuberculosis (bTB)-free cattle. **(A)** Peripheral blood mononuclear cells (PBMCs) stimulated by PPD-B for 6 h. **(B)** PBMCs stimulated by CE for 6 h. **(C)** PBMCs stimulated by PET for 6 h. PBMCs were isolated from 10 cows naturally infected with *M. bovis* and 5 cows free of bTB, and these cells stimulated with PPD-B, CE, PET, or phosphate-buffered saline (PBS). RNA was isolated to measure the levels of interferon gamma (IFN-γ), IFN-γ-induced protein 10 (IP-10), IL-6, IL-12, IL-17A, and tumor necrosis factor alpha (TNF-α) gene expression using the $2^{-\Delta\Delta CT}$ method, with PBS-stimulated PBMCs as a calibrator and *β-actin* as the reference gene. The significance between cytokines transcript levels in *M. bovis*-infected and bTB-free cattle was determined using an unpaired t test. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$. Data are mean ± SEM values (for *M. bovis*-infected cattle, $n = 10$; for bTB-free cattle, $n = 5$).

TABLE 3 | Correlations between interferon gamma (IFN-γ), IFN-γ-induced protein 10 (IP-10), IL-6, IL-12, IL-17A, and tumor necrosis factor alpha (TNF-α) (mRNA) induced by PPD-B and CE.

Correlation coefficient (Spearman *r*)	PPD-B-stimulated peripheral blood mononuclear cells (PBMCs)						CE-stimulated PBMCs					
	IFN-γ	IP-10	IL-6	IL-12	IL-17A	TNF-α	IFN-γ	IP-10	IL-6	IL-12	IL-17A	TNF-α
IFN-γ		0.75*	0.53*	0.70*	0.73*	0.80*		0.65*	0.66*	0.47	0.79*	0.88*
IP-10	0.75*		0.41	0.71*	0.56*	0.65*	0.65*		0.44	0.15	0.79*	0.61*
IL-6	0.53*	0.41		0.46	0.83*	0.62*	0.66*	0.44		0.58*	0.83*	0.66*
IL-12	0.70*	0.71*	0.46		0.55*	0.84*	0.47	0.15	0.58*		0.50	0.45
IL-17A	0.73*	0.56*	0.83*	0.55*		0.79*	0.79*	0.79*	0.83*	0.50		0.74*
TNF-α	0.80*	0.65*	0.62*	0.84*	0.79*		0.88*	0.61*	0.66*	0.45	0.74*	

*PBMCs were isolated from naturally Mycobacterium bovis infected and bovine tuberculosis-free cattle, and stimulated with PPD-B, CE, PET, or phosphate-buffered saline (PBS). RNA was isolated to measures levels IFN-γ, IP-10, IL-6, IL-12, IL-17A, and TNF-α gene expression using the $2^{-\Delta\Delta CT}$ method, with PBS-stimulated PBMCs used as a calibrator, and β-actin as the reference gene. Correlation coefficients were determined using Spearman's two-tailed correlation test. *p < 0.05, significant correlation. Bovine Tuberculin PPD, 300 μg/ml (Prionics AG, Schlieren, Switzerland). CE: CFP-10-ESAT-6, 20 μg/ml, expressed and purified in our lab, with a Trx-His-S tag at the N-terminus, with endotoxin at a concentration less than 10 EU/mg.*

better correlation with IFN-γ ($r = 0.88$) and IL-17A ($r = 0.74$) levels than that with those of IL-6 ($r = 0.66$) or IP-10 ($r = 0.61$).

PPD-B- or CE-induced IP-10 and IL-17A transcripts were significantly increased in *M. bovis*-infected cows relative to those in uninfected ones, and these levels were also higher than those of IL-6, IL-12, and TNF-α, and correlated well with the levels of IFN-γ. Therefore, the levels of PPD-B- and CE-induced IP-10 and IL-17A mRNA in PBMCs from animals experimentally infected with *M. bovis* 68002 were analyzed. The levels of PPD-B- and CE-induced IP-10 and IL-17A protein in plasma from animals experimentally infected with *M. bovis* 68002 were also analyzed.

IP-10 and IL-17A Response to *M. bovis* Infection and Comparison with that of IFN-γ

To verify whether PPD-B- or CE-induced IP-10 and IL-17A expression was related to *M. bovis* infection, three Luxi beef calves were intravenously injected with *M. bovis* 68002, and three other calves were injected PBS as uninfected controls. The three *M. bovis* 68002-infected calves were confirmed to be *M. bovis* infected by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA and CE-based IGRA (Figures S1 and S2 in Supplementary Material), and the PBS-inoculated calves were confirmed to be uninfected with *M. bovis* (Figures S1 and S2 in Supplementary Material). The mRNA and protein expression levels of IFN-γ, IP-10, and IL-17A induced by PPD-B, CE, PET, or PBS were measured by real-time PCR and ELISA before injection and at 6, 26, 30, and 58 weeks post-infection. The mRNA and protein expression levels of IFN-γ, IP-10, and IL-17A induced by PET in PBMCs from both *M. bovis* 68002-infected and uninfected calves were close to those induced by PBS (data not shown). The levels of IFN-γ, IP-10, and IL-17A (mRNA and protein) induced by PET and PBS in *M. bovis* 68002-infected calves and uninfected calves were not significantly different (data not shown). After *M. bovis* 68002 infection, the responses of IP-10 and IL-17A (mRNA and protein) to PPD-B and CE showed kinetics similar to those of IFN-γ responses (**Figures 2** and **3**). The mRNA transcript levels of IFN-γ, IP-10, and IL-17A induced by PPD-B or CE in PBMCs from *M. bovis*-infected calves were significantly higher than those from uninfected calves between 6 and 58 weeks post-infection (**Figure 2**). However, the levels of IFN-γ (induced by CE) and

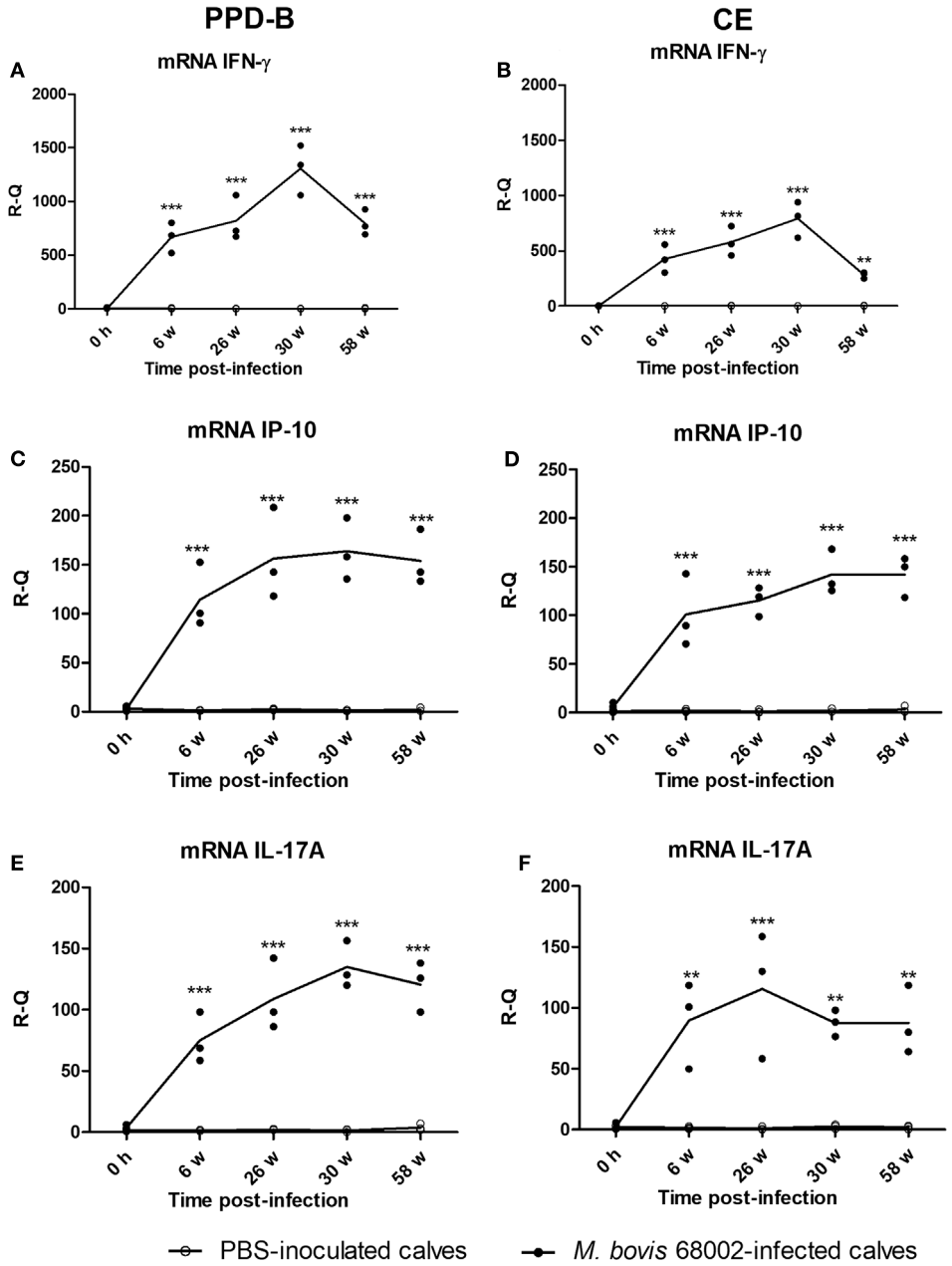

FIGURE 2 | Levels of cytokines mRNA in *Mycobacterium bovis*-infected cattle. **(A)** Interferon gamma (IFN-γ) induced by PPD-B. **(B)** IFN-γ induced by CE. **(C)** IFN-γ-induced protein 10 (IP-10) induced by PPD-B. **(D)** IP-10 induced by CE. **(E)** IL-17A induced by PPD-B. **(F)** IL-17A induced by CE. Peripheral blood mononuclear cells (PBMCs) were isolated from calves experimentally infected with *M. bovis* ($n = 3$) and those inoculated with phosphate-buffered saline (PBS) (uninfected controls, $n = 3$) at 0 h and 6, 24, 30, and 58 weeks post-infection. PBMCs were then stimulated with PPD-B, CE, or PBS for 6 h. RNA was isolated to measure IFN-γ, IP-10, and IL-17A gene expression levels using the $2^{-\Delta\Delta CT}$ method, with PBS-stimulated PBMCs used as a calibrator, and *β-actin* as the reference gene. The significance of differences between cytokine transcript levels in *M. bovis* 68002-infected calves and uninfected controls was determined at each time point using a two-way analysis of variance followed by Bonferroni post-test. $^{**}p < 0.001$; $^{***}p < 0.0001$. Data are each replicate and means connected by lines (for *M. bovis*-infected cattle, $n = 3$; for uninfected cattle, $n = 3$).

IP-10 (induced by CE) protein at 58 weeks post-infection and IL-17A (induced by PPD-B or CE) protein at 58 weeks post-infection in *M. bovis*-infected calves showed no differences from those in uninfected calves (**Figure 3**). The IFN-γ (mRNA and protein) elicited by PPD-B and CE in *M. bovis*-infected calves were higher than those of IP-10 and IL-17A. The protein levels of IFN-γ ranged from 0 to 22 ng/ml, whereas IP-10 and IL-17A levels ranged from 0 to 7 ng/ml. Correlations between IFN-γ, IP-10, and IL-17A levels were determined at all treatments and all time points ($n = 30$ [6 cattle × 5 time points]). As shown in

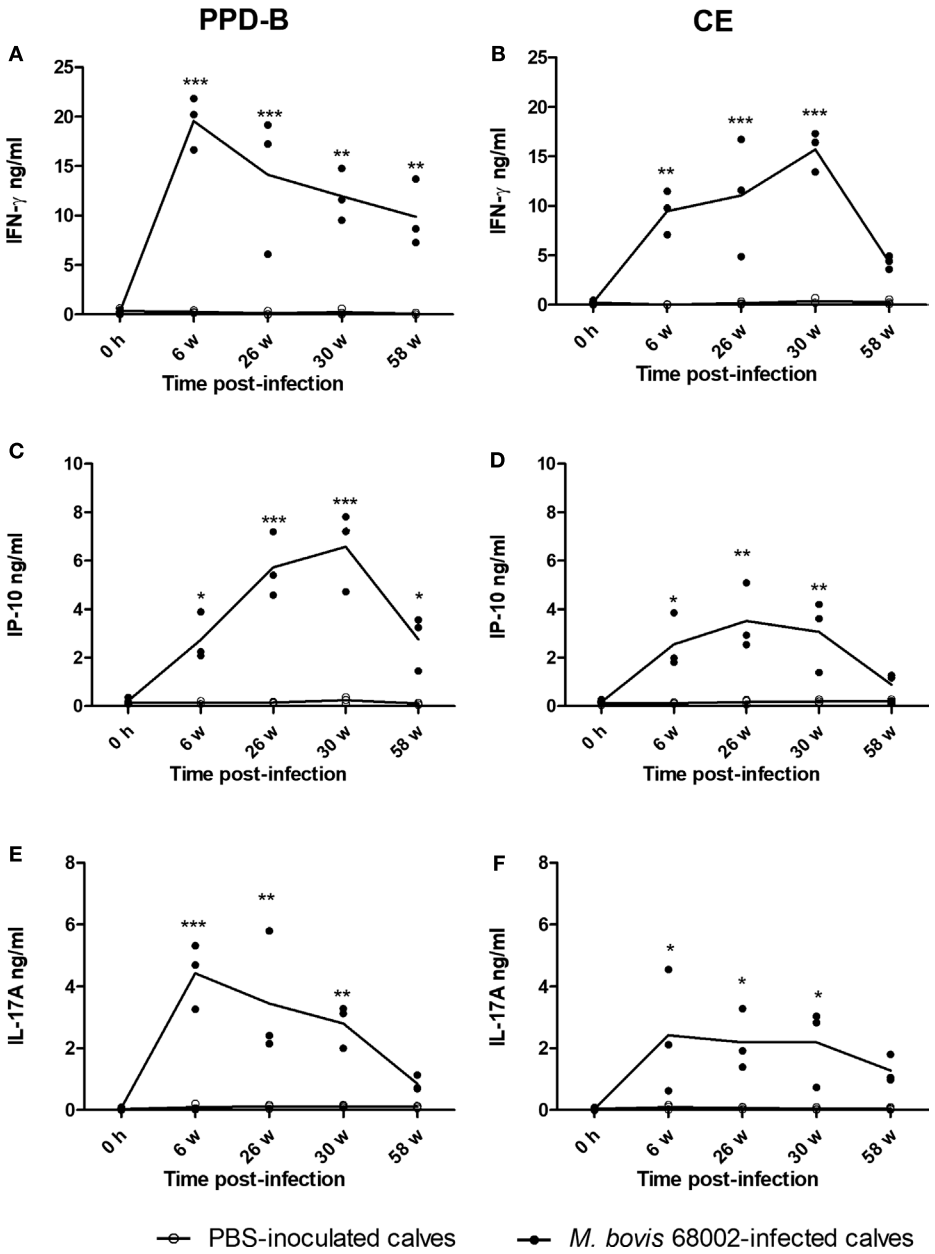

FIGURE 3 | Level of cytokine proteins in *Mycobacterium bovis*-infected cattle. **(A)** Interferon gamma (IFN-γ) induced by PPD-B. **(B)** IFN-γ induced by CE. **(C)** IP-10 induced by PPD-B. **(D)** IP-10 induced by CE. **(E)** IL-17A induced by PPD-B. **(F)** IL-17A induced by CE. Whole blood was collected from three *M. bovis* 68002-infected and three phosphate-buffered saline (PBS)-inoculated calves, and then stimulated with PPD-B, CE, or PBS for 24 h, and at 0 h and 6, 26, 30, and 58 weeks post-infection. Plasma was harvested to measure IFN-γ, IP-10, and IL-17A expression levels using commercial kits. The significance of differences between cytokine expression levels in *M. bovis* 68002-infected calves and uninfected controls was determined at each time point using a two-way analysis of variance followed by Bonferroni post-test. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$. Data are each replicate and means connected by lines (for *M. bovis*-infected cattle, $n = 3$; for uninfected cattle, $n = 3$).

Table 4, IFN-γ, IP-10, and IL-17A levels showed good correlation with each other ($r > 0.65$). IFN-γ showed better correlation with IP-10 than with IL-17A. Therefore, PPD-B- and CE-induced IP-10 and IL-17A mRNA expression could be used to differentiate *M. bovis*-infected calves from uninfected ones between 6 and 58 weeks post-infection, whereas PPD-B- and CE-induced IP-10 and IL-17A protein expression could be used to differentiate *M. bovis*-infected calves from uninfected ones between 6 and 30 weeks post-infection.

To confirm infection with *M. bovis* 68002, three *M. bovis* 68002-infected calves were slaughtered after experiment. Only one calve showed typically tubercles on the lungs, and there were no typical tubercles on the livers, spleen or other organs. So, we collected three pieces of each tissue (lung, liver, kidney, spleen,

TABLE 4 | Correlations between interferon gamma (IFN-γ), IFN-γ-induced protein 10 (IP-10), and IL-17A (mRNA and proteins) induced by PPD-B and CE.

Correlation coefficient (Spearman *r*)	PPD-B-stimulated peripheral blood mononuclear cells (PBMCs) (mRNA)			CE-stimulated PBMCs (mRNA)			PPD-B-stimulated whole blood (protein)			CE-stimulated whole blood (protein)		
	IFN-γ	IP-10	IL-17A	IFN-γ	IP-10	IL-17A	IFN-γ	IP-10	IL-17A	IFN-γ	IP-10	IL-17A
IFN-γ		0.82	0.77		0.86	0.83		0.76	0.69		0.85	0.73
IP-10	0.82		0.73	0.86		0.87	0.76		0.72	0.85		0.66
IL-17A	0.77	0.73		0.83	0.87		0.69	0.72		0.73	0.66	

PBMCs were isolated from three Mycobacterium bovis 68002-infected and three phosphate-buffered saline (PBS) inoculated calves, and then stimulated with PPD-B, CE, PET, or PBS for 6 h, at 0 h and 6, 26, 30, and 58 weeks post-infection. RNA was isolated to measure levels of IFN-γ, IP-10, and IL-17A gene expression using the $2^{-\Delta\Delta CT}$ method, with PBS-stimulated PBMCs as a calibrator, and β-actin as the reference gene. Whole blood was collected from three M. bovis 68002-infected and three PBS-inoculated calves, and then stimulated with PPD-B, CE, PET, or PBS for 24 h, at 0 h and 6, 26, 30, and 58 weeks post-infection. Plasma was harvested to measure levels of IFN-γ, IP-10, and IL-17A expression using commercial kits. Correlation coefficients were determined using Spearman's two-tailed correlation test. All correlations were significant ($p < 0.05$). The comparisons included all treatments and all time points ($n = 30$ [6 cattle × 5 time points]). PPD-B: Bovine Tuberculin PPD, 300 μg/ml (Prionics AG, Schlieren, Switzerland). CE, CFP-10-ESAT-6, 20 μg/ml, expressed and purified in our lab, with a Trx-His-S tag at the N-terminus, with endotoxin at a concentration less than 10 EU/mg.

and bronchial lymph nodes) for nested PCR, *Mycobacterium* culture, and hematoxylin–eosin staining. The lung and liver tissues were positive by nested PCR and *Mycobacterium* culture, hematoxylin–eosin staining showed no pathological changes in the livers, kidneys, or spleens.

Comparisons of Cytokines between Nested PCR-P Cattle and PCR-N Cattle

To assess whether IP-10 and IL-17A proteins could be used to detect all naturally *M. bovis*-infected cattle, more than 1,000 cows were detected by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, and CE-based IGRA. 151 *M. bovis*-infected cows from dairies where bTB was prevalent and 50 uninfected cows from bTB-free dairies were selected for follow-up study. Nasal swabs from these 201 selected cows were collected and tested by nested PCR. 35 of 151 cows were determined positive by nested PCR, and 21 of these cows were confirmed positive by *Mycobacteria* culture and classified as PCR-P. The remaining 116 of 151 cows were determined negative by nested PCR and classified as PCR-N (data not shown). 21 PCR-P cows, 21 PCR-N cows, and 20 uninfected cows (NC) were randomly selected for additional analysis. Heparinized whole blood was collected from each cow and then stimulated by PPD-B, PPD-A, CE, or PBS for 24 h. The levels of IFN-γ, IP-10, and IL-17A protein in stimulated plasma were determined by commercial kits (**Figure 4**). PPD-B-induced IFN-γ, and IL-17A levels in the PCR-P and PCR-N groups were higher than those induced by CE, and significantly higher than those in the NC group. IFN-γ and IP-10 levels induced by CE in the PCR-P and PCR-N groups were significantly higher than those in the NC group. PBS-induced IP-10 levels were significantly higher in the PCR-N group than in the PCR-P and NC groups. PBS-induced IFN-γ levels were similar in the PCR-P, PCR-N, and NC groups. However, CE- and PBS-induced IL-17A levels were significantly higher in the PCR-N group than in the PCR-P and NC groups. Correlations among IFN-γ, IP-10, and IL-17A protein levels were calculated. This comparison included all individuals and all treatments ($n = 62$ [21 PCR-P cows + 21 PCR-N cows + 20 NC cows]). As shown in **Table 5**, IFN-γ, IP-10, and IL-17A induced by PPD-B or CE were significantly correlated with one other. PBS-induced IFN-γ levels showed no correlation with those of PBS-induced IP-10 ($r = 0.12$, $p > 0.05$) and IL-17A ($r = -0.10$, $p > 0.05$), whereas PBS-induced IP-10 showed a weak correlation with IL-17A ($r = 0.38$, $p < 0.05$).

To confirm infection, one naturally *M. bovis*-infected cow that was found positive by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, CE-based IGRA, and nested PCR, was slaughtered after the experiment. There were no typical tubercles on the lung, kidney, and spleen, and hematoxylin–eosin staining of lung, kidney, spleen, and bronchial lymph node tissues indicated no pathological changes. However, the nasal swab and BAL fluid were positive by nested PCR.

DISCUSSION

CE-Induced IL-17A Was Not a Reliable Indicator of Mycobacteria Shedding in Cattle

The airborne route of infection is considered as the most common for the transmission of *M. bovis*, and more than 15% of cattle with bTB shed the mycobacteria, mainly at an early stage in infection. Nested PCR based on the amplification of *mpb*70 can be used to detect *Mycobacteria* in milk, nasal exudates, and BAL fluid. Previous studies using nested PCR showed that 26% of TST-reactors shed *M. bovis* in nasal exudates (12). Furthermore, Flores-Villalva et al. found that in a high-prevalence herd, 60.3% (38/63) of TST-positive and 63.4% (40/63) of ESAT-6-CFP-10-based skin test-positive cattle were confirmed positive for *M. bovis* infection by nested PCR, whereas in a low-prevalence herd, 87.5% (7/8) of the ESAT-6-CFP-10-based skin test-positive cattle were confirmed positive by nested PCR (11). However, 24.84% (205/825) of *M. bovis*-infected cattle were positive by nested PCR in our previous study (9), and we found 23.18% (35/151) of *M. bovis*-infected cattle were positive by nested PCR in current study. The lower positivity rate in our study may be related to a difference in the bTB prevalence at diaries or the greater number of animals included in this study. There are few data on the differences in immune responses or immunopathology between cattle determined PCR positive and negative for *M. bovis* infection (*M. bovis*-infected animals

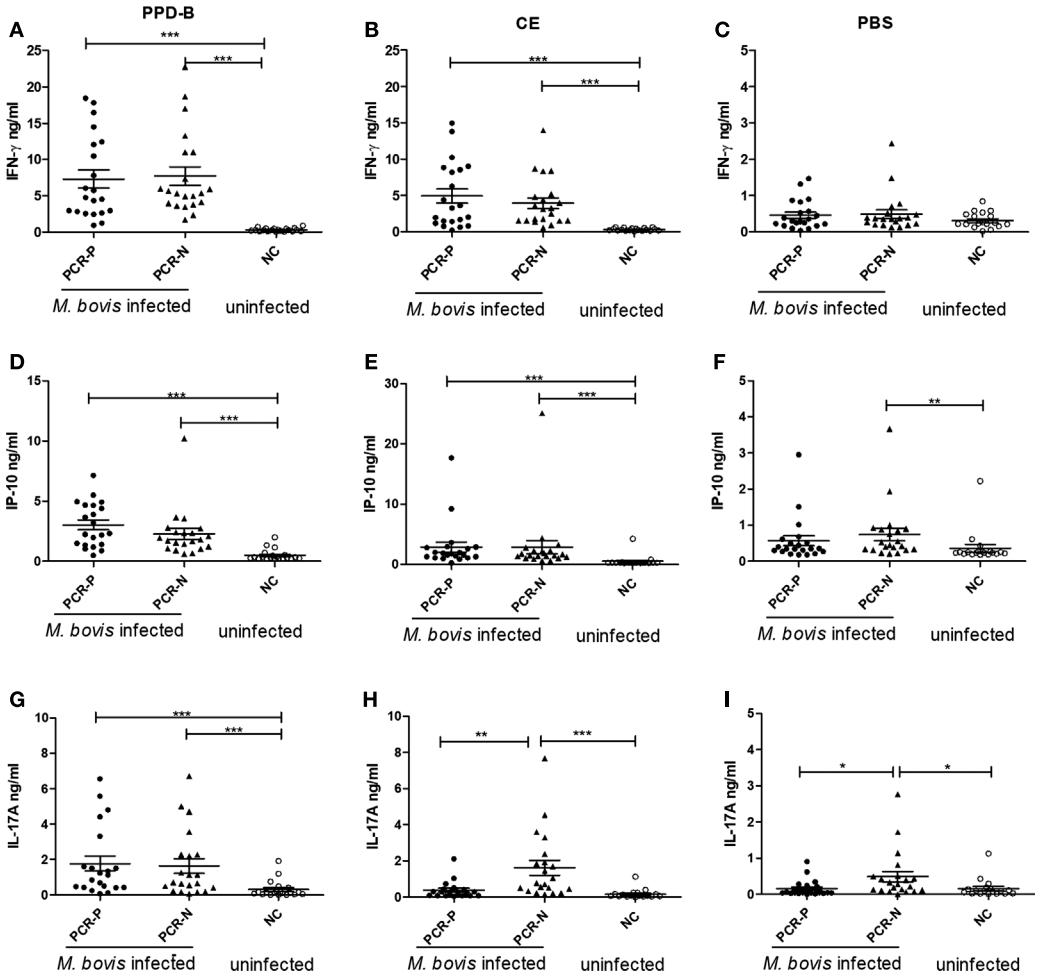

FIGURE 4 | The levels of cytokine protein expression in PCR-P, PCR-negative (PCR-N), and NC. **(A)** Interferon gamma (IFN-γ) induced by PPD-B. **(B)** IFN-γ induced by CE. **(C)** IFN-γ induced by phosphate-buffered saline (PBS). **(D)** IFN-γ-induced protein 10 (IP-10) induced by PPD-B. **(E)** IP-10 induced by CE. **(F)** IP-10 induced by PBS. **(G)** IL-17A induced by PPD-B. **(H)** IL-17A induced by CE. **(I)** IL-17A induced by PBS. PCR-P (nested PCR positive): 21 cattle from a dairy where bovine tuberculosis (bTB) was prevalent and *Mycobacterium bovis* infection was detected by tuberculin skin test (TST), CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, CE-based IGRA, and nested PCR. PCR-N (nested PCR-N): 21 cattle from a dairy where bTB was prevalent and *M. bovis* infection was detected by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, and CE-based IGRA, but not detected by nested PCR. NC (uninfected control): 20 cattle from a bTB-free diary determined *M. bovis* negative by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, CE-based IGRA, and nested PCR. Whole blood was collected and stimulated with PPD-B, CE, or PBS for 24 h. Plasma from each well was harvested to measure levels of IFN-γ, IP-10, and IL-17A expression using commercial kits. The significance of differences between cytokine expression levels in PCR-P, PCR-N, and NC was determined using a one-way analysis of variance (Kruskal–Wallis test) followed by Dunn's multiple comparison. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$.

diagnosed by TST or IGRA), but we did find the CE-induced and uninduced IL-17A (protein) levels were significantly higher in PCR-N cattle than in PCR-P and uninfected cattle (**Figure 4**). It indicated that *M. bovis*-infected cattle could be divided into two stages by using TST or IGRA and nested PCR results. One cow naturally infected with *M. bovis* in the PCR-P group was slaughtered and showed a low level of CE-induced IL-17A in plasma and no lesions in the lungs. The reason for this phenomenon is not known, but recent studies have shown that IL-17A (mRNA and protein) may be predictive of both vaccine efficacy and lesion severity when measured after vaccination and during infection, respectively (13, 14). Blanco et al. also found that increased PPD-B induced IL-17A transcripts in PBMC is associated with pathology in *M. bovis*-infected cattle: cattle with macroscopic lesions showed a higher level of IL-17A transcripts than animals without macroscopic lesions (13). Therefore, we speculate that *M. bovis*-infected cattle that were PCR positive and those that were negative may be at different stages in the progression of bTB and might show different immune responses to *M. bovis*-specific antigens. More studies are needed to test this hypothesis.

Our study also showed that the levels of PPD-B-stimulated IL-17A (mRNA and protein) were significantly higher in cattle naturally or experimentally infected with *M. bovis* than in those that were uninfected. The levels of PPD-B-induced IL-17A (mRNA and protein) could be used to differentiate *M. bovis*-infected calves from uninfected ones for 6 to 30 weeks post-infection. However, CE-induced IL-17A (protein) was not a reliable indicator of *M. bovis* infection in cattle that were confirmed positive for infection

TABLE 5 | Correlations between interferon gamma (IFN-γ), IFN-γ-induced protein 10 (IP-10), and IL-17A (proteins) induced by PPD-B and CE.

Correlation coefficient (Spearman *r*)	PPD-B-stimulated whole blood (protein)			CE-stimulated whole blood (protein)			Phosphate-buffered saline (PBS)-stimulated whole blood (protein)		
	IFN-γ	IP-10	IL-17A	IFN-γ	IP-10	IL-17A	IFN-γ	IP-10	IL-17A
IFN-γ		0.59*	0.62*		0.65*	0.52*		0.12	0.38*
IP-10	0.59*		0.54*	0.65*		0.45*	0.12		−0.10
IL-17A	0.62*	0.54*		0.52*	0.45*		0.38*	−0.10	

*Whole blood was isolated from 21 PCR-P (nested PCR positive), 21 PCR-N (nested PCR negative) and 20 NC (uninfected control) cattle and then stimulated with PPD-B, CE, or PBS for 24 h. Plasma was harvested to measure levels of IFN-γ, IP-10, and IL-17A protein expression using commercial kits. Correlation coefficients were determined using Spearman's two-tailed correlation test. *p < 0.05, significant correlation. Comparisons included all individuals and all treatments (n = 62 [21 PCR-P + 21 PCR-N + 20 NC]). PCR-P: cattle from a dairy where bTB was prevalent, and M. bovis infection was detected by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, CE-based IGRA, and nested PCR. PCR-N: cattle from a dairy where bTB was prevalent, and M. bovis infection was detected by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA and CE-based IGRA, but undetected by nested PCR. NC: cattle from a bTB-free diary and determined M. bovis negative by TST, CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, IGRA, CE-based IGRA, and nested PCR. PPD-B: Bovine Tuberculin PPD, 300 μg/ml (Prionics AG, Schlieren, Switzerland). CE: CFP-10-ESAT-6, 20 μg/ml, expressed and purified in our lab, with a Trx- His-S tag at the N-terminus, with endotoxin at a concentration less than 10 EU/mg.*

by nested PCR, and levels of IL-17A (mRNA and protein) were lower than those of IFN-γ. Together, these findings suggest that IL-17A is not suitable for the diagnosis of bTB.

Limitations of IP-10 As Biomarker of bTB

Several recent studies have shown that mycobacterial antigen (PPD-B, CFP-10-ESAT-6) -stimulated IP-10 levels are higher in patients with active TB and latent TB compared with healthy controls and to IFN-γ (15, 16). Unlike IFN-γ, IP-10 levels were not age dependent, and more TB cases were identified in children aged <5 years when this chemokine was used for detection (17–19). This chemokine can even be used to detect TB in patients with HIV and those undergoing tuberculosis therapy (20–22). Therefore, IP-10 can be used as a biomarker for TB. Similarly, Goosen et al. found that in buffaloes naturally infected with *M. bovis*, the levels of IP-10 protein expression were significantly elevated in whole blood stimulated with ESAT-6/CFP-10 and higher than those of IFN-γ, suggesting its potential as a biomarker for bTB in African buffaloes (7, 23). Waters et al. found that PPD-B specific IP-10 mRNA showed a pattern of expression similar to that IFN-γ mRNA over the entire course of *M. bovis* infection and could be a biomarker for bTB, and IP-10 protein showed a poor correlation with IFN-γ in cattle experimentally infected with *M. bovis* (24). By contrast, Parsons et al. found that levels of mycobacterial-specific IP-10 protein strongly correlated with those of IFN-γ in cattle naturally infected with *M. bovis*, and that the differential release of IP-10 induced by PPD-B and PPD-A could be used to distinguish between *M. bovis*-infected cattle and bTB-free cattle with a sensitivity of 100% (95% Cl, 86–100%) and specificity of 97% (95% Cl, 85–100%) (25). In our study, the levels of IP-10 mRNA and protein induced by PPD-B or CE were significantly higher in cattle naturally or experimentally infected with *M. bovis* compared with those that were bTB free, which is consist with Parsons's results but contrasts with the results of Waters. This may because a bovine IP-10 ELISA kit was used in our study and the study by Parsons et al. to determine IP-10 levels, whereas a human IP-10 ELISA kit was used in the Waters et al. study.

Previous studies have shown that levels of IP-10 induced by PPD-B or CE were significantly higher than those in IFN-γ in patients with TB and in *M. tuberculosis*-infected monkeys (26), whereas, in our study, levels of antigen-induced IP-10 were lower than those of IFN-γ in *M. bovis*-infected cattle. Because the amount of IP-10 in *M. bovis*-infected cattle and healthy cattle is rarely reported, the differences in results between humans, monkeys, and cattle may be related to differences in the species tested or the limited number of animals included in our study. The lower levels of PPD-B- or CE-induced IP-10 protein relative to that IFN-γ limit the utility of IP-10 for the diagnosis of bTB. The levels of PPD-B- or CE-induced IP-10 transcripts, however, should be analyzed further for their value in bTB diagnosis.

We also found that levels of PPD-B- or CE-stimulated IP-10 in PCR-P animals were similar to those in PCR-N animals, and we could not differentiate PCR-P from PCR-N animals with this chemokine. However, levels of unstimulated IP-10 (PBS exposed) in PCR-N animals were significantly higher than those in uninfected animals but similar to PCR-P animals. Similarly, studies on human TB have shown that antigen-stimulated IP-10 in plasma cannot be used to distinguish between patients with active and latent TB, whereas serum IP-10 concentrations were higher in patients with active TB than in those with latent TB. However, IP-10 is likely to be a non-specific marker of inflammation and also elevated in other bacterial, viral, and parasitic infections, whereas unstimulated serum IP-10 levels primarily reflect a pro-inflammatory state, therefore, unstimulated IP-10 may be limited in its use for detection of PCR-N cattle.

One drawback of this study is that we did not test every cow naturally or experimentally infected with *M. bovis* by nested PCR. Thus, we could not evaluate the mRNA transcript levels of cytokines in PCR-P and PCR-N animals. Another limitation is that it was impossible to slaughter all cattle used in this study to confirm the stages of bTB progression in the *M. bovis*-infected cattle (either PCR positive or negative). Therefore, more work is needed to determine the differences in immune responses and immunopathology between PCR-P and PCR-N cattle.

CONCLUSION

CE-induced IL-17A protein cannot detect all *M. bovis*-infected cattle, and PPD-B- or CE-induced IP-10 and IL-17A protein in *M. bovis*-infected cattle were lower than IFN-γ protein. IL-17A

and IP-10 proteins are not suitable as biomarkers for bovine tuberculosis, but the levels of PPD-B- or CE-induced IP-10 mRNA transcripts should be analyzed further for their potential to be used in the diagnosis of bTB.

AUTHOR CONTRIBUTIONS

HZ, TX, JD and HJ designed the experiments. TX, XG, HY, PL, QL, XS, SH, XG and WY performed the experiments and analyzed the data. TX and XG wrote and revised the paper.

ACKNOWLEDGMENTS

The authors are especially grateful to the personnel who collected samples at each of the farms involved in this study.

FUNDING

This work was supported by the Beijing Natural Science Foundation (no. 6164039), National Key Research and Development Program (no. 2016YFD0500902), National Science Foundation for Young Scientists of China (no. 31602086) Agricultural Science and Technology Innovation Program (no. ASTIP-IAS-11), and Fundamental Research Funds for IAS-CAAS (no. 2014ywf-zd-1).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fvets.2018.00028/full#supplementary-material.

FIGURE S1 | *Mycobacterium bovis* 68002 infection confirmed by skin test. **(A)** *M. bovis* 68002 infection confirmed by tuberculin skin test (TST). **(B)** *M. bovis* 68002 infection confirmed by CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test. Three *M. bovis* 68002-infected calves and three PBS-inoculated calves were tested using TST and CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test at 8 and 32 weeks post-infection. The TST was performed as the Chinese diagnostic standard for bovine tuberculosis (GB/T 18645-2002), and the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test was previously established in our laboratory. PPD-B (PPD-B, 2,500 IU/cattle) and the CFP-10/ESAT-6/TB10.4 protein cocktail were intradermally injected (0.1 ml each) into two sites on the same side of a cow's neck. Differences in skin thicknesses (mm) pre- and 72 h post-injection were calculated. With the GB/T 18645-2002, if the difference in skin thicknesses was ≥4 mm, the cattle were considered as *M. bovis*-infected; if the difference in skin thicknesses was <2 mm, the cattle were considered *M. bovis*-uninfected. For the CFP-10/ESAT-6/TB10.4 protein cocktail-based skin test, if the difference in skin thicknesses was ≥1.1 mm, the cattle were considered *M. bovis* infected; if the difference in skin thicknesses was <1.1 mm, the cattle were considered free from bTB. PPD-B: Bovine tuberculin (Harbin Pharmaceutical Group, Heilongjiang Province, China), 2,500 IU/cattle. CFP-10/ESAT-6/TB10.4 protein cocktail: 0.5 mg/ml, endotoxin less than 10 EU/mg, prepared in the Institute of Animal Sciences (IAS-CAAS).

FIGURE S2 | *Mycobacterium bovis* 68002 infection confirmed by IGRA. **(A)** *M. bovis* 68002 infection confirmed by IGRA [interferon gamma (IFN-γ) release assay], differences in OD value PPD-B- and PPD-A-stimulated blood plasma. **(B)** *M. bovis* 68002 infection confirmed by IGRA, differences in OD value PPD-B- and phosphate-buffered saline (PBS)-stimulated blood plasma. **(C)** *M. bovis* 68002 infection confirmed by CE-based IGRA (CFP-10/ESAT-6-based IFN-γ release assay), differences in OD value CE- and PBS-stimulated blood plasma. Three *M. bovis* 68002-infected calves and three PBS-inoculated calves were tested using IGRA and CE-based IGRA before injection, and at 8, 24, 48, 72, and 96 h, and 2, 4, 6, 8, 12, 16, 20, 26, 30, 34, 38, 45, 50, and 58 weeks post-infection. For IGRA, if PPD-B-simulated blood plasma having an OD value more than 0.100 above that of plasma stimulate with PPD-A and PBS indicated cattle were *M. bovis* infected. For CE-based IGRA, if CE-stimulated blood plasma having an OD value more than 0.100 above that of plasma stimulated with PBS indicated cattle were *M. bovis*-infected. PPD-B: Bovine Tuberculin PPD, 300 μg/ml (Prionics AG, Schlieren, Switzerland). CE: CFP-10-ESAT-6, 20 μg/ml, expressed and purified in our lab, with a Trx-His-S tag at the N-terminus, with endotoxin at a concentration less than 10 EU/mg.

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Conflict of Interest Statement: 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 June 2017
doi: 10.3389/fvets.2017.00107

Generation of Monoclonal Antibodies against Ag85A Antigen of *Mycobacterium tuberculosis* and Application in a Competitive ELISA for Serodiagnosis of Bovine Tuberculosis

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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 March 2017
Accepted: 20 June 2017
Published: 30 June 2017

Citation:
Xu Z, Hu T, Xia A, Li X, Liu Z, Min J, He J, Meng C, Yin Y, Chen X and Jiao X (2017) Generation of Monoclonal Antibodies against Ag85A Antigen of Mycobacterium tuberculosis and Application in a Competitive ELISA for Serodiagnosis of Bovine Tuberculosis. Front. Vet. Sci. 4:107. doi: 10.3389/fvets.2017.00107

The Ag85 complex functions as the main secretory protein of *Mycobacterium tuberculosis* (*M. tuberculosis*) and BCG. This complex is composed of the proteins, Ag85A, Ag85B, and Ag85C, with Ag85A thought to play the largest role within the complex. However, the lack of commercially available monoclonal antibodies (mAbs) against Ag85A still hinders the biological and applicative research on this protein. In this study, we developed and identified anti-Ag85A mAbs, and five hybridoma cells were established. Using the indirect immunofluorescence test, we found that two anti-Ag85A mAbs did not cross-react with Ag85B and/or Ag85C. In addition, we showed that all of the mAbs tested in this study are able to react with endogenous Ag85A protein in BCG and rBCG:Ag85A using indirect ELISA and Western blot analyses. A competitive ELISA (cELISA) based on mAb 3B8 was developed, the analyses of clinic serum samples from cattle with bovine tuberculosis (TB) and healthy cattle demonstrated that the sensitivity of the cELISA was 54.2% (26/48) and the specificity was 83.5% (167/200). This study demonstrated that the mAbs against Ag85A will provide useful reagents for further investigation into the function of the Ag85 complex and can be used for serodiagnosis of bovine TB.

Keywords: Mycobacterium tuberculosis, Ag85A, *Monoclonal antibody*, cross-react, competitive ELISA, bovine tuberculosis

INTRODUCTION

Tuberculosis (TB) is an infectious disease that is widely prevalent throughout the entire world. Approximately one-third of the population of the world is infected with *Mycobacterium tuberculosis* (*M. tuberculosis*). In 2015, there were an estimated 10.4 million new cases of active TB across the world, and it was responsible for an estimated 1.4 million deaths globally in the same year (1–3).

The majority of secreted proteins found in *M. tuberculosis* culture filtrate have been shown to be generated by the Ag85 complex, a complex comprised three proteins from a 30- to 32-kDa protein family (Ag85A, Ag85B, and Ag85C). These three proteins are secreted into the culture medium in a 2:3:1 ratio (4, 5). The proteins of the Ag85 complex are encoded by three paralogous genes, *fbpA*, *fbpB*, and *fbpC*, all of which have been shown to be localized to distinct regions of the bacterial genome.

M. tuberculosis Ag85A, Ag85B, and Ag85C are highly homologous on the DNA and amino acid level, with approximately 77% of amino acids shared between Ag85A and Ag85B, and about 71% amino acids shared between Ag85A and Ag85C (6).

The proteins comprising the Ag85 complex have been shown to be abundantly secreted in *M. tuberculosis*. These proteins play a key role in the final step of cell wall assembly and the maintenance of the bacterial cell envelope integrity by catalyzing the transfer of mycolic acid to the cell wall component, arabinogalactan. In addition, this complex has been shown to play an important role in the synthesis of trehalose dimycolate (7–9). Numerous studies to date have focused on the potential of Ag85 complex in vaccine development, diagnostics, and as a therapeutic drug target (10). Because the Ag85 complex has been shown to play a role in the catalysis of the biosynthesis of abundant cell envelope components, including TMM and TDM, there is great interest in Ag85 as a novel target for drug development (11). The *M. tuberculosis* Ag85 complex has been demonstrated to stimulate a strong humoral- and cell-mediated immune response (12, 13). Thus, Ag85A is considered to be one of the most popular TB vaccine candidates (14–16). In addition, the abundance of serum antibodies generated against the Ag85 complex in active TB patients provides further support that the Ag85 complex could also function as a promising diagnostic marker (5, 17).

There were already some works describing the production of monoclonal antibodies (mAbs) to Ag85 complex, nine mAbs were produced against *M. tuberculosis* Ag85 complex using isoelectric focusing combined with Western blot analysis, the results showed that one antibody was found to be specifically directed only against Ag85B (18). A method to select antibodies against any Ag85 complex using a novel combination of phage and yeast display was described (19). And antibodies to Ag85B of *M. tuberculosis* were produced and subsequently used to develop ELISA technique for detecting Ag85 in the culture filtrate (20). Up until this point, there have been no commercial specific mAbs available against Ag85A. The widely used anti-Ag85 mAb HYT 27 reacts strongly with *M. tuberculosis* Ag85C and weaker with Ag85A and Ag85B (21, 22). The rabbit polyclonal antibody against *M. tuberculosis* Ag85B is only specific for the Ag85B protein (23, 24). Thus, it is necessary to first develop a specific mAb against Ag85A to be used in both basic biological research and Ag85A applicative research.

In this study, we developed mAbs against recombinant Ag85A protein. We showed that all of the generated mAbs exhibit good reactivity with both recombinant Ag85A and endogenous Ag85A *via* indirect ELISA and Western blot techniques. And mAbs 1C6 and 3B8 were specific only for Ag85A, mAbs 2E6 and 2F2 cross-reacted with Ag85B or Ag85C, while mAb 3D9 react with Ag85A, Ag85B, and Ag85C. A competitive ELISA (cELISA) based on mAb 3B8 was developed, and the diagnostic specificity and sensitivity were 54.2% (26/48) and 83.5% (167/200), respectively. We anticipate that the mAbs generated against Ag85A will prove to be a valuable tool for the study of the biological function of the Ag85 complex. In addition, these antibodies hold great promise as tools that can be used toward the development of diagnostic methods and drug development for *M. tuberculosis*.

MATERIALS AND METHODS

Construction of Recombinant Expression Vector

The *fbpA* gene was PCR amplified from chromosomal DNA isolated from the *M. tuberculosis* H37Rv strain. The sequences of the primer used for PCR amplification are as follows: sense primer, 5′-AAGCGGATCCATGTTTTCCCGGCCGGGCTTG-3′, antisense primer, 5′-AGTCGAATTCTGTTCGGAGCTAGGCG CCCTGGG-3′. Amplification reactions were carried out at 95°C for 5 min followed by 30 cycles at 94°C for 45 s, annealing at 55°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 30 min. The generated gene fragments were then ligated to the T-cloning site of a pMD20-T vector (Takara, Japan). This was then isolated by digestion with *Bam*HI and *Eco*RI. The *fbpA* gene was then ligated into pET-30a and pGEX-6p-1 vectors to generate recombinant plasmids.

The genes *fbpA*, *fbpB*, and *fbpC* were fused genetically to GFP gene to generate GFP-*fbpA*, GFP-*fbpB*, and GFP-*fbpC* fragments, respectively, by the splice overlap extensioning PCR technique. Briefly, the GFP gene fragment was amplified from the EGFP plasmid by PCR using primers GFP-F1 and GFP-R1. The *fbpA*, *fbpB*, and *fbpC* genes were amplified from the genomic DNA of *M. tuberculosis* H37Rv strain by PCR, using primers *fbpA*-F2, *fbpA*-R2, *fbpB*-F2, *fbpB*-R2, *fbpC*-F2, and *fbpC*-R2. The sequence for the four pairs of primers was summarized in **Table 1**. After the first rounds of PCR using GFP-F1/GFP-R1, *fbpA*-F2/*fbpA*-R2, *fbpB*-F2/*fbpB*-R2, and *fbpC*-F2/*fbpC*-R2, the PCR products were gel purified. In a second round of PCR, using the resulting PCR products as templates, the GFP-*fbpA*, GFP-*fbpB*, and GFP-*fbpC* fusion genes were created by overlap PCR using primers GFP-F1/*fbpA*-R2, GFP-F1/*fbpB*-R2, and GFP-F1/*fbpC*-R2. The sequence GGGGSGGGGS was incorporated at the junction of the GFP fragment and *fbpA*/*fbpB*/*fbpC* gene as a flexible linker. The fusion genes were cloned into the pcDNA3.1(+) vector (Invitrogen, USA) between the *Eco*RI and *Xba* I restriction sites to generate pcDNA3.1-GFP-*fbpA*, pcDNA3.1-GFP-*fbpB*, and pcDNA3.1-GFP-*fbpC* constructs and confirmed by restriction endonuclease digestion and DNA sequencing.

TABLE 1 | Primer sequences.

Primers	Sequences
GFP-F1	5′-TAGAATTCGCCACCATGGTGA GCAAGGGCGAGGAGCTG-3′
GFP-R1	5′-CACCGCCGCTTCCACCGCCACC CTTGTACAGCTCGTCCATGCCGAG-3′
fbpA-F2	5′-GTGGAAGCGGCGGTGGCGGAAGC ATGCAGCTTGTTGACAGGGTTC-3′
fbpA-R2	5′-TATCTAGAGTTGTGTCTGTT CGGAGCTAGGC-3′
fbpB-F2	5′-GTGGAAGCGGCGGTGGCGGAAGC ATGACAGACGTGAGCCGAAAGA-3′
fbpB-R2	5′-TATCTAGAAACCCTTCGGTTGATCCCGTCA-3′
fbpC-F2	5′-GTGGAAGCGGCGGTGGCGGAAGC ATGACGTTCTTCGAACAGGT-3′
fbpC-R2	5′-TATCTAGAGATGCTGGCTTGCTGGCTCA-3′

Expression and Purification of Recombinant Ag85A Protein

The pET-30a-*fbpA* and pGEX-6p-1-*fbpA* constructs were transformed into *Escherichia coli* strains BL21(DE3) and BL21, respectively. Transformed *E. coli* cells were cultured and recombinant protein expression was induced using 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were then harvested and lysed by sonication on ice. The recombinant proteins, rHis-Ag85A and rGST-Ag85A, were purified from the lysate using the His-binding purification kit (Novagen, Germany) and GST-binding purification kit (GE, USA) according to the manufacturer's instructions, respectively.

Immunization of Mice and Establishment of Hybridomas

BALB/c mice (females, 6 weeks old) were injected subcutaneously with 80 μg of purified rGST-Ag85A protein mixed with Freund's complete adjuvant (Sigma-Aldrich, USA) in a 1:1 volumetric ratio. A secondary immunization with the same antigen was also mixed with Freund's incomplete adjuvant (Sigma-Aldrich, USA) in a 1:1 volumetric ratio, and it was given at 2-week intervals. One week following the secondary immunization, the serum titer was determined using an indirect ELISA method with purified rHis-Ag85A protein. The final booster immunization with the same antigen was injected intravenously 3 days prior to cell fusion, without any adjuvants. Finally, the splenocytes of BALB/c mice were collected and fused with SP2/0 myeloma cells using standard hybridoma methods (25). The mice were housed, handled, and immunized at our animal biosafety facilities, and all procedures were approved by the Institutional Animal Experimental Committee of Yangzhou University. All experiments were performed according to the national guidelines for animal welfare.

Screening of Hybridoma Cells

To screen for positive hybridomas, titers of the hybridoma supernatants were determined using an indirect ELISA method with purified rHis-Ag85A protein. In these experiments, a polyclonal antibody against Ag85A protein was used as positive control, and the supernatant from SP2/0 myeloma cells was used as negative control. After subcloning cells two to three times, hybridoma cells that stably secreted antibody were established. Hybridoma cells were then intraperitoneally injected into BALB/c mice to induce the generation of ascites containing mAbs against Ag85A protein.

Isotype and Titer Analysis

Both the class and subclass of the mAbs against Ag85A produced were determined using a mouse monoclonal antibody isotyping kit (Sigma-Aldrich, USA). Protocols were carried out according to the manufacturer's instructions.

ELISA plates (Nunc, Denmark) were coated with purified rHis-Ag85A protein (1 μg/ml) and left overnight at 4°C. The next morning, plates were washed three times with PBST and blocked with phosphate-buffered saline (PBS) containing 2% bovine serum albumin (BSA) at 37°C for 2 h. Following the blocking step, the plates were washed three times with PBST. Plates were then incubated with serially diluted culture supernatant and ascites. Plates were incubated with primary antibody for 1 h at 37°C. HRP-conjugated goat anti-mouse IgG antibody (1:8,000 dilution) (Sigma-Aldrich, USA) was then added to each well (100 μl/well) and incubated for 1 h at 37°C. Finally, the TMB substrate was then added to the plates and incubated for 10 min, after which the plates were read at 450 nm.

Western Blot Analysis

Purified rHis-Ag85A, rGST-Ag85A, wtBCG, and rBCG:Ag85A (26) were separated on 12% SDS-PAGE gels. Protein was then transferred to PVDF membrane. The membrane was then blocked for 2 h at room temperature with 2% BSA in PBS. The membrane was then washed three times with PBS containing 0.05% Tween 20 (PBST). Following the washes, the membrane was incubated with anti-Ag85A mAbs (1:1,000) at room temperature for 1 h. The membrane was then washed again three times in PBST, followed by incubation with HRP-conjugated goat anti-mouse secondary antibody (1:2,000) (Sigma-Aldrich, USA). Finally, the membrane was developed using 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, USA) and visualized using X-ray film.

Indirect ELISA Assay

ELISA plates (Nunc, Denmark) were coated with MPT63, RpfE, CFP10-ESAT6, and Ag85A protein (1 μg/ml), respectively, and left overnight at 4°C. The next morning, plates were washed three times with PBST and blocked with PBS containing 2% BSA at 37°C for 2 h. Following the blocking step, the plates were washed three times with PBST. Plates were then incubated with the anti-Ag85A mAbs (1:2,000 dilution). Plates were incubated with primary antibody for 1 h at 37°C. HRP-conjugated goat anti-mouse IgG antibody (1:8,000 dilution) (Sigma-Aldrich, USA) was then added to each well (100 μl/well) and incubated for 1 h at 37°C. Finally, the TMB substrate was then added to the plates and incubated for 10 min, after which the plates were read at 450 nm.

Transient Transfection and Indirect Immunofluorescence Test

One day prior to transfection, HEK293T cells were plated in 24-well plates in complete DMEM at a density of 1×10^5 cells/well. Cells were permitted to attach to the plate overnight at 37°C in a 5% CO_2 atmosphere. The following day, HEK293T monolayers were transfected with the pcDNA3.1-GFP-*fbpA*, pcDNA3.1-GFP-*fbpB*, and pcDNA3.1-GFP-*fbpC* constructs using Lipofectamine reagent 3000 (Invitrogen, USA) according to the manufacturer's instructions.

Twenty-four hours posttransfection, HEK293T cells were washed twice with PBS. Cells were then fixed with methanol for 15 min at room temperature. Following fixation, the cells were incubated with anti-Ag85A mAbs for 2 h at room temperature. The cells were then washed with PBS and incubated with Alexa Fluor® 586 conjugated goat anti-mouse IgG secondary antibody (Life Technology, USA) for 1 h. Cell staining was observed using fluorescence microscopy.

Detection of Ag85A Protein in BCG and rBCG:Ag85A

ELISA plates (Nunc, Denmark) were first treated with 5% glutaraldehyde (100 μl/well) and incubated at 37°C for 2 h. Plates were then washed three times with PBST. Following the washes, plates were coated with 1×10^6 colony-forming units of BCG or rBCG:Ag85A bacteria (100 μl/well) and left overnight at 56°C. The plates were washed with PBST and blocked with PBS containing 2% BSA at 37°C for 1 h. Then, the plates were washed and incubated with the mAbs for 2 h at 37°C. HRP-conjugated goat anti-mouse IgG antibody (1:8,000 dilution) (Sigma-Aldrich, USA) was added to each well (100 μl/well) and incubated for 1 h at 37°C. Finally, the TMB substrate was then added to the plates and incubated for 10 min, after which the plates were read at 450 nm.

Protocol of cELISA Assay

ELISA plates (Nunc, Denmark) were coated with rHis-Ag85A protein (1 μg/ml) overnight at 4°C. The next morning, plates were washed three times with PBST and blocked with PBS containing 2% BSA at 37°C for 2 h. Following the blocking step, the plates were washed three times with PBST. Test and control sera samples were diluted 1:100 in PBS and added to each well (50 μl/well), and HRP-labeled anti-Ag85A antibody 3B8 were diluted (1:1,000) and added to each well (50 μl/well), followed by incubation at 37°C for 2 h and washing. Finally, the TMB substrate was then added to the plates and incubated for 10 min, after which the plates were read at 450 nm. Results were calculated based on the OD450 values of the negative control serum sample (N) and test serum sample (S) using the following formula: % inhibition $= [(N - S)/N] \times 100\%$. A total of 200 healthy bovine serum samples were tested for determination of cutoff point. The mean inhibition rate + 3× SD was cutoff point (Cn). If the inhibition rate ≥Cn, the bovine TB antibody reaction is positive, if the inhibition rate <Cn, the bovine TB antibody reaction is negative.

Evaluation of cELISA for Detection of Anti-Ag85A Antibody

Total of 298 serum samples were harvested from dairy farms, of which 76 samples are positive and 222 samples are negative identified by PPD skin test, 70 samples are positive and 228 samples are negative identified by interferon-gamma release assay (IFN-γ assay) (Prionics, Switzerland). Bovine serum samples were diluted 100-fold and the inhibition rate was measured using cELISA method based on the criteria described above to determine the sensitivity and specificity of the assay.

Statistical Analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using a Student's t-test. A value of $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ was considered statistically significant.

RESULTS

Isotype and Titer Analysis of mAbs

Following two to three rounds of cell subcloning and detection, hybridoma cells producing anti-Ag85A mAbs were established and named 1C6, 2E6, 2F2, 3B8, and 3D9. Isotype analysis of the mAbs produced against Ag85A protein was determined using a mouse mAb isotyping kit. These results demonstrated that all nine mAbs belong to the IgG1 isotype (**Table 2**). In addition, ELISA results suggested that both hybridoma supernatant and ascites possessed high titer levels (**Table 2**).

Western Blot Analysis

The reactivity of anti-Ag85A mAbs against recombinant Ag85A protein was studied using Western blot analysis. We show that all anti-Ag85A mAbs react with rHis-Ag85A and rGST-Ag85A protein (**Figure 1**), with all the mAbs exhibiting good reactivity.

Indirect ELISA Assay

In order to evaluate the specificity of anti-Ag85A mAbs, the cross-reactivity of anti-Ag85A mAbs against other protein from mycobacteria was detected by indirect ELISA assay. The results showed that anti-Ag85A mAbs 1C6, 2E6, 2F2, 3B8, and 3D9 did not react with MTP63, RpfE, and CFP10-ESAT6 (**Figure 2**). All the mAbs did not react with selected mycobacteria protein, suggesting these mAbs are specific to Ag85A protein.

Indirect Immunofluorescence Test

In order to evaluate the cross-reactivity of anti-Ag85A mAbs with the Ag85 complex, HEK293T cells were transfected with pcDNA3.1-*fbpA*-GFP, pcDNA3.1-*fbpB*-GFP, and pcDNA3.1-*fbpC*-GFP constructs. Cells were then stained with the anti-Ag85A mAbs. We show that following staining with mAbs 1C6 and 3B8, only HEK293T cells transfected with pcDNA3.1-*fbpA*-GFP exhibited yellow fluorescence (**Figures 3A,D**). However, HEK293T cells transfected with pcDNA3.1-*fbpB*-GFP and/or pcDNA3.1-*fbpC*-GFP also exhibited yellow fluorescence following staining with the mAbs 2E6, 2F2, and 3D9 (**Figures 3B,C,E**). The HEK293T cells in the control group did not exhibited yellow fluorescence (**Figure 3F**). This suggests that a subset of anti-Ag85A mAbs cross-react with Ag85B and/or Ag85C. The mAbs 2F2 and 2E6 cross-react with Ag85B and Ag85C, respectively, and the mAbs 3D9 can react with Ag85A, Ag85B, and Ag85C.

Detection of Ag85A Protein in BCG and rBCG:Ag85A

To determine whether the generated mAbs can also combine with endogenous Ag85A protein in BCG, we used Western blot and indirect ELISA methods to detect BCG and rBCG:Ag85A. We showed that all anti-Ag85A mAbs reacted with endogenous Ag85A protein in BCG and rBCG:Ag85A, the 31-kDa Ag85A

TABLE 2 | Identification and characteristic of anti-Ag85A monoclonal antibodies (mAbs).

mAbs	Isotype	Supernatant titer	Ascites titer
1C6	IgG1	1:81,902	1:8,192,000
2E6	IgG1	1:10,240	1:16,384,000
2F2	IgG1	1:327,680	1:16,384,000
3B8	IgG1	1:20,480	1:1,024,000
3D9	IgG1	1:40,960	1:8,192,000

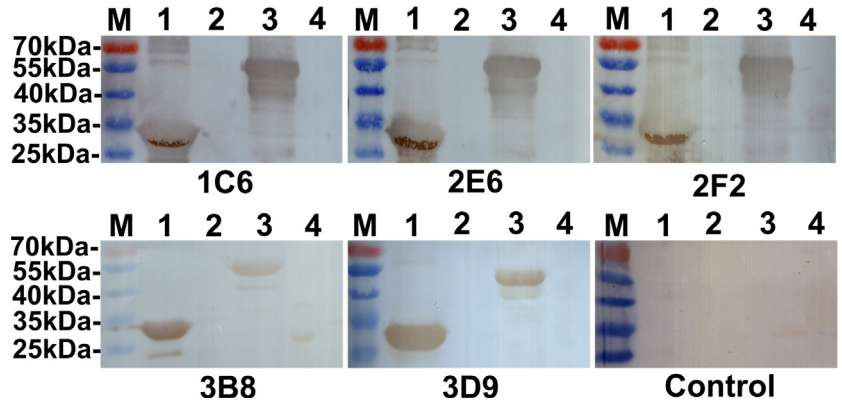

FIGURE 1 | Western blot analysis. All anti-Ag85A monoclonal antibodies (mAbs) recognized rHis-Ag85A protein (lane 1) and rGST-Ag85A protein (lane 3). Anti-Ag85A mAbs did not react with BL21(DE3) (pET-30a) (lane 2) and BL21(pGEX-6p-1) (lane 4). The control group was recombinant protein react with SP2/0 ascites.

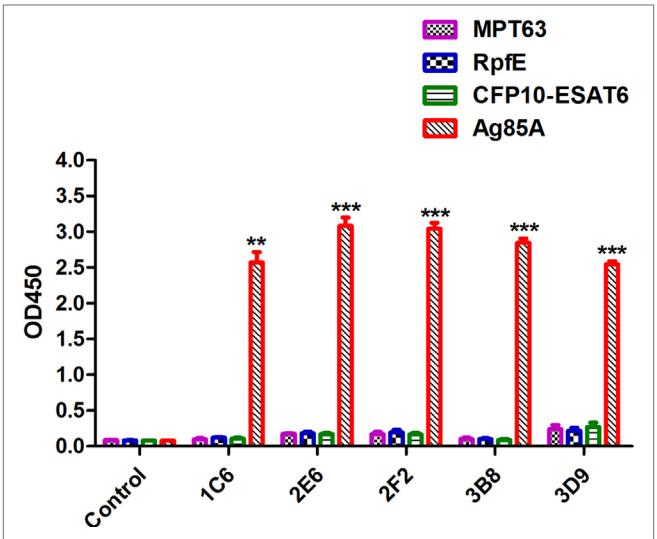

FIGURE 2 | Indirect ELISA assay. The specificity of anti-Ag85A monoclonal antibodies (mAbs) was detected by indirect ELISA. All anti-Ag85A mAbs strongly recognized Ag85A protein, but did not react with MPT63, RpfE, and CFP10-ESAT6 protein. The control group was recombinant protein react with SP2/0 ascites. The control group was SP2/0 ascites control. Data depicted are the mean values ± SEM. Statistical significance was determined by a Student's *t*-test (**$P < 0.01$, ***$P < 0.001$).

protein levels are increased twofold in rBCG:Ag85A compared with those of BCG (**Figures 4A,B**). We also note that these mAbs reacted with other protein bands, which could represent Ag85B and/or Ag85C protein (**Figure 4B**), respectively.

Development of cELISA for Detection of Anti-Ag85A Antibody

A cELISA for detection of anti-Ag85A antibody was established. The rHis-Ag85A protein was coated on the ELISA plate as capture antigen, and anti-Ag85A antibody clone 3B8 was used as a detection antibody. The OD_{450} value was detected for 200 healthy bovine serum samples, the mean inhibition rate was 0.162 with a SD of 0.029, the Cn value was 0.249 (≈25%). A total of 248 serum samples (48 bovine TB positive samples and 200 bovine TB negative samples detected by both skin test and IFN-γ assay) were detected, the inhibition rate of 26 samples were above 25%, and the inhibition rate of 167 samples were below 25%, the results indicated that the diagnostic specificity and sensitivity were 54.2% (26/48) and 83.5% (167/200), respectively. A total of 298 serum samples (76 positive by skin test; 222 negative by skin test) were analyzed, the cELISA results were compared with those of the skin test. 45 skin test positive serum samples were also positive by cELISA, with a positive coincidence of 54.2% (90/166). And 177 skin test negative serum samples were negative by cELISA, with a negative coincidence of 82.3% (354/430). Therefore, the total coincidence of the cELISA method and skin test was 74.5% (**Table 3**). And the results were also compared with the IFN-γ assay, 298 serum samples (70 positive by IFN-γ assay; 228 negative by IFN-γ assay) were detected, 38 serum samples were also positive by cELISA with a positive coincidence of 47.5% (76/160), and 176 serum samples were also negative by cELISA with a negative coincidence of 80.7% (352/436). Therefore, the total coincidence of the cELISA assay and IFN-γ assay was 71.8% (**Table 4**).

DISCUSSION

The Ag85A protein is a member of the Ag85 complex, a 30- to 32-kDa family of three proteins (Ag85A, Ag85B, and Ag85C). All three members of the Ag85 complex have been shown to exhibit mycolyltransferase activity (27). Interestingly, Ag85A has also been shown to stimulate production of Th1 cytokines and CTL activity. Because this protein generates a strong immune response, it is thought to be an ideal candidate antigen for the development of novel vaccines (28, 29).

However, there were still no commercial mAbs available against Ag85A. Thus, it may hinder the biological research and applicative research of Ag85A. The widely used commercial anti-Ag85

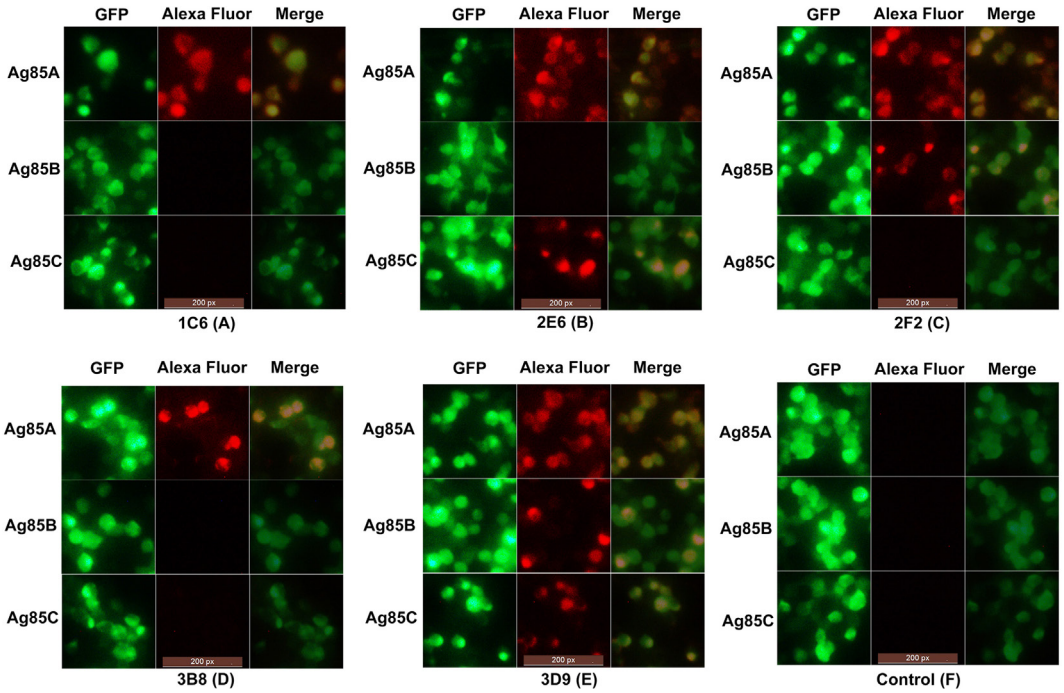

FIGURE 3 | Indirect immunofluorescence test. HEK293T cells transfected with pcDNA3.1-*fbpA*-GFP, pcDNA3.1-*fbpB*-GFP, and pcDNA3.1-*fbpC*-GFP constructs were stained with anti-Ag85A monoclonal antibodies (mAbs) and Alexa Fluor 568-conjugated anti-mouse IgG antibody, respectively. Following staining with mAbs 1C6 and 3B8, only HEK293T cells transfected with pcDNA3.1-*fbpA*-GFP exhibited yellow fluorescence **(A,D)**. However, HEK293T cells transfected with pcDNA3.1-*fbpB*-GFP and/or pcDNA3.1-*fbpC*-GFP also exhibited yellow fluorescence following staining with mAbs 2E6, 2F2, and 3D9 **(B,C,E)**. The control group was HEK293T cells stained with SP2/0 ascites **(F)**.

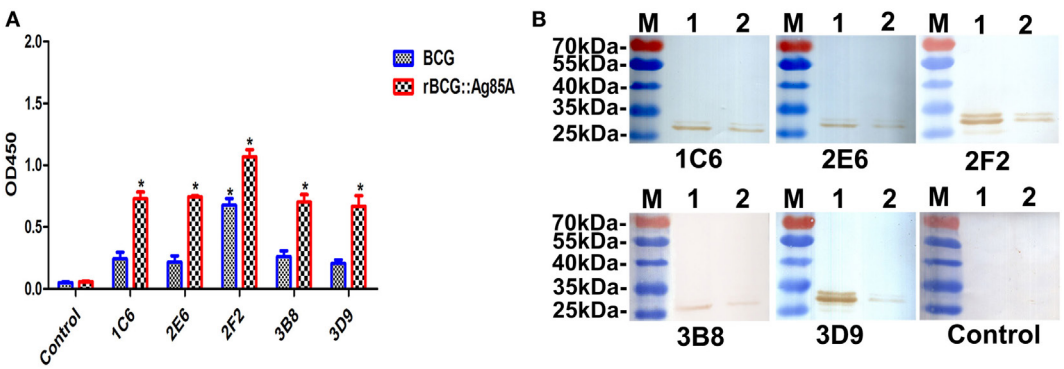

FIGURE 4 | Detection of Ag85A protein in BCG and rBCG:Ag85A. BCG and rBCG:Ag85A were detected using indirect ELISA and Western blot. All anti-Ag85A monoclonal antibodies (mAbs) were able to react with endogenous Ag85A protein in BCG and rBCG:Ag85A group **(A)**. All of the mAbs were able to recognize endogenous Ag85A protein in rBCG:Ag85A [**(B)** lane 1] and BCG [**(B)** lane 2]. The control group was SP2/0 ascites control. Data depicted are the mean values ± SEM. Statistical significance was determined by a Student's *t*-test (*$P < 0.05$).

TABLE 3 | Comparative result of competitive ELISA (cELISA) assay and skin test.

		cELISA assay		Total
		P	*N*	
Skin test	*P*	45	31	76
	N	45	177	222
Total		90	208	298

TABLE 4 | Comparative result of competitive ELISA (cELISA) assay and IFN-γ assay.

		cELISA assay		Total
		P	*N*	
IFN-γ assay	*P*	38	32	70
	N	52	176	228
Total		90	208	298

antibody HYT 27 only reacts strongly with *M. tuberculosis* Ag85C protein. The rabbit polyclonal antibody against *M. tuberculosis* Ag85B is only specific for the Ag85B protein.

Here, we used rGST-Ag85A protein as an immune antigen and rHis-Ag85A protein as a detective antigen. A total of five mAbs were established, and named as 1C6, 2E6, 2F2, 3B8, and 3D9. All nine mAbs used in this study were determined to be of the IgG1 isotype. Analysis of the generated mAbs showed that both hybridoma supernatant and ascites had a high titer. In addition, we show through Western blot analysis that all of the mAbs generated in this study are able to react with recombinant Ag85A protein. All the mAbs did not react with selected mycobacteria protein, suggesting these mAbs are specific to Ag85A protein.

Mycobacterium tuberculosis Ag85A, Ag85B, and Ag85C are highly homologous on the DNA and amino acid level (6). In order to evaluate the cross-reactivity of anti-Ag85A mAbs with the Ag85 complex, the HEK293T cells transfected with pcDNA3.1-*fbpA*-GFP, pcDNA3.1-*fbpB*-GFP, and pcDNA3.1-*fbpC*-GFP constructs were detected by indirect immunofluorescence, respectively. The mAbs 1C6 and 3B8 were specific only for Ag85A, the mAbs 2F2, 2E6, and 3D9 cross-react with Ag85B and/or Ag85C, respectively. The results suggested that a subset of the anti-Ag85A mAbs is able to cross-react with Ag85B and/or Ag85C. It is likely that some mAbs could recognize the same epitopes of Ag85A, Ag85B, and Ag85C protein. It may be beneficial to the wide employment of anti-Ag85A mAbs.

In previous works, we developed many mAbs using recombinant protein expressed in *E. coli*. All of these mAbs may have good reactivity against recombinant proteins but may not be reactive against the natural proteins (30). As a result, we identified the reactivity of anti-Ag85A mAbs against BCG. The indirect ELISA and Western blot analysis showed that all of the generated mAbs are able to react with endogenous Ag85A protein in BCG and rBCG:Ag85A. Currently, several serological tests with promising accuracy have recently emerged, many ELISA technology has been intensively established for the detection of serum antibodies of bovine TB (31–34). In this research, a cELISA based on mAb 3B8 was developed, the analyses of clinic serum samples from cattles with bovine TB and healthy cattles demonstrated that the diagnostic specificity and sensitivity were 54.2% (26/48) and 83.5% (167/200), respectively, and the total coincidence of the cELISA method with skin test and IFN-γ assay was 74.5 and 71.8%, respectively. Although the poor sensitivity of antibody-based ELISA methods has prevented widespread use of these assays for early detection of bovine TB, such as the sensitivity of an iELISA established with rM70-83-E6 as the diagnostic antigen was 69.4% (59/85) and the specificity was 96.0% (96/100) (32), an ELISA technology that detects antibody to *M. bovis* antigens MPB83 and MPB70 was established, and the sensitivity and specificity of the ELISA assay with naturally infected cattle were 63 and 98%, respectively (34). However, antibody responses to *M. bovis* well correlate with *M. bovis*-elicited pathology and *M. bovis* antigen burden (35), antibody response-based assays may be used in a wide range of applications and a supplemental test to cell-mediated response-based assays.

Overall, the mAbs generated against Ag85A will prove to be a valuable tool for the study of the subcellular localization and biological function of the Ag85 complex; in addition, these antibodies could act as important materials for the development of a diagnostic method for TB, as well as identification of novel drug candidates for the treatment of this infectious disease.

AUTHOR CONTRIBUTIONS

ZX, XC, and XJ designed the experiments. ZX, TH, AX, XL, ZL, JM, JH, and CM performed the experiments and analyzed the data. YY, XC, and XJ contributed reagents/materials/analysis tools. ZX, XC, and XJ wrote and revised the paper.

FUNDING

This work was supported in part by the National Key Research and Development Program of China (2017YFD0500300), the National Natural Science Foundation of China (31602031), the Science and Technology Program of Jiangsu (BE2015343, BK20160466, 16KJB230003), and the Qinglan Project and Priority Academic Development Program of Jiangsu Higher Education Institutions (PADP).

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Conflict of Interest Statement: 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: 30 May 2018
doi: 10.3389/fvets.2018.00088

Prevalence, Diagnosis, and Vaccination Situation of Animal Chlamydiosis in China

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Since the first case of *Chlamydia* infection in duck had been reported in 1956 and the first case from domestic animal had been reported in 1979 in China, the chlamydia prevalence in China was heavily according to the published data. The *Chlamydia* in avian prevalence has been reported at least 11 provinces, *Chlamydia* in sheep and goats at least 11 provinces, in swine at least 15 provinces, in cows at least 13 provinces and in yaks at least 5 provinces with result of IHA detection. Different diagnostic method such as CFT, ELISA and ABC-ELISA (avidin-biotin-complex ELISA) had been established besides IHA. The inactivated vaccines have been developed with isolated strains from sheep, goats, swine and cows. These inactivated vaccines have been used since 1980s and *Chlamydia* prevalence in China has been successfully controlled in domestic animal. However, the inactivated vaccines of Chlamydia isolated from avian species have not been successful, although a series of experimental vaccine have been done. Due to the unsustainable eradication plan of *Chlamydia* in China, sporadic outbreak in animal would happen if the vaccinations were suspended and economy lose in some farmers. Although Chlamydia prevalence in China has a long history, however, almost all published studies are in Chinese, which, in some degree, blocked scientists in other countries to understand the prevalence situation and control measures of *Chlamydia* in China.

Keywords: prevalence, diagnosis, vaccine, *Chlamydia*, China

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: *20 December 2017*
Accepted: *06 April 2018*
Published: *30 May 2018*

Citation:
Zhou J, Li Z, Lou Z and Fei Y (2018) Prevalence, Diagnosis, and Vaccination Situation of Animal Chlamydiosis in China. Front. Vet. Sci. 5:88. doi: 10.3389/fvets.2018.00088

INTRODUCTION

Chlamydiae, zoonotic and obligate intracellular Gram-negative bacteria, have a worldwide distribution and cause a wide range of diseases in human hosts, livestock, companion animals, wildlife, exotic species (1), and poultry (2). Chlamydiosis, a disease caused by *Chlamydia,* has been found in many countries around the world where sheep-rearing is practiced, ranging from Europe to Africa, North America (3), and Asia (4). These include France (5), Poland (6), Spain (7), Australia (8), the United Kingdom (9), Ireland (10), China (11) and Switerland (12).

Vaccination is one of the best ways to control Chlamydiosis. Formalin-inactivated, egg-grown vaccine of *Chlamydia abortus* was developed (13) in the U. K. The inactivated vaccine of *C. abortus* was used in sheep although the efficacy of the vaccine against EAE was unstisfatory (14). After the first reported cases of avian *Chlamydia* in China in 1959 (15) and reports of infection in domestic animals in Qinghai province in 1981 (4), China began developing inactivated vaccines using different isolates from sheep, swine, and cow, which successfully controlled the prevalence

of *Chlamydia* in China. However, almost all these achievements were reported only in Chinese, rather than in English, which presents a challenge for scientists all over the world to understand the situation of *Chlamydia* prevalence and control measures in China.

CHLAMYDIA PREVALENCE

Following the first report of *Chlamydiosis* in China from a duck in Beijing in 1959 (15), prevalence of avian *Chlamydia* has been reported in 10 provinces, including Beijing, Tianjin, Jiangsu, and Guangdong due to lack of effective vaccines (16).

The first instance of livestock *Chlamydiosis* was found in ruminants in the Qinghai province by scientists from the Lanzhou Veterinary Research institute (LVRI), Chinese Academy of Agricultural Sciences (CAAS), in 1979 (17). *Chlamydia* was detected in sheep and goats in at least 11 provinces, in swine in 15 provinces, in bovines in 13 provinces, and in yaks in 5 provinces, using the indirect hemagglutination assay (IHA) test; this prevalence if the disease caused a huge economic loss in China (**Figure 1**). Although the seropositive rate was high in different animals in China, similar seropositive rates existed in other countries. For example, a German study reported *Chlamydia* antigen prevalence in sheep to range between 5 and 71% (18) and a Polish study reported the prevalence of *Chlamydial* infection in birds in Europe to range between 6.3

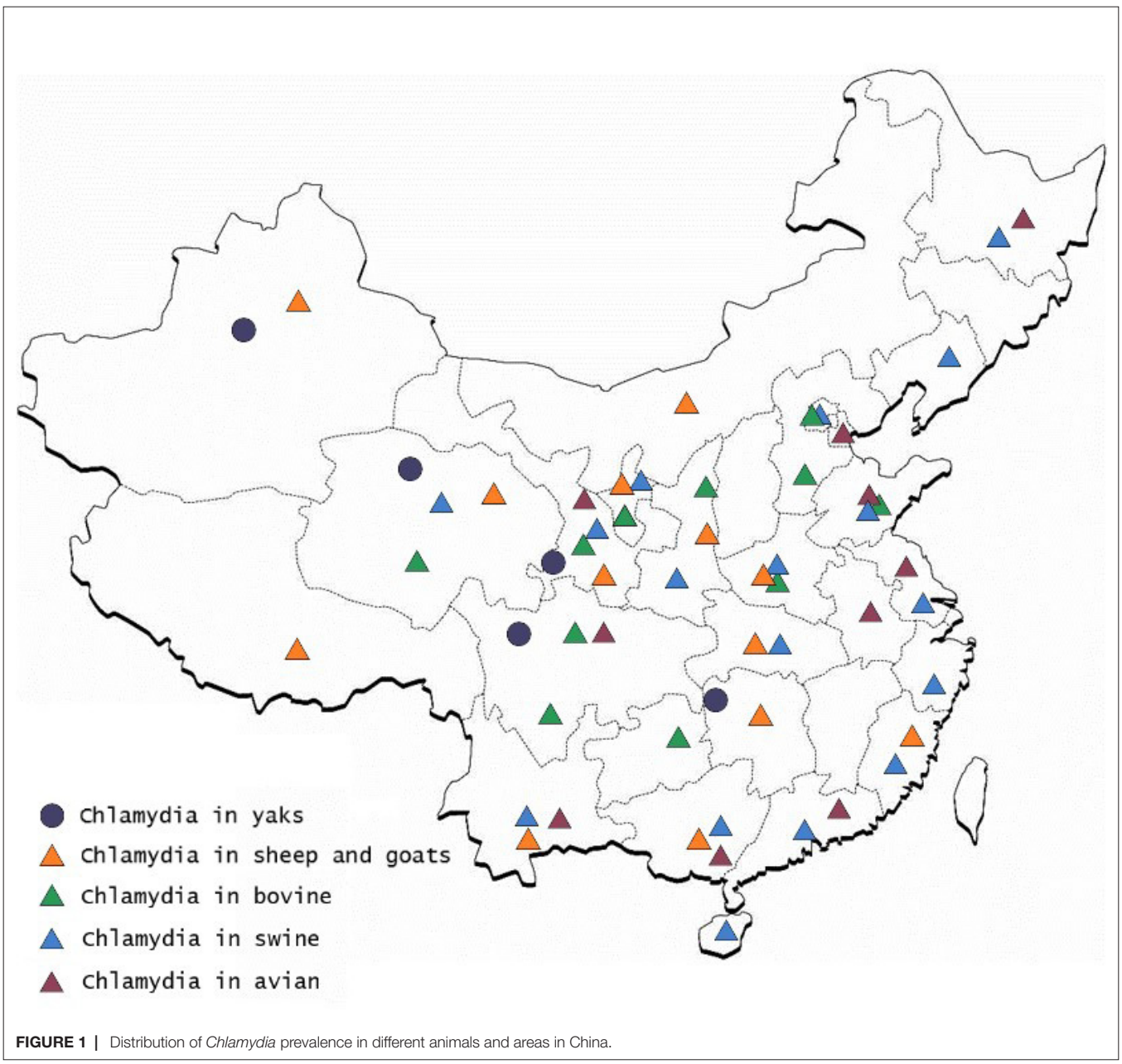

FIGURE 1 | Distribution of *Chlamydia* prevalence in different animals and areas in China.

TABLE 1 | Avian *Chlamydia* seroprevalence in China.

Year	Positive rate	Area	Reference (published in Chinese)
1991	21.8%	Shandong	Wu *et al.* (1990:61) (23)
1988–1989	39.2%	Sichuan	Xu *et al.* (1991:18–19) (24)
1991	26.91%	Gansu	Wang *et al.* (1991:18) (25)
1994	20–40%	Jiangsu	Yu *et al.* (1994:13–15) (20)
1994	22.7%	Yunnan	Wang *et al.* (1997:10–12) (26)
1998	5.1%	Heilongjiang	Jiang *et al.* (1998:25–6) (27)
2001	20.66%	Guangxi	Liu *et al.* (2001:13) (28)
2003	10–30%	Beijing Tianjin	Shi *et al.* (2003:217–21) (21)
2003	59.9%	Guangdong	Zhang *et al.* (2003:29) (29)
2012	20–45.4%	Anhui	Ou *et al.* (2012:61-3) (30)
2013	3.9%	Qinghai	Ma *et al.* (2013:213–5) (31)
2012–2014	36.97%	Tianjin	Zhu *et al.* (2016:148–50) (32)
2016	43.2%	Sichuan	Ouyang *et al.* (2016:46–51) (22)

and 19.2% (19). There was uncertainty about the accuracy of the results of *Chlamydia* detected in animals using the IHA technique in China. However, compared with the results from other countries, which used McCoy cell culture isolation, the IHA technique may reflect the real prevalence of *Chlamydia* in China.

Using IHA, *Chlamydia* was found to be prevalent in chicken, ducks, pigeons, geese, and other avian species in 11 provinces, with seropositive rates of 10 to 60% in last three decades (20–22) (**Table 1**). Likewise, *Chlamydia* was prevalent in cows, yaks, sheep, goats, and pigs with seropositive rates of 2–40% in goats and sheep (4, 33–38) (**Table 2**), 5–53% in swine (50–55) (**Table 3**), 3–35% in bovine (67–71) (**Table 4**), and 2–30% in yak (11, 76–79) (**Table 5**). The disease was found in almost the entire country through IHA detection.

TABLE 2 | Sheep and goat *Chlamydia* seroprevalence in China.

Year	Positive rate	Area	Reference (published in Chinese)
1981		Qinghai	Yang *et al.* (1981:13–15) (4)
1987–1989	6.9%	Zhejiang	Wang *et al.*(1990:11) (39)
1988–1989	2.95%	Hubei	Zhang *et al.* (1992:34) (40)
1998	5.78%	Hunan	Qiu et al.(1998:3-5) (41)
1996–1998	1.98%	Guangxi	Wu *et al.* (2000:41) (36)
1991–1995	26.12%	Yunan	Wang *et al.* (2000:465–6) (42)
2003	19.3%	Ningxia	Bao *et al.* (2003:13–14) (43)
2009	7.57%	Neimeng	Wang *et al.* (2009:154) (44)
2010–2011	36.12%	Guizhou	Hong *et al.* (2012:127–9) (37)
2012	1.4%	Xinjiang	Lei *et al.* (2012:28-9) (45)
2013	20.9%	Xizang	Huang *et al.* (2013:243–5) (in English) (46)
2014	40.3%	Qinghai	Zhang *et al.* (2014:38–9) (47)
2014	60%	Hubei	Cheng *et al.* (2015:472–4) (48)
2014	42.86%	Anhui	Cheng *et al.* (2015:472–4) (48)
2014	52.75%	Shandong	Cheng *et al.* (2015:472–4) (48)
2014	40.13%	Xinjiang	Cheng *et al.* (2015:472–4) (48)
2014	51.91%	Jilin	Cheng *et al.* (2015:472–4) (48)
2014	30.56%	Sichuan	Cheng *et al.* (2015:472–4) (48)
2014	67.74%	Ningxia	Cheng *et al.* (2015:472–4) (48)
2014	90%	Gansu	Cheng *et al.* (2015:472–4) (48)
2012–2015	13.09%	Qinghai	Su.(2016:29-30) (35)
2015–2016	12.5%	Henan	Gao *et al.* (2017:66–9) (49)
2013–2017	11.87%	Qinghai	Zha *et al.* (2017:60–1) (38)

TABLE 3 | Swine *Chlamydia* seroprevalence in China.

Year	Positive rate	Area	Reference (published in Chinese)
1983–1984	29.72%	Hubei	Jiang *et al.* (1985:32–4) (50)
1985	33.3%	Qinghai	Diao *et al.* (1990:21–2) (56)
1991	20.4%	Guangxi	Yi *et al.* (1991:6–9) (55)
1997	13.65%	Shandong	Ji *et al.* (2003:39) (57)
1998–2000	34.91%	Gansu	Gao *et al.* (2001:13–14) (58)
2003	2.3%	Henan	Lang *et al.* (2004:29) (59)
2003	5.16%	Liaonin	Wang (2004:26) (60)
2005	49.49%	Hainan	Suo *et al.* (2005:31–2) (51)
2005	41.41%	Shanghai	Jin *et al.* (2005:23) (61)
2005	6.82%	Zhejiang	Jin *et al.* (2005:23) (61)
2004–2006	14.97%	Guangdong	Zhu *et al.* (2007:26–7) (62)
2006–2007	27.71%	Fujian	Zhou *et al*. (2008:30–5) (63)
2011	18.5%	Yunnan	Bi *et al.* (2011:134–6) (52)
2012	7.6%	Shaanxi	Wang *et al.* (2013:9–10) (64)
2013	53.30%	Qinghai	Ma *et al.* (2013:213–5) (31)
2014	58.59%	Jiangxi	Jang *et al.* (2014:27–28) (65)
2014	57.0%	Qinghai	Zhang *et al.* (2014:38–9) (47)
2014	18.88%	Yunnan	Li *et al.* (2014:29–30) (66)
2015	18.4%	Yunnan	Su *et al.* (2015:155–6) (53)
2013–2015	11.3%	Henan	Ma *et al.* (2016:119–22) (54)

DIAGNOSTIC METHOD

Before 1984, antibodies to *Chlamydia* were detected by using the complement fixation (CF) method in China. However, this technique was cumbersome and time consuming (88). Thus, a new technique, IHA, was developed. The IHA technique had a higher sensitivity and specificity than CF, and has been used to determine the prevalence of *Chlamydia* in domestic animals in China since the 1980s (88). It has also been used as a high throughput method of seroprevalence detection. Positive results are detectable within 2 h, but false positive and negative readings are possible, since scoring is subjective to the researcher's observation (88). Besides

TABLE 4 | Bovine *Chlamydia* seroprevalence in China.

Time	Positive rate	Area	Published in original journal
1988	26.81%	Hubei	Yang *et al.* (1988:5–6) (72)
1997–1998	16.13%	Shandong	Zhou *et al.* (2000:14–15) (67)
1997–1998	23.1%	Hebei	Zhou *et al.* (2000:14–15) (67)
1997–1998	43.18%	Shaanxi	Zhou *et al.* (2000:14–15) (67)
1997–1998	25.71%	Henan	Zhou *et al.* (2000:14–15) (67)
1997–1998	20.68%	Gansu	Zhou *et al.* (2000:14–15) (67)
1997–1998	25.6%	Sichuan	Zhou *et al.* (2000:14–15) (67)
1997–1998	16.8%	Ningxia	Zhou *et al.* (2000:14–15) (67)
1997–1998	15.49%	Qinghai	Zhou *et al.* (2000:14–15) (67)
2012	24.14%	Ningxia	Xie *et al.* (2012:102–4) (68)
2010–2011	2.86%	Guizhou	Hong *et al.* (2012:127–9) (37)
2013	21.3%	Chongqing	Huo (2013:67) (73)
2013	26.4%	Qinghai	Ma *et al.* (2013: 213-5) (31)
2013	17.71%	Gansu	Tan *et al.* (2015:1283–7) (74)
2013	38.97%	Ningxia	Tan *et al.* (2015:1283–7) (74)
2014	36.8%	Qinghai	Zhang *et al.* (2014:38–9) (47)
2012–2015	8.25%	Qinghai	Su.(2016:29–30) (35)
2015	26.31%	Qinghai	Wang *et al.* (2016:16–17) (69)
2015	37.43%	Henan	Li *et al.* (2017:22–4) (71)
2015–2016	1.75%	Qinghai	Chen *et al.* (2017:33–5) (75)
2013–2017	9.13%	Qinghai	Zha *et al.* (2017:60–1) (38)

TABLE 5 | Yak *Chlamydia* seroprevalence in China.

Year	Positive rate	Area	Reference (published in Chinese)
1988	29.0%	Qinghai	Shuai *et al.* (1988:76–81) (76)
1993	21.03%	Qinghai	Dong *et al.* (1993:25) (80)
1996	2.1%	Xinjiang	Wang *et al.* (1996:46) (77)
2000	20.69%	Gansu	Zhou *et al.* (2000:14–15) (67)
2000	15.49%	Qinghai	Zhou *et al.* (2000:14–15) (67)
2000	25.6%	Sichuan	Zhou *et al.* (2000:14–15) (67)
2004	19.23%	Qinghai	Ma *et al.* (2004:14) (81)
2009	9.81%	Qinghai	Zhang *et al.* (2009:14–15) (82)
2010	17.39%	Qinghai	Hou(2011:10) (78)
2010–2012	2.8%	Qinghai	Kong *et al.* (2012:51–51) (83)
2012	4.13%	Qinghai	Li *et al.* (2013:126) (84)
2013	27.7%	Qinghai	Xie(2013:33) (85)
2012–2013	15.9%	Gansu	Qin(2015:8) (in English) (79)
2014	25.08%	Gansu	Yin(2014:281–285) (86)
2009–2014	7.68%	Qinghai	Fu *et al.* (2016:50–51) (87)
2015	23.81%	Qinghai	Li *et al.* (2015:1–6) (in English) (11)

IHA, enzyme linked immunosorbent assay (ELISA) (89) and avidin-biotin-complex ELISA (ABC-ELISA) (90) techniques were developed. Although the ABC-ELISA and ELISA methods have higher sensitivity than the IHA method, they have not been used in clinical studies due to high cost.

For pathogen diagnosis, chicken egg isolation and Giemsa staining were combined to detect *Chlamydia*. The yolk sac membranes from dead chicken embryos were spread on slides and fixed with methanol or through heating, and Giemsa stain was used to stain them for half an hour or overnight. *Chlamydia* elementary body (EB) and inclusion were detected by light microscopy (91).

The PCR and real-time PCR tests, although highly sensitive and used to detect *Chlamydia* in different animals in other countries (92–96) have seldom been used on a large scale due to high cost. The *omp1* gene, which is very conservative, was used as a target gene to detect *Chlamydia* in different animals when IHA results were ambiguous (16, 97). In China, the PCR-RFLP method was developed and used only to identify *Chlamydia* species isolated from animals (11), while recombinase polymerase amplification (RPA) was used to identify *C. abortus* (98). However, IHA is considered as a simple, safe, and reliable means of testing *C. abortus* antibodies, and has been employed in previous serological investigations (79, 99).

PREVENTION AND CONTROL OF CHLAMYDIOSIS

Vaccination

Vaccination is one of the most important methods of disease prevention in animals. The inactivated vaccine of *C. abortus* plays a huge role to control the spreading of the disease in China. Simply, the process for developing inactivated vaccine is as follows: *Chlamydia* seeds were isolated from the yolk sac membranes of 7-day-old chicken embryos between the 4 and 8th days after inoculation. The titer of *Chlamydia* used for vaccines was at least 10^{12} ELD_{50}/0.4 ml. The yolk sac membrane was disrupted with a homogenizer and filtered through a mesh strainer. Formalin (0.3%) was used to inactivate *Chlamydia* with an equal volume of 206 adjuvant (SEPPIC, France) and mixed under 40 Mpa of pressure. After 7 days of inactivation with formalin, the safety and efficacy of the vaccine were tested using specific pathogen free (SPF) embryos and mice (100–102).

Chlamydia inactivated vaccines for sheep and goat have been used since 1981–1986 (100). During that time, 120,000 sheep and goats in Qinghai, Gansu province, and Xinjiang Uygur Autonomous region were vaccinated and the abortion rate declined sharply. No abortion happened due to vaccination in pregnant sheep and goats. A total of 2,000,000 ml (about 700,000 doses) of inactivated vaccine was produced and used in 1988 (100). The duration of immunity was at least 2 years, but 75% of the sheep and goats were protected from infection in the 4th year after vaccination (100, 103). Besides, the inactivated vaccine could be used after a 2 year storage period at 4°C (100). A similar inactivated vaccine of *Chlamydia* isolated from goat was studied by Zhang in the Inner Mongolia Autonomous Region (33). According to this report, 5,099 goats belonging to 51 groups were vaccinated with inactivated vaccine and at least 90% of the goats were protected. Other regions, such as Huachi County, Gansu province, showed similar results of very high prevalence of *Chlamydia* in goats during 1981–1986, with abortion rate in goats being 20–40%. The disease was controlled when inactivated vaccine was used, reducing the abortion rate of the vaccinated groups to 3.3–6.5%, compared with the control group abortion rate of 14.03–14.3%, during 1986–1988 (102).

Chlamydia of swine was also detected and isolated in Qinghai province and the GuangxiZhuang Autonomous Region (55, 56, 88). According to these reports, abortion happened among sows and the highest positive rate of abortion was 56.1% in one of the groups in which *Chlamydia* was detected using the complement fixation test (CFT). Two strains were isolated using 7-day-old SPF eggs, and the inactivated vaccine was produced and tested (101). A total of 1,080 sows were vaccinated in two farms and each sow was immunized subcutaneously with 3 ml of inactivated vaccine (101). After 3 months, 482 sows that had been vaccinated and 439 sows that were not vaccinated were studied; only 1.45% abortion rate was observed in the vaccinated group, while 29.53% abortion rate was observed in the non-vaccinated group (101). These results showed that the inactivated vaccine provided good infection protection (101). Subsequently, 10,594 sows were vaccinated in Qinghai, Shanxi province, and Guangxi Zhuang Autonomous regions. The duration of immunity was at least 1 year when 2 ml of vaccine was injected (104). The vaccine remained active after storage at 4–8°C for 1 year (101).

Chlamydia in bovines was reported firstly in China in 1986 (70). In 1990, 2 strains (CCS10 and CCS15) were isolated from cows in a farm in Shaanxi province (105). However, the strain used for the inactivated vaccine was isolated in 2006 (102). This isolated strain (SX5) from a farm in Shaanxi province was tested and the LD_{50}/0.4 ml remained at 10^{-12} after at least 5 times propagation in SPF eggs. The minimal effective dosages for the vaccine was 3 ml for adult dairy cow and 1.5 ml for calf. The average protection was about 94.4%, while the duration of immunity was 10 months (102).

Although formalin-inactivated *C. abortus* vaccines have been used in China since 1985, their production was discontinued because of lack of good manufacturing practices (GMP), causing

sporadic outbreaks in sheep (46), yaks (106), and other animals. However, there is no information about *Chlamydia* prevalence in recent years from Dulan county (100), Delingha (56), Qinghai province, and Huachi county (101), Gansu province, where *Chlamydia* was first isolated and animals were vaccinated with inactivated vaccine. On the contrary, farms near the original places, such as in Wulan county, Guinan county, Haiyan county, Gonghe county, Huzhu county, Huangyuan county, and Tianjun County, Qinghai province, reported that *Chlamydia* caused huge abortion among sheep, goats, and yaks in recent years (11, 35, 38, 68, 75, 84, 87, 107–109). Interestingly, during the investigation, shepherds reported that dead Tibetan antelopes were found and their eyes were obviously blind (personal communication). They also reported that *Chlamydia* spread among different groups of sheep that had never been in contact with each other, suggesting that wild animals may play a very important role to spread *Chlamydia* to domestic animal. Therefore, based on this information, we can conclude that the original source of *Chlamydia* infections in China is wild animals in Qinghai province.

Besides inactivated vaccine, the subunit vaccine for *Chlamydia* in ewes, which has three different doses of major outer-membrane protein from genetically engineered *Chlamydia psittaci,* was developed by the State Key Laboratory of Pathogen and Biosecurity, Institute of Microbiology and Epidemiology, Academy of Military Medical Sciences, China. The study analyzed the antibody responses in ewes vaccinated with the subunit vaccine of rCps-MOMP. The sera of ewes were detected before vaccination and at different times post-vaccination. Experimental results indicated that multilocus intramuscular injection in the neck region with a dose of 0.5 mg per ewe could stimulate good immune response (110). Because of the good security and immunity protection, a new veterinary drug certificate was awarded by the Ministry of Agriculture of the People's Republic of China (111). However, there are few reports of the promotion and application of the submit vaccine in China (112).

Although some successes were obtained for controlling *Chlamydia* in livestock in China since the inactivated vaccines were introduced, each year huge economic losses are caused by avian *Chlamydia* in chicken production (28). A subunit vaccine and a recombinant adenovirus live vector vaccine have been developed (16, 113). However, the recombinant adenovirus vector vaccine has not been approved by the government due to potential biological concerns, and no further data about the subunit vaccine has been published. Avian *Chlamydia* is still serious in China due to a lack of effective and safe vaccine. Moreover, the disease is a potential risk for human health. Therefore, further investigation into the development of vaccines is necessary.

TREATMENT

Antibiotics such as tetracycline, oxytetracycline, and penicillin sodium are used to treat Chlamydiosis in animals in China. However, since 2014, to regulate the use of veterinary antibiotics in China, the government established a veterinary prescription drug management system, including measures for administration of veterinary prescription and over-the-counter drugs. The quality of antibacterial drug products has been improving every year. The quality of sampling inspection is maintained at more than 95%, whereas the rate of residues of veterinary drugs in livestock and poultry products remains stable at more than 97%. Thus, the use of antibiotics has been greatly reduced.

FUTURE PERSPECTIVE

Although IHA plays a very important role in detecting *Chlamydia* in animals in China, it does not reflect the real situation of Chlamydiosis prevalence in animals. However, Hagemann JB (114) reported that aborting sheep exhibited a strong antibody response to surface (MOMP, MIP, and Pmp13G) and virulence-associated (CPAF, TARP, and SINC) antigens. While the latter disappeared within 18 weeks following abortion in majority of the animals, antibodies to surface proteins persisted beyond the duration of the study. In contrast, experimental non-abortion infected sheep developed antibodies mainly to surface antigens (MOMP, MIP, and Pmp13G), all of which did not persist. This indicates that new diagnostic methods need to be established to improve the accuracy of disease diagnosis and provides scientific basis for controlling animal Chlamydiosis.

CONCLUSION

Since the first case of avian *Chlamydia* was reported in China, *Chlamydia* infection has been observed in different animals in most areas of China, which causes serious economic losses each year. Several diagnostic techniques, including CF, IHA, ELISA, ABC-ELISA, egg isolation, and PCR, have been studied and used in China. Formalin inactivated vaccines of *Chlamydia* from sheep, goat, swine, and cow were developed and have been used since 1981 in those areas where animals are threatened by *Chlamydia*. Because Chlamydiosis was considered an unimportant disease in animals by the Chinese government and no eradiation plan has implemented, there are sporadic outbreaks of *Chlamydia* in domestic animals in some areas, especially where vaccination has been suspended. However, the abortion rate was down sharply when inactivated vaccines for *Chlamydia* were used in domestic animals. This may have contributed to the lack of large-scale outbreak of Chlamydiosis in domestic animals in the last 30 years. The most important problem now is avian Chlamydiosis, which has a seropositive rate of 10–50% with IHA and easily spreads from birds to humans. Due to lack of effective vaccines, avian Chlamydiosis *may* become a public health problem in China.(Reorder)

AUTHOR CONTRIBUTIONS

Substantial contribution to the references of the work: ZLi, ZLou, YF. Draft and/or critical revision of the manuscript: JZ. Final approval of the version to be published: JZ, ZLi, ZLou, YF.

FUNDING

This work was supported by the National Key Research and Development Plan (2017YFD0500905).

ACKNOWLEDGMENTS

Special thanks to Xueli Yang, Yingcai Li, Yongyu Shuai, and Changqing Qiu who devoted their life to researching *Chlamydia* and controlling its prevalence in China.

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Conflict of Interest Statement: 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: 14 December 2017
doi: 10.3389/fvets.2017.00221

A Promising Recombinant Herpesvirus of Turkeys Vaccine Expressing PmpD-N of *Chlamydia psittaci* Based on Elongation Factor-1 Alpha Promoter

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The obligate intracellular Gram-negative bacterium *Chlamydia psittaci* often causes avian chlamydiosis and influenza-like symptoms in humans. However, the commercial subunit *C. psittaci* vaccine could only provide a partial protection against avian chlamydiosis due to poor cellular immune response. In our previous study, a recombinant herpesvirus of turkeys (HVT)-delivered vaccine against *C. psittaci* and Marek's disease based on human cytomegalovirus (CMV) promoter (rHVT-CMV-*pmpD*) was developed and provided an effective protection against *C. psittaci* disease with less lesions and reduced chlamydial loads. In this study, we developed another recombinant HVT vaccine expressing the N-terminal fragment of PmpD (PmpD-N) based on human elongation factor-1 alpha (EF-1α) promoter (rHVT-EF-*pmpD*) by modifying the HVT genome within a bacterial artificial chromosome. The related characterization of rHVT-EF-*pmpD* was evaluated *in vitro* in comparison with that of rHVT-CMV-*pmpD.* The expression of PmpD-N was determined by western blot. Under immunofluorescence microscopy, PmpD-N protein of both two recombinant viruses was located in the cytoplasm and on the cell surface. Growth kinetics of rHVT-EF-*pmpD* was comparable to that of rHVT-CMV-*pmpD*, and the growth rate of rHVT-EF-*pmpD* was apparently higher than that of rHVT-CMV-*pmpD* on 48, 72, and 120 h postinfection. Macrophages activated by rHVT-EF-*pmpD* could produce more nitric oxide and IL-6 than that activated by rHVT-CMV-*pmpD*. In this study, a recombinant HVT vaccine expressing PmpD-N based on EF-1α promoter was constructed successfully, and a further research *in vivo* was needed to analyze the vaccine efficacy.

Keywords: *Chlamydia psittaci*, Marek's disease, herpesvirus of turkeys, PmpD-N, elongation factor-1 alpha promoter

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: *23 September 2017*
Accepted: *04 December 2017*
Published: *14 December 2017*

Citation:
Liu S, Sun W, Huang X, Zhang W, Jia C, Luo J, Shen Y, El-Ashram S and He C (2017) A Promising Recombinant Herpesvirus of Turkeys Vaccine Expressing PmpD-N of Chlamydia psittaci Based on Elongation Factor-1 Alpha Promoter. Front. Vet. Sci. 4:221. doi: 10.3389/fvets.2017.00221

INTRODUCTION

Chlamydia psittaci is an emerging zoonotic pathogen of global significance, which can cause avian chlamydiosis (1), and infection in human with pneumonia, encephalitis, endocarditis, and even death (2). Study at home, abroad and in our laboratory had indicated that the seropositivity rate of *C. psittaci* was extremely high in poultry flocks (3–6). Currently, no efficacious commercial *C. psittaci*

vaccine is available because the cellular and humoral immunity are both necessary to protect animals from this obligate intracellular bacterial infection (7).

The major outer membrane protein (MOMP) and the autotransported polymorphic membrane protein D (PmpD) of *C. psittaci* are proved to be good vaccine candidates (8–10). PmpD has more merit than MOMP because PmpD is conserved and can elicit early immune-mediated neutralization of an ongoing chlamydial infection (11, 12). The specific neutralizing antibody triggered by N-terminal fragment of PmpD (PmpD-N) may provide humoral immune protection against early infection (12).

A herpesvirus of turkeys (HVT) vector-based vaccine can deliver antigens to the surface of cells, and further effectively stimulate cellular immunity and humoral immunity (13). This characteristic is very useful for the development of an effective vaccine for the prevention and control of *Chlamydia* infection (14). Furthermore, a great many of HVT-based recombinant vaccines expressing protective antigens of avian pathogens, such as Newcastle disease virus, infectious bursal disease virus, and highly pathogenic avian influenza, were constructed, and excellent and long-term protective effect in chickens against both pathogens were proved (15–18).

In our previous study, a recombinant HVT expressing PmpD-N based on cytomegalovirus (CMV) promoter (rHVT-CMV-*pmpD*) was constructed to successfully elicit an exceptional cellular and humoral immunity, and finally proved to confer a partial protection in chickens against *C. psittaci* challenge infection (10). In this study, we developed another recombinant HVT vaccine expressing PmpD-N based on elongation factor-1 alpha (EF-1α) promoter (rHVT-EF-*pmpD*), and its morphological and immunological characterization was analyzed in comparison with rHVT-CMV-*pmpD in vitro*.

MATERIALS AND METHODS

Chicken Cells and *C. psittaci* Strain

Primary chicken embryo fibroblast (CEF) cells were prepared from 10-day-old specific-pathogen-free embryos (Vital Merial Experimental Animal Co., Ltd., Beijing, China) (15). The HD11 chicken macrophage cell line was maintained in RPMI-1640 medium with 10% FBS (v/v). The DNA of *C. psittaci* strain CB7 was extracted and stored in our lad as reported previously (10).

Generation of Recombinant HVT Expressing *pmpD*-N Gene Based on EF-1α Promoter

The recombinant HVT was generated as described previously (10). The recombinant HVT bacterial artificial chromosome (BAC) was based on EF-1α promoter, which was from the vector of pEF6/V5-His (Invitrogen, Carlsbad, CA, USA). A pair of primers used to amplify the expression cassette with the EF-1α promoter at the 5′ end and the BGH polyadenylation (poly A) site at the 3′ end was designed using Oligo 7 (Molecular Biology Insights, USA). The forward primer was 5′-GGTTAATTAACCTTCTAG GTCTTGAAAGGAGTGGGA-3′, and the reverse primer was 5′-GGTTAATTAAGCCTCAGAAGCCATAGAGCCCACC-3′. Both primers contained a *Pac*I restriction site at their 5′ termini. The recombinant virus was designated as rHVT-EF-*pmpD*.

Plaque Assays and One-Step Growth Kinetics

The plaque size, morphology, and plaque-forming unit (PFU) of rHVT-EF-*pmpD* were compared with those of the same passage of rHVT-CMV-*pmpD* by the immunohistochemical assay as recorded previously (15). Briefly, recombinant viruses were 10-fold diluted and inoculated into the CEF cells, which were seeded in six-well plates. Four days later, ice-clod acetone was added into each well to fix the cells. Cells were washed by phosphate-buffered saline (PBS) and then blocked by blocking buffer (PBS containing 0.1% BSA). Cells were incubated with the wild type HVT-specific polyclonal antibodies raised in chickens (diluted 1:100), and subsequently reacted with horseradish peroxidase-labeled goat-anti-chicken IgG (1:4,000) (Sigma-Aldrich, Shanghai, China). Cells containing the conjugated antibody were identified by incubation at 37°C for 1 h in developing solution containing 1% (w/v) 3-amino-9-ethylcarbazole (Sigma) and 0.02% (v/v) H_2O_2 in 0.1 M sodium acetate (pH 4.8). Plaques were counted using an inverted microscope. Moreover, the growth rates of the recombinant viruses were studied on CEF cells by calculating the virus plaques at 12, 24, 48, 72, 96, and 120 h postinfection (16).

Identification of the Recombinant Viruses Using Immunoblot and Immunofluorescence

Immunoblot analysis was carried out as described previously (10). Briefly, 3,000 PFU of rHVT-CMV-*pmpD*, rHVT-EF-*pmpD*, and parental HVT were used to infect CEF cells seeded in T25 flasks. The cells were washed twice with PBS when the cytopathic effect occurred. Lysates of cells were subjected to 12% SDS-PAGE and electroblotted to polyvinylidene fluoride membranes, followed by 0.25% trypsin for 2 min. Membranes were incubated with the *C. psittaci* strain 6BC-specific polyclonal antibodies (diluted 1:100) previously prepared by our own lab, and then reacted with horseradish peroxidase-labeled goat-anti-chicken IgG (1:4,000) (Sigma-Aldrich, Shanghai, China). The PmpD-N glycoprotein bands were visualized using the enhanced DAB reagents (Tiangen, Beijing, China) according to the manufacturer's instructions.

The expression and distribution of PmpD-N were determined in rHVT-CMV-*pmpD*-infected cells and rHVT-EF-*pmpD*-infected cells by immunofluorescence. First, CEF cells were infected with 3,000 PFU rHVT-CMV-*pmpD* and rHVT-EF-*pmpD*, respectively. The cells were probed with mouse anti-PmpD-N polyclonal serum of *C. psittaci* (previously prepared in our lab) and anti-HVT polyclonal serum (IVDC, Beijing, China) at a dilution of 1:100, and then overlaid with a mixture of goat anti-mouse IgG labeled with Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) and goat anti-chicken IgY labeled with Alexa Fluor 568 (1:600 dilution) (Invitrogen, Carlsbad, CA, USA). Moreover, cell nuclei were stained with 1:10,000 dilution of 4,6-diamidino-2-phenylindole (DAPI) for 1 min or longer. Finally, the rinsed cover slips were mounted with Vectashield mounting medium

(Vector Laboratories, Burlingame, CA, USA) and examined using a confocal microscope (Nikon, Tokyo, Japan).

Measurement of Nitric Oxide (NO) and IL-6

HD11 cells were infected by 400 PFU rHVT-CMV-*pmpD* and rHVT-EF-*pmpD*, respectively. Cell culture supernatants were collected on day 1, 2, and 4 postinfection. OD values of supernatants were measured at 540 nm. NO production by activated HD11 cells was assessed as nitrite content in conditioned media using Griess reagent as illustrated previously (19). Sodium nitrite was used as the standard.

The infected cells treated above were also collected on day 1, 2, and 4 after inoculation. The cells were washed twice with PBS. Total RNA of cells on different day's postinfection was extracted. The quantity of IL-6 was measured by relative quantitative real time RT-PCR, and the internal control gene was GAPDH as described previously (20).

Statistical Analysis

Data were analyzed using the one-way ANOVA. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Generation of Recombinant HVT Expressing *pmpD*-N Gene Based on EF-1α Promoter

The *pmpD*-N gene with EF-1α promoter was successfully inserted into the HVT BAC region between UL45 and UL46. The recombinant HVT BAC DNA based on EF-1α promoter was transfected into CEF cells. Four days after transfection, typical plaques appeared in the CEF monolayers, these plaques being similar in size and morphology compared with those formed by rHVT-CMV-*pmpD* (**Figure 1**). The viral titration by staining plaques revealed no significant difference between rHVT-EF-*pmpD* and rHVT-CMV-*pmpD*.

Expression of PmpD-N in rHVT-EF-*pmpD* Infected Cells

To confirm the expression of the PmpD-N protein, CEF cells infected with rHVT-EF-*pmpD*, rHVT-CMV-*pmpD*, and parental HVT were analyzed by immunoblot and immunofluorescence. A 43-kDa band corresponding to the PmpD-N polypeptide was identified with polyclonal antibodies generated against *C. psittaci* 6BC strain by immunoblot analysis (**Figure 2A**). Under immunofluorescence microscopy, PmpD-N protein of rHVT-EF-*pmpD* and rHVT-CMV-*pmpD* was both expressed in the cytoplasm and on the cell surface (**Figures 2B,C**).

One-Step Growth Kinetics

Growth trends of both recombinant viruses were similar as revealed by calculating the virus plaques at various times postinfection (**Figure 3**). Growth rates displayed a significant increase between 24 and 96 h, and a sharp decrease from 96 to 120 h. Except for 96 h postinfection, the growth rate of the rHVT-EF-*pmpD* was consistently higher than that of rHVT-CMV-*pmpD*, with a consequential difference appearing on 48, 72, and 120 h postinfection.

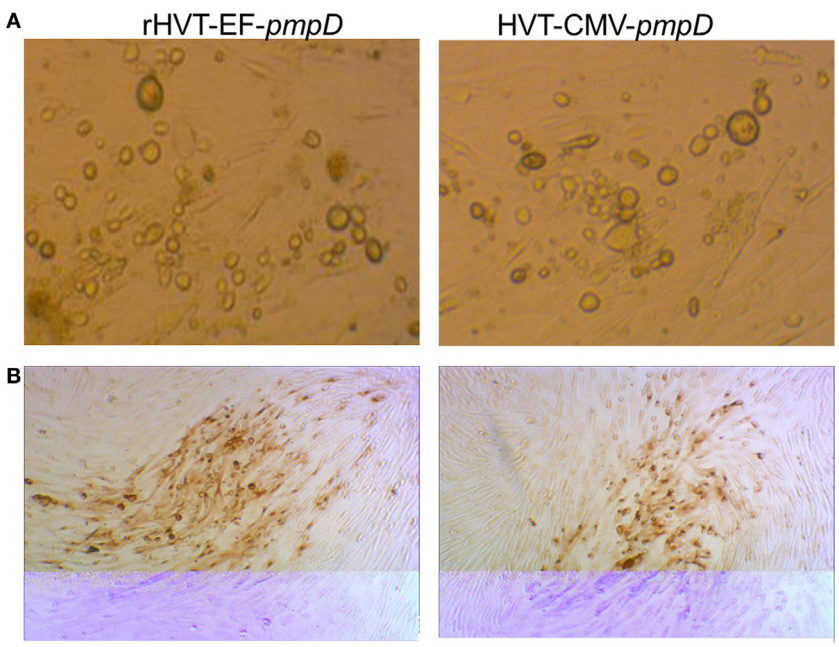

FIGURE 1 | Cytopathic effect of rHVT-EF-*pmpD* and rHVT-CMV-*pmpD* on chicken embryo fibroblast (CEF) cells. **(A)** Morphology of the infected CEF cells induced by rHVT-EF-*pmpD* or rHVT-CMV-*pmpD* (magnifications 100×). **(B)** Immunohistochemical staining of CEF cells post inoculation with rHVT-EF-*pmpD* or rHVT-CMV-*pmpD* (magnifications 100×).

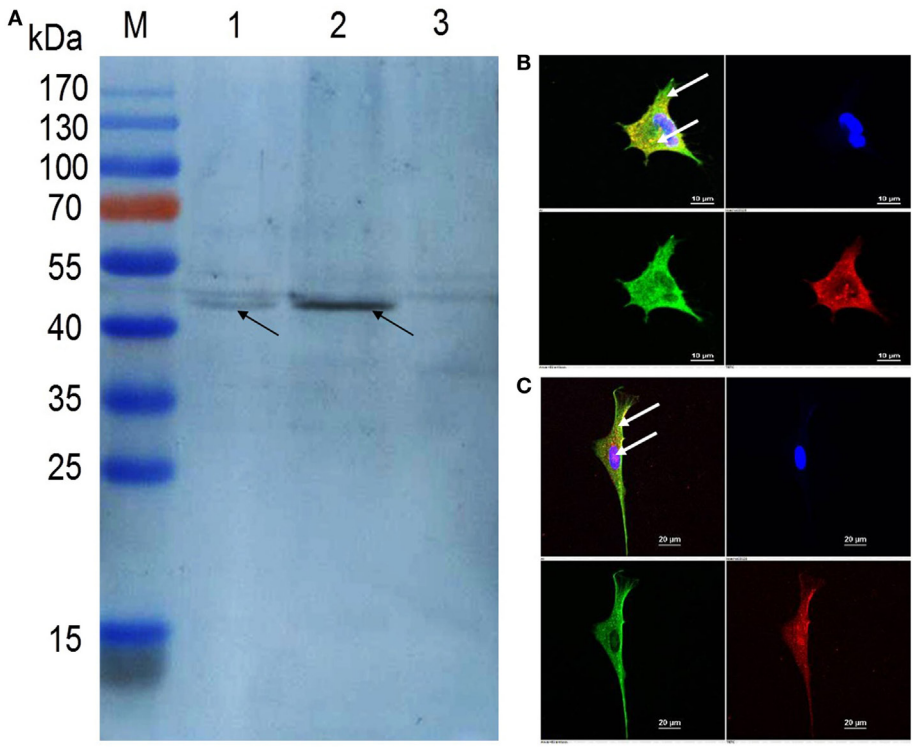

FIGURE 2 | Confirmation of PmpD-N protein expression in herpesvirus of turkeys (HVT) vector by immunoblotting assay and indirect immunofluorescence. **(A)** The PmpD-N expression in rHVT-EF-*pmpD* was detected by immunoblot using *Chlamydia psittaci* strain 6BC-specific polyclonal antibodies. Lane M, pre-stained protein ladder; Lane 1, cell lysate post inoculation with rHVT-EF-*pmpD*; Lane 2, cell lysate post inoculation with rHVT-CMV-*pmpD*; Lane 3, cell lysate post inoculation with parental HVT. The black arrow indicates the approximate size of 43 kDa. **(B)** Indirect immunofluorescence analysis of PmpD-N expression in chicken embryo fibroblast (CEF) cells. CEF cells on glass coverslips were infected with rHVT-EF-*pmpD*, then incubated with mouse anti-PmpD-N polyclonal antibody of *C. psittaci* and chicken anti-HVT polyclonal serum, and then subsequently reacted with the goat anti-mouse IgG conjugated with Alexa Fluor 488 (green fluorescence, shown in the lower left panel) and goat anti-chicken IgY labeled with Alexa Fluor 568 (red fluorescence, shown in the lower right panel), respectively. Finally, cell nuclei were stained with diamidino-2-phenylindole (blue fluorescence, shown in the top right panel). The merged image is shown in the top left panel. The expression of the targeted protein is indicated by white arrows in the top left panel. **(C)** rHVT-CMV-*pmpD* control. CEF cells on glass coverslips were infected with rHVT-CMV-*pmpD*, and then the process of test and the panel meaning are the same as those shown in panel **(B)**.

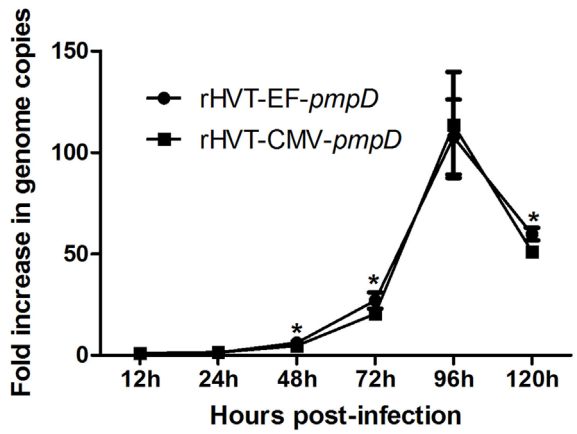

FIGURE 3 | One-step growth kinetics of rHVT-EF-*pmpD* and rHVT-CMV-*pmpD in vitro*. The virus growth was calculated as the fold increase at different time points compared with the 12th hour postinfection. The asterisk indicates significant differences of the growth kinetics between rHVT-EF-*pmpD* and rHVT-CMV-*pmpD* ($P < 0.05$). The values were shown as means ± SD.

Measurement of NO and IL-6

The quantity of NO and IL-6 on HD11 cells infected by rHVT-CMV-*pmpD* and rHVT-EF-*pmpD* was assayed on days 1, 2, and 4. The NO production of rHVT-EF-*pmpD* was significantly higher than those of rHVT-CMV-*pmpD* on day 2 and day 4 ($P < 0.05$) (**Figure 4**). The IL-6 level of rHVT-EF-*pmpD* was apparently higher than that of rHVT-CMV-*pmpD* on day 4 (**Figure 5**).

DISCUSSION

Chlamydia psittaci, an obligate intracellular Gram-negative bacterium, often causes avian chlamydiosis and influenza-like symptoms in humans. At present, five serovars of *C. psittaci* have been found in chickens, which are genotypes B, C, D, F, and E/B (3, 21–23). The development of an efficacious vaccine against *C. psittaci* is needed as a high seropositivity rate was found in chickens (3–6). In this study, a recombinant HVT vaccine expressing PmpD-N based on EF-1α promoter was developed and was proved to be more efficient than that of CMV-based promoter.

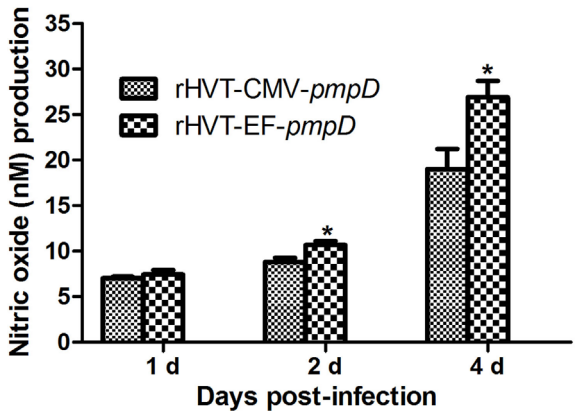

FIGURE 4 | Nitric oxide (NO) responses of HD11 cells to rHVT-EF-*pmpD*. HD11 cells were subjected to 400 plaque-forming unit recombinant viruses for 1, 2, and 4 days. The supernatants were collected for NO analysis. Data were expressed as mean ± SE ($n = 3$) and were analyzed by Student's *t*-test. Significance (*) was considered as $P < 0.05$ when compared with rHVT-CMV-*pmpD*.

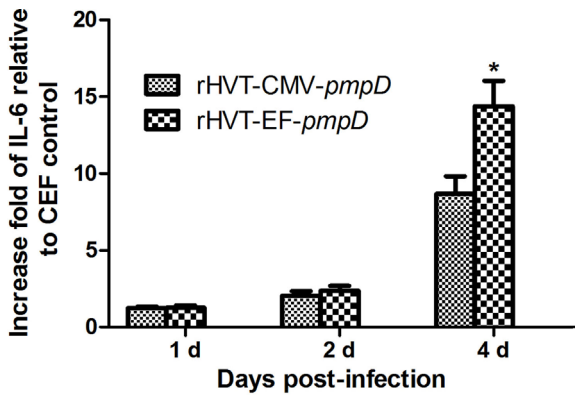

FIGURE 5 | IL-6 responses of HD11 cells to rHVT-EF-*pmpD*. HD11 cells were subjected to 400 plaque-forming unit recombinant viruses for 1, 2, and 4 days. The cells were collected for IL-6 analysis using relative quantitative real time RT-PCR. The IL-6 increase fold of the recombinant viruses relative to chicken embryo fibroblast (CEF) cells was analyzed. Data were expressed as mean ± SE ($n = 3$) and were analyzed by Student's *t*-test. Significance (*) was considered as $P < 0.05$ when compared with rHVT-CMV-*pmpD*.

To stimulate better immune protection, the constructed recombinant virus can deliver the target protein to the surface of the cell. Confocal analysis showed that the HVT vector could deliver PmpD-N protein to the surface of the cell in the rHVT-EF-*pmpD*, same as rHVT-CMV-*pmpD*. Plaque assay further showed that compared with rHVT-CMV-*pmpD*, the rHVT-EF-*pmpD* did not change the lesion status, plaque size and proliferation rate.

Macrophage, which belongs to phagocyte, participates in innate and cellular immunity in the body of animals. Macrophages display remarkable plasticity and can change their physiology in response to environmental cues. These changes can give rise to different populations of cells with distinct functions. Macrophages play an important role in monitoring, phagocytosis, uptake of antigens, secretion of various proinflammatory cytokines, and control and elimination of infections (24). In birds, macrophages play a central role in resistance to microbial infections and the pathogenesis of viruses, bacteria, and parasitic infections. Research shows that avian macrophages exposed to pathogens can be activated to produce proinflammatory cytokines, chemokines, reactive oxygen species (ROS), and NO, while ROS and NO are considered as antimicrobial arsenal that can control and remove pathogens. The HD11 cell line, which is obtained from avian macrophage like cell lines transformed by the avian encephalomyelitis virus, has the ability of phagocytosis and expressing Fc receptors and macrophage surface antigens (25). HD11 cells, as chicken macrophages, are widely used in the study of immune function *in vitro*. In this study, HD11 cells were used to analyze the macrophage that was impacted by the recombinant virus rHVT-EF-*pmpD* and rHVT-CMV-*pmpD*, respectively. Our results have shown that rHVT-EF-*pmpD* can significantly stimulate the macrophage to produce more proinflammatory cytokines IL-6 than rHVT-CMV-*pmpD* from day 2, and significantly stimulate the macrophage to produce more NO than rHVT-CMV-*pmpD* from day 4. Furthermore, the rHVT-EF-*pmpD* can stimulate the macrophage to kill more pathogens and promote more immune response than rHVT-CMV-*pmpD*.

In summary, the recombinant HVT expressing PmpD-N based on EF-1α promoter is more effective than our former constructed HVT based on CMV promoter *in vitro*. Further study is needed to analyze whether the rHVT-EF-*pmpD* could produce better protective immune response than rHVT-CMV-*pmpD in vivo*.

AUTHOR CONTRIBUTIONS

SL and CH designed the study. SL, WS, and XH performed experiments. SL, WZ, CJ, JL, and YS collected test data. SL and WS performed the date analysis and drafted the manuscript. CH and SE-A revised the manuscript. All the authors read and approved the final manuscript.

FUNDING

This study was supported by Tongren science and technology project (Grant No. copper science research [2016] 17-5), National major innovation ability construction project of emerging industries in the west area (Grant No. Fagaibangaoji [2016] 2195), the National Natural Science Foundation of China (Grant No. 31172305 and 31372420), Ministry of Science and Technology (MoST) (Grant No. 2012BAD31B05), and Beijing Natural Science Foundation (Grant No. 6122018).

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