EMERGING SWINE VIRUSES

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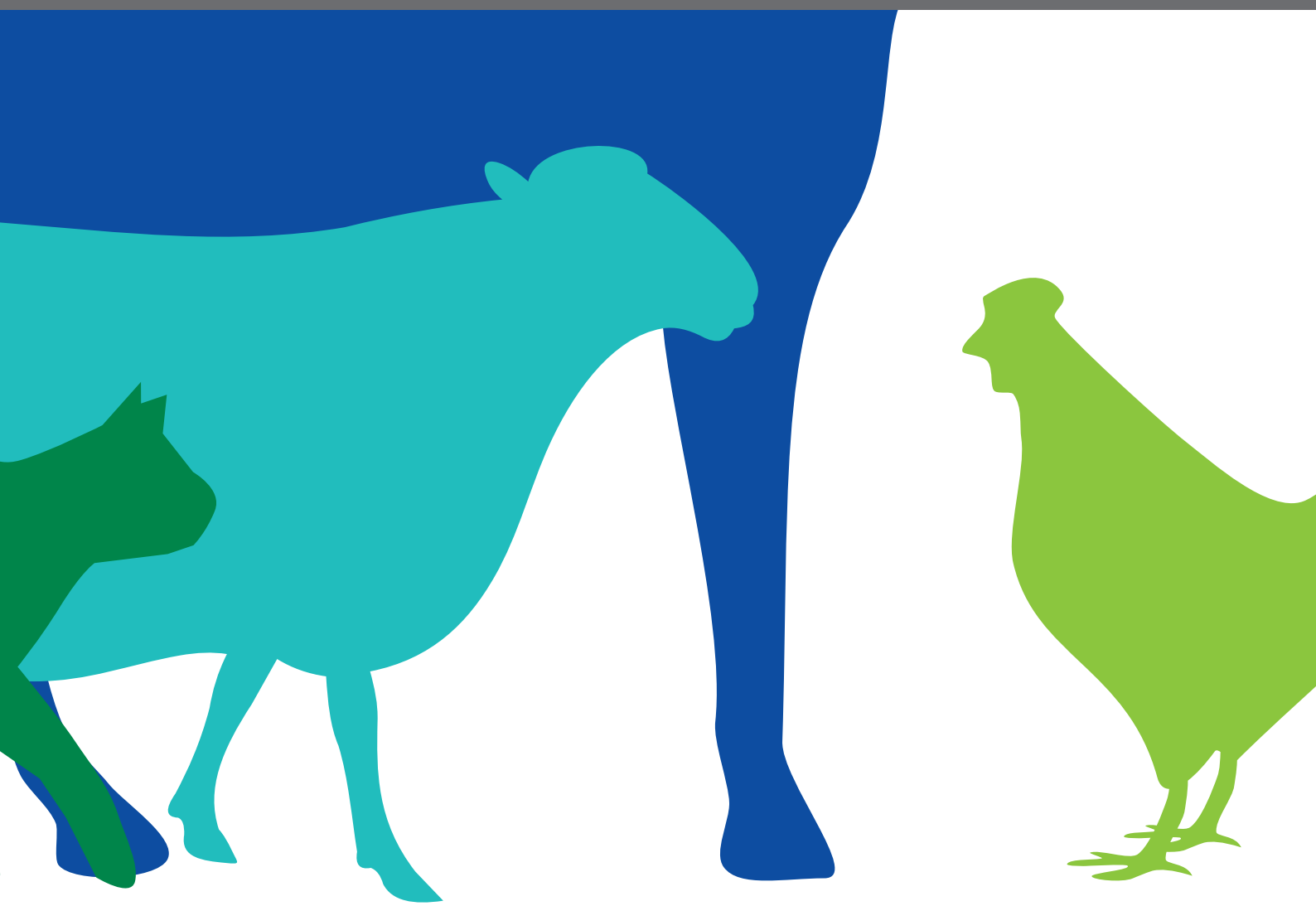

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EMERGING SWINE VIRUSES

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EDITORIAL
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Editorial: Emerging Swine Viruses

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Editorial on the Research Topic

Emerging Swine Viruses

Over the last 30 years, diseases caused by emerging swine viruses (ESV) have acquired great relevance, more than in other species. Diseases caused by porcine reproductive and respiratory syndrome virus (PRRSv), high pathogenicity porcine epidemic diarrhea virus (PEDv), porcine circovirus type 2 (PCV-2), and influenza virus H1N1pdm09 had great economic impact. Others, however, such as porcine enteroviruses, porcine toroviruses (PToV), porcine sapelovirus (PSV), porcine bocavirus (PBoV), porcine kobuvirus (PKBV), and porcine Torque teno sus virus (TTSuV) are mostly subclinical in swine herds. Furthermore, novel emerging viruses, such as SENECA virus, atypical porcine pestivirus (APPV), PCV-3, SADS-CoV, influenza D, and others with regional or worldwide distribution constitute a new challenge for researchers and practicing veterinarians.

Emerging viruses should be considered to occur when there are changes in the relationship between the agent, the host and the enviroment. The response to how and why the ESV have emerged can be explained through several factors.

First, interspecies transmission means the presence of a potentially pathogenic agent into a new host, such as between aquatic migratory birds and human beings for influenza A. Bats are the source of Nipah virus, and swine acute diarrhea syndrome (SADS coronavirus). Both have limited distribution to Asia or TGE and PED. Currently PCV-3 have been found with high homology with bats PCV-1.

Secondly, changes in the virulence (mutation, reassortant, recombination) of the agents in the same host, particularly the RNA and single strand DNA viruses that have a high mutation rate (10–4/10–5 nucleotides per replication cycle), that facilitate its adaptation to the innate immune response. The absence of enzymes (transcriptase) in infected cells that correct errors in reading RNA synthesis and segmented RNA chains favor reassortant. A population of RNA viruses does not consist of a single genotype, but a "set or cloud" of related viruses that interact with each other called "quasispecies". Relevant examples are HP PRRSv, influenza A H1N1pdm09 and PEDv.

Next, the viruses have been present for a long period of time as subclinical infections and have been discovered with the development of metagenomic techniques [Next Generation Sequencing NGS, Lawrence Livermore Microbial Detection Array (LLMDA or virochip)], or exogenous factors as most emerging viruses do not grow in traditional culture media. Viruses such PCV-3, SADS-CoV, and LINDA virus have been characterized by the aforementioned techniques.

Now we come to change in the production system. The presence of farms of good health, large size and homogeneous genetics favors the fitness, and the ability of a particular population of viruses to multiply and spread in a specific environment.

Our fifth point is the recognition and sensitization of practicing veterinaries of "abnormal" cases or syndromes through the routine postmortem monitoring of pigs that die "unexceptionally" on the farm, as well as the syndromes surveillance at the slaughterhouse.

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Finally, Specialized diagnostic laboratories that offer new and accessible diagnostic tools for the diagnoses of known and unknown emerging viruses.

This Research Topics comprises ten review articles are related by: Next Generation Sequencing (NGS) plus *in situ* Hybridization (ISH); porcine hemagglutinating encephalomyelitis coronavirus (PHE-CoV); porcine circovirus type 3 (PCV-3); classical swine fever virus (CSF); porcine torovirus (PToV); porcine respiratory reproductive syndrome virus (PRRSV); human influenza A; Nipah virus in humans and pigs swine; atypical porcine pestivirus (APPV); porcine epidemic diarrhea coronavirus (PEDV); and porcine delta coronavirus (PDCoV) pathogenesis. These subjects provide a discussion on the broad field of emerging swine viruses infections and its control.

Three original research articles about phylogeny and genome composition of novel Seneca virus (SVA) isolated in China; estimation of time to porcine epidemic diarrhea coronavirus (PED-CoV) removal in Ontario herds and a longitudinal serological and RT-PCR fecal studies of hepatitis E virus (HEV) were published.

REVIEW ARTICLES

Resende et al. provide comprehensive information about the results of the combination of NGS-ISH for the diagnosis of known and unknown emerging pathogens in tissues by NGS, and its relationship with specific lesions where it is visualized in active infection through detection of mRNA by ISH. Results on the application of PCV-2, PPV-2, Seneca virus, and *Mycoplasma hyorhinis* are comments.

Mora-Díaz et al. review the disease produced by porcine hemagglutinating encephalomyelitis coronavirus (PHE-CoV), a neurotropic virus affecting piglets <4 weeks old. Subjects such as: characteristic of the virus, history of the emergence of PHE-CoV, global distribution, clinical signs, pathogenesis, lesions, and diagnosis are discussed. As the infection is endemic in most swine herds, and no current vaccines are available, early exposure to old or young sows to induced maternal immunity is the only way to prevent the disease.

Klaumann et al. discuss the current knowledge on a new circovirus named porcine circovirus type 3 (PCV-3). Originally this was identified by metagenomic analyses from an outbreak of PDNS in sows associated with reproductive failure, myocarditis, and multisystemic inflammation. Thereafter it was found associated with respiratory, digestive and nervous signs in healthy pigs and wild boars. Retrospective studies detected PCV-3 as early as the 1990s. Coinfection with several virus and bacteria were reported. The authors emphasize the need of studies related to pathogenesis, the role of coinfections and their association or not with certain clinically pathological entities.

Zhou reviews classical swine fever (CSF) in China. The author discusses the epidemiology, and the geographical distribution of genotypes where 2.1, 2.1b, and 2.1c are currently dominant in China. The first one persists in an immune population by natural infection due to the high mutation rate of the enveloped glycoprotein E2. For eradication of CSF it is necessary to distinguish between the naturally infected and vaccinated animals by live attenuated marked vaccine. An experimental E2 subunit vaccine was developed in China. Besides preventive vaccination, we need culling strategies, skilled veterinarians, up-to-date diagnostic and monitoring technology, and biosecurity.

Hu et al. review the progress in the knowledge on porcine torovirus (PToV) a single-stranded RNA enteric virus found in piglets with diarrhea in North America, Africa, Asia, and Europe. The authors describe the virus morphology, the genomic structure and genotypes division, although chimeric strains with genes from porcine and bovine ToV has been identified as well as recombination with enterovirus. For epidemiological studies an indirect ELISA based in recombinant N protein expressed in baculovirus is available. Other methods included RT-PCR; qRT-PCR; and nested PCR. Prevalence of PToV is quite variable according with the country.

Montaner-Tarbes et al. analyze numerous gaps in PRSSv knowledge. Related with the biology, the scarce whole genome sequencing from different geographical origins hinder understanding the virus evolution/mutation. The function and the complex interaction of viral non-structural proteins with the target cells are reviewed. The mechanisms of the virus to avoid the innate and acquired immune response through recognition and antibody neutralization are reviewed. The new known mechanisms of dissemination mediated by cell to cell connected nanotubules and extracellular vesicles are thoroughly discussed. Later on, the development of exosomes, as a novel vaccine is analyzed.

Rajao et al. review the role of pigs in the interspecies transmission and how their susceptibility to different viruses can affect the overall epidemiology of swine influenza. The factors that have been implicated in the interspecies transmission of influenza such as receptor-binding specificity/affinity, balance between HA and NA content, host temperature and host-specific immune factors are analyzed. Surveillance of IAV in swine has shown that human viruses are transmitted to pigs more frequently than from pigs to humans. The result is the establishment of several human-origin virus lineages, antigenic diversity and failure of current swine vaccines.

McLean and Graham provide an update about Nipah virus (NiV), an RNA paramyxovirus that causes a severe neurological disease in humans. In suckling pigs, NiV infection causes high mortality and in older pigs, respiratory and neurological signs. The natural host of NiV is a fruit bat of the genus *Pteropus,* and pigs act as an "amplifying host". The disease has been found in Asia in people with close contact to pigs. Recombinant NiV mutant, attenuated and subunit vaccine using several viral vectors have been studied. Currently, neither a human nor a pig vaccine has been licensed.

Gatto et al. review the information around a new RNA Pestivirus named atypical porcine pestivirus (APPV), detected in pigs with congenital tremors (CT) type AII, and splayed legs in offspring from sows by NGS technology. Viral genomes were detected in semen, preputial swabs and fluids highlighting the importance of AI in APPV epidemiology. Horizontal transmission can be made by surviving CT in healthy new-born boars, piglets and adult pigs. The virus exhibits high genetic

diversity. Recently, APPV was detected in wild boars. Until the development of a vaccine, the authors recommend feedback on reproductive management in sows with CT cases.

Koonpaew et al. review the emergence of highly pathogenic porcine epidemic diarrhea (PED) and porcine delta coronavirus (PDCoV) as agents of watery diarrhea in suckling piglets. The authors describe aspects related to the biology pathogenesis and the host innate immune response of gastrointestinal tracts against those enteric coronaviruses. The agents evade recognition by host pattern recognition receptors (PRRs), present in resident antigen present cells (APCs) and located in gut associated lymphoid tissue (GALT), through the inhibition or blocking of interferon (IFN) induction and the signaling cascade, respectively. This knowledge will profit the development of immune modulators as well as effective vaccines.

ORIGINAL RESEARCH

Sun et al. analyze the phylogeny and genome compositions of 17 novel Seneca virus (SVA) isolated in China in 2017 and compare them with the genomic sequences deposited in the GenBank. SVA is a single stranded positive-sense RNA virus associated with porcine idiopathic vesicular disease (PIVD), and sudden neonatal dead reported in six countries in Asia and America. The isolated strain clustered into three distinct groups: A, B, and C, not related with the previously SVA identified in China and different from SVA identified in other countries. More effort should be directed to SVA monitoring, rapid and specific diagnosis and vaccination strategies.

Perri et al. studied the estimation of time to eliminate porcine epidemic diarrhea coronavirus (PED-CoV) in Ontario herds based in large-scale disease control program database (DCP). The analysis takes into consideration the time between the initial infection, and the confirmation of PED-CoV freedom at the minimum level of 10%. The median time to elimination varied from 23 weeks in nursery herds, to 43 weeks in farrow-to-feeder herds. Farrow-to-wean herds had the highest hazard of PED-CoV elimination. Type of herds, season and year of original diagnosis were associated with the time of negativity and reflect the complexity of the infection control practices.

Krog et al. work to determine the dynamics of infection of hepatitis E virus (HEV) by carrying out a longitudinal serological and RT-PCR fecal studies. Sows and their progeny from 2 weeks to slaughter were sampled. Antibodies were only detected in offspring born from sows with high levels of maternal antibodies (MAbs) and a few of them became shedders. All pigs seroconverted at 13–17-week-olds. By PCR 65.5% of pigs were positive at least one time during the weeks 13, 15, and 17. In 3 out of 10 slaughter pigs, HEV was detected in feces and organs. As MAbs reduced the shedder of HEV, sow's vaccination might be an option.

As a summary, this Research Topic provides a comprehensive review about the results of the combination of NGS-ISH for the diagnosis of known and unknown emerging pathogens. It's also an important discussion of potentially emerging viruses such as PCV-3, torovirus, atypical pestivirus, reemerging viruses such as PHECoV and transboundary viruses such as classical swine fever. The mechanisms used by PRRS to circumvent the host's innate immune and vaccine immune response are updated along with the development of vaccines to exosomes. The immune response against enteric coronaviruses such as PED and PDCoV and innovative vaccines for both viruses are analyzed. The role of pigs as an amplifier of the Nipah virus is reviewed, as well as the importance of vaccination to pigs for the prevention of this infection in man. The repeated transmission of human seasonal viruses to pigs has resulted in the establishment of several human-origin virus lineages globally and the failure of the current pig vaccines. A research study indicated that isolated strain Seneca virus from vesicular fluid of sows, clustered into three distinct groups, A, B, and C not related with the previously SVA identified in China, and highlight that different genotypes of SVA co-exist and spread. A study of the estimation of time to porcine epidemic diarrhea coronavirus (PED-CoV) elimination in Ontario Type of herds was carried out. The year and season of original diagnoses are associated with the time of negativity and reflect the complexity of the infection control practices. Finally, a study shows that hepatitis E virus infection is widespread in the herd, and pigs spread virus during the final stages of life, and there's a strong chance to find infected pigs at slaughter. As maternal antibodies reduce, the shedder of virus vaccinating sows might be an option.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Current Knowledge on *Porcine circovirus 3* (PCV-3): A Novel Virus With a Yet Unknown Impact on the Swine Industry

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Porcine circovirus 3 (PCV-3) is a recently described virus belonging to the family *Circoviridae*. It represents the third member of genus *Circovirus* able to infect swine, together with PCV-1, considered non-pathogenic, and PCV-2, one of the most economically relevant viruses for the swine worldwide industry. PCV-3 was originally found by metagenomics analyses in 2015 in tissues of pigs suffering from porcine dermatitis and nephropathy syndrome, reproductive failure, myocarditis and multisystemic inflammation. The lack of other common pathogens as potential infectious agents of these conditions prompted the suspicion that PCV-3 might etiologically be involved in disease occurrence. Subsequently, viral genome was detected in apparently healthy pigs, and retrospective studies indicated that PCV-3 was already present in pigs by early 1990s. In fact, current evidence suggests that PCV-3 is a rather widespread virus worldwide. Recently, the virus DNA has also been found in wild boar, expanding the scope of infection susceptibility among the *Suidae* family; also, the potential reservoir role of this species for the domestic pig has been proposed. Phylogenetic studies with available PCV-3 partial and complete sequences from around the world have revealed high nucleotide identity (>96%), although two main groups and several subclusters have been described as well. Moreover, it has been proposed the existence of a most common ancestor dated around 50 years ago. Taking into account the economic importance and the well-known effects of PCV-2 on the swine industry, a new member of the same family like PCV-3 should not be neglected. Studies on epidemiology, pathogenesis, immunity and diagnosis are guaranteed in the next few years. Therefore, the present review will update the current knowledge and future trends of research on PCV-3.

Keywords: *Porcine circovirus 3*, domestic pig, wild boar, infection, epidemiology

INTRODUCTION

The evolution of emerging diseases is associated with factors embedded in the concept "host-agent-environment triangle" (1). To infect the host and cause disease, the pathogen needs to evade host defenses, which may occur through single point mutations, genome rearrangements, recombination and/or translocation (2). Genetic uniformity generated through genetic selection of the host (3) and the fact that demographic changes, intensification of farming, and international commerce have occurred markedly over the last decades, must be also considered as essential factors for the development of emerging diseases (4–6).

As well as in humans, emerging diseases drastically affect animal populations, especially food-producing animals. Livestock production in large communities (i.e., pig farms or poultry flocks) represents an excellent environment to facilitate the transmission and maintenance of huge viral populations, contributing to the pathogen evolution (through mutation, recombination and reassortment, followed by natural selection) (7–9). The intensification of livestock during the last four decades has probably been one of the main factors that contributed to the emergence of new pathogens and/or pathogen variants, leading to changes in the epidemiology and presentation of diseases (10).

The number of viral infectious diseases in swine has significantly increased in the last 30 years. Several important worldwide distributed viruses have been reported in this period, including *Porcine reproductive and respiratory syndrome virus* (PRRSV, family *Arteriviridae*), *Porcine circovirus 2* (PCV-2, family *Circoviridae*) and *Porcine epidemic diarrhea virus* (PEDV, family *Coronaviridae*). In addition to those worldwide widespread viruses, an important number of novel swine pathogens causing different types of diseases has been described (11, 12). Although their economic impact might be variable, they are considered significant infection agents and their monitoring is nowadays performed in some parts of the world. Among others, relevant examples are *Porcine deltacoronavirus* (associated with diarrhea) (12), *Senecavirus A* (causing a vesicular disease and increased pre-weaning mortality) (11), *Porcine sapelovirus* (found in cases of polioencephalomyelitis) (13), *Porcine orthoreovirus* (assumed to cause diarrhea) (14), *Atypical porcine pestivirus* (cause of congenital tremors type II) (15) and HKU2-related coronavirus of bat origin (associated with a fatal swine acute diarrhea syndrome) (16).

Besides overt emerging diseases of swine, many other novel infectious agents have been detected in both healthy and diseased animals, and their importance is under discussion. This group of agents is mainly represented by *Torque teno sus viruses*, *Porcine bocavirus*, *Porcine torovirus* and *Porcine kobuvirus*, which are thought to cause subclinical infections with no defined impact on production (13, 17, 18). An exception may be represented by *Hepatitis E virus* (HEV); although it seems fairly innocuous for pigs, it is considered an important zoonotic agent (19, 20). Recently, a novel member of the *Circoviridae* family named *Porcine circovirus 3* (PCV- 3), with unknown effects on pigs, has been discovered (21, 22).

Porcine circovirus 3 (PCV-3) was first described in 2015 in North Carolina (USA) in a farm that experienced increased mortality and a decrease in the conception rate (21). Sows presented clinical signs compatible with porcine dermatitis and nephropathy syndrome (PDNS) and reproductive failure. In order to identify the etiological pathogen, aborted fetuses and organs from the affected sows were collected for further analyses. Whilst histological results were consistent with PCV-2-systemic disease, both immunohistochemistry (IHC) and quantitative PCR (qPCR) methods to detect PCV-2 yielded negative results. Samples were also negative for PRRSV and *Influenza A virus*. Homogenized tissues from sows with PDNS-like lesions and three fetuses were tested through metagenomic analysis, revealing the presence of an uncharacterized virus (21). Further analyses using rolling circle amplification (RCA) followed by Sanger sequencing showed a circular genome of 2,000 nucleotides. Palinski et al. (21) also performed a brief retrospective study through qPCR on serum samples from animals clinically affected by PDNS-like lesions (but negative for PCV-2 by IHC) and pigs with porcine respiratory diseases. Results revealed PCV-3 qPCR positivity in 93.75 and 12.5% of the analyzed samples, respectively (21).

Interestingly, almost concomitantly, another research group from the USA reported a clinical picture pathologically characterized by multi-systemic and cardiac inflammation of unknown etiology in three pigs of different ages ranging between 3 and 9 week-old (22). Several tissues from these animals were tested by next-generation sequencing (NGS) methods and PCV-3 genome was found. Beyond NGS, *in situ* hybridization was performed in one out of these three pigs, confirming PCV-3 mRNA in the myocardium (cytoplasm of myocardiocytes and inflammatory cells mainly, although to a very low frequency).

Based on these two initial works, the name PCV-3 was proposed as the third species of circoviruses affecting pigs, since pairwise analysis demonstrated significant divergence with the existing PCVs. The novel sequences showed <70% of identity in the predicted whole genome and capsid protein amino acid (aa) sequence compared to the other members of the *Circovirus* genus (22). Taking into account the economic importance and the well-known effects of PCV-2 on the swine industry, a new member of the same family like PCV-3 should not be neglected. Studies on epidemiology, pathogenesis, immunity and diagnosis are guaranteed in the next few years, but the scientific community is still in its very beginning on the knowledge of this new infectious agent. Therefore, the objective of the present review is to update the current knowledge and forecast future trends on PCV-3.

MOLECULAR ORGANIZATION OF PORCINE CIRCOVIRUSES

Porcine circovirus 3 (PCV-3) belongs to the family *Circoviridae*, genus *Circovirus*. Until 2016, the *Circoviridae* family was divided into two different genera named *Circovirus* and *Gyrovirus* (23); however, on the basis of the viral structure and genome, a new taxonomical grouping has been recently established by the

International Committee on Taxonomy of Virus. The genus *Gyrovirus* has been removed from the family *Circoviridae* and reassigned into the *Anelloviridae* family, and the new taxon *Cyclovirus* has been included into the *Circoviridae* family (24). This new genus is closely related with *Circovirus* genus members, with some differences in the genomic structure such as the orientation of the major open reading frames (ORFs). Moreover, viral sequences of the genus *Cyclovirus* have been reported in both vertebrates and invertebrates, including humans and other mammals (25–29), birds (30), and insects (31). Members of the *Circovirus* genus have been detected in vertebrates (32); most recently one study reported the presence of a *Circovirus* genome in invertebrates (33). One of the first *Circovirus* discovered, *Psittacine beak and feather disease virus*, was described in avian species (34) and, subsequently, several reports revealed the presence of similar virions in other species such as swine (35), fishes (36), bats (37–39), chimpanzees (40), dogs (41) humans (40), and minks (42). Since 2016, three species of porcine circoviruses have been formally accepted, including *Porcine circovirus 1* (PCV-1), PCV-2 and PCV-3 (21, 22).

Structurally, circoviruses are small single-stranded DNA (ssDNA) viruses (43), characterized by a non-enveloped virion with icosahedral symmetry, and a circular genome with a diameter ranging from 13 to 25 nm. Members of this family are constituted by 60 capsid protein subunits organized in a dodecahedral pentamer clustered unit (44). PCV-1 has a genome size ranging from 1,758 to 1,760 nucleotides (nt) (45–47), while the circular genomes of PCV-2 and PCV-3 consist of 1,766–1,769 and 1,999–2,001 nt, respectively (21, 46, 48–50).

Porcine circoviruses contain three major ORFs arranged in the strands of the replicative form (RF) (21). For PCV-1, a total of seven putative ORFs capable to encode proteins larger than 5 kDa have been predicted on both DNA strands (47), being six of them larger than 200 nt (51, 52). PCV-2 contains, besides the three major ORFs, eight more predicted ones, but just ORF4 has been characterized in more detail (53–55). PCV-3 contains so far three identified ORFs, but only ORF1 and ORF2 have been characterized. The general characteristics of the three major ORFs of PCVs are summarized in **Table 1**.

ORF1 encodes for Rep and Rep′ proteins involved in replication initiation, of 312 and 168 aa, respectively, in PCV-1, and of 314 and 297 aa, respectively, for PCV-2 (56). ORF1 apparently codes for a single replicase protein in PCV-3, of 296–297 aa (21, 22). ORF1 is located on the positive strand and considered the most conserved region of the circovirus genome (57). The origin of replication (*ori*), constituted by a conserved non-anucleotide motif [(T/n)A(G/t)TATTAC], is located on the same strand as ORF1 and, consequently, this frame is involved in rolling circle replication (RCR) (58).

ORF2 encodes the only structural protein (Cap). It consists of 230–233 aa for PCV-1, 233–236 aa for PCV-2 (56, 59, 60) and 214 aa for PCV-3 (21, 22). ORF2 is located on the negative DNA viral strand and Cap protein is considered the most variable (46, 61, 62), and most immunogenic (63) viral protein. Nucleotide similarity of 67% in Cap protein between PCV-1 and PCV-2 was detected through phylogenetic analyses (64); moreover, the similarity in this protein is much lower (24%) among PCV-1 and PCV-3 (22) while being 26–37% between PCV-2 and PCV-3 (21, 22).

The ORF3 is oriented in the opposite direction of ORF1, also in the negative strand, which codifies for a non-structural protein with apoptotic capacity (56, 65). The ORF3 protein consists of 206 aa for PCV-1, 104 aa for PCV-2 and 231 aa for PCV-3 (21, 66). The apoptotic activity of ORF3 protein has been described both *in vitro* and *in vivo* for PCV-1 and PCV-2 (67, 68), while its putative function in PCV-3 is still unknown.

Lastly, ORF4, also located in the negative strand, has only been described in the PCV-2 genome. This gene codifies for a protein of approximately 60 aa with anti-apoptotic function (53, 54).

Table 2 summarizes the nucleotide and amino acid raw distances (calculated by means of the median pairwise distances) among and within porcine circoviruses.

The similarity between PCV-3 sequences ranges from 97 to 100% throughout the analyzed years and tested countries (48, 69–71). Phylogenetic analyses suggested two main groups classified as PCV-3a and PCV-3b and several sub-clusters (48, 72, 73), based on differences found between both groups in the aa sites 122 and 320 (S122A and A320V). In fact, certain antigenicity differences among groups have been proposed (74), although it is still too early to discuss about potential different genotypes or subgroups for PCV-3. Additionally, the progressive increase in sequence availability is revealing the presence of other branching patterns, which hardly fit with the "two genotype" classification. Therefore, similarly to PCV-2, a higher heterogeneity might be found in the future. A phylogenetic tree including full-length sequences of PCV-3 is depicted in **Figure 1**.

EPIDEMIOLOGY

After the first description reported from the USA, several countries located in Asia, Europe and South America (**Figure 2**) have demonstrated the presence of PCV-3 genome in domestic pig (70, 73, 75–80).

PCV-3 genome has been detected at all tested ages, including sows, mummified fetuses and stillborn (21, 79, 81). The frequency of viral detection found by PCR in pigs is variable according to the collected samples around the world (**Table 3**). A lower frequency of PCV-3 PCR positivity has been detected in lactating pigs when compared with nursery and fattening ones; the highest prevalence was found in animals after weaning (48, 77, 82). However, these studies included different pigs from fairly limited age-groups and not the same animals over time. In a very recent work performed on longitudinally sampled pigs in Spain (83), PCV-3 DNA was found at all age-groups in four tested farms, and the frequency of infection was not clearly dominant at any age. Also, PCV-3 has been detected at moderate to high rate in sera pools from sows in Poland (77) and Thailand (84).

PCV-3 genome has been detected by PCR in oral fluids and nasal swabs (76, 82) as well as in feces (85, 95), semen (70), and colostrum (84). Kedkovid et al. (84) found a positive correlation between detection in serum samples and in colostrum, suggesting that the colostrum is influenced by the viremic stage of the sow. No specific studies have been performed on the virus detection

TABLE 1 | Summary of characteristics of the three major ORFs in PCV-1, PCV-2, and PCV-3.

Porcine circovirus	Size (nt)	ORF1		ORF2		ORF3	
		Protein	Size (aa)	Protein	Size (aa)	Protein	Size (aa)
PCV-1	1,758–1,760	Rep Rep′	312 168	Cap	230–233	NS	206
PCV-2	1,766–1,769	Rep Rep′	314 297	Cap	233–236	NS	104
PCV-3	1,999–2,001	Rep	296–297	Cap	214	Unknown	231

NS, Non-structural protein; nt, nucleotides; aa, amino acids.

TABLE 2 | Median of pairwise genetic and amino acid distance calculated for all available PCV-1, PCV-2, and PCV-3 sequences.

		Complete genome			Cap			Rep		
		PCV-1	PCV-2	PCV-3	PCV-1	PCV-2	PCV-3	PCV-1	PCV-2	PCV-3
DNA	PCV-1	0.011 [0.000–0.026]	0.228 [0.220–0.271]	0.533 [0.528–0.543]	0.017 [0.000–0.043]	0.332 [0.314–0.352]	0.598 [0.586–0.611]	0.006 [0.000–0.070]	0.174 [0.116–0.194]	0.500 [0.491–0.527]
	PCV-2	0.228 [0.220–0.271]	0.037 [0.001–0.102]	0.525 [0.518–0.544]	0.332 [0.314–0.352]	0.057 [0.000–0.172]	0.547 [0.539–0.569]	0.174 [0.116–0.194]	0.022 [0.000–0.056]	0.495 [0.485–0.520]
	PCV-3	0.533 [0.528–0.543]	0.525 [0.518–0.544]	0.009 [0.000–0.024]	0.598 [0.586–0.611]	0.547 [0.539–0.569]	0.014 [0.000–0.028]	0.500 [0.491–0.527]	0.495 [0.485–0.520]	0.006 [0.000–0.034]
Amino acid	PCV-1	NA	NA	NA	0.028 [0.000–0.071]	0.303 [0.283–0.346]	0.748 [0.732–0.760]	0.006 [0.000–0.075]	0.147 [0.088–0.194]	0.583 [0.577–0.607]
	PCV-2	NA	NA	NA	0.303 [0.283–0.346]	0.055 [0.000–0.177]	0.689 [0.681–0.736]	0.147 [0.088–0.194]	0.009 [0.000–0.075]	0.574 [0.564–0.602]
	PCV-3	NA	NA	NA	0.748 [0.732–0.760]	0.689 [0.681–0.736]	0.012 [0.000–0.035]	0.583 [0.577–0.607]	0.574 [0.564–0.602]	0.003 [0.000–0.031]

The distance range is reported between brackets after removal of the lower and upper 0.1 percentile. This measure was selected to exclude extreme values, which could be due to poor quality of some sequences challenging to be detected during alignment inspection. NA, non-applicable.

in the environment, but one study indicates that the virus was found in 2 out of 4 sponges used for sampling pig transporting trucks after sanitation (89).

Besides domestic pigs, PCV-3 infects wild boar. Viral DNA sequences retrieved from wild boar showed more than 98% similarity with the available sequences from domestic pigs (95, 96). The prevalence found in tested serum samples was similar or higher than that found in domestic pigs, ranging from 33 to 42.66%. Additionally, infection susceptibility was associated with the age in both studies; juvenile animals were statistically less often PCV-3 PCR positive than the older ones. In fact, a potential reservoir role of the wild boar with respect to PCV-3 infection has been suggested (95, 96).

PCV-3 seems to be restricted to *Suidae* species. However, PCV-3 genome has been found in 4 out of 44 (9.09%) serum samples of dogs from China. The authors suggested that the virus might infect, therefore, non-porcine species (97). To date, there is no further evidence regarding susceptibility to PCV-3 infection in other species.

DISEASE ASSOCIATION WITH PCV-3

PCV-3 has been detected in pigs with different clinical/pathological conditions, such as respiratory, reproductive, gastrointestinal and neurological disorders; however, the virus has been also detected in apparently healthy animals (21, 71, 98). The conditions in which PCV-3 has been found are summarized in **Table 4**. Noteworthy, in most of these scenarios there are not complete diagnostic studies, but only the detection of the viral genome in a number of pigs affected by different clinical signs. Even though the viral genome was detected, it is worthy to state that it does not imply a causative role of PCV-3 in the observed condition. Thus, this section compiles the peer-reviewed papers, reporting PCV-3 DNA detection in different disease scenarios.

The amount of viral DNA in serum samples (10^2-10^7 copies/mL) (21) and tissues (10^4-10^{11} copies/mg) (86, 91) in postweaning pigs and adults was rather variable, as well as in stillborn or fetal tissues (10^6-10^9 copies/mg) (21, 75). In most of these cases, the number of PCV-3 genome copies should be considered moderate to low (21, 91). In addition, detection was possible in some instances, but the viral load was below the limit of quantification of the qPCR, which may emphasize the subclinical nature of the infection in these cases (48, 81). An association between high viral load and severity has been demonstrated for other porcine circovirus (PCV-2), especifically under PCV-2-SD (102) and PCV-2-reproductive disease (103) scenarios. However, the meaning of a given genome

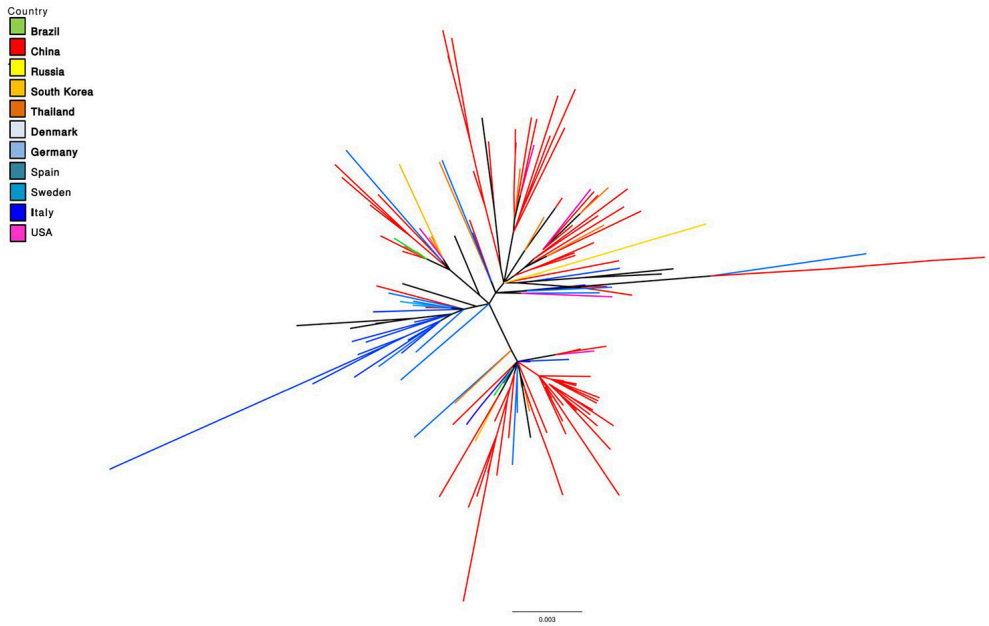

FIGURE 1 | Maximum Likelihood unrooted phylogenetic tree reconstructed based on all available PCV-3 complete genome sequences (retrieved on October 2018). Tree terminal branches have been color coded according to the corresponding collection country. Black color in terminal branches indicates other countries not included in the list.

FIGURE 2 | Countries in red are those that have been so far reported PCV-3 PCR positive samples in domestic pig.

viral load for PCV-3 in healthy or diseased pigs is still to be elucidated.

Reproductive Disease

PCV-3 genome was initially retrieved from sows with clinical signs compatible with PDNS in USA. In the affected farm, a decrease of 0.6% in the conception rate was found while the sow mortality showed a 10.2% increase (21). In China, PCV-3 was found in serum samples from sows with reproductive problems characterized by acute loss of neonatal piglets (70). Moreover, a comparative study between healthy sows and sows with a clinical picture characterized by chronic reproductive failure (including increase in abortion and sow mortality rates) revealed that PCV-3 positivity was higher in affected sows (39 out of 84, 46.42%) than in healthy ones (23 out of 105, 21.9%) (69). Viral genome has also been found in tissues from stillborn in farms experiencing reproductive failure in China (69–71) and Korea (94).

TABLE 3 | Reports describing PCV-3 frequency of detection on different countries and sample types.

References	Country	Sample type	PCV-3 positive (*n*)	Tested samples (*n*)	Frequency of detection (%)
Collins et al. (85)	Ireland	Tissue and feces	52	313	16.61
Fu et al. (73)	China	Tissue and stillborn	76	285	26.67
Kwon et al. (82)	South Korea	Oral fluid	159	360	44.17
Ku et al. (70)	China	Tissue, stillborn, semen and serum	77	222	34.68
Palinski et al. (21)	USA	Serum	47	150	31.33
Stadejek et al. (77)	Poland	Serum	55	215	25.58
Xu et al. (86)	China	Tissue and serum	53	170	31.18
Zhai et al. (87)	China	Tissue and serum	84	506	16.60
Zheng et al. (71)	China	Tissue	132	222	59.46
Wen et al. (88)	China	Tissue and serum	50	155	32.26
Klaumann et al. (81)	Spain	Serum	75	654	11.47
Franzo et al. (89)	Italy	Sponge sample	2	4	50.00
Franzo et al. (76)	Denmark	Tissue and serum	44	78	56.41
Franzo et al. (76)	Italy	Tissue and serum	36	91	39.56
Franzo et al. (76)	Spain	Serum (pools)	14	94	14.89
Hayashi et al. (90)	Japan	Tissue	7	73	9.59
Kedkovid et al. (84)	Thailand	Colostrum	17	38	44.74
Kedkovid et al. (91)	Thailand	Tissues and serum	33	103	32.04
Sun et al. (78)	China	Tissue	13	200	6.50
Zou et al. (69)	China	Serum	62	190	32.63
Zhao et al. (92)	China	Tissue	40	272	14.71
Ye et al. (93)	Sweden	Tissue	10	49	20.41
Kim et al. (94)	Korea	Serum	37	286	17.9
Kim et al. (94)	Korea	Tissue	20	296	6.8

Respiratory Disease

PCV-3 DNA was also detected in pigs with respiratory disorders, as already indicated in the first report of this virus (21). Two more studies reported PCV-3 genome in animals from China with abdominal breathing and lesions including lung swelling and congestion (87, 99). More recently, the viral genome has been detected in fattening pigs from Thailand suffering from porcine respiratory disease complex (PRDC), characterized by coughing, dyspnea, fever and anorexia; the prevalence was higher in diseased animals (60%; 15 out of 25) than in healthy ones (28%; 7 out of 25) (91).

Other Conditions

Multisystemic inflammation and myocarditis were initially linked with the presence of PCV-3 (22). One single study described PCV-3 in weaned pigs that suffered from gastro-intestinal disorders (diarrhea), showing higher prevalence in pigs with clinical signs (17.14%, 6 out of 35) compared to those with non-diarrhea signs (2.86%; 1 out of 35) (87). In another report, animals with congenital tremors were analyzed and PCV-3 was the only pathogen found in the brain, with high amount of viral DNA (101).

Healthy Animals

A number of studies found PCV-3 in apparently healthy animals (69, 76, 81, 87, 93), which makes much more complicated the overall interpretation of this virus as potential causative agent of disease.

Co-infections

Whilst the initially PCV-3 PCR positive cases were negative for three of the most important swine infectious agents (PCV-2, PRRSV, and *Porcine parvovirus*, PPV) (21, 22, 87), subsequent studies revealed frequent co-infection with other viruses. All pathogens found in co-infections with PCV-3 are summarized in **Table 5**.

It is still too early to establish the overall picture of PCV-3 infection, since it is a widespread virus in healthy animals. Therefore, the likelihood of disease may not depend on its presence only, but other factors may serve as illness triggering factors or up-regulate its replication under disease scenarios.

LABORATORY TOOLS TO DETECT PCV-3

The detection of the virus is currently based on molecular techniques such as conventional PCR and qPCR and its characterization by Sanger sequencing or NGS. In fact, the first PCV-3 complete genome was identified by NGS, and subsequently Sanger sequencing has been systematically applied to obtain novel PCV-3 sequences. Several primer

TABLE 4 | Clinical signs reported in PCV-3 PCR positive animals according to production phase in different clinical/pathological scenarios.

Disorders	Production phase	Clinical signs - disease	Control group-healthy animals	Reference
Reproductive	Sows	• Increase in the sow mortality; decrease in the conception rates; mummified fetuses	NA	Palinski et al. (21)
		• Aborted fetuses, stillborn	NA	Faccini et al. (75)
		• Abortion, mummified fetuses; reproductive failure; decrease of neonatal rate	NA	Ku et al. (70)
Respiratory	Lactation	• Dyspnea	NA	Phan et al. (22)
	Weaning	• Anorexia, fever, icterus, abdominal breathing	NA	Shen et al. (99)
	Weaning	• Cough, softly panting, abdominal breathing	Yes*	Zhai et al. (87)
	Fattening	• Respiratory signs	NA	Phan et al. (22)
	Fattening	• Porcine respiratory disease complex (PRDC)	NA	Kedkovid et al. (91)
Cardiovascular	Weaning	• Anorexia, weight loss, swollen joints	NA	Phan et al. (22)
Gastrointestinal	Weaning	• Diarrhea	Yes*	Zhai et al. (87)
Systemic	Weaning	• Wasting	Yes*	Stadejek et al. (77)
		• Periweaning failure-to-thrive syndrome (PFTS)	Yes*	Franzo et al. (100)
Neurological	Lactation	• Neurological signs	NA	Phan et al. (22)
	Lactation	• Congenital tremors	NA	Chen et al. (101)
Others	Fattening	• Rectal prolapse	NA	Phan et al. (22)
	Sows	• PDNS	NA	Palinski et al. (21)

*NA, not available in the published study; *, PCV-3 positivity in lower frequency than diseased animals.*

pairs and probes have been designed for these molecular techniques (21, 89, 101). Moreover, a duplex qPCR for the simultaneous detection of PCV-2 and PCV-3 has been also attempted (105).

In situ hybridization, a technique used to detect viral genome on histological tissue sections, has been performed in two studies (22, 91). However, the technique is not yet completely standardized, since it is still used in minimal number of laboratories worldwide and a thorough description of the infected cell types is still missing.

A minimum number of studies showed the development and validation of serological tests. Two reports have published limited information about indirect enzyme-linked immunosorbent (ELISA) tests using recombinant PCV-3 Cap protein (21, 106). More recently, a PCV-3 specific monoclonal antibody has been produced, presumably working on formalin-fixed, paraffin-embedded tissues by means of immunohistochemistry (72).

Infection of cell cultures with PCV-3 tissue homogenates has been attempted in PK-15 (21, 75) and swine testicle cells (ST) (21) without success. The cells were observed for cytopathic effects and monitored by qPCR for viral growth. However, the Ct-values did not increase at each cellular passage and no cytopathic effect was observed (21, 75). Therefore, there is not any PCV-3 isolate so far available.

Definitely, in order to elucidate the PCV-3 pathogenesis, further establishment of laboratory techniques such as viral isolation, serology, and detection of viral components in tissues is needed. In consequence, the potential association of PCV-3 with any clinical condition, if any, is difficult to be demonstrated due to existing technical limitations.

KNOWLEDGE GAPS OF PCV-3 INFECTION

PCV-3 As a Cause of Disease

Porcine circoviruses (PCVs) are ssDNA ubiquitous viruses, widespread worldwide in the domestic pig population (107). Two species were known to infect *Suidae* species before 2015: PCV-1, considered non-pathogenic, and PCV-2, the cause of one of the most devastating porcine diseases, PCV-2-SD. PCV-3 represents an expansion of the swine virosphere within the *Circoviridae* family, but the up-to-date knowledge is still very limited and there is not yet any clue on its potential pathogenesis or disease causation role. It is at least curious that 20 years ago there were serious doubts about PCV-2 as a cause of an overt disease characterized by severe lesions and high mortality (108), while nowadays PCV-3 has been found within a number of clinical conditions and putative association has been established from the very beginning (21, 22).

Current literature has already reported the presence of PCV-3 in animals affected by different clinical pictures, although just few of them included healthy control groups (71, 76, 87, 91). In all studies, the frequency of PCV-3 detection in diseased animals was higher; although these results did not prove any disease causality, at least open the avenue to definitively ascertain its role in clinical/pathological manifestations. Further studies on potential disease association of PCV-3 are needed.

Pathogenesis

No data is available regarding the pathogenesis of PCV-3 infection. The lack of virus isolation has impeded the establishment of an infection model to date. It is known that PCV-3 can be found in different tissues of domestic pig and wild boar (86, 87, 95), indicating the systemic nature of the

TABLE 5 | Pathogens present in PCV-3 PCR positive samples/cases.

Pathogen	Frequency of co-infection (percentage)	Reference
PCV-2	38/200 (19%)	Sun et al. (78)
	28/40 (70%)	Zhao et al. (92)
	35/222 (15.77%)	Ku et al. (70)
	13/46 (28.26%)	Kim et al. (104)
	1/8 (12.5%)	Kedkovid et al. (91)
	11/57 (19.3%)	Kim et al. (94)
PRRSV	1/8 (12.5%)	Kedkovid et al. (91)
	25/57 (43.86%)	Kim et al. (94)
Torque teno sus virus (TTSuV1 and 2)	66/132 (50%)	Zheng et al. (78)
Classical swine fever virus (CSFV)	108/200 (54%)	Sun et al. (78)
Porcine bocavirus (PBoV)	NA	Chen et al. (101)
Porcine epidemic diarrhea virus (PEDV)	NA	Chen et al. (101)
Atypical porcine pestivirus (APPV)	NA	Chen et al. (101)
Porcine deltacoronavirus (PDCoV)	NA	Chen et al. (101)
Porcine kobuvirus (PKV)	NA	Chen et al. (101)
Porcine pseudorabies virus (PRV)	NA	Chen et al. (101)
Porcine sapelovirus (PSV)	NA	Chen et al. (101)
Porcine parvovirus (PPV)	NA	Franzo et al. (100)
Ungulate bocaparvovirus 2 (BoPV2)	NA	Franzo et al. (100)
Pasteurella multocida	NA	Kedkovid et al. (91)
Haemophilus parasuis	NA	Phan et al. (22)
Streptococcus suis	NA	Phan et al. (22)
Mycoplasma hyorhinis	NA	Phan et al. (22)

NA, not available in the published study.

infection. However, the point of viral entry, primary replication, organic distribution and persistence are still unsolved issues. PCV-3 has been found in feces, nasal swabs, oral fluids, and trucks transporting pigs (82, 85, 95), which allows speculating that horizontal transmission through direct contact is probably an important route. Detection of viral genome in fetuses and stillborn from farms with history of reproductive failure (21, 70, 75), as well as in semen and colostrum, points out also to vertical transmission as another likely route. Definitively, more studies are needed to ascertain the potential excretion routes of this virus.

Co-infections

Co-infection of PCV-3 with both PCV-2 and PRRSV has been reported (70, 78, 91, 92, 94). In fact, this was expected since both well-known pathogens are widespread in the pig population (109–111). Noteworthy, it is known that both PCV-2 and PRRSV are able to affect the immune system and, therefore, co-infections with these viruses are not unusual (112, 113). Other pathogens were also detected in PCV-3 PCR positive samples (78, 114). Very recently, PCV-3 has been found by NGS approach in pigs affected by periweaning failure-to-thrive syndrome in co-infection with PPV and *Ungulate bocaparvovirus 2* (100). Since experimental and field studies demonstrated that co-infection with PPV increase the effect of PCV-2 in causing PCV-2-SD (115), at this point it cannot be ruled out that a similar effect may occur with PCV-3. Further investigations are needed to determine whether PCV-3 might act as a secondary agent up-regulating its replication once pigs are immunosuppressed or immunomodulated, or whether the frequency of co-infection is independent of the immune system affection.

Age of Infection and Transmission

Although PCV-3 genome has been detected at higher prevalence in weaned pigs (48, 77, 82), only one study has monitored PCV-3 infection longitudinally (83). In this study, PCV-3 was found in pigs at all ages with a similar frequency. This infection dynamics contrasts with that of PCV-2, which infects pigs mainly between five and 12 weeks of age, and rarely in animals at the lactation phase (116–118). This is explained by the fact that colostrum antibodies are protective against infection and then decline during the lactation and weaning phases. Once maternally derived antibodies waned, an infection is followed by active seroconversion (117–119). This seroconversion usually occurs between 9 and 15 weeks of age and the antibodies may last until 28 weeks of age at least (117, 120–122). Regrettably, information about infection in sows, maternally derived immunity and how protective the immunity might be against PCV-3 is completely lacking at this moment. It is known that PCV-3 can be found in colostrum (84), implying the possibility of vertical transmission (sow to piglet) and emphasizing the potential importance of early infections. Again, available information regarding these issues on PCV-3 is still to be generated.

Persistent or Long Lasting Infection

One study performed in samples from captured and re-captured wild boar revealed long-lasting infection (potential persistent infection), since the virus was detected during a period of at least 5–7 months in few animals (95). Susceptibility of wild boar to PCV-3 was not a surprise, since this species shows susceptibility to several pathogens that affect humans and animals (123), including PCV-2; moreover, the wild boar can also develop PCV-2-SD (124). Taking into account the potential long period of infection observed in some animals and even a higher overall prevalence in wild boar when compared with domestic pigs, such potential reservoir role deserves further investigations (95, 96).

Spectrum of Species Infected and Public Health Issues

Infection of PCV-3 in other non-*Suidae* species is, at this point, still to be demonstrated. Although PCV-3 DNA has been found in sera from dogs in China (97), the lack of other detection techniques able to confirm a true infection with this virus prevents the assumption of multiple species susceptibility.

Another interesting aspect yet currently unknown is the potential impact of PCV-3 on public health. DNA from PCV-1 and PCV-2 has been found in vaccines intended for use in humans (125), probably associated to the use of reagents from

swine origin in the vaccine manufacturing. At this point, no information regarding PCV-3 and its role as a contaminant of human medicines do exist. On the other hand, porcine circoviruses belong to a group of microorganisms that still has not been fully addressed in terms of risk evaluation for xenotransplantation (126), so, PCV-3 should be also *a priori* added to such list.

Origin, Evolution, and Phylogeny

Palinski et al. (21) conducted a brief study in paraffin fixed tissues from 2010 to 2016 in North America and results showed a high percentage of PCR positivity in these samples, suggesting that the virus emerged before the year of its discovery. In fact, PCV-3 has been already demonstrated retrospectively in Sweden in 1993 (93) and Spain (81) and China in 1996 (78), indicating that this is not a new virus and it has been circulating during several decades in domestic pigs. Moreover, PCV-3 has been detected in the oldest samples so far tested in these studies, suggesting that this virus could have been infecting pigs for even a longer period. However, these findings cannot be assumed as a proof of non-pathogenicity, especially when mirroring another closely-related circovirus, PCV-2. Although this latter virus was initially detected in association with disease by mid-late 1990s, retrospective studies showed evidence of pig infection a number of decades before (120, 127–129). In fact, in most of these investigations, evidence of PCV-2 infection coincided with the very first investigated year, suggesting again that PCV-2 might be even an older circulating virus. In addition, a retrospective study on PCV-3 conducted in samples of wild boar from Spain during a 14-year period (95) detected the virus in the first tested year (2004). Overall, obtained data confirmed that PCV-3 is not a new virus and has been circulating for a fairly, non-determined long time in swine and wild boar populations. In fact, the most common ancestor of PCV-3 was estimated to be originated approximately in 1966 (73, 130).

Genetic characterization of PCV-3 is mainly done through Sanger sequencing. Phylogenetic analyses of PCV-3 genomes available from the GenBank indicate they are part of different clusters. However, nucleotide identity among these sequences is really high (>97%). In consequence, it seems that PCV-3 has remained fairly stable over the years without an independent molecular evolution according to specific areas of the world. Moreover, these findings do not point out a high mutation rate as has been suggested (48, 131). If such mutation rate were high, it would have generated a higher genomic heterogeneity, which should have been detected at least in the performed retrospective studies accounting for more than 20 years. Further studies on the evolution on PCV-3 are crucial to solve out these controversies.

The first metagenomics sequence available from PCV-3 revealed low identity with *cap* and *rep* genes of PCV-1 and PCV-2 and a closer identity with other Circoviruses such as *Canine circovirus* (21, 22) and *Barbel circovirus* (71). The *Circovirus* genus members are able to infect a wide range of hosts, and cross-species transmission has also been reported (40). Franzo and collaborators (132) hypothesized the possibility of PCV-3 being the product of recombination related with a host jump. The analysis of genome composition of PCV-3 found the *rep* gene closely related with that of bat circoviruses and *cap* gene with that of avian ones (132). Recently, novel circoviruses isolated in civets, showing higher similarity in terms of aa sequence in Rep protein with PCV-3, have been described (133). The increasing new data should be useful to clarify the relationships and origin of this virus. On the other hand Fux et al. (48) found nucleotide changes, which resulted in two aa alterations in ORF1/ORF2 and ORF3 (A24V and R27K), between the two proposed genotypes (PCV-3a and PCV3b). Li et al. (131) also suggested two groups with two individual subclades termed PCV-3a-1 and PCV-3a-2. The aa site 24 from ORF2, predicted to be under positive selection, was suggested to be located in a potential epitope region. The presence of possible genotypes was also suggested in other studies (73, 76). However, considering the high similarity found in partial or complete PCV-3 sequences (>98% in most of the cases), the importance of determining genotypes or groupings at this stage seems poorly relevant. Due to the sensitivity limitations of Sanger sequencing, it must be emphasized the need to apply NGS technology to discover minor variants, which might unravel the presence of quasispecies undetected by the currently used technology.

CONCLUSIONS

Porcine circovirus 3 is a recently discovered virus widespread in both domestic pigs and wild boar population. The virus can be found at all tested ages and few animals may display a persistent infection. Although the virus has been found in several clinical and pathological conditions, a definitive proof of its pathogenicity is still lacking. Phylogenetic information available to date indicates a low genetic variability of PCV-3 in comparison with other single stranded-DNA viruses and indicates that the virus genome has been relatively stable across the years.

AUTHOR CONTRIBUTIONS

FK and JS did the majority of the writing and communicated with the coauthors to coordinate the document editing. JS designed the outline of the manuscript. GF provided the phylogenetic analyses. FC-F, GF, MS, and JN revised the manuscript, did partial writing and approved the final version for publication.

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Phylogenetic and Genome Analysis of 17 Novel Senecavirus A Isolates in Guangdong Province, 2017

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Senecavirus A (SVA), an emerging RNA virus, is considered to be associated with porcine idiopathic vesicular disease (PIVD). From February to September 2017, 17 novel SVA strains were isolated from samples with the vesicular disease from Guangdong Province, China. Full-length genomes and individual genes of the 17 new SVA isolates were genetically and phylogentically analyzed. Results showed that complete genomes, VP1, 3C, and 3D genes of these 17 novel SVA isolates revealed 96.5–99.8%, 95.1–99.9%, 95.6–100%, and 96.9–99.7% nucleotides identities, respectively. Phylogenetic analyses based on sequences of full-length genomes, VP1, 3C, and 3D genes indicated that 17 novel SVA isolates separated to three well-defined groups. Meanwhile, phylogenetic analysis for all available Chinese SVA strains demonstrated that 45 Chinese SVA strains clustered into five distinct groups with no significant relationship between strains from different provinces and/or years, including a newly emerging branch in China. This is the first comprehensive study of phylogenetic analysis for all available Chinese SVA strains, indicating the appearance of a new type of SVA strains and the complicated circulations with at least five different types of SVA strains in pigs in China.

Keywords: Senecavirus A, genome, phylogenetic analysis, Guangdong province, China

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INTRODUCTION

Senecavirus A (SVA), first discovered as a cell contaminant in 2002, is a non-enveloped, single-stranded and positive-sense RNA virus. It belongs to the family *Piconaviridae* and is the only member of the genus *Senecavirus* (1, 2). The genome of SVA is approximately 7.2 kb in length and encodes four structural proteins (VP1 to VP4) and eight non-structural proteins (L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D) (2). After large scale outbreaks of vesicular disease in sows as well as sudden neonatal death loss in Brazil which started at late 2014, SVA is identified to be the etiological agent of porcine idiopathic vesicular disease (PIVD) (3, 4). As a new causative agent, SVA has spread quickly and its clinical signs are difficult to be distinguished with infections of other viruses, including food-and-mouth disease virus (FMDV), swine vesicular disease virus (SVDV), vesicular exanthema of swine virus (VESV) and vesicular stomatitis virus (VSV), which have resulted in significant economic losses (5, 6). So far, there are six countries in Asian and American continents that have documented SVA associated with the vesicular disease in pigs (7–12) (**Figure 1**).

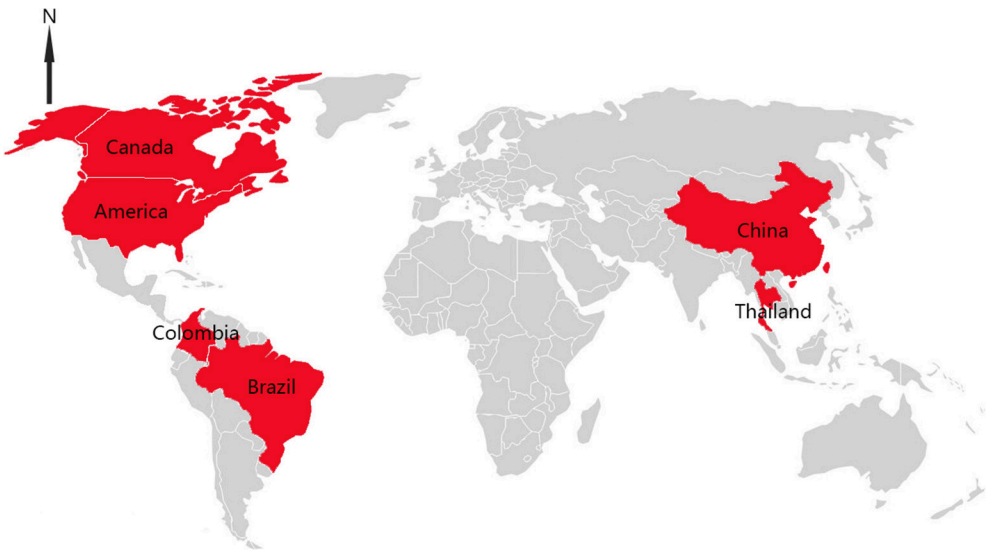

FIGURE 1 | Distributions of SVA infection cases in the world.

In China, the first SVA strain was isolated from pigs with classical symptoms of PIVD in Guangdong Province in 2015, and since then, increasing cases of SVA infections have emerged in more provinces, including Heilongjiang, Hebei, Henan, Hubei, Anhui, Fujian, and Guangxi (13–17). At present, nearly 30 full-length genomes of SVA reported from China are available in Genbank (access date: 19 August, 2018). Here, we report 17 novel SVA strains isolated in Guangdong Province from February to September in 2017 that are genetically separated into three distinct groups, and give a deep insight of the phylogenetic relationship between all available Chinese SVA strains.

MATERIALS AND METHODS

Ethics Approval and Consent to Participate

This study was carried out in accordance with the recommendations of National Standards for Laboratory Animals of the People's Republic of China (GB149258-2010). The protocol was approved by Animal Research Committees of South China Agricultural University. Pigs used for the study were handled in accordance with good animal practices required by the Animal Ethics Procedures and Guidelines of the People's Republic of China.

Sample Collection and Virus Detection

Seventeen vesicular and tissue samples of sows and piglets associated with vesicular disease were collected from 16 pig farms in five cities in Guangdong Province between February to September 2017 (**Table 1**). Viral RNA was extracted from tissue homogenates by using AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, United States) according to the manufacturer's instructions. All RNA samples were stored at −80°C. SVA was detected by RT-PCR with primers described by Wu et al. (15).

Virus Isolation

PK-15 cells that were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Thermo Scientific) were employed to isolate SVA. The tissue homogenates or vesicular fluids with phosphate-buffered saline (PBS; 20% w/v) were centrifuged for 15 min at 10,000 × g; then filtered suspended samples were inoculated into a 25-cm^2 flask containing PK-15 cells at 80% confluency. The inoculated cells were incubated at 37°C in 5% CO_2 and observed daily for cytopathic effect (CPE). When CPE appeared in 70% of the cells, viruses were harvested and the presence of virus was examined by RT-PCR.

Genome Amplification and Sequence Analysis

Seven pairs of primers were utilized for the entire genome sequencing as previously described by Wu et al. (15). RNA samples were reverse-transcribed into cDNA and amplified using a one-step RT-PCR kit (TaKaRa, Dalian, China). The RT-PCR assay was performed with the following cycling conditions: 50°C for 30 min and 94°C for 5 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products were purified by a Gel Band Purification Kit (Omega Bio-Tek, United States) and then cloned into the PMD-19T vector (TaKaRa, Dalian, China) and transformed *E. coli* DH5α competent cells. The recombinant plasmids were sequenced by the Beijing Genomics Institute (Shenzhen, Guangdong, China) Genomic sequences were assembled and aligned using the DNASTAR program (DNAStar V7.1, Madison, WI, United States). Phylogenetic trees were constructed using the neighbor-joining method in MEGA 7.0 software with bootstrap analysis of 1,000 replicates. Percentages of replicate trees in which the associated taxa clustered are shown as nearby branches (18–20).

RESULTS

Seventeen new SVA genomic sequences studied in this work were submitted to GenBank with the following accession numbers MG765550 to MG765566. The length of the complete genome was 7,286 bp. Nucleotide identities between these 17 new SVA strains ranged from 96.5 to 99.8%, and all 46 Chinese SVA strains showed 95.7–100% nucleotide identities. Except sharing low sequence identities with the prototype SVA strain SVV-001 (93.5–94.1%) and the strain ATCC PTA-5343 (93.1–94%), these 17 new Chinese strains were 97.1–98.6% identical to other USA strains. When compared to full-length sequences of Colombian, Brazilian, Canadian and Thai, the new 17 SVA strains shared 97.1–98.3%, 96.9–97.9%, 95.3–97.4%, and 94.8–95.8% complete genomic identities, respectively. Sequence analyses for individual genes of 17 new SVA strains showed that of the 12 genes, VP1, 3C and 3D genes had relatively low identities each with 95.1–99.5%, 95.9–99.7%, and 97–99.7%, while other nine genes were more conserved possessing 99.5–100% nucleotide identities.

Phylogenetic analysis based on complete genomic sequences indicated that these 17 new SVA strains clustered into three distinct groups. The group A only contained sequences reported from Guangdong Province in 2017, including six new strains SVA/CHN/01/2017 to SVA/CHN/06/2017 and eight previously reported sequences. Meanwhile, the group A had a close relationship with two US strains identified in 2015. Fourteen SVA sequences in this group shared 97.7–100% identities with each other. Three new strains SVA/CHN/07/2017 to SVA/CHN/09/2017 and three other sequences that each were collected from Anhui, Hebei and Guangdong Province in 2016 and 2017 formed group B. Genomic sequences within this group shared 98.1–99.5% nucleotides identities. The remaining eight new strains and six previous Chinese sequences identified between 2015 and 2017 belonged to group C, and nucleotide identities in this group ranged from 97.2 to 99.9%. Besides these three groups, there were another two well-defined branches that only contained SVA strains in China. One group (D) included the first published Chinese SVA strain, CH-01-2015. Most of the strains in group D were reported in 2015 and 2016, but there was also a strain GD04-2017 (MH316113) which was identified in 2017. The last group (E) only included three sequences that were identified from Fujian and Henan provinces in 2017, and had a close relationship with three US strains identified in 2015. Nucleotide identities within and between each group were listed in **Table 2**. Compared with sequences in these five distinct groups, the remaining two Chinese strains, SVA/HLJ/CHA/2016 and AH02-CH-2017, separated from other Chinese SVA strains and each clustered with different US stains (**Figure 2**).

Phylogenetic analyses based on sequences of VP1, 3C, and 3D genes also indicated the presence of five well-defined groups for Chinese SVA strains. For members in each group as described above, VP1 tree showed same results with the full-length genomic tree. The sequence GD04-2017 that belonged to group E based on VP1 genes and complete genomes was clustered into group B in the phylogenetic tree of 3D genes. This change also occurred in the phylogenetic tree of 3C genes. Besides this, in the tree of 3C genes four new SVA strains SVA/CHN/03/2017 to SVA/CHN/06/2017 and other five previous sequences were separated from other strains in group A and had a close relationship with strains in group B (**Figures 3–5**).

TABLE 1 | Details of 17 new SVA strains isolated in Guangdong Province, China.

Name of sample	Sampling date	Sampling location	Sample	Pig group
SVA/CHN/01/2017	27-Jun-17	Farm 1, Yangjiang	Viscera, lymph node	Sow
SVA/CHN/02/2017	25-Jul-17	Farm 2, Yangjiang	Vesicular fluid	Sow
SVA/CHN/03/2017	7-Jul-17	Farm 3, Zhaoqing	Vesicular fluid	Sow
SVA/CHN/04/2017	25-Jun-17	Farm 4, Qingyuan	Vesicular fluid	Sow
SVA/CHN/05/2017	2-Sep-17	Farm 5, Qingyuan	Vesicular fluid	Sow
SVA/CHN/06/2017	1-Sep-17	Farm 5, Qingyuan	Vesicular fluid	Sow
SVA/CHN/07/2017	19-Jul-17	Farm 6, Foshan	Lung, tongue, lymph node	Sow
SVA/CHN/08/2017	15-Jun-17	Farm7, Shaoguan	Vesicular fluid	Sow
SVA/CHN/09/2017	29-Aug-17	Farm 8, Shaoguan	Vesicular fluid	Sow
SVA/CHN/10/2017	3-May-17	Farm 9, Qingyuan	Vesicular fluid	Sow
SVA/CHN/11/2017	3-May-17	Farm 10, Zhaoqing	Vesicular fluid	Sow
SVA/CHN/12/2017	26-Apr-17	Farm 11, Qingyuan	Lung, lymph node	Piglet
SVA/CHN/13/2017	27-Apr-17	Farm 12, Qingyuan	Hoof	Sow
SVA/CHN/14/2017	26-Apr-17	Farm 13, Qingyuan	Hoof	Sow
SVA/CHN/15/2017	19-Apr-17	Farm 14, Qingyuan	Hoof, Viscera	Sow
SVA/CHN/16/2017	7-Jul-17	Farm 15, Qingyuan	Vesicular fluid	Sow
SVA/CHN/17/2017	16-Feb-17	Farm 16, Shaoguan	Vesicular fluid	NA

NA, not available.

TABLE 2 | Nucleotide identities within and between five distinct groups.

Group name	A	B	C	D	E
A	97.7–100%*	97–98.3%	96.5–97.5%	95.7–96.6%	97.3–97.9%
B		98.1–99.5%*	96.7–97.6%	96.2–97.7%	97.4–98.2%
C			97.2–99.9%*	95.9–97.6%	96.9–97.7%
D				97.7–100%*	96.2–96.8%
E					99.7–99.8%*

* *shows the nucleotide identities within each group.*

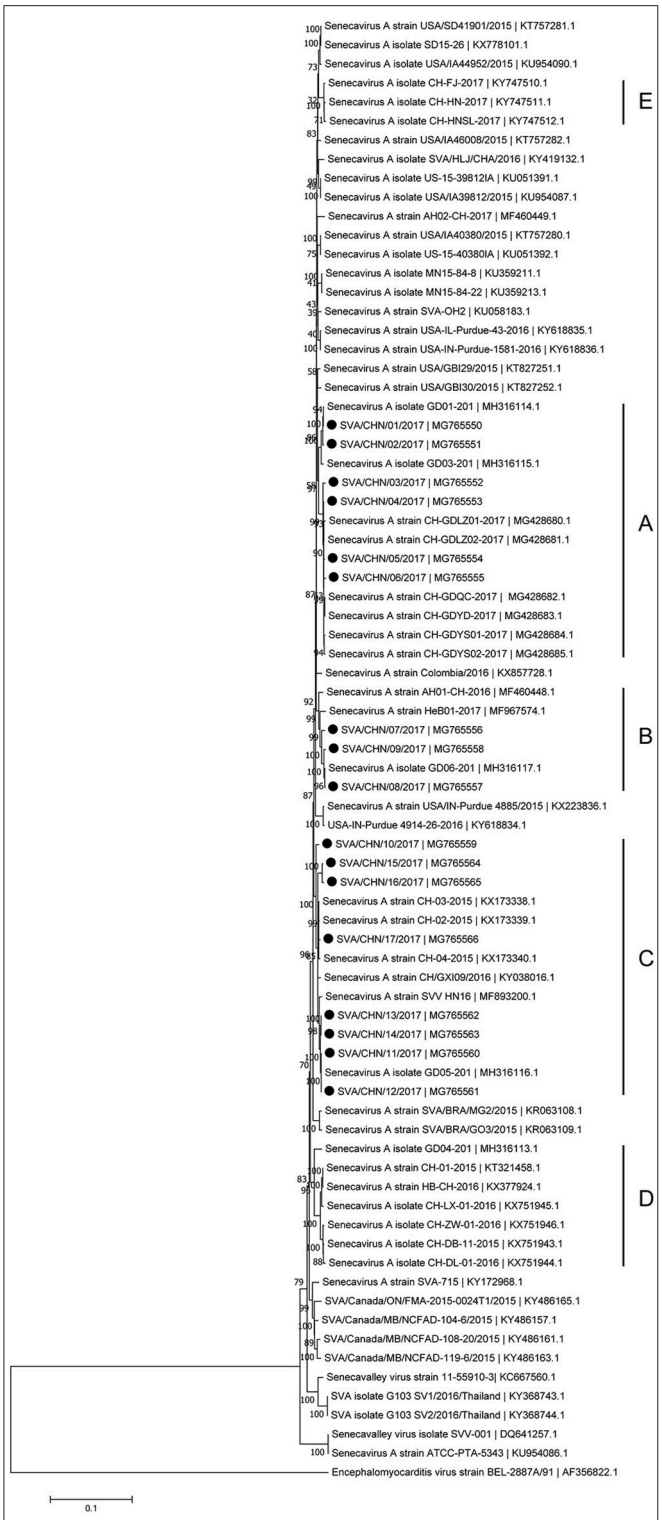

FIGURE 2 | Phylogenetic relationships based on the complete genomes of 77 SVA strains identified from different countries and years. The tree was constructed using MEGA 7.0 software with neighbor-joining methods and 1,000 replicate sets on bootstrap analysis. 17 new complete genomes sequences studied in this work were indicated with "black blots".

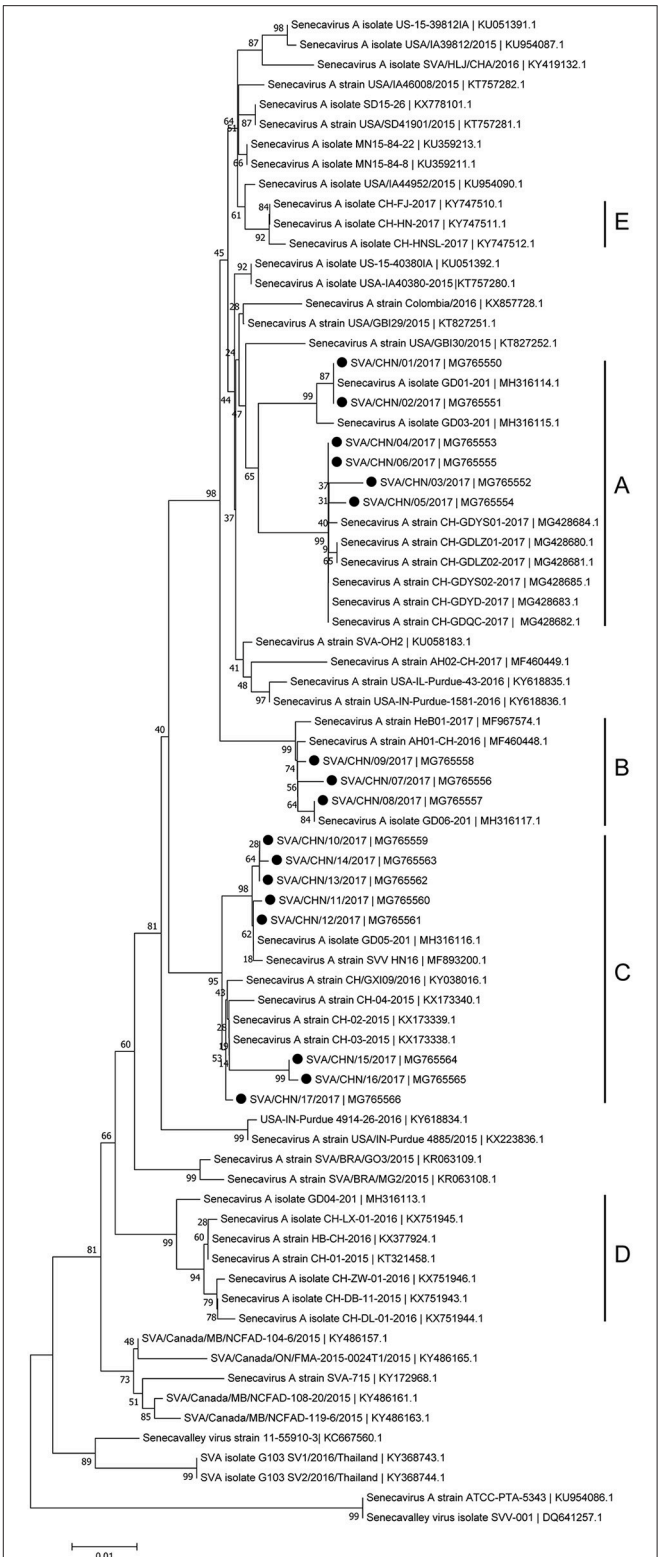

FIGURE 3 | Phylogenetic relationships based on VP1 genes of SVA. The tree was constructed with the same method described previously. 17 new sequences of VP1 genes studied in this work were indicated with "black blots".

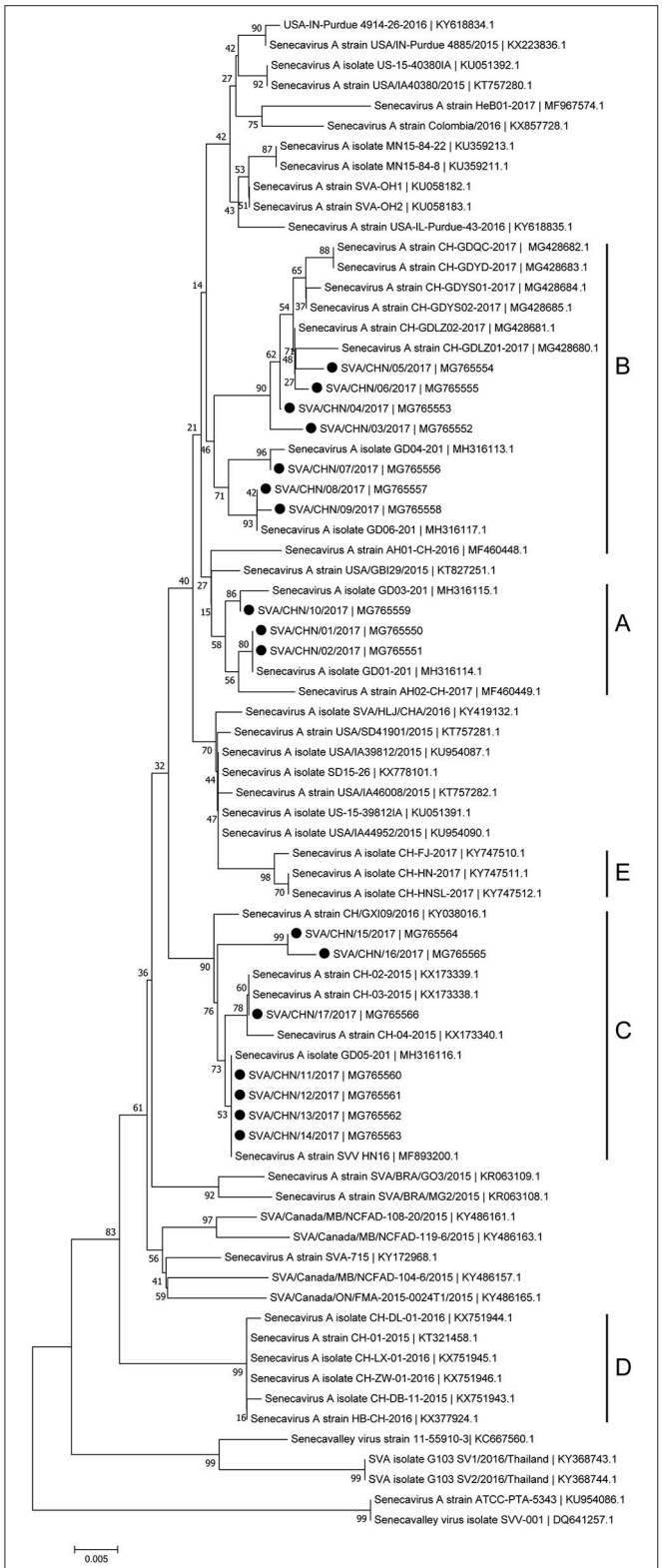

FIGURE 4 | Phylogenetic relationships based on 3C genes of SVA. The tree was constructed with the same method described previously. 17 new sequences of 3C genes studied in this work were indicated with "black blots".

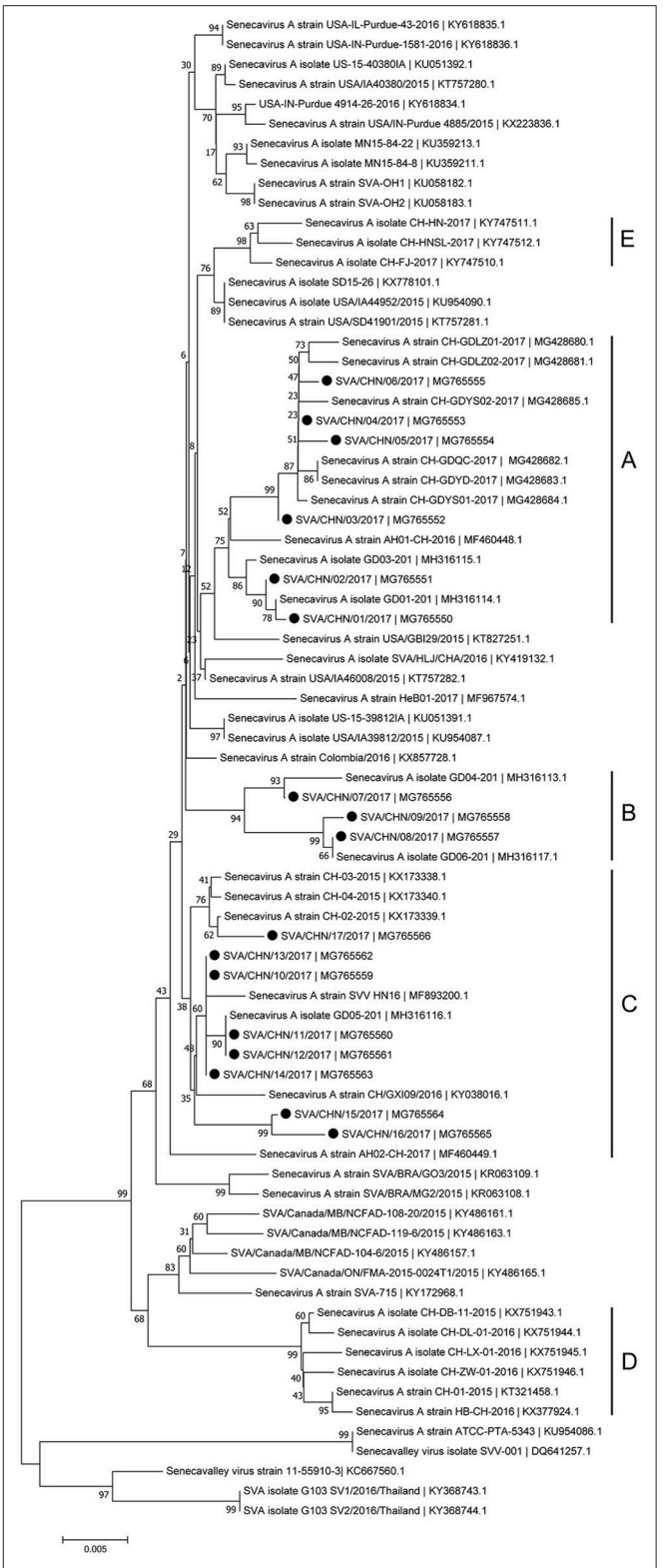

FIGURE 5 | Phylogenetic relationships based on 3D genes of SVA. The tree was constructed with the same method described previously. 17 new sequences of 3D genes studied in this work were indicated with "black blots".

DISCUSSION

Since the first Chinese SVA infection case emerged in Guangdong Province on March 2015, 35 SVA genomic sequences including 17 new stains in this study has been reported from Guangdong Province which account for over 70% of Chinese isolates [(8, 12, 15); this paper]. Based on complete genomes and three individual genes of SVA, our results showed that these 17 new isolates and some other reported Chinese sequences clustered to three distinct groups with no significant relationship between strains from different provinces and/or years. Only a few of new SVA strains clustered with previous strains identified from Guangdong Province in 2017, while most of new strains clustered with sequences identified outside Guangdong Province in 2015–2017. 17 new SVA isolates in our study were determined from 16 pig farms, so the documented number of SVA infected farms in Guangdong Province has increased to 20 (12, 15). This indicates that since 2015 SVA infections have been rapidly expanding in Guangdong Province. Our results also showed that except one strain SVA/CHN/12/2017 collected from the piglet, most new strains were achieved from sows, which was consistent with findings of Zhang et al. (6) that SVA cases in 2017 in China were mainly identified in adult pigs.

Besides strains identified from Guangdong Province, there still are 10 SVA strains reported from other seven provinces in China. Combined our results and previous findings, by now there have been five different groups of SVA strains, which indicates different genotypes of SVA strains co-exist in China (8, 17). One group including three novel SVA strains SVA/CHN/07/2017 to SVA/CHN/06/2017 is a newly emerging group, implying the appearance of a new type of SVA strains and the complicated prevalence of SVA in pigs in China. Zhang et al. (6) reported that the recent Chinese SVA strains were closely related to current US strains. Our phylogenetic analyses based on 3C and 3D genes for all available Chinese strains showed that SVA strains identified in 2017 were all close to US strains identified in 2015, which is consist with Zhang et al.'s observations. However, the results based on complete genomes and VP1 genes indicated that a strain GD04-2017 were separated from other sequences identified in 2017 and clustered into a group which was close to Brazilian sequences and contained the first Chinese strain CH-01-2015. Three strains CH-01-2015, HB-CH-2016 and CH-LX-01-2016 in this group were employed in the phylogenetic analysis of Xu et al. (11) to investigate relationships between 33 genomes of SVA strains from US, Brazil, Canada, and China, and they obtained that these SVA sequences clustered in four groups according to different countries. However, according to our results there was no significant relationship between sequences from different countries, especially for SVA strains in China. Therefore, to better understand the diversity and evolutionary relationships of SVA strains, it's important to use more whole-genome sequences or variable individual genes sequences.

By now, five, seven and 33 genomes of SVA strains have been reported in China in 2015, 2016, and 2017, respectively. Continuous descriptions of SVA infections in China especially plenty of reports in 2017 suggests that SVA may spread across the country in the future. Better understanding spreading routes of SVA is crucial for its control, while information on SVA transmission remains sparse. In our investigations we have found that cohabitation and contamination of feed or pigs carriage contribute to the disseminations of SVA, which is consistent with previous findings (5). Although pigs are a natural host of SVA, this virus has been detected in mice and houseflies (21), the role of non-swine animals in SVA transmission requires further studies. Meanwhile, more efforts are warranted to focus on SVA monitoring and managements, e.g., rapid and specific diagnostics, isolations of infected animals and vaccination strategies.

CONCLUSIONS

In conclusion, we reported 17 novel SVA isolates collected from Guangdong Province in 2017 and analyzed the phylogenetic relationships of all available Chinese SVA strains based on sequences of complete genomes, VP1, 3C, and 3D genes. Our results indicated the circulations of five different types of SVA strains in pigs in China, including a newly emerging type. Further studies based on more information of molecular epidemiology will help better understanding origin, evolution and transmission patterns of SVA in pigs in China.

AUTHOR CONTRIBUTIONS

J-YM conceived and designed the study, and critically revised the manuscript. YS conducted data analysis and wrote the manuscript. JC performed the experiments. R-TW helped in genomic data analyzed. Z-XW, J-WC, and YL helped in experimental implementation. Q-MX helped in study design. All authors read and approved the final manuscript.

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REVIEW
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Adaptation of Human Influenza Viruses to Swine

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A large diversity of influenza A viruses (IAV) within the H1N1/N2 and H3N2 subtypes circulates in pigs globally, with different lineages predominating in specific regions of the globe. A common characteristic of the ecology of IAV in swine in different regions is the periodic spillover of human seasonal viruses. Such human viruses resulted in sustained transmission in swine in several countries, leading to the establishment of novel IAV lineages in the swine host and contributing to the genetic and antigenic diversity of influenza observed in pigs. In this review we discuss the frequent occurrence of reverse-zoonosis of IAV from humans to pigs that have contributed to the global viral diversity in swine in a continuous manner, describe host-range factors that may be related to the adaptation of these human-origin viruses to pigs, and how these events could affect the swine industry.

Keywords: influenza A virus, swine, human, interspecies, adaptation, host range

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INTRODUCTION

Influenza is one of the most devastating respiratory pathogens of pigs and humans and continues to threat animal and public health with the continuing possibility of outbreaks or a pandemic. The intricacies of influenza A viruses (IAV) at the human-swine interface dates back to the 1918 pandemic. For several decades, it was hypothesized that pigs played a role in the origin of the 1918 H1N1 pandemic virus (1). Although there is evidence suggesting that the pandemic virus did not originate from pigs and that the classical swine H1N1 virus was in fact derived from the 1918 human virus (2), the bias perceiving swine as the source of IAV to humans still remains.

The ecology of IAV is complex and involves a broad range of avian and mammalian host species. IAVs are enveloped, segmented RNA viruses in the family *Orthomyxoviridae* (3). The virus genome is composed of eight negative-sense, single-stranded viral RNA (vRNA) segments that encode between 10 and 17 viral proteins depending on the strain (4–6). Each RNA segment forms the viral ribonucleoprotein complexes (vRNPs) with the nucleoprotein (NP) and the three polymerase proteins (PB2, PB1, and PA). Two major glycoproteins are projected on the virus envelope, hemagglutinin (HA), and neuraminidase (NA) (7). Based on the antigenic properties of the HA and NA, IAV are divided into 18 HA subtypes (H1–H18) and 11 NA subtypes (N1–N11) (7–9).

Influenza viruses have high mutation rates and are constantly changing, which enables the virus to quickly adapt to changes in the host environment, as is the case during interspecies transmission. The rapid evolution results from two mechanisms: reassortment and point mutations (10). Reassortment occurs when two different strains infect the same cell of a given host, allowing for exchange of intact gene segments. When reassortment involves either the HA or NA segments, it is termed antigenic shift. Point mutations occur due to an error prone polymerase devoid of

a proof-reading and correction mechanism. When point mutations are fixed in the HA or NA segments, usually a result of escape from immune pressure, it is termed antigenic drift. Both of these mechanisms play pivotal roles in the emergence of novel influenza viruses that could jump the host barrier. Once the virus jumps into a new host, it must adapt and change to be able to spread and become established in the new population.

In this review, we describe the role of pigs in the interspecies transmission of influenza and how their susceptibility to different viruses can affect the overall epidemiology of swine influenza. We discuss the factors that have been implicated in the interspecies transmission of influenza with an emphasis on the human-swine interface. We then provide an overview of human-to-swine IAV spillover events that significantly affected the epidemiology of viruses circulating in swine and how these viruses can have a negative effect on the control of influenza in pigs.

WHY PIGS BECOME INFECTED WITH VIRUSES FROM OTHER SPECIES?

To result in a successful replicative cycle, influenza viruses must efficiently infect the host cell, replicate, and produce functional virus progeny that will be released and infect new cells. The first step for infection is the attachment of the HA protein to the cell receptor. The HA is a type I transmembrane glycoprotein, present as a homotrimer on the virus' surface, each monomer carrying a transmembrane anchor and a small cytoplasmic tail. The proteolytic cleavage of the precursor HA0 produces two subunits, HA1 (globular head) and HA2 (stem). The receptor binding site (RBS) forms a shallow pocket at the distal tip of the HA1 head and consists of a base of four highly conserved amino acid residues (Y98, W153, H183, and Y195, numbering based on the H3 subtype) that are bordered by the 130-loop, the 190-helix and the 220-loop (11–13).

Through the RBS, influenza viruses bind to terminal sialic acid (SA, N-acetylneuraminic acid) moieties in glycoprotein or glycolipid receptors on the host cell surface. The SAs are usually bound to the penultimate galactose (Gal) in two major conformations: α2,3SA or α2,6SA (13). Differences in the type of SA linkage found in receptors expressed in different host species have a major impact on the host restriction of IAVs. Sialic acids with α2,3-linkage are predominantly expressed on epithelial cells in the intestinal and respiratory tracts of birds while the epithelial cells in the upper respiratory tract of humans contains predominantly α2,6-linked SA receptors (14–17) (**Figure 1**). Most avian influenza viruses preferentially bind to α2,3-SA, whereas human and other mammalian influenza viruses preferentially recognize α2,6- SA receptors (21–23).

Pigs have been historically believed to be intermediary hosts, or "mixing vessels," of influenza viruses due to their susceptibility to infection with both human-origin and avian-origin IAV and their propensity for the generation of reassortant viruses (24–27). Pigs have a similar distribution to humans of α2,3-SA and α2,6-SA receptors in the respiratory tract (**Figure 1**). As in humans, α2,6-linked SA receptors predominate in the upper respiratory tract of pigs, but α2,3-SA receptors are present in low quantities in swine tracheas, and the frequency increases toward the lower respiratory tract (18, 19) (**Figure 1**). The presence of both types of SA receptors in swine airways supports the potential role of pigs as "mixing vessels." However, such distribution of α2,3- and α2,6-SA receptors is similar in swine and humans (15, 18), and it must be noted that avian viruses do not usually transmit from pig-to-pig as is also the case in humans (28, 29). Humans can also become infected with avian-origin IAVs directly from avian sources and could potentially provide the environment for the adaptation of avian viruses (30–32). Hence, generation of reassortant viruses with pandemic potential may not require swine as intermediate hosts. However, as highlighted by the 2009 pandemic (33), while swine are not required, they may serve as intermediate hosts for generation of reassortant viruses with the ability to cause human pandemics. The 2009 pandemic has led to an increased concern about the transmission of swine viruses to humans. However, improved surveillance of swine IAV after the pandemic has shown that human viruses are transmitted to pigs, and have resulted in sustained onward transmission, far more frequently than swine viruses have infected humans (34). This lower host barrier observed for human viruses in pigs can be explained in part by the similar receptor distribution in both species and the shared preference for α2,6-linked SA receptors between human and swine viruses (22, 26).

WHAT ARE THE MECHANISMS FOR ADAPTATION OF HUMAN INFLUENZA VIRUSES TO PIGS?

Although IAV transmission events from humans to pigs are continually detected globally and despite the similarities of receptor preference and distribution between the two species, whole human IAV rarely become established in swine. Typically, these viruses reassort and emerge with only some of the human-origin viral gene segments persisting, often with marked genetic differences from the precursor strain (34–36). This implies that adaptation factors other than the receptor linkage-type specificity are required for human-origin viruses to be transmitted and subsequently become endemic in swine populations.

The adaptation of influenza viruses between humans and pigs is likely driven by selective pressures or bottlenecks imposed to the virus population during IAV host jump, as a result of the changes in the host environment (37, 38). Several factors may affect these selective pressures during interspecies transmission, either within the virus or the host. Receptor-binding specificity and affinity, balance between HA and NA content, temperature of the host, and host-specific immune factors may be some of these factors. However, the differences in the selective pressure between humans and swine and how they may differently affect virus adaptation are not entirely understood, and some of the currently know differences are discussed below.

Binding Determinants of Host Range

Specific amino acid residues at the influenza HA are required for binding to either α2,3-SA or α2,6-SA receptors and specific

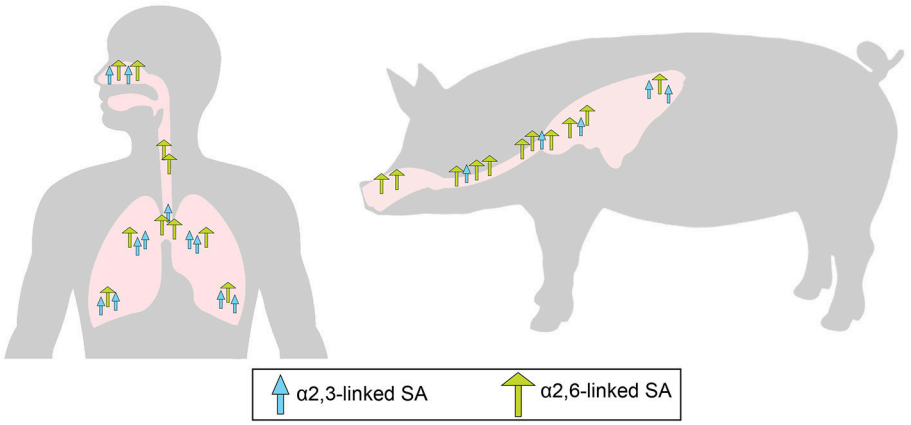

FIGURE 1 | Overall distribution of α2,6-linked sialic acid (SA; green long arrow) and α2,3-linked SA (blue short arrow) in the epithelium of the respiratory tract of pigs (18, 19) and humans (14, 15). Adapted from de Graaf and Fouchier (20).

amino acid substitutions at the RBS of the HA can alter receptor-binding specificity and facilitate host jump (**Figure 2A**). In H1 subtype viruses, positions 190 and 225 were shown to have an impact in receptor specificity. The combination of E190/G225, E190/D225, or D190/G225 in the RBS of the HA, found in avian viruses and late stage 2009 pandemic H1N1 strains, results in dual receptor-binding specificity, whereas D190/D225 and D190/E225, combinations found in seasonal human viruses, results in human-type receptor specificity (40–42). As for H3 and H9 viruses, positions 226 and 228 in the HA are critical for receptor specificity. Avian-adapted viruses usually present Q226/G228 and show dual-binding or α2,3-SA preference, but amino acid substitutions Q226L/G228S leads to receptor specificity switch to human-type receptor preference and is, therefore, more commonly found in human viruses (22, 43). Analysis of H1, H3, and H9 virus sequences from swine using the Influenza Research Database (44) revealed that swine viruses have mostly D190/D225 in H1 viruses, a fairly equal distribution between Q226/G228 and L226/G228 in H9 viruses, and the unique combination of amino acids in H3 viruses V226/S228 (**Figure 3**).

Receptor-binding specificity of influenza HA is not only mediated by changes in the sialic acid linkage, the structural length and topology of the glycans can also determine the binding specificity and affinity of IAV. Avian viruses were shown to bind to α2,3-linked SA carrying a shorter carbohydrate chain whereas human viruses bind preferentially to long α2,6-linked SA (45, 46). Moreover, avian HA binds to narrow α2,3-SA in a "cone-like" topology and human HA binds to long α2,6-SA in an "umbrella-like" topology, which are predominantly expressed in the human upper respiratory tissues (47). In general, human and swine viruses have been shown to recognize similar glycan structures on glycan microarrays, mainly branched α2,6-SA (48, 49).

NA and M as Determinants of Host-Range

While the HA is involved with binding to SA receptors, the NA cleaves α2-3 and α2-6-linked SA residues from cellular surfaces and mucus through its sialidase enzymatic activity and mediates the release of newly synthesized viruses from the host cells (7). For an optimal viral replication, balanced activities between the HA binding affinity and the NA enzymatic function are expected. The ideal HA-NA balance seems to be an important factor in host adaptation (**Figure 2B**). The HA-NA balance was shown to be crucial for the adaptation of the 2009 pandemic H1N1 virus to humans, since balanced HA and NA activities were seen in the human strains but not in precursor swine viruses (50) and this balance resulted in increased replication and transmissibility in ferrets (51). Additionally, adaptation of H5 and H7 viruses from wild birds to chickens led to selective changes in both HA and NA, maintaining a balance between binding and cleavage that was important for replication and transmission in the new host (52). These chicken-adapted H5 and H7 viruses possess a shorter NA due to the deletion of several residues in the stalk domain that were shown to enhance replication and virulence in chickens but block respiratory transmission in ferrets (53, 54).

In addition to the NA, the matrix (M) gene segment has been shown to be a critical determinant of respiratory transmission efficiency of IAV in new hosts. The M segment was implicated with the increased transmissibility of the 2009 pandemic H1N1 virus in animal models (55, 56), suggesting it played an important role on the spread of the virus in humans. In pigs, the combination of the NA and M genes from the 2009 pandemic virus was essential to facilitate efficient replication and transmissibility (57). Interestingly, reassortant H1 and H3 swine-origin viruses containing the M gene of the 2009 pandemic virus have caused almost yearly zoonotic outbreaks in humans, more frequently than was observed prior to the pandemic, confirming that the M gene plays a role in adaptation and transmission of swine viruses in humans (58–60).

Temperature Determinants of Host-Range

The virus polymerase (comprised of viral proteins PB1, PB2, and PA) was also shown to be a major determinant for host range of influenza viruses (61) (**Figure 2C**). This host restriction has

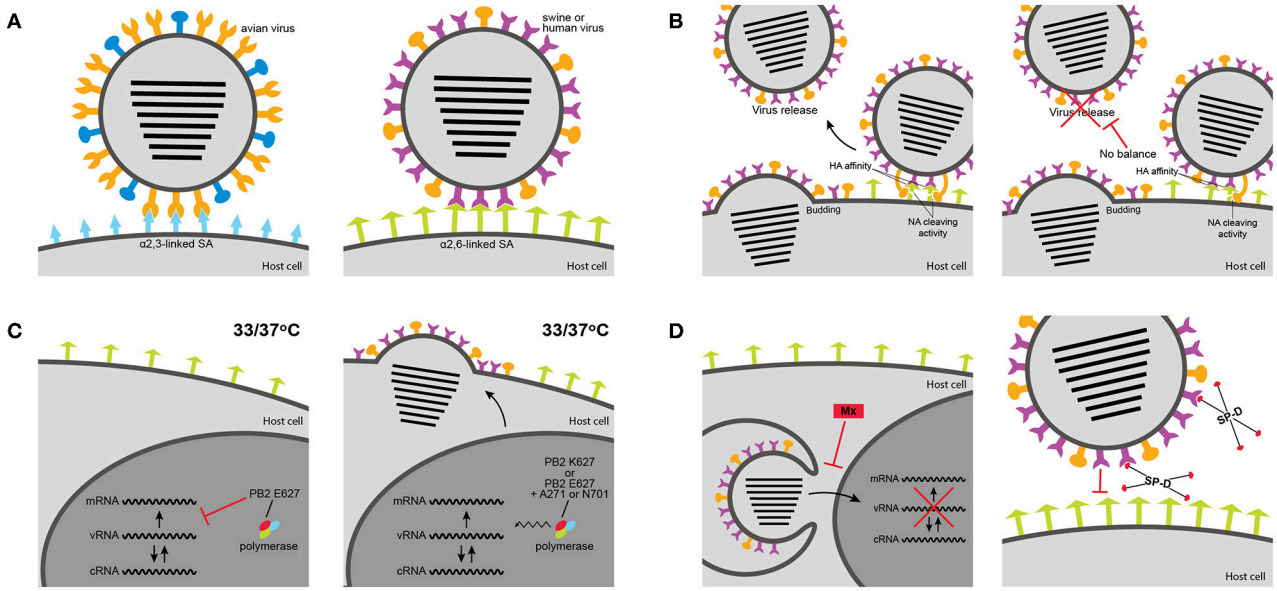

FIGURE 2 | Host range determinants of influenza A viruses (IAV). **(A)** Avian influenza virus HA protein recognize short α2,3-linked sialic acid (blue), whereas HA from human and swine IAV recognize long α2,6-linked sialic acid (green). **(B)** The balance between the HA binding affinity and the NA activity to cleave sialic acid receptors is important for replication and adaptation to a new species. If a virus has strong biding affinity but low cleavage activity replication may be reduced. **(C)** The PB2 polymerase has an impact in the optimal replication temperature of IAV and can restrict host range. K627 increases replication at the low temperature of human or swine upper airway. E627 decreases replication at low temperatures, unless in combination with A271 or N701. **(D)** The sensitivity of a virus to host-specific innate immune factors can restrict interspecies transmission of IAV. To be able to replicate and spread in a new host, IAV must become resistant to the antiviral activity of interferon-induced Mx protein or to the neutralizing activity of surfactant protein D (SP-D) from that particular host. Adapted from Cauldwell et al. (39).

been attributed to a single residue in the PB2 gene, amino acid 627, and is largely associated with the optimal temperature of replication of IAVs (62, 63). While the human upper respiratory tract temperature is around 33°C, in the avian intestinal tract the temperature is closer to 41°C. Therefore, enhanced replication at lower temperature should correlate with enhanced replication in the upper airway of humans and consequently improve transmission. Lysine (K) at position 627 in PB2, present in the vast majority of human viral isolates (64), was correlated with increased polymerase activity, virus replication and transmission in mammals (65–67), including enhanced replication of an avian virus in pigs (68). Replication and polymerase activity of different avian viruses, which predominantly possess glutamic acid (E) at position 627, were reduced at low temperature in mammalian cells (65, 66, 69).

The temperature of the upper respiratory tract of pigs is approximately 37°C and higher (approximately 39°C) in the lower respiratory tract. Interestingly, most swine isolates that have a PB2 of avian-origin retain the avian signature E627, including the predominant North American triple reassortant internal gene (TRIG) constellation viruses, the predominant Eurasian avian-like viruses, and even the 2009 pandemic H1N1 viruses (70, 71). The presence of the avian-like E627 in swine viruses usually does not result in the temperature sensitivity observed for avian viruses in mammalian cells (69), suggesting that these viruses can replicate at temperatures of avian intestines and human airways. Other residues, such as A271 and N701, were shown to compensate for the absence of K627 in these swine or swine-origin viruses and contribute to virus growth and transmission in swine and other mammalian species, including humans (71–74).

Immune Determinants of Host-Range

Following influenza infection in respiratory epithelial cells, acute inflammation leads to activation of the innate immune response through pro-inflammatory cytokines or chemokines (75). Type-I interferons (IFN-α/β) are cytokines quickly secreted after IAV infection. Type-I IFN mediated responses to IAV results in the expression of several antiviral proteins (76, 77). The Mx proteins are a family of large GTPases that are central to the antiviral activity of IFN against IAV by blocking nuclear entry of the vRNPs (78, 79). Sensitivity to interferon-induced Mx varies among different IAV strains and represents a barrier against transmission of avian influenza viruses to mammals: avian isolates are more susceptible to the antiviral action of murine Mx and human MxA proteins than human viruses (80, 81). The Mx sensitivity was shown to be determined by a cluster of surface-exposed amino acids on the viral NP (81, 82). Interestingly, serial passage in mice of a virus that is sensitive to murine/human Mx activity leads to a single amino acid adaptive NP mutation that results in escape from the Mx activity, and the same mutation is also seen in human H7N9 isolates (83). Not surprisingly, some swine IAV strains with avian-origin NP tend to have a higher sensitivity to mouse Mx1 than human isolates (84). However, the 1918 pandemic H1N1 and the 2009 pandemic H1N1 viruses acquired resistance-associated substitutions on the NP protein

FIGURE 3 | Proportion of amino acids found in influenza A viruses circulating in pigs globally at the HA receptor-binding site positions previously shown to impact receptor-specificity for H1, H3, and H9. Analysis was performed using the Influenza Research Database Sequence Variation (SNP) tool (44). Sequences with 100% identity were removed resulting in a set of 8076 H1 HA, 2287 H3 HA, and 46 H9 HA swine IAV sequences. The amino acids previously shown to change receptor-binding specificity are displayed on the right.

that allow escape from human Mx (82). The functional Mx1 protein is expressed in the lungs of pigs experimentally infected with IAV (85). It seems that the precursor of the 2009 pandemic H1N1 virus acquired Mx-resistance mutations driven by the porcine Mx1 during its circulation in pigs prior to the pandemic, being able to partially resist the human MxA (82). The Eurasian avian-like viruses are similarly resistant to human MxA, however different mutations were attributed to this phenotype (86). It remains unknown whether human and swine viruses would have different sensitivities to the porcine Mx protein.

Surfactant protein D (SP-D) is a collectin of the innate immune system that also has early strong antiviral activity against IAVs. SP-D binds to carbohydrate moieties on the surface of influenza viruses (HA and/or NA), blocking attachment to epithelial cells and inducing phagocytic responses, resulting in non-specific virus neutralization and clearance (87). The susceptibility of different IAV to SP-D activity was shown to be dependent on the glycosylation pattern of the virus, particularly on the HA (88–90). Influenza strains of the H3 subtype tend to acquire and accumulate more glycosylations on the HA head as a mechanism to evade the antibody response in humans, but this in turn may make them more susceptible to the antiviral effect of SP-D. Interestingly, porcine SP-D has a higher affinity to bind IAV glycans than human or rat SP-D, resulting in stronger neutralization activity (91, 92). Therefore, differences in susceptibility to Mx or SP-D could be an important component in host restriction of influenza viruses that needs to be overcome, usually by changes in specific viral proteins, in order for a virus to adapt to a new species (**Figure 2D**).

The IAV NS1 protein plays an important role as an antagonist of the host IFN response by preventing the activation of retinoic acid-inducible gene 1 (RIG-I) or inhibiting processing of mRNA (93). Differences between the NS1 amino acid sequences may affect the functional IFN-antagonistic properties of the NS1 (94, 95). Consequently, NS1 and its ability to control IFN response could play a role in host range of IAV. Indeed, although the avian NS1 protein was able to control IFN-α/β response in human cells, the human type I IFN response appeared to limit the replication of the avian viruses, suggesting that the NS1 also contributes to the host specificity of IAV (96).

The adaptive immunity of an individual or population can also have a role in host range restriction of IAV. Even when a novel virus contains an ideal combination of factors that allow replication in the new host, as discussed above, previous cross-protective immunity might block even the initial infection. In some cases, the level of cross-protective immunity of the

population may still allow infection but might block virus dissemination; however, naïve individuals will be at a higher risk for infection and may serve as sources of transmission. That was the case for the zoonotic infections with swine-origin viruses in recent years, in which the majority of affected individuals were children (58, 59). For these outbreaks, infection was observed in people with close contact with pigs, and transmission from human-to-human was rare, which was attributed to low levels of cross-protective immunity in the human population due to previous exposure to seasonal viruses (97). In pigs, however, there is a continuous introduction of naïve individuals and the majority of the population does not have previous immunity to viruses circulating in humans, increasing the chances of those viruses that have ability to infect pigs to become widespread.

HOW DO HUMAN VIRUSES RELATE TO THE EVOLUTION OF SWINE INFLUENZA VIRUSES?

Human-origin viruses have been repeatedly transmitted to swine worldwide and have had a major role on the epidemiology of swine IAV (34) (**Figure 4**). The classical swine H1N1 virus that emerged around the 1918 pandemic remained relatively antigenically stable for eight decades without causing major problems to swine producers. A novel triple-reassortant virus with human seasonal H3N2 surface genes emerged in the late 1990's in North America (27, 98) and led to reassortment with the classical viruses and subsequently gave rise to different antigenically distinct H3N2, H1N1, and H1N2 strains (99, 100). The triple-reassortant internal gene (TRIG) constellation, containing gene segments from a complex reassortment history among swine-, human- and avian-origin IAVs, became the predominant backbone of the viruses circulating in pigs in the U.S. (101, 102). Shortly after, two additional introductions of human-origin H1N1 resulted in the establishment of two new lineages of H1N1 and H1N2 viruses after reassortment with the TRIG strains, termed δ-lineages (103). After the spread in humans, the H1N1 pandemic 2009 virus (H1N1pdm) was quickly transmitted to swine in North America (104). And recently, a novel virus derived from 2010 to 2011 human seasonal H3 IAV led to establishment of a new H3-lineage that is genetically and antigenically distinct from previously circulating strains (105). The current scenario for the epidemiology of IAV circulating in North American swine consists of a highly diverse pool of viruses, with 14 phylogenetic clades of HA co-circulating (36, 101, 105, 106). It is clear how impactful the human-to-swine transmissions were to this current epidemiology: at least 10 of these phylogenetic clades have evolved from a human virus. If considering the hypothesis that the classical swine virus originated from the human 1918 pandemic virus, all of those clades should be considered of human-origin.

In Europe, a human-origin H3N2 virus descendent from the 1968 pandemic virus was introduced in the 1980's. This virus became widespread after reassorting with an avian-origin H1N1 virus that was introduced to European swine in 1979 and remains endemic to date (107, 108). Another human-origin virus, an H1N2, was detected in 1994, containing the H1 that evolved from a 1980 human seasonal H1N1 virus and a human-origin N2 that is distinct from the previously introduced H3N2 human-like virus. This virus acquired the internal gene constellation of the 1979 avian-like virus after reassortment and is now endemic in Europe (109, 110). As in the U.S., the H1N1pdm virus has been transmitted from humans to pigs in Europe establishing a new endemic lineage (108, 111). Recently, a triple-reassortant H3N2 virus with a human-origin HA from a 2004–2005 seasonal virus, N2 from endemic swine viruses, and the internal genes from H1N1pdm has spread in Denmark swine herds (112). In China and other countries in Asia, importation of live animals has resulted in the co-circulation of both European (or Eurasian) and North American TRIG virus lineages that contain human-origin genes (113–115). Additionally, reassortant genotypes between these lineages containing HA and/or NA genes from H1N1 and H3N2 human viruses have been detected in Asia since the 1960's and have become established in pigs (34, 116, 117).

Human-origin IAVs have been reported circulating in pigs in other countries where surveillance is limited (34, 118–120), including countries with large swine populations like Brazil (121, 122), Vietnam (123), Mexico and Chile (124). But, even in some of these cases where human-origin viruses or viral genes were reported in swine, it is not possible to infer if they have become endemic or predominant. However, in several cases, such as in Latin America, the human-origin swine viruses were most closely related to human seasonal strains that circulated many years earlier and were separated by long phylogenetic branches, suggesting that these viruses have circulated undetected in pigs for years prior to their recent detection (34, 118, 121, 124). Considering the frequency of human-to-swine transmissions in highly surveilled areas, it is likely that additional human-origin viruses have gone undetected in countries with low surveillance efforts.

In addition to the recurrent seasonal virus spillover events into swine populations, the H1N1pdm has been repeatedly transmitted from humans to swine globally (125). The H1N1pdm virus originated in Mexico from the reassortment between Eurasian and North American swine viruses and this novel virus may have circulated undetected for approximately 10 years before it gained the ability to infect humans (33). Soon after the initial spread of the H1N1pdm in the human population, the H1N1pdm virus was detected in pigs and since then transmitted from human to pigs throughout the world (104, 126–131). The virus has now become endemic in humans and circulates as a seasonal strain, increasing the possibility of spillovers to swine populations during influenza season each year. Owing to its swine-origin and yearly circulation in humans, continuous and frequent detection of the H1N1pdm virus in pigs has been reported globally (125, 132). The constant circulation and re-introduction of H1N1pdm globally has led to reassortment with endemic swine viruses and changed the genotypic characteristics of swine IAV by contributing several genes, most commonly the internal genes. In the U.S., the surface genes of the H1N1pdm are not frequently maintained, however most genotypes of H1 and H3 viruses contain at least one internal gene of pandemic lineage (133, 134). In Europe, the H1N1pdm virus has reassorted with

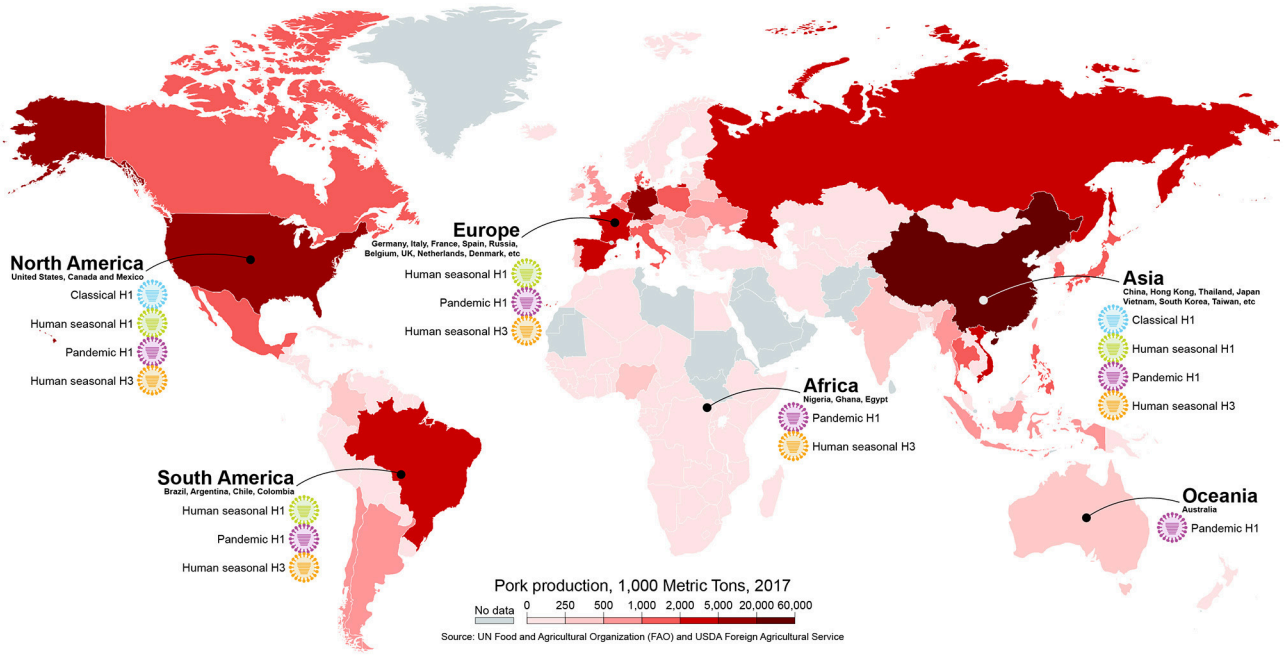

FIGURE 4 | Different subtypes/lineages of human-origin influenza viruses circulating in swine in different continents. The map is colored according to pork production in 1,000 metric tons. Map created with mapchart.net.

endemic European viruses and gave rise to genotypes containing the internal genes from pandemic origin and some genotypes have maintained one or both surface genes of pandemic lineage (135). Interestingly, there is recent evidence of the independent antigenic evolution of the swine H1N1pdm virus in European pigs (136). In China, although Eurasian and North American viruses circulated prior to the 2009 pandemic without substantial evidence of reassortment, the introduction of the H1N1pdm led to the establishment of reassortant genotypes containing several internal genes from pandemic lineage (117). The H1N1pdm has been reported throughout the world in swine with frequent reassortment (137–139), even in countries that were previously considered influenza free like Australia and Norway (128, 140).

HOW DO HUMAN-ORIGIN VIRUSES AFFECT CONTROL OF INFLUENZA IN SWINE?

The repeated transmission of human seasonal viruses to pigs has resulted in the establishment of several human-origin virus lineages globally, adding to the antigenic diversity of swine viruses. Global antigenic characterization has revealed that the antigenic diversity of H1 and H3 viruses circulating in pigs was largely a result of the frequent introductions of human-origin IAV into swine (35). These viruses then evolved antigenically, independent from human strains and often confined to their geographic areas, contributing to the overall global diversity, which consequently contributes to the challenges for effective vaccination programs in swine. Most vaccines used against influenza in swine are whole inactivated virus (WIV) vaccines combined with oil-in-water adjuvants typically given to sows to allow transfer of maternally derived antibodies to piglets (141). Recently, two novel platforms were licensed for use in pigs in the U.S. as alternatives to improve the efficacy of swine vaccines, a non-replicating alphavirus RNA vectored-vaccine and a live-attenuated influenza virus (LAIV) vaccine (142, 143).

Because most vaccines rely on the effective stimulation of the immune response against the surface HA glycoprotein, any changes that lead to antigenic drift, such as the incursion of novel human-origin viruses, can lead to vaccine mismatch. It was demonstrated that changes in only 6 amino acids in the HA account for major antigenic changes of swine H3 influenza viruses, and a single amino acid change can lead to significant antigenic drift (144, 145). Amino acids in similar positions at the HA were also associated with antigenic characteristics of H1 viruses (36). It is not surprising, therefore, that when novel human-origin viruses become established in pigs there are considerable antigenic differences from the circulating swine strains (105), and any vaccines available at the time are unlikely to provide immunity against these novel viruses. In addition to the lack of protection, vaccine mismatch can also have detrimental effects. When the vaccine stimulates a cross-reactive antibody response that fails to neutralize the virus, it can result in severe immune-mediated disease termed vaccine-associated enhanced respiratory disease (VAERD). Therefore, more effective vaccine technologies and vaccination strategies that improve the breadth of the immune response and avoid any negative effects are needed

to increase protection against the antigenically diverse human-origin viruses that are continuously introduced in pigs.

CONCLUDING REMARKS

Since the 2009 pandemic, renewed attention has been given to the interspecies transmission of influenza viruses between pigs and humans, bringing back the attention to the theory that pigs can serve as "mixing-vessels" of influenza viruses. However, it is not entirely clear if swine are in fact more susceptible to infection with avian viruses than humans. There is compelling evidence, though, that human viruses are frequently transmitted to pigs, and have had a significant impact on the diversity of viruses that circulate in pigs globally. Additional surveillance is necessary to understand the diversity of IAVs circulating in different regions and the participation of human-origin strains in this overall diversity. Surveillance is also critical for antigenic characterization of the strains that are circulating in a particular area to allow an accurate selection of representative vaccine strains that will provide an optimal protection. Moreover, despite the increasing evidence of the important role that human seasonal viruses have played in driving the genetic and antigenic diversity of IAV in swine, vaccine and sick leave policies for swine industry workers are not consistently employed but should be considered. Furthermore, understanding the mechanisms involved with host-range specificity and the adaptation to swine allows assessment of the risks posed by the introduction of novel viruses into the swine population, which is crucial for preparedness and to improve biosecurity measures to reduce the IAV burden to the swine industry.

AUTHOR CONTRIBUTIONS

DR, AV, and DP contributed to the conceptualization of the ideas, drafting and critical revision of the manuscript, and final approval. DR designed figures.

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Vaccine Development for Nipah Virus Infection in Pigs

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Nipah virus (NiV) causes a severe and often fatal neurological disease in humans. Whilst fruit bats are considered the natural reservoir, NiV also infects pigs and may cause an unapparent or mild disease. Direct pig-to-human transmission was responsible for the first and still most devastating NiV outbreaks in Malaysia and Singapore in 1998–99, with nearly 300 human cases and over 100 fatalities. Pigs can therefore play a key role in the epidemiology of NiV by acting as an "amplifying" host. The outbreak in Singapore ended with the prohibition of pig imports from Malaysia and the Malaysian outbreak was ended by culling 45% of the country's pig population with costs exceeding US$500 million. Despite the importance of NiV as an emerging disease with the potential for pandemic, no vaccines, or therapeutics are currently approved for human or livestock use. In this mini-review, we will discuss current knowledge of NiV infection in pigs; our ongoing work to develop a NiV vaccine for use in pigs; and the pig as a model to support human vaccine development.

Keywords: Nipah virus, pigs, zoonosis, epidemiology, pathogenesis, vaccine development

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NIPAH VIRUS IS AN EMERGING PATHOGEN WITH THE POTENTIAL FOR PANDEMIC

Nipah virus (NiV) is an enveloped, single stranded, negative sense RNA paramyxovirus, genus *Henipavirus*. The natural hosts and wildlife reservoirs of NiV are Old World fruit bats of the genus *Pteropus* (1). Both Nipah and the related Hendra virus possess a number of features that distinguish them from other paramyxoviruses. Of particular note is their broad host range which is facilitated by the use of the evolutionary conserved ephrin-B2 and –B3 as cellular receptors (2). The NiV attachment glycoprotein (G) is responsible for binding to ephrin-B2/-B3 (3). Following receptor binding, the G protein dissociates from the fusion (F) protein. Subsequently, the F protein undergoes a series of conformational changes which in turn initiates fusion of the viral and host membrane allowing entry (4). During viral replication, the F protein is synthesized and cleaved into fusion active F1 and F2 subunits. These subunits are subsequently transported back to the cell surface to be incorporated into budding virions, or facilitate fusion between infected and adjacent uninfected cells (5). This cell-to-cell fusion results in the formation of multinucleated cells called syncytia, and greatly influences the cyopathogenicity of NiV as it allows spread of the virus, even in the absence of viral budding (5, 6).

NiV infection is currently classed as a stage III zoonotic disease, meaning it can spill over to humans and cause limited outbreaks of person-to-person transmission (7, 8). NiV outbreaks have been recognized yearly in Bangladesh since 2001 as well as occasional outbreaks in neighboring India (**Figure 1**). These outbreaks have been characterized by person-to-person transmission

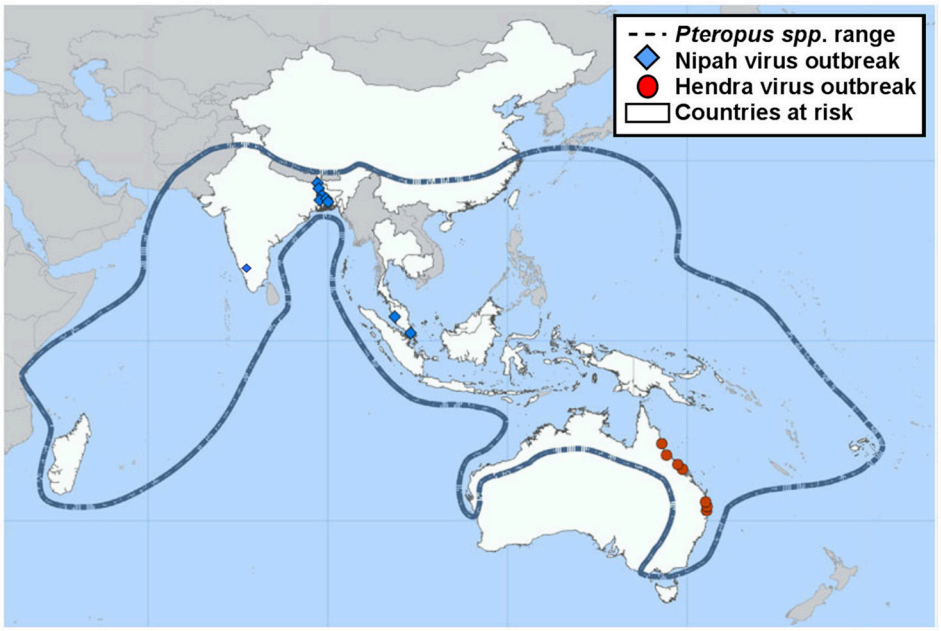

FIGURE 1 | Previous locations of Henipavirus infection outbreaks. Nipah and Hendra virus distribution map highlighting the range of the natural wildlife reservoir, *Pteropus* spp. bats [adapted from (9)].

and the death of over 70% of infected people (10, 11). In May 2018, the first ever outbreak in southern India was reported. A total of 19 NiV cases, of which 17 resulted in death, were reported in the state of Kerala. *Pteropus giganteus* bats from areas around the index case in Kozhikode, Kerala, were tested at the National High Security Animal Diseases Laboratory at Bhopal. Of these, 19% were found to be NiV positive by RT-PCR (12). Characteristics of NiV that increase the risk of it becoming a global pandemic include: humans are already susceptible; many NiV strains are capable of person-to-person transmission; and as an RNA virus, NiV has a high mutation rate (13). NiV has been found to survive for up to 4 days when subjected to various environmental conditions, including fruit bat urine and mango flesh (14). Whilst survival time was influenced by fluctuations in both temperature and pH, the ability for NiV to be spread by fomites could play a role in outbreak situations.

The first and still most devastating NiV outbreak occurred in peninsular Malaysia from September 1998 to May 1999 (15, 16). The link to pigs in this outbreak was obvious as 93% of the infected patients had contact with pigs (17). If a NiV strain were to become human-adapted and infect communities in Southeast Asia where there are high human and pig densities and pigs are a primary export commodity, infection could rapidly spread and humanity could face its most devastating pandemic (8, 11, 18).

THE ROLE OF PIGS IN THE 1998/99 NIPAH VIRUS OUTBREAK

In September 1998, there was an outbreak of severe febrile encephalitis among pig farmers in the state of Perak, Malaysia, that was associated with a high mortality rate. A total of 265 cases of encephalitis, of which 105 resulted in death, were confirmed. These deaths were initially thought to be due to Japanese encephalitis (JE), an endemic disease in Malaysia. However, with most cases occurring in men who worked with pigs, the epidemiological characteristics of this disease were distinct from those of JE, where ∼75% of cases occur in children aged 0–14 years (19–21). The epidemiological link was from fruit bats infecting pigs that then served as amplifier hosts, resulting in transmission to humans through close contact (22). As a result of movement of infected pigs and humans to other states in Malaysia, by February 1999 similar diseases were recognized in both pigs and humans in new outbreak areas (23). In the following month, there were 11 cases of respiratory illness and encephalitis amongst Singapore abattoir workers who had handled pigs imported from the outbreak areas in Malaysia (15). Due to this, the importation of pigs from Malaysia ceased which in turn ended the outbreak in Singapore. The outbreak in Malaysia ended when 1.1 million pigs (45% of the country's pig population) were culled from outbreak and surrounding areas (17, 24). The NiV outbreak incurred significant economic costs and long-term damage to the Malaysian pig industry: US$582 million in direct costs and lost market revenue, including US$97 million in compensation to farmers for the 1.1 million pigs slaughtered and 36,000 jobs lost (25). To this date, Malaysian pig farming is only permitted in "identified pig farming areas."

NIPAH VIRUS INFECTION IN PIGS

Pigs also suffered during the 1998/99 Malaysian outbreak, but this was only diagnosed as part of the investigation following the human cases. The severity of symptoms of NiV infection in

pigs varied with age. In suckling pigs (<4 weeks old), mortality could be high (up to 40%) and labored breathing and muscle tremors were evident. In growing pigs (1 to 6 months), an acute febrile (>39.9°C) illness was observed with respiratory signs ranging from increased or forced respiration to a harsh, loud non-productive cough, open mouth breathing, and epistaxis (26). In some cases these respiratory signs were accompanied by one or more of the following neurological signs: trembles, neuralgic twitches, muscle fasciculation, tetanic spasms, incoordination, rear leg weakness, or partial paralysis. Pigs of this age had high morbidity and low mortality (<5%) (26–28). Some animals over 6 months of age died rapidly (within 24 h) without signs of clinical disease. Respiratory signs were reported in adult pigs, as with younger animals, although these were less obvious (labored breathing, bloody nasal discharge, increased salivation) and neurological signs included head pressing, bar biting, tetanic spasms and convulsions. First trimester abortions were also reported (26–28).

In an experimental infection study, pigs were inoculated subcutaneously with a NiV isolate from the central nervous system of a fatally infected human patient. Infection elicited respiratory and neurological symptoms consistent with those observed in naturally infected Malaysian pigs, which included febrile illness, incoordination, mucosal nasal discharge, and persistent cough (29). Pigs inoculated orally with the same dose did not show clinical signs although they still shed virus. In a second study, piglets were inoculated oronasally with a human NiV isolate (30). All infected animals showed a transient increase in body temperature between 4 and 12 days post-infection. Two of these animals developed transient respiratory signs, mild depression and a hunched stance. Both these studies concluded that NiV infection in pigs had no pathognomonic features i.e., the clinical signs observed were non-specific. This can make field diagnosis of NiV infection in pigs difficult, as observed in the outbreak in Malaysia (16, 28).

The name proposed for the disease caused by NiV infection of pigs was "porcine respiratory and neurological syndrome" (also known as "porcine respiratory and encephalitis syndrome"), or, in peninsular Malaysia, "barking pig syndrome" (28). NiV infection was included as the sixth pig disease notifiable to the OIE World Organization for Animal Health (31). The OIE approve diagnostics and recommends preventative and control measures for a range of transboundary livestock diseases.

CURRENT STATE OF NIV VACCINE DEVELOPMENT

Despite the importance of NiV as an emerging disease with the potential for pandemic, no therapeutics or vaccines are approved for use in humans or livestock species. Due to the lethal nature of NiV infection, producing a safe, live attenuated vaccine with no potential for reversion is difficult. However, recombinant NiV mutants, attenuated in hamster and ferret models, have been shown to generate strong neutralizing antibody responses (32, 33). More commonly, NiV vaccine approaches have focused individual candidate antigens delivered as subunit vaccines or using viral vectors. The most studied vaccine candidate is the soluble form of the G protein (sG) from the related Hendra virus (HeV). HeV and the NiV Malaysia strain share between 68 and 92% amino acid homology between their proteins; with F and G proteins sharing 88 and 83% homology, respectively (34). Both F and G envelope glycoproteins are regarded as vaccine candidate antigens since they are the targets of NiV neutralizing antibodies (35).

An adjuvanted HeV sG protein subunit-based vaccine (Equivac® HeV, Zoetis) has been licensed in Australia to protect horses against HeV and to reduce the zoonotic risk to humans (36). Equivac® HeV protects ferrets and African green monkeys (AGMs) after experimental challenge with NiV, as well as HeV (37, 38). Surprisingly, this vaccine failed to protect pigs from experimental NiV challenge (39). Since the vaccine induced cross-neutralizing antibodies but not measurable T cell responses, the authors concluded that both arms of the adaptive immune response may be required for protection against NiV and HeV. These studies also potentially highlight that adjuvants can have species specific effects and tailoring of adjuvants to the target species may be required or considered in the context of preclinical models. The experimental viral vectored vaccine candidates for NiV include vesicular stomatitis virus, rabies virus, canarypox virus (ALVAC strain), adeno-associated virus (AAV), measles virus, Newcastle disease virus (NDV) and Venezuelan equine encephalitis virus (40). ALVAC expressing NiV G or F (ALVAC-G and ALVAC-F) was found to protect pigs against NiV challenge 2 weeks after the second immunization (41). High titres of NiV neutralizing antibodies were induced with the ALVAC-G vaccine, while despite the low levels of neutralizing antibodies induced by the ALVAC-F; all vaccinated pigs were protected against virulent NiV challenge. Recombinant attenuated NDV expressing NiV glycoproteins have been shown to induce long lasting NiV-specific nAbs in pigs, with the vector expressing NiV G performing better than NiV F (42). However, no challenge was performed in this study and it remains to be determined whether these paramyxovirus-based vaccine candidates are efficacious. Compared to canarypox vectors, NDV-based vectors have a number of advantages including their high titer propagation in chicken eggs removing the requirement for cell culture (41, 42). Despite these encouraging results and the continued threat posed by NiV, no vaccine candidate has progressed toward market for either pigs or humans.

THE DEVELOPMENT OF A NIV VACCINE FOR PIGS

The promising performance of experimental NiV and HeV vaccines in animal models and the licensure of Equivac® HeV, as a "One Health" vaccine to safeguard animal and human health, strongly support the proposition that a safe and effective NiV vaccine may be developed for pigs to reduce the severe economic consequences of NiV outbreaks and the threat to public health. With partners, we have initiated a project that aims to develop such a vaccine. We are systematically analyzing the immunogenicity and protective efficacy of three

NiV vaccine candidates in pigs: (1) an adjuvanted NiV sG protein (orthologous to the Equivac® HeV vaccine), (2) NiV G protein delivered by a replication-deficient simian adenoviral vector (ChAdOx1 NiV G), and an adjuvanted, molecular clamp stabilized NiV F (mcsF) protein. ChAdOx1 is a multispecies vector with an established human and livestock safety profile (43). ChAdOx1 offers the potential for both single dose efficacy and thermostabilization (44, 45). The molecular clamp is a proprietary stabilization domain that preserves the F protein in its native "pre-fusion" form, which should enhance immunogenicity and thermostability. In depth analyses of T cell and antibody responses are being conducted to identify correlates of vaccine-induced protection. We will examine the durability of NiV-neutralizing antibodies and other immune responses associated with protection, including a comparison of a single-shot vs. homologous prime-boost immunization regimes. In-contact animals will be introduced to assess transmission of challenge virus from vaccinates or unvaccinated control animals.

The sporadic nature of NiV outbreaks means that the commercial development of NiV vaccines for use in pigs (other livestock or humans) is limited and animal health companies are of the opinion that NiV vaccines will have limited marketability. Our ongoing studies should help facilitate this by developing a safe and efficacious prototype NiV vaccine that is amenable to "surge production" and discrimination of infection in vaccinated animals (DIVA) capability. Subsequent development and licensure of this vaccine will require engagement with international, regional, and national agencies and the creation of dependable markets via the establishment of NiV vaccine banks. The OIE World Fund manages vaccine banks and the delivery of vaccines for avian influenza, rabies, foot-and-mouth disease, and peste de petit ruminants (46). Vaccine banks ensure the procurement and delivery of high quality vaccines mass-produced in line with OIE intergovernmental standards. Critically these vaccine banks can be rapidly deployed when required and this model appears most appropriate in the context of reactive emergency vaccination programmes to aid NiV outbreak control. Vaccines can play a major component in an emergency response against emerging infectious disease, with the main aim to reduce virus spread between susceptible hosts (47). The precise decisions on control strategies will be complex and vary for different regions. Factors such as: herd density, production systems, the presence of susceptible wildlife, the impact on export trade and current opinions on economic vs. ethical factors will likely play a role. One strategy to halt a NiV outbreak would be to deploy a stockpiled vaccine for ring vaccination around the NiV affected area. This approach was utilized in the 2016 Ebola outbreak in Guinea and showed great promise in terms of disease containment and elimination (48). For such a strategy, a vaccine with single-dose efficacy and a rapid onset of immunity preventing virus transmission would be preferential. This is likely to be best achieved with a viral-vectored (45) or mRNA vectored vaccine (49). The highly unpredictable nature of NiV outbreaks means that it is highly unlikely that NiV vaccines would be used routinely by pig producers. One strategy that could help ensure that immunity to NiV is maintained in pig herds could involve the engineering of NiV G into a live attenuated viral vaccine, such as pseudorabies, which are widely used in countries at-risk.

THE PIG AS A MODEL FOR HUMAN NIV

The recent Ebola and Zika epidemics highlighted how poorly prepared we were to deal with these new and emerging diseases. There has therefore been a global drive to develop vaccines against these diseases and improve preparedness. The Coalition for Epidemic Preparedness Innovation's (CEPI's) was established in 2016 with a mandate of financing and coordinating the development of new human vaccines to prevent and contain infectious disease epidemics. CEPI selected NiV, Lassa virus and Middle East respiratory syndrome-coronavirus, three pathogens from the WHO's list of priority diseases needing urgent R&D attention as its initial focus (50, 51). The WHO's list of priority diseases is part of the R&D Blueprint, which identifies priority diseases and addresses gaps in the global scientific community to increase preparedness for future outbreaks. The main aim of the Blueprint is to fast-track the availability of effective tests, vaccines, and medicines that can be used to save lives and avert large scale crises (51).

In 2002, the US Food and Drug Administration (FDA) established the "Animal Rule" for regulatory approval of vaccines and therapeutics for which efficacy testing in humans is impossible, therefore requiring relevant animal models that represent a disease model similar to that of the human disease (52). Vaccine efficacy studies in animal models aim to identify specific vaccine-induced correlates of protection including neutralizing antibodies or cell-mediated responses (53). In 2015, a vaccine to protect against anthrax was the first to be approved through the "animal rule" (54). The licensing pathway for the "Animal Rule" requires that immunogenicity results from clinical trials must be consistent with previously identified immune correlates associated with protection (52). Therefore, identifying reliable markers of vaccine-generated immunity becomes critically important for pathogens such as NiV. Large animal models have been shown to more accurately predict vaccine outcome in humans in comparison to small animal models (55) therefore defining correlates of vaccine-induced protection in pigs, may play an important role in supporting subsequent human vaccine licensure under the "Animal Rule."

Animal models can be validated for a particular disease according to a number of different criteria, which include "face" and "predictive" validity. For face validity there must be similarities in the pathology and clinical symptoms between the animal model and the human disease (56). As discussed above, NiV infection of pigs causes a similar respiratory and neurological syndrome as seen in human infections. Although, disease severity in pigs may be considered lower than in humans. The predictive validity of a model means that clinically effective interventions demonstrate a similar effect in the animal model (56). No clinical trials of NiV vaccine candidates have been reported to compare with vaccine performance in animal models, including the pig. As noted above, the success of the

Equivac® HeV vaccine in horses and other animal models was not replicated in swine (38, 39), highlighting a potential issue of predicative validity when comparing NiV vaccines between animal species, which may extend to humans. On the other hand, pigs have been used successfully as models to study many human infectious diseases (57–63), including NiV infection (64). There is also a growing appreciation that pigs provide a superior animal model for influenza A virus infection and immunity and should play a more prominent role as a model for human influenza vaccine development (65). The success of the pig as an experimental animal model is partly due to their similarities with humans in terms of anatomy, immunology, and physiology, but also due to their manageable behavior and size, and by the general ethical acceptance of using pigs for experimental purposes instead of non-human primates (55, 63, 66).

CONCLUSIONS

The NiV outbreaks in Malaysia and Singapore demonstrated that pigs can play a key role in the epidemiology of NiV by acting as an amplifier host. The region most at risk of NiV infection has some of the highest pig population densities found anywhere in the world, which are rising fast due to the demand of a growing human population. This increases the risk of NiV transmission to pigs and humans. The development of a NiV vaccine for use in pig populations would decrease the major risk NiV poses to the developing pig industries, as well as to the livelihoods of poor livestock keepers in Southeast Asia. The use of non-human animal models is crucial for vaccine development against diseases such as NiV since efficacy testing in humans is impossible. The pig model may therefore contribute to human vaccine development, supporting human vaccine licensure under the Animal Rule.

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

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REVIEW
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Key Gaps in the Knowledge of the Porcine Respiratory Reproductive Syndrome Virus (PRRSV)

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The porcine reproductive and respiratory syndrome virus (PRRSV) is one of the most important swine diseases in the world. It is causing an enormous economic burden due to reproductive failure in sows and a complex respiratory syndrome in pigs of all ages, with mortality varying from 2 to 100% in the most extreme cases of emergent highly pathogenic strains. PRRSV displays complex interactions with the immune system and a high mutation rate, making the development, and implementation of control strategies a major challenge. In this review, the biology of the virus will be addressed focusing on newly discovered functions of non-structural proteins and novel dissemination mechanisms. Secondly, the role of different cell types and viral proteins will be reviewed in natural and vaccine-induced immune response together with the role of different immune evasion mechanisms focusing on those gaps of knowledge that are critical to generate more efficacious vaccines. Finally, novel strategies for antigen discovery and vaccine development will be discussed, in particular the use of exosomes (extracellular vesicles of endocytic origin). As nanocarriers of lipids, proteins and nucleic acids, exosomes have potential effects on cell activation, modulation of immune responses and antigen presentation. Thus, representing a novel vaccination approach against this devastating disease.

Keywords: porcine reproductive and respiratory syndrome virus, PRRSV, virus biology, immunology, vaccinology, extracellular vesicles

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ECONOMIC IMPACT

PRRSV is responsible for respiratory disease in weaned and growing pigs, as well as reproductive failures in sows. It is considered one of the most important swine diseases worldwide, with an economic impact estimated at $664 million in losses every year to U.S. producers, representing an increase of 18.5% in the last 8 years (1, 2). In Europe, the situation is similar and economic disease models have been carried out to determine the economic burden in the best and worst case scenario combining reproductive failure and respiratory disease, estimating annual losses from a median of €75,724, if the farm was slightly affected during nursing and fattening, to a median of €650,090 if a farm of 1,000 sows is severely affected in all productive phases (3). Nevertheless, there is scarce of information about the economic impact of this disease as a consequence of multiple

factors (vaccination, treatment, respiratory symptoms, reproductive failure, and other PRRSV-related diseases) making a difficult task to quantify exactly this parameter under field conditions. Thus, the exact economic impact of PRRSV remains a key gap in the knowledge for this disease.

BIOLOGY OF PRRSV

The porcine reproductive and respiratory syndrome virus (PRRSV) was first isolated in the early 1990s in Europe and North America (4, 5). It is an enveloped single-stranded positive-sense RNA virus of the family *Arteriviridae,* Genus *Porarterivirus* according to the International Committee of Taxonomy of Viruses (6). Presently, there are four distinct species included in this Genus (*Porarterivirus)*, PRRSV-1 and PRRSV-2 (with 30–45% variation in nucleotide sequences), along with other two viruses that do not affect pigs (Lactate dehydrogenase-elevating virus and Rat Arterivirus 1) (7). The genome size of PRRSV is about 15 kb with 10 open reading frames (ORFs), with replicase genes located at the 5′-end followed by the genes encoding structural proteins toward the 3′-end (8). The majority of the genome (∼60–70%) encodes non-structural proteins involved in replication (ORF1a and ORF1ab), whereas ORFs 2–7 encodes structural proteins (N, M, GP2-GP5, E) (**Figures 1A,B**) (9). Using ORF5 in molecular epidemiological studies, an enormous genetic variability has been described (10). Yet, data on whole genome sequencing is scarce and constitute another important gap in the knowledge of this virus and its evolution (**Box 1**).

PRRSV replicase genes consist of two ORFs, ORF1a and ORF1b, which occupy the 5′ proximal three-quarters of the genome (**Figure 1A**). Both are expressed from the viral genome, with expression of ORF1b depending on a conserved ribosomal frameshifting mechanism. Subsequently, extensive proteolytic processing of the resulting pp1a and pp1ab polyproteins yields at least 14 functional non-structural proteins (nsps), specifically nsp1 to nsp12, with both the nsp1 and nsp7 parts being subject to internal cleavage (giving origin to nsp1α and nsp1β, and nsp7α, and nsp7β, respectively), most of which assemble into a membrane-associated replication and transcription complex (11). Recently, a programmed ribosomal frameshift encoding an alternative ORF that generates two extra proteins, nsp2TF and nsp2N, was discovered in PRRSV and other *Arteriviruses* (12, 13). These nsps, described for PRRSV, have proven to

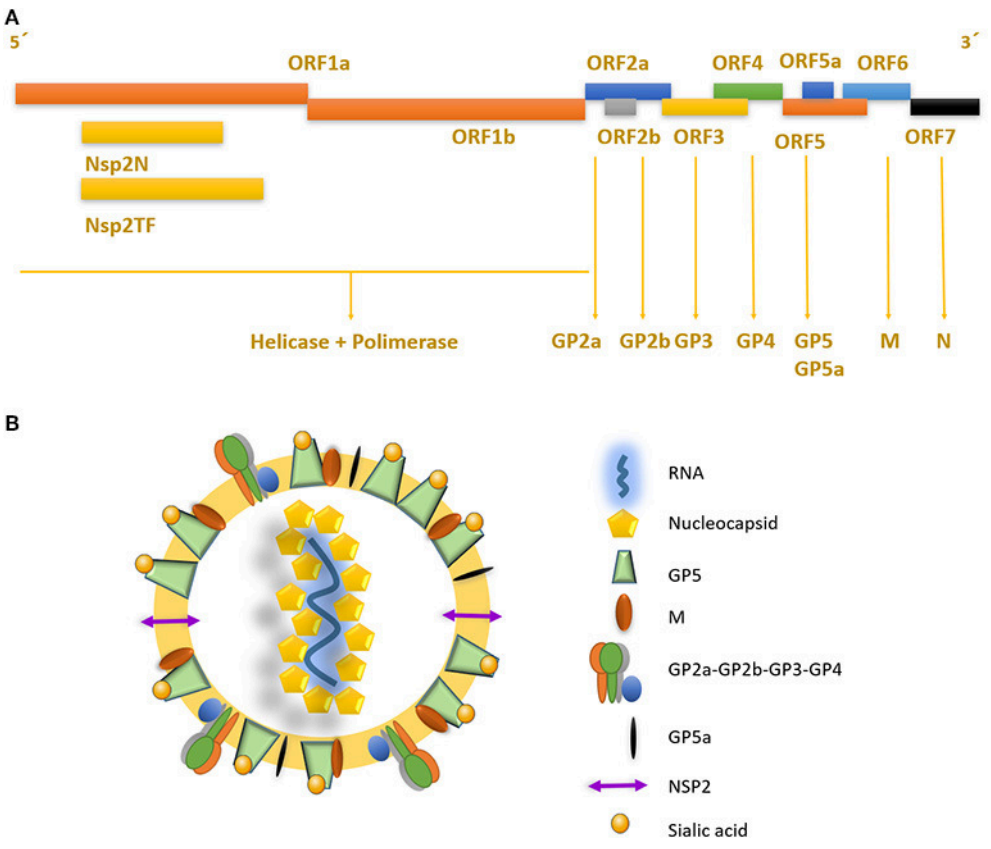
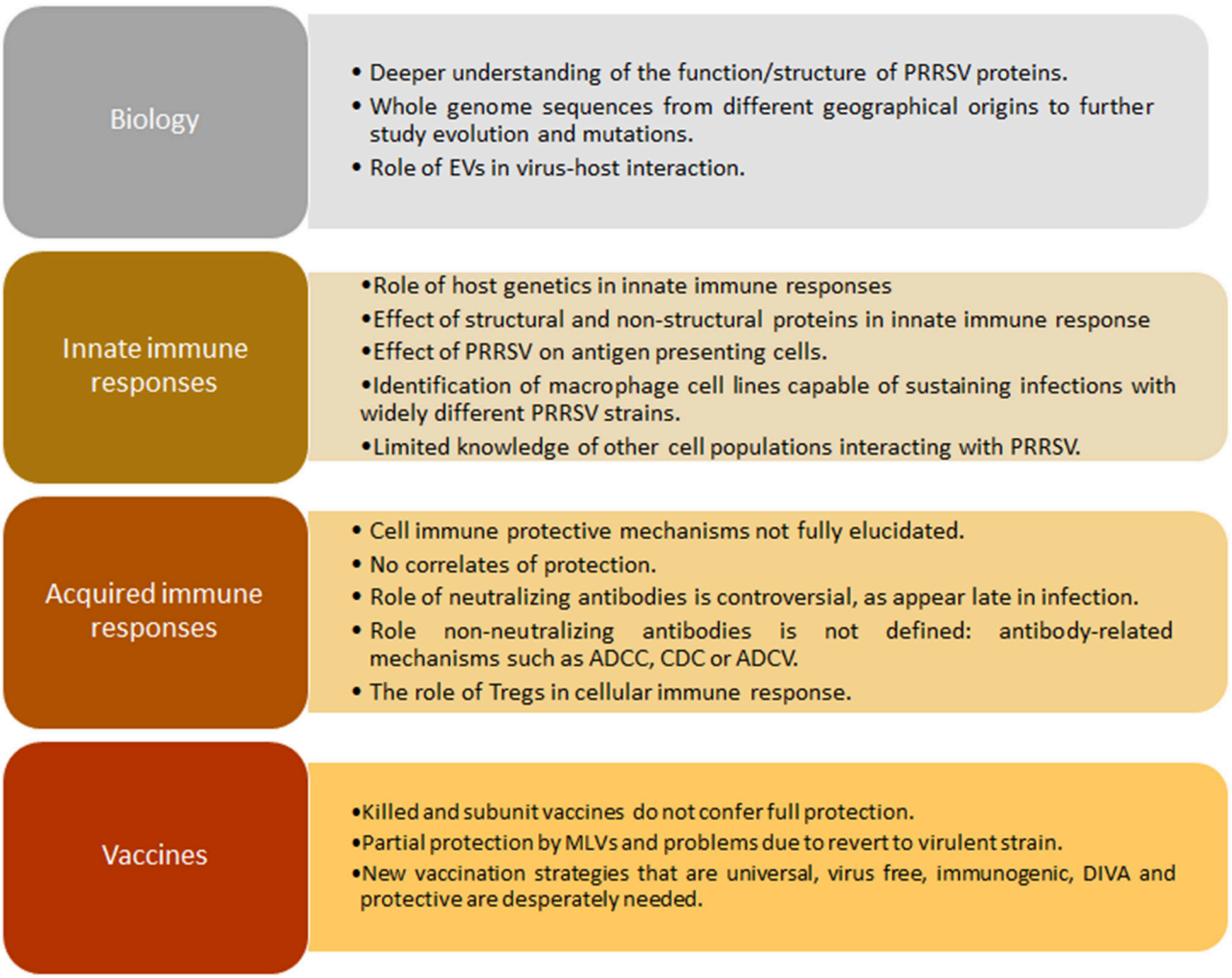


FIGURE 1 | Genome structure and mature viral particle of PRRSV virus. **(A)** Non-structural proteins are located in the 5′ end of the genome, codifying for two different polyproteins pp1a and pp1ab that are cleaved into at least 14 nsps (nsp1 to nsp12 and nsp1α and nsp1β, and nsp7α, and nsp7β). Structural proteins located near the 3′ end, are associated to the viral envelope and RNA packaging. **(B)** PRRSV mature viral particle, composed of a lipid bilayer envelop with viral receptor glycoproteins involved on infection and cell internalization. Single stranded positive RNA is associated with nucleocapsid protein in the internal layer of the virus.

BOX 1 | Gaps in knowledge in PRRSV.



be necessary and sufficient for the induction of membrane modifications resembling those found in infected cells (14). Most importantly, all positive RNA viruses seem to induce one of two basic morphotypes of membrane modifications: invaginations or double-membrane vesicles.

PRRSV also has a set of 8 structural proteins, including a small non-glycosylated protein and a set of glycosylated ones: GP2a-b, GP3, GP4, GP5, and GP5a, M and N proteins (15). However, nsp2, traditionally classified as a non-structural protein, has been found to be incorporated in multiple isoforms within the viral envelope (Ovarian tumor domain protease region, hypervariable region and C-terminal region) (16), giving new insights into the structure of this virus (**Figure 1B**). First, the nucleocapsid protein (N), as one of the most important parts of the mature viral particle, has been deeply characterized on PRRSV, finding important features shared in most non-segmented RNA viruses. The N protein consists of 123 amino acids for genotype 2 and 128 amino acids for genotype 1. The viral envelope glycoproteins (GP2 to GP5) are the first interactors with host cell receptors to initiate infection and are exposed to the immune system when viral particles are in blood and lymphoid tissue circulation (**Figure 2**). There is also another protein that contribute to virion structure, M protein, that is required during viral entry to interact with heparan sulfate cell receptor on macrophages. Later, GP5 is thought to bind to sialoadhesin and virus internalization and uncoating is triggered by a formation of a viral heterotrimer (GP2a, GP3, and GP4) with scavenger receptor CD163 (**Figure 2**) (17, 18). GP5 is the most abundant glycoprotein. First, it interacts with two cell entry mediators, heparan sulfate glycosaminoglycans and sialoadhesin/CD169 (17, 18) to favor viral entry and then possibly with the N protein and its MHC-like domain to carry N-Viral RNA complex to the budding site (**Figure 2**). GP2, GP3, and GP4 are protected with glycan shields, like most PRRSV membrane proteins, to avoid antibody recognition and neutralization. GP2 has two glycosylation sites, GP3 have seven and GP4 have four,

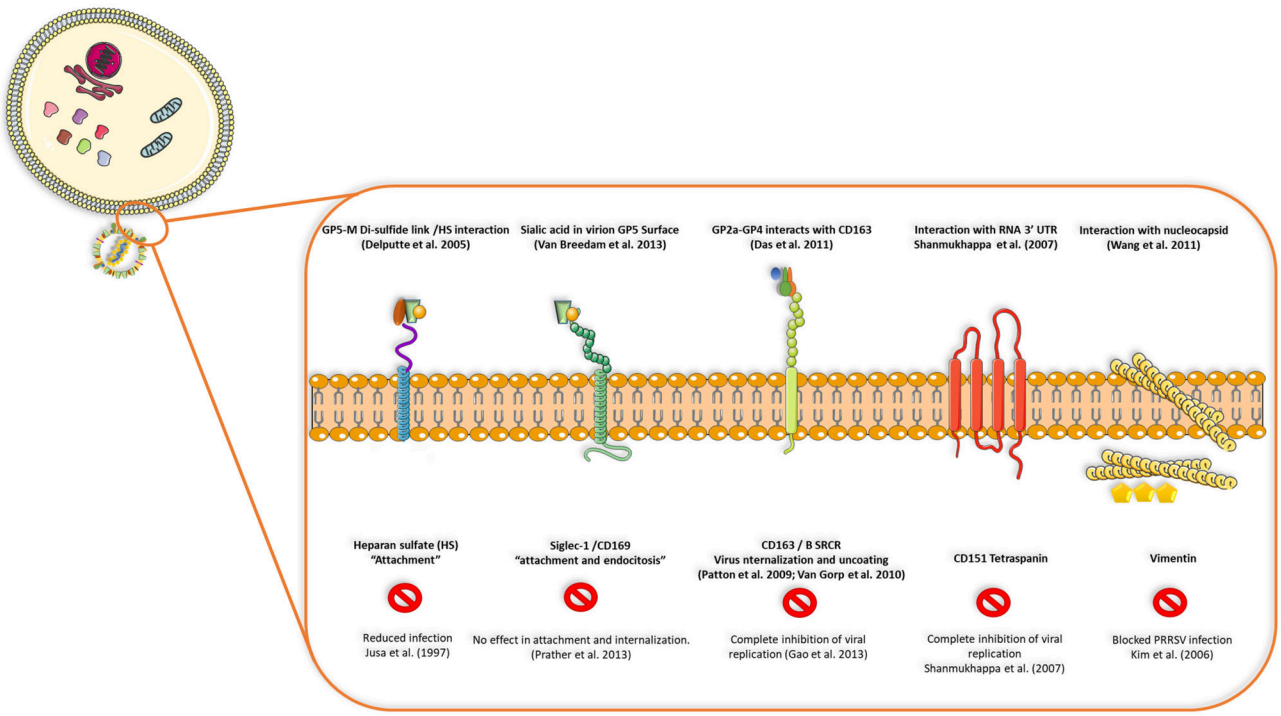


FIGURE 2 | Interactions between viral proteins and cell receptors for virus attachment, entry, uncoating and release of genetic ssRNA to cell cytoplasm. Blocking CD163, CD151 tetraspanin or vimentin seems to inhibit viral replication or infection in the host cell, but reduced replication or no effect is seen when receptors such as heparan-sulfate or siglec-1 are blocked, demonstrating that some viral proteins and cell receptors are indispensable in terms of production of infectious viral progeny and dissemination in the host.

three of which are directly related to virus survival, causing lethal damage in virus production when more than two of these sites are mutated (19) (**Figure 2**).

VIRUS REPLICATION AND ENTRY MECHANISMS IN HOST CELLS

Viral replication starts by interaction of viral glycoproteins with different cellular receptors (**Figure 2**) (17). CD163 and CD169 play a main role during infection, uncoating of the viral particle, activation of clathrin-mediated endocytosis and release of viral genome in the cytoplasm (20). CD163 has been defined as the main receptor for viral infection by evaluating the effect of PRRSV on CD163 knockout pigs, where there is complete resistance to infection (21). Cysteine-rich domain 5 in this receptor seems to be necessary to establish interactions with PRRSV-1 species, since its deletion by CRISPR/Cas9 system (exon 7 of the gene encoding this region) implies protection for a large panel of these viruses demonstrated by *in vitro* challenge of edited-pig macrophages and *in vivo* experiments with ΔSRCR5 animals (22–24). More important, edited pigs show no side effects when kept under standard husbandry conditions and CD163 seems to maintain its biological function (hemoglobin-haptoglobin scavenger) regardless the lacking cysteine-rich 5 domain, nevertheless, other unknown functions could be impaired by this modification. In conclusion, gene-edited pigs lacking SRCR5 region of CD163 could be an important asset to confront PRRSV epidemics with the final goal of eradication.

CD169 seems to be related only to co-interactions with sialic acid in the virion surface, however, knockout pigs for either exon 1, 2, or 3 of CD169 were not protected from infection and viral load as well as antibody responses were similar to heterozygous ($CD169^{+/-}$) or wild type pigs ($CD169^{+/+}$) (25). The former experiments suggested that other unknown mechanisms could be involved in PRRSV infection such as other receptors, new unknown susceptible cell types different from macrophages or possible leaking of CD169 expression in the knockout model.

Other molecules are also involved in viral entry, such as CD151 (26) and vimentin (27); blocking of any of these four molecules (CD163, CD169, CD151, and vimentin) had an effect on viral infection, either on internalization or complete inhibition of viral replication (17). After cell entry, PRRSV causes a series of intracellular modifications to complete its replication cycle, which includes rearrangements of intracellular membrane organelles to generate the replication complex. These include the formation of perinuclear double membrane vesicles apparently derived from endoplasmic reticulum, synthesis of genomic RNA (gRNA), transcription of segmented RNA (sgRNA) and expression of viral proteins (20, 28). At late stages of replication,

the mature virions accumulate in the intracellular membrane compartments and they are then released into the extracellular space through exocytosis (29).

A non-classical spread pathway has been detected in several viruses including PRRSV where virus dissemination is mediated by cell to cell nanotubules (30). It was reported that almost all PRRSV proteins interact with myosin and actin (especially F-actin and Myosin IIA) where nanotubules connected cells allowing the movement of structural proteins and RNA, infecting naïve cells in a non-classical way even in the presence of neutralizing antibodies in the cell media. In addition, this non-classical pathway demonstrated that PRRSV cell entry receptors were not necessary to establish infection, as non-permissive cells became infected when were contacted by infected cells via nanotubes. This spreading strategy has been proposed as a mechanism to facilitate infection either by surfing of viral particles between adjacent cell membranes or as a receptor-independent mechanism for infection (31); Importantly, has been reported for other viruses such as HIV-1 where nanotube number on macrophages increases after infection (32) and Herpesvirus transmission between bovine fibroblasts (33). Interestingly, although several viral proteins were detected in nanotubules (nsp1β, nsp2, nsp2TF, nsp4, nsp7, and nsp8, GP5 and N), GP4 was detected in only a few nanotubes. In particular, the role of GP4 in this non-classical spread pathway is not fully understood and it will be interesting to further evaluate GP4 interaction with other cellular components to elucidate the reason why GP4 is not transported to new recipient naïve cells. Altogether these data indicate that PRRSV has evolved different pathways to spread even though, *in vivo*, the virus shows narrow cell tropism for monocytes and macrophages (34, 35) (**Box 1**).

IMMUNOLOGY OF PRRSV AND MECHANISMS INVOLVED IN IMMUNE EVASION

Innate Immune Response

The innate immune response is the first system any given pathogen encounters, specially to prevent viral replication and invasion into mucosal tissues (respiratory tract in the case of PRRSV) and, importantly, to initiate the strong adaptive immune response to fight against intracellular infectious agents (7). Type I interferons (IFN α/β) comprise one of the most potent mechanisms against invading viruses in the first stages of infection, triggering an array of IFN-stimulated genes (ISG) (36). Generally speaking, all nucleated cells have the ability to produce IFN α/β, but plasmacytoid DC (pDC) are the most potent producers of this family of cytokines (37). PRRSV has evolved a set of mechanisms for suppressing IFN α/β *in vivo*, maintaining low expression levels of this cytokines on infected pigs (38) during almost all time-course of infection shortly after transient elevation in the lungs (39). Suppression of IFN α/β also takes place *in vitro* in PRRSV infected MARC-145 and porcine alveolar macrophages (38, 40, 41). Further studies have shown that IFN type I suppression is a major strategy of PRRSV to modulate host antiviral defense. In fact, several viral proteins have been identified as IFN antagonists (nsp1α, nsp1β, nsp2, nsp4, nsp11, and N) (7, 42–44). As an example for N protein, upon dsRNA stimulation, IFN-β production was shown to decrease proportionally with increasing levels of N expression and additionally it was found to downregulate IFN-dependent gene production by dsRNA interfering with dsRNA-induced phosphorylation and nuclear translocation of IRF3 (45).

Among PRRSV non-structural proteins with type I IFN modulation capacity, nsp1 has been considered as the strongest antagonist of IFN-β production by acting on interferon regulatory factor 3 (IRF3) phosphorylation and nuclear translocation. Almost all nsps, excepting nsp1, have been related to the perinuclear region, associated with intracellular membranes, supposedly derived from the endoplasmic reticulum (ER), which are modified into vesicular double-membrane structures with which the viral replication and transcription complex (RTC) is thought to be associated with (14, 46, 47). Nsp1 translocates to the nucleus during the first hours of infection, where it is capable of inhibiting IRF3 association with CREB-binding protein (CBP), promoting CBP degradation by a proteasome-dependent mechanism, without which the transcription enhanceosome may not assemble the transcription machinery for the interferon expression (15, 46). Recently, post-transcription protein expression of IFN β was shown to be regulated by PRRSV by means of upregulating cellular miRNA in porcine alveolar macrophages (48)

Nsp2 is the largest (mature) PRRSV protein and contains at least four distinct domains: The N-terminal CP/OTU domain, a central hypervariable region, a putative transmembrane domain, and a C-terminal region of unknown function that is rich in conserved cysteine residues. This protein is unique in the context of PRRSV due to its genetic heterogeneity, its participation in diverse roles supporting the viral replication cycle, and its packaging within the PRRSV virion (16, 49). Previous studies suggest that nsp2 has different roles related to immune evasion mechanisms. It has been determined that nsp2 OTU domain (thiol-dependent deubiquitinating domain) inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) by interfering with the polyubiquitination process of IkBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor) and, subsequently, preventing the degradation of the IkBα protein (50). Moreover, viable deletion mutants in nsp2, when infecting cells, caused a downregulation of cytokines (IL-1β and TNF-α) mRNA expression, in comparison with that of parental virus, suggesting that certain regions of nsp2 might contribute to the induction of a virus-specific host immune response and that deletion of such a region could produce a more virulent virus (51).

There are several isoforms of nsp2, sharing a consistent core set between viral strains, which are integrated into mature virion at the final stage of replication (**Figure 1B**), although some of them could be strain-specific. Inclusion of nsp2 within the PRRSV virion suggests that it may function in previously unknown roles related to extracellular function, entry, or immediate-early viral replication events (16). Truncated

forms of nsp2 have also been identified, named nsp2TF and nsp2N, with apparent roles in modulation of immune evasion. When deletion mutants for those forms were used to infect cells, there was a significant change in gene expression, a strong activation of those involved in cytokine-cytokine receptor interaction, TNF signaling, toll-like receptor signaling, NOD-like receptor signaling, NF-κB signaling, RIG-I-like receptor signaling, chemokine signaling, JAK-STAT signaling, cytosolic DNA-sensing, and NK cell mediated cytotoxicity (13), suggesting that an active role (direct or indirect) is played by these truncated forms in modulating host cells innate immune response, making PRRSV infectious cycle more complicated than it was initially thought.

Nsp11, is a *Nidovirus* conserved endoribonuclease with an uridylate-specific endonuclease (NendoU). It has been demonstrated *in vitro* that overexpression of nsp11 enhanced viral titter (52). Moreover, nsp11 antagonizes type I IFN, specifically IFNβ production, activated by the retinoic acid inducible gene 1 like receptor, showing substrate specificity toward Mitochondrial Antiviral Signaling proteins (MAVS) and RIG-I (transcripts and proteins), and demonstrating that this activity was associated to the endoribonuclease activity of this protein in which transfection mutant viruses were unable to degrade MAVS mRNA and impair IFNβ production (53). Another mechanism whereby this protein limits antiviral response is related to inflammasome and synthesis of IL-1β, due to its important role in both the innate and adaptive immune response and in pathological mechanisms. It has been shown that PRRSV could activate NLRP3 inflammasome in early stages of infection but induce host's immunosuppression later as measured by determining the levels of pro-IL-1β and procaspase-1 mRNA and the mature IL-1β protein in porcine alveolar macrophages (PAM) (54). It is not surprising that nsp11 also interacts with the RNA-silencing complex (RISC), as it has been demonstrated *in vitro* in a MARC-145 cell line that this protein and nsp1α are responsible for inhibiting RISC and downregulating argonaute-2 protein expression increasing viral titter significantly, which demonstrates a direct relationship between this silencing complex and viral replication at least *in vitro* (55).

Other non-structural proteins have been studied but there is an important gap on information about *in vivo* and *in vitro* functions and interaction in signaling pathways. Additionally, the enormous variation among strains makes it difficult to characterize all protein variants and interactions with cell systems (macrophages, Dendritic cells "DCs," monocytes and others) (**Box 1**).

Recently, a body of evidence associates host genetics with different outcomes following PRRSV infection in the respiratory and reproductive form of the disease (56–60). Although pathways and mechanisms involved in specific disease-resistance traits have not yet been fully characterized, it is clear that the genetic variation in disease resilience is polygenic, regulating aspects of both innate resistance and acquired immunity (56). In connection with innate response, the average daily gain (ADG) after PRRSV infection was associated with a single genomic region in chromosome 4 (SSC4) which is best represented by the SNP tag marker WUR, located in the 3′ non-coding region of the interferon-inducible guanylate-binding protein 1 (GBP1) gene (61). The pig genetic resistance to PRRSV infection has been historically overlooked in PRRSV research probably generating a confounding factor in immune response studies. A key gap in the knowledge of PRRSV is linked the pig genetic variability after PRRSV infection with the enormous variability of the virus itself (**Box 1**).

In pigs, PRRSV replicates in cells belonging to the innate immune system. PAMs are the primary cells to be infected in the lungs as well as other cells of the monocyte/macrophage lineage, which later could disseminate the virus to other tissues or support replication to release viral particles into the bloodstream (17) (**Figure 2**). Moreover, PRRSV is thought to be able to infect professional antigen presenting cells such as DCs and monocyte derived dendritic cells, (MoDC) impairing their normal antigen presentation ability by inducing apoptosis, down-regulating the expression of IFN-α, MHC class I, MHC class II, CD11b/c and CD14, upregulating the expression of IL-10 and inducing minimal Th1 cytokine secretion (62–65). Nevertheless, new evidence suggest by *in vivo* and *in vitro* experiments that specifically lung cDC1, cDC2, and MoDCs are not infected by PRRSV-1 viruses from subtypes 1 and 3 and one possible explanation is the lower expression of CD163 and CD169 in those 3 DC subtypes, associating previous results of infection in DCs to culture conditions of monocytes *in vitro* that could cause a sensibilization to infection by certain strains as Lena (66). In addition, these findings were also tested in tonsil cDC and tracheal cDC1 and cDC2 observing that those cell populations are not infected by PRRSV virus (67, 68).

Moreover, a new type of PAM has been characterized and named porcine intravascular macrophages (PIM) due to its association to endothelial lung capillaries and not to the alveoli, presenting strong capacity to phagocytised blood-related particles (69). Importantly, when infected PIM cells gave similar results of viral load to those derived from infected PAM, but significantly upregulates of TNFα and non-significantly IL-6 and IL-8 expression after infection when compared to normal alveolar macrophages, indicating that these cells have an important pro-inflammatory role during PRRSV infection in the lungs (69). New interactions between cells and the virus need to be further explored to unravel possible immunological features that leads to correlates of protection.

Recently, it has been shown that a domain within Nsp1α is able to stimulate the secretion of CD83, which in turn inhibits MoDC function *in vitro,* impairing the ability of MoDC to stimulate T cell proliferation (70). Production of IFN α/β and the mechanisms for cell activation by pDC are severely suppressed during PRRSV infection, although these cells are not permissive to PRRSV infection (71, 72). However, this phenomenon is strain dependent, as other PRRSV strains are able to stimulate pDC for IFN α/β production in large quantities (73). Again, there is an enormous variability between PRRSV strains in relation with their effect on antigen presenting cells which prevent scientists from finding common mechanisms. It might be of interest to link this key gap of knowledge

for PRRSV with host genetics (**Box 1**). Moreover, in PRRSV-infected cells, N is abundantly expressed benefiting from the discontinuous transcription mechanism (74). This protein is also distributed in the nucleus, induced by two nuclear localization signals called cryptic NLS or NLS-1 and functional NLS or NLS-2 (positions 10–13 and 41–47, respectively) (75). The effect of N protein has been examined in PAMs and MoDCs using transfection, finding a significant upregulation of IL-10 gene expression.

Natural killer (NK) cells constitute another powerful arm of the innate immune system against PRRSV, particularly when considering the high percentage of circulating NK cells in pigs (76). The cytotoxic function of NK cells is reduced in PRRSV infected pigs from day 2 after infection up to 3–4 weeks (38, 77, 78). Initial studies using *in vitro* systems demonstrated that the stimulation of porcine NK cells with proinflammatory cytokines (IL-2 and IL-15) was capable of activating NK cells and inducing them to express high levels of IFN-γ and perforins to cause lysis of infected cells, but a different scenario appears if cells are evaluated post-infection, indicating that a virus such as PRRSV is capable of impairing NK cell cytotoxicity (79). *In vitro,* the NK cytotoxicity against PRRSV-infected PAMs was decreased and degranulation of NK cells inhibited (80). *In vivo,* the immune response is the same as that observed *in vitro*, with some studies reporting that approximately half of viremic pigs had a reduction >50% in NK cell-mediated cytotoxicity and enhanced secretion of IL-4, IL-12, and IL-10 and reduced frequency of cytotoxic T-cells ($CD4^{-}CD8^{+}$ T) and double positive T cells ($CD4^{+}CD8^{+}$ T) and upregulated frequency regulatory T- cells (Tregs) (81).

Acquired Immune Responses

Innate immune responses against PRRSV are obstructed by different mechanisms as are adaptive responses. The modest and delayed B cell mediated neutralizing antibody response is one of the main characteristics associated to PRRSV acquired immune responses. Even though PRRSV specific antibodies appear early at 7–9 days post-infection, the efficacy of those antibodies remains unclear. Neutralizing antibodies take longer, appearing nearly 1 month after infection (34). However, passive transfer of these neutralizing antibodies conferred almost full protection in a PRRSV reproductive model (95% of offspring alive after challenging pregnant sows with high neutralizing antibody titter). Nevertheless, in another experiment using the reproductive model, when the presence of PRRSV was examined after the transfer of neutralizing antibodies, lungs, tonsils, buffy coat cells, and peripheral lymph nodes contained replicating PRRSV similar to infected controls, although pigs were apparently protected against infection. In summary, passive transfer of high neutralizing antibody titter conferred protection to gilts and offspring (not detectable viremia), but did not eliminate the presence of viral particles in peripheral tissues nor transmission to animals they were in contact with (82–84). Curiously, the role of neutralizing antibodies in the protection against the respiratory form of the disease is a key gap of knowledge for PRRSV. This point is critical to define precisely targets for improved vaccines based on the humoral immune response against this virus (**Box 1**).

N protein is involved in several mechanisms for immune evasion and is also one of the most immunogenic structural proteins (75). Antibodies against N appear early during acute infection, together with those against M and GP5 proteins, but are non-neutralizing and could be involved in antibody dependent enhancement (85, 86).

There are other "antibody-related mechanisms" that do not necessarily involve neutralizing activity. Antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent complement-mediated cytotoxicity (CDC) and antibody-dependent complement mediated virolysis (ADCV) have been examined in the context of PRRSV, although none of these mechanisms were evident during infection or have not been deeply investigated on *in vitro* and *in vivo* models of this virus (87). It is important to note that neutralizing antibodies appear late in PRRSV infection and other immune mechanisms (cellular or antibody mediated immune response) might be acting to suppress viral replication in blood, causing the virus to be isolated in lymphoid tissues and maintaining suboptimal replication that will finally end in viral clearance. For type PRRSV-2 it has been demonstrated that immunization of pigs with ectodomain peptides from GP5/M complex did not induce neutralizing antibodies (88) although those ectodomain-specific antibodies generated were capable of binding virus.

An important feature that makes difficult to validate the location of neutralizing epitopes is the number of glycosylations in or around it. For PRRSV-1 strains, up to 3 glycosylations may be found in, or flanking the GP5 neutralizing epitope that is located between amino acids 37–45 (89), whereas for PRRSV-2 strains there are four potential glycosylation sites (90). When tested, PRRSV with mutations in GP5 glycosylation sites (either at N44 or in the hypervariable region, upstream the neutralizing epitope) enhanced immunogenicity with increased concentration of antibodies directed to this epitope 5–10 fold higher compared with those induced by the wild type strains (89). Same results were obtained when administering another deglycosylation mutant (double deglycosylation in the putative glycosylation moieties on GP5) twice, which conferred better protection against homologous challenge (91). In addition, when this protein is expressed early during infection, it stimulates production of early neutralizing antibodies and IFN-β, two main antiviral mechanisms, demonstrating its role in induction of self-protection mechanisms from the host (92). Available data about neutralizing antibodies induced by this protein are controversial, which may be due to the high variation among PRRSV strains (93) and, as previously commented, the host genetics. ORF5 is also complemented by a small frameshift of the subgenomic mRNA called ORF5a, encoding a type I membrane protein consisting primarily of alpha helix with a membrane-spanning domain (called GP5a) that is incorporated into virions as a very minor component, playing a role in viral replication, as mutation in the initiation codon or premature termination related to expression for this protein leads to non-efficient viral replication and lower titter (94, 95). This protein is capable of eliciting specific antibody immune response

in natural infections and after immunizations, although those are not neutralizing neither protective in a challenge trial after infection, making difficult to define the role of this particular small protein in the whole immune response and viral clearance of PRRSV infection (96). In summary, the role of humoral immunity remains elusive in PRRSV infection (neutralizing and non-neutralizing antibodies) and a better characterization will be required to overcome this relevant gap of knowledge (**Box 1**).

Treg typically increase in number in chronic viral diseases to prevent a persistent inflammatory response and pathological damage associated to viral infections. Conversely, Tregs are described as key contributors in modulating the host immune response to viral infection. This cell population is an important component in regulating the magnitude of the immune response to infection (in viruses such as HIV and HCV), thus preventing excessive inflammation and tissue damage. However, they can also be inappropriately induced by viruses to switch the balance of the immune response in favor of maintaining viral replication (97). In PRRSV, the role of Tregs remains unclear and appears to be a consequence of IL-10 induction of some strains as early as 2 days post infection (81). In some experiments, *in vitro* infected DCs with PRRSV-1 exhibited an unbalanced ability to stimulate T cell immune responses in a strain-dependent manner, but no Tregs were detected, at least *in vitro*, as measured by expression of CD25 and FoxP3 markers (98). When using PRRSV-2 strains, the case seems to be different, as the virus was capable of stimulating IL-10 production with concomitant generation of Tregs (99) which was associated to nucleocapsid protein expression in the *in vitro* system. This group also suggested that IL-10 production and Treg could be related to impaired gamma interferon (IFN-γ) production and altered development of protective T-cell response by inhibiting T-cell proliferation as seen in the early stage of infection with viruses such as HCV. Vaccine strains currently in use in the United States do not provide adequate heterologous protection, one possible explanation could lay on their inability to induce an adequate IFN-γ response due to their ability to stimulate Tregs, at least *in vitro* (100). Structural conformation, but not nuclear localization, of the expressed N protein was suggested as essential for the ability to induce IL-10 that, in consequence, causes induction of Tregs as measured by markers CD4+CD25+Foxp3+ (99). It should be noted that when the role of the nuclear localization signal was evaluated using deletion mutants, results suggested that NLS-2 was not essential for virus survival, although pigs developed a significantly shorter duration of viremia and higher neutralizing antibodies than those of wild-type PRRSV-infected pigs (101). The role of Tregs cells in the immune response against PRRSV is a key gap of knowledge in order to develop more efficacious PRRSV vaccines (**Box 1**).

Moreover, reports have highlighted the impact of PRRSV infection on thymic cellularity mainly as a loss of $CD4^+/CD8^+$ cells in the thymus of PRRSV-infected pigs. Acute lymphopenia, thymic atrophy, and lymphadenopathy associated with the presence of PRRSV antigen in the thymus are some of the mechanisms whereby PRRSV suppresses the immune response. In addition, presence of PRRSV antigens in the thymus could also induce tolerance and presents a mechanism that could explain the presence of Tregs during PRRSV infection (93). Nevertheless, the picture is not complete and basic knowledge about the effect of PRRSV on cell development in the thymus would be of great interest to understand the effect of this viruses in the host.

PRRSV immunology thus remains an unsolved puzzle due to complex interactions between different viral strains and the host. Similar immune responses could be the key feature of this virus, such as persistence viremia, a strong inhibition of innate cytokines (IFN-α/β, TNF-α, IL-1β, IFN-γ), dysregulation of NK cell function (cytotoxicity and degranulation), rapid induction of non-neutralizing antibodies, delayed appearance of neutralizing antibody, late and low CD8+ T-cell response, and induction of regulatory T cells (Tregs) (102). As a whole, neutralizing antibodies and PRRSV-specific IFN-γ secreting cells do not fully depict the immune effector functions related to protective immunity, as the viral targets related to them are unknown. As a consequence, correlates of protection remain elusive for this infection due to the laborious work *in vitro* and *in vivo* and the enormous genetic diversity that causes confusion and makes it difficult to predict how immune responses against one isolate or strain could be applied to another in a cross-protective immune prediction model (103, 104). Without any doubt, the most important gap of knowledge for PRRSV is the lack of correlates of protection that makes extremely difficult to have robust models to check vaccines efficacy against this disease (**Box 1**).

Vaccination Strategies in PRRSV. Classical and Novel Vaccines

Since the beginning of PRRSV outbreaks in Europe and the USA, the development of efficacious PRRSV vaccines has been a challenge. Classical approaches are not working properly for several reasons: viral mutation can lead to more pathogenic strains, there is a lack of knowledge on how the porcine immune system interacts with all PRRSV proteins, and most importantly, there is no robust parameter (surrogate marker) that can be unequivocally linked with viral clearance. Thus, there is no relationship between complete homologous or heterologous protection and classic immunological parameters such as an increase/decrease in particular cell population (105), IFN-γ production, neutralizing antibodies (106), non-neutralizing antibodies and clinical outcome (107). In addition, highly divergent strains make it more difficult to develop a universal vaccine for this virus (28).

Several different vaccines against PRRSV have reached the market and have been reviewed recently (108). Most of these vaccines rely upon modified live virus (Porcilis PRRS from Merck, Ingelvac PRRSFLEX EU from Boehringer Ingelheim, Amervac-PRRS from Hypra, Pyrsvac-183 from Syva) against PRRSV-1, as well as some to control PRRSV-2 (Fostera PRRS from Zoetis, Ingelvac PRRS MLV/Ingelvac PRRSATP from Boehringer Ingelheim). There is also evidence that most MLV vaccines of both PRRSV-1 and PRRSV-2 species elicit specific humoral and cell-mediated immune (CMI) responses, as they confer protection to homologous parental strains and partial

protection to heterologous strains. Although it is possible to control some PRRSV outbreaks by use of MLV in combination with good practices, there are major safety issues such as a high mutation rate leading to reversion to virulence and recombination among vaccine and wild type strains. Cases have been reported in which new viruses have been introduced as a consequence of MLV vaccines. For example, nucleotide sequence identities of atypical Danish isolates were between 99.2 and 99.5% with the vaccine virus RespPRRS and 99.0–99.3% with VR2332, which is the parental virus to the vaccine virus, supporting the conclusion that the introduction of PRRSV-2 in Denmark was due to the spread of vaccine virus (109). In China a recombination event was reported in which a PRRSV variant with nucleotide deletions and insertions in the non-structural protein 2 (nsp2) gene also showed a possible recombination event between a MLV strain and a prototype Chinese field strain (110).

Current inactivated vaccine approaches are not highly effective since elicited immune responses are not enough to prevent spreading of the virus. However, this type of vaccine can augment anamnestic virus neutralizing antibodies and virus-specific IFN-γ responses following a wild-type virus infection or PRRSV-MLV vaccination which can contribute to viral clearance (111, 112). Thus, the combination of modified live vaccines with inactivated ones can be a reasonable approach to control the disease under field conditions (113) but unfortunately, there is no robust data comparing this approach with other options available on the market. On the other hand, most inactivated vaccines are not approved for use in the United States due to the poor efficacy showed in challenge trials (114) as measured by production of PRRSV specific neutralizing and non-neutralizing antibodies and low cellular immune responses leading to their failure in the porcine market. According to the Centre for Food Security and Public Health of Iowa State University, only

BOX 2 | Exosomes and therapeutic applications in PRRSV.

Pan et al., 1983; Harding et al., 1983.	•Discovered in 1983. Known as a garbage-disposal mechanism in the maturation of reticulocytes to erythrocytes.
Raposo et al., 1996	•Exosomes released from B-lymphocytes are capable of antigen presentation as contain MHC-II in their surface and induce antigen-specific MCH-II restricted T-cell responses.
Zhu et al., 2014	•First use of exosomes in PRRSV. Application of exosome for delivering artificial microRNAs targeting sialoadhesin and CD163 receptors.
Montaner-Tarbes et al., 2016.	•Serum-derived exosomes from non-viremic animals previously exposed to the porcine respiratory and reproductive virus (PRRSV) contain antigenic viral proteins.
Lener et al., 2015	•Guidance of International Society of Extracellular Vesicles regarding the most important aspects of safety and regulatory requirements that must be considered for pharmaceutical manufacturing and clinical application of extracellular vesicles including strategies to promote the therapeutic application of EVs in future clinical studies.
Montaner-Tarbes et al. 2018.	• First targeted-pig trial using serum derived extracellular vesicles as a new vaccination platform for PRRSV virus.
FDA clinical trials for veterinary diseases	• To the best of our knowledge, no clinical trials in veterinary medicine have been approved by FDA

"BIOSUIS PRRS Inact EU+Am" is approved to be used in the US. However, new strategies are being evaluated to overcome these problems (115), including nanoparticle entrapped antigens (116–119), plant based approaches (120) or vectored vaccines (121).

Several attempts have been made to use structural proteins to develop vaccines against PRRSV because they are specific targets of neutralizing antibodies. For this reason, one may hypothesize that antibodies against those proteins could be the main key to inhibit viral replication and spread as it is common for many viruses. Approaches such as VLPs combining different structural proteins have been tested (122–124), finding that anamnestic response is possible (boosted IgG and IFN-γ producing cells) in previously vaccinated or infected pigs but not in the pre-challenge period. These structural proteins are able to prime the immune system, but no reduction of viremia was observed after challenge (123). Those results suggest that other viral proteins may be targeted to induce a protective response in pigs. A plausible explanation for this finding may be based on the presence of few neutralizing epitopes in their sequences, most of which are located in variable regions of the proteins, to the phenomena of glycan shielding for epitopes and to the high variability observed between PRRSV virus strains. Again, a critical gap of knowledge for PRRSV is to precisely characterize common epitopes that are present in all PRRSV strains. Epitopes responsible for generating an efficient immune response eliciting cross-protective immunity remained elusive. Taken together, this evidence points to the need for new vaccination approaches that comply with a pathogen free strategy, capable of eliciting effective cellular and antibody responses with mid to long term protection against homologous strains and preferable to heterologous challenge as well.

Extracellular Vesicles As a New Vaccination Approach

Extracellular vesicles(EVs) are gaining increased scientific attention as novel vaccines against infectious diseases, including animal diseases of veterinary importance by its capacity of self-antigen presentation, activation of host cell and antibody immune responses and more important, to induce protection in lethal challenge trials (125–131) (**Box 2**). In the case of PRRSV, artificial microRNAs (amiRNA) were initially synthetized to try suppressing expression of sialoadhesin (Sn) or CD163 by recombinant adenoviral vectors to be contained in exosomes, causing a subexpression of Sn and CD163 at mRNA and protein level, and reducing viral titter when porcine macrophages were pre-treated with amiRNA thus providing new evidence supporting the hypothesis that EVs can also serve as an efficient small RNA transfer vehicle for pig cells (132). More recently, PRRSV viral proteins associated to extracellular vesicles (EVs) in the size range of exosomes, were reported (129). Moreover, a targeted-pig trial using EVs from sera of infected pigs who had overcome the disease, demonstrated that EVs are capable of inducing specific IFN-γ secreting cells after a prime-boost strategy, are safe, free-of-virus and can differentiate infected from vaccinated animals (133), moreover, it was demonstrated that those EVs contained antigenic viral proteins recognized by pig immune sera and not by the pre-immune one. Of interest, however, a recent article indicated that PRRSV derived EVs are capable of transmitting the virus from one cell to another (134). Whether these discrepancies are due to *in vivo* vs. *in vitro* experimental work and methods applied to isolate EVs from serum samples or culture supernatant, remains to be determined.

EVs have also been explored as novel control strategies in other viral diseases. For example, in respiratory syncytial virus infection, EVs are released with a selected modified cargo when compared with uninfected epithelial cells. When analyzed in detail, several viral proteins and diverse species of RNA were detected and capable of activating innate immune responses through induction of cytokine and chemokine release (135). Similar scenarios of viral proteins exported in EVs have been observed and extensively reviewed for HIV/HCV/HTLV-1 (136), EBV (137), and other viral diseases. Moreover, viral products of various origin and size including Ebola Virus VP24, VP40, and NP, Influenza Virus NP, Crimean-Congo Haemorrhagic Fever NP, West Nile Virus NS3, and Hepatitis C Virus NS3, when fused with Nef C-terminal domain through DNA vectors, were directed to the EVs membrane or packaged into them and remained stable after fusion. More importantly, when injected in mice, DNA vectors expressing the diverse fusion products elicited a well detectable antigen- specific CD8+ T cell response associating with a cytotoxic activity potent enough to kill peptide-loaded and/or antigen-expressing syngeneic cells, proving its promising results as a cytotoxic T lymphocyte vaccine (138).

Concluding Remarks

PRRSV is a complex disease and several gaps in the knowledge of its economic impact, biology and evolution, genetic polymorphism, mechanism of viral infections, elicitation of protective immune responses and novel control strategies, have been reviewed here (**Box 1**). Since the late 1980's, different approaches have permitted to examine more closely this virus allowing the discovery of new features of the complex replication cycle, the identification of proteins and nucleic acids playing a role together with extracellular vesicles and nanotubules in facilitating spreading, and a better understanding of immune evasion (non-neutralizing antibodies, glycan shielding, mutation, recombination events, among others) to further vaccine development. Presently available PRRSV vaccines have many limitations in terms of heterologous protection, but some efforts have been made by combining new adjuvant formulations with modified live viruses, DNA and peptide vaccines, as well as extracellular vesicles a new vaccination approach. Advancing in all these gaps in knowledge, will eventually accelerate eliminating and eventually eradicating this devastating veterinary disease of such huge economic importance.

AUTHOR CONTRIBUTIONS

SM-T: wrote the first draft of the manuscript. MM, HdP, and LF: wrote sections of the manuscript. All authors

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REVIEW
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Atypical Porcine Pestivirus (APPV) as a New Species of *Pestivirus* in Pig Production

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The genus *Pestivirus*, which belongs to the family *Flaviviridae*, includes ssRNA+ viruses responsible for infectious diseases in swine, cattle, sheep, goats, and other domestic and wild animals. Recently, several putative pestiviruses species have been discovered and characterized in mammalian species (giraffe pestivirus, antelope pestivirus, HoBi virus, Bungowannah virus, and Linda virus); one of these is a genetically distinct pestivirus, named atypical porcine pestivirus (APPV), discovered using the next-generation sequencing technology. APPV has been detected in piglets with congenital tremor (CT) from four different continents, including North America, South America, Europe, and Asia. There is strong evidence that experimental inoculation and *in field* outbreaks involving APPV induce CT in piglets. Additionally, splay leg (SL) syndrome has been observed concurrently with CT, and it was induced by APPV in experimental studies and some field cases. Animals with a persistent and/or chronic infection condition can shed the virus over time. Viral-RNA is frequently detected in different tissues from CT-piglets; however, high loads of APPV are detected most consistently in central nervous tissue. Moreover, the APPV genome has been recently detected in semen and preputial swabs from boar studs, as well as in serum and tissue samples from wild boars and domestic adult pigs, all known to be clinically healthy. Phylogenetic analysis revealed that the APPV sequence (complete or partial polyprotein) exhibits high genetic diversity between viral strains detected in different countries and formed independent clusters according to geographic location. Additional studies are needed to evaluate the molecular detection and sero-prevalence of APPV around the world. Lastly, more research is needed to understand clinical presentations associated with APPV infection, as well as the economic losses related to the virus in pig production worldwide.

Keywords: atypical porcine pestivirus, congenital tremor, pestiviruses, pig production, pre-weaning mortality

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INTRODUCTION

Piglet pre-weaning mortality is a major problem in pig farms around the world. On average, pre-weaning mortality rates of live-born piglets can be as high as 23% and starvation and crushing are the main causes of death (1). Different etiologies may be involved in piglet pre-weaning mortality including atypical porcine pestivirus-associated congenital tremor (CT) (2–4).

Historically, CT was first reported in the literature 97 years ago, when Kinsley (5) described "dancing pigs." Subsequently, it was characterized as tremors of intent that ceases when piglets are at rest (6). The syndrome is classified into five types according to the etiology (Type AI–AV) (**Table 1**); however, most contemporary CT outbreaks had been attributed to an unidentified virus, Type AII (7, 8).

Since atypical porcine pestivirus (APPV) was first identified in 2015 (9), several studies have linked this new *Pestivirus* with the occurrence of CT in newborn piglets. It was usually described as a temporary condition, lasting several weeks to months, and characterized by tremors of the head, limbs, and body, varying in severity and intensity. However, the clinical signs were reduced or absent during inactivity or sleep (2, 3, 10).

In general, CT is not the cause of death in affected piglets; however, their survival may be threatened because of inadequate colostrum, or milk intake, leading to severe growth retardation and death by starvation or crushing due to impairment of evasive actions (2, 7). Furthermore, APPV is capable of inducing neurological disorders, such CT, increasing piglet pre-weaning mortality and reducing reproductive performance in affected pigs (2–4, 10).

Although the impact of most exotic diseases in animal production and global economy is known (11), a more comprehensive understanding of the epidemiology, genetic variability, and economic losses associated with the role of APPV in pig production is required.

ETIOLOGY

Pestiviruses are highly variable RNA viruses causing economically relevant diseases in domestic animals. The genus *Pestivirus*, which belongs to the family *Flaviviridae* (ssRNA +), includes 11 recognized species: *Pestivirus* A (bovine viral diarrhea virus type 1), *Pestivirus* B (bovine viral diarrhea virus type 2), *Pestivirus* C (classical swine fever virus), and *Pestivirus* D (border disease virus), *Pestivirus E* (pronghorn pestivirus), *Pestivirus F* (Bungowannah virus), *Pestivirus G* (giraffe pestivirus), *Pestivirus H* (Hobi-like pestivirus), *Pestivirus I* (Aydin-like pestivirus), *Pestivirus J* (rat pestivirus), and *Pestivirus K* (atypical porcine pestivirus) (12).

Additionally, three atypical pestiviruses have been characterized in pigs: Bungowannah virus (causing myocarditis), APPV and Linda virus (causing lateral shaking) (9, 13, 14). Nevertheless, several reports (experimental conditions or field cases) have demonstrated that APPV is a prominent cause of APPV-CT type II in newborn piglets around the world (2–4, 10, 15). However, no studies have provided an efficient protocol for APPV isolation in cell culture (3, 9, 16, 17), and Koch's postulate couldn't be established.

Further characterization of APPV, as well as other porcine pestiviruses linked to severe clinical diseases in pigs, is needed (18, 19). Remembering that, the continued expansion of the genus *Pestivirus* and its high genetic diversity constitute a worldwide concern.

GEOGRAPHIC DISTRIBUTION

APPV has been detected in four different continents, including North America, South America, Europe, and Asia, and in countries such as the United States (first report) (9, 10), the Netherlands (2), Germany (15, 16), Sweden (20), Spain (2, 21), Austria (3), China (22), South Korea (23), Brazil (4, 24, 25), Canada (26), Hungary (27), Great Britain (28, 29), Italy, the Republic of Serbia, Switzerland, and Taiwan (29). Considering all these reports, APPV has wide distribution in the world (**Figure 1**). Postel et al. (29) have suggested that APPV must be regarded as a pig pestivirus of likely worldwide relevance.

EPIDEMIOLOGY

APPV-associated CT has been reported to be more prevalent in litters of gilts than sows (2, 4), suggesting that the immune status of the dam is likely the key in disease development in piglets (4). During outbreaks, CT morbidity varies within and between litters; a few pigs in one or all pigs in several litters may be affected (2–4, 10). Overall, CT is observed in both males and females (2) and its prevalence within the litters ranges from < 10 to 100% (2–4, 10). Moreover, CT severity in piglets seems to vary within litters (10). Several recent studies have linked APPV with CT occurrence in piglets and sporadic detection in domestic and wild boars. Detailed information regarding APPV studies can be found in **Table 2**.

Adult domestic pigs (15, 16, 21) and wild boar are susceptible to APPV infection (34, 35). Recently, APPV was detected in boar preputial fluids (2) and semen (3); both sample types came from boars that had clinical signs consistent with CT at birth. Still, APPV has been detected in semen, preputial swabs, and preputial fluids from commercial boar studs in the United States (32) and it is highly improbable that these boars had CT at the time of birth, suggesting that either transiently infected or persistently infected (PI) boars with no clinical history of CT could be shedding APPV in semen.

A retrospective analysis of cerebellum samples from Germany and another retrospective study with fresh and formaldehyde-fixed paraffin-embedded tissue samples from Hungary showed the presence of APPV in CT-affected piglets from over a decade ago (15, 27). Similarly, a study from Spain confirmed the presence of APPV nearly two decades prior to its first discovery (21).

Wild boar is also susceptible to APPV infection, although its role in the virus epidemiology is unknown (35). Limited information regarding APPV route of transmission, ecology, pathogenesis, carriage, spread, and epidemiology is available. However, piglets presenting with CT, surviving CT syndrome piglets, boars without CT (at birth), and clinically healthy adult domestic pigs, can shed moderate to high loads of virus, playing a relevant role in virus epidemiology; similar to a chronically and/or PI animal (2, 3, 32, 34).

Regarding to diagnosis, Postel et al. (17) described the presence of viral genome in serum samples with different levels of antibodies, suggesting a degree of antibody protection; and, samples with absence of antibodies and viral genome loads,

TABLE 1 | Etiology of congenital tremor types in piglets.

	Congenital tremor type				
	A-I	**A-II**	**A-III**	**A-IV**	**A-V**
Etiology	Classical swine fever virus	Atypical porcine pestivirus	Genetic sex-linked recessive	Genetic autosomal recessive	Chemical trichlorfon
Breed	All	All	Landrace	Saddleback	All
Affected litters	High	High in gilts Low in sows	Low	Low	High
Mortality of CT-piglets	Moderate-high	Low-moderate	High	High	High

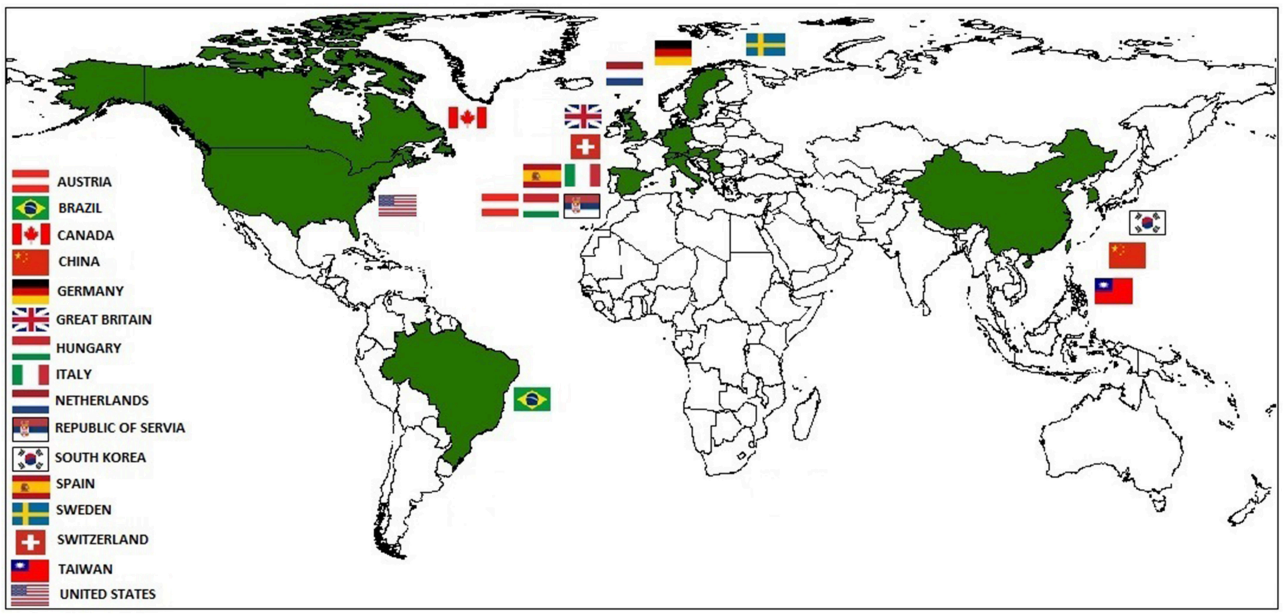

FIGURE 1 | Geographic distribution of Atypical Porcine Pestivirus (APPV) around the world. The geographic information system ArcGIS 10.5.1 was used to generate the map.

indicating acutely or PI animals. Similarly, Muñoz-González et al. (21) also suggested the PI condition, which could help the APPV spread. On the other hand, the presence of systemic levels of type I Interferon in newborn piglets could lead to the activation of the immune system by APPV (21). Based on these results, more research is needed in order to better understand the role of the innate immune system response to APPV infection.

APPV PATHOGENESIS AND PATHOLOGY

The newly discovered APPV (9) is capable of inducing neurological disorders, reducing reproductive performance, and increasing pre-weaning mortality (2–4, 10). Two independent research groups have experimentally reproduced CT using an inoculum containing APPV and observed a subset of piglets with concurrent splayleg (SL) (2, 10) and that the affected CT-litters presented as weak piglets with an abnormal posture (2). Field studies have also reported the occurrence of SL in litters with CT sporadically affecting the same piglet (3, 4). SL prevalence ranged from 6 to 55% within the affected CT litters (4).

APPV has a wide distribution in tissue samples, excretion and secretion fluids (2, 10, 15, 21, 22, 32). According to Gatto et al. (4), central nervous and lymphoid tissues appear to be suitable sites for viral replication; however, the cerebellum was the most consistently positive sample type from CT piglets and could constitute a target for APPV replication. Although a specific target of replication has not been determined (10), this may suggest that viral replication occurs systemically and has a predilection for certain types of tissues. However, the primary replication sites remain unknown. The histological findings of a number of studies are described in **Table 3**.

IMPACT ON PIG PRODUCTION

The economic relevance of an APPV-outbreak loss in pig production worldwide remains undetermined; however, it is estimated that the number of weaned piglets per sow decreases by > 10%, affecting reproductive performance. Additionally, mortality increased up to 30%, when CT-affected new-born piglets died of malnutrition in a farm in Austria (3).

TABLE 2 | Summary of atypical porcine pestivirus studies.

Year	Country	Serology	Strength(s)	References
2015	United States	+	Virus discovered by next-generation sequencing	(9)
2016	United States	−	Experimental inoculation in fetuses (45 and 62 days of gestation)	(10)
2016	Netherlands	−	Experimental intramuscular inoculation (32 days of gestation)	(2)
2016	Germany	−	Detection of APPV genome by fluorescent *in-situ* hybridization/Detection in adult domestic pigs/Virus isolation was attempted (failed)	(15)
2016	Germany	−	First indication of a cell culture isolate is provided/Detection in adult domestic pigs	(16)
2017	Austria	+	Persistent infection condition was suggested/Virus isolation (inefficient)	(3)
2017	China	−	Viral strains showed highly genetic diversity	(30)
2017	China	−	Suggested APPV origin and dissemination/Virus isolation attempted (failed)	(22)
2017	Great Britain	−	First detection in the country	(28)
2017	Spain	+	Retrospective study (virus was identified at least since 1997)/Detection in adult domestic pigs	(21)
2017	Switzerland/China/Great Britain/Germany/Italy/Republic of Serbia/Taiwan	+	Geographically wide distribution of genetically highly variable APPV and high APPV genome detection	(29)
2017	South Korea	−	First detection in the country	(23)
2018	China	−	Suggested a novel APPV strain in China	(31)
2018	Brazil	−	First detection in Brazil/Formalin-fixed paraffin-embedded samples were used	(4)
2018	Brazil	−	Sequencing and analysis of the partial NS5B gene	(24)
2018	Brazil	−	High lethality and coinfection with porcine teschovirus (PTV)	(25)
2018	United States	−	Detection in semen, preputial swabs, and preputial fluids from boar studs	(32)
2018	China	−	Identification and characterization of two possible strains	(33)
2018	Germany/Republic of Servia	+	First detection in wild boars/Detection of APPV-antibodies in wild boars from the Republic of Serbia	(34)
2018	Hungary	+	First detection in this country/Distinct lineages were reported, suggesting multiple introduction events of the virus	(27)
2018	Canada	−	First detection in the country	(26)
2018	Spain	−	Detection in wild boars/Low prevalence	(35)
2018	Brazil	−	APPV-associated with pathological lesions	(36)

de Groof et al. (2) reported 26% mortality in CT-litters affected by APPV, with 60% of these deaths attributable to CT in one farm. Additionally, they showed that under experimental conditions, the affected CT-litters presented weak piglets with an abnormal posture (bent back [kyphosis] and ears on the neck) (2). The SL syndrome has been observed concomitant with some CT cases (2–4, 10), limiting the locomotion of the CT-piglets and increasing the percentage of crushing.

In China, Shen et al. (33) reported a mortality rate of 60% in CT-piglets and Dessureault et al. (26) reported an average mortality rate of 24.6% in CT-litters in Canada. According to Gatto et al. (4) the case fatality of affected CT-piglets in Brazilian pig farm production was 30%. Early data from the United States, Germany, Italy, China, and Taiwan have suggested a relatively high abundance (2.3–22%) of APPV genomes in apparently healthy pigs (9, 15, 16, 29). In addition, Gatto et al. (32) detected APPV genomes in semen (up to 34% prevalence), preputial swabs (up to 23%), and preputial fluids (up to 28%) from commercial boar studs in the United States, which could play an important epidemiological role in virus transmission route and spread. Thus, artificial insemination could constitute a potential APPV transmission route and should be considered an important factor when developing and implementing biosecurity measures to prevent APPV-infections.

The impact of APPV infection on pig production seems to be indirect, since the mortality of CT piglets is the only loss reported so far, and it is mainly attributed to secondary factors. So, further studies evaluating its impact on pig production should

TABLE 3 | Histopathological and histochemical findings from Congenital Tremor (CT) cases.

Country	Histopathological and histochemical findings	References
Germany	No significant findings in the central and peripheral nervous system, as well as skeletal muscles. Luxol fast blue staining revealed mildly reduced staining intensity accentuated in the lateral white matter of the spinal cord	(15)
Austria	Vacuoles in cerebellar white matter; moderate hypomyelination in the white matter of the cerebellum and thoracic spinal cord; detection of oligodendrocytes; and increased staining intensity	(3)
Brazil	Moderate vacuolization of the white matter of the cerebellum and brain stem. Luxol fast blue staining did not reveal a decrease in the amount of myelin in the cerebellum; however, mild myelin loss was noted in the white matter found in the spinal cord and sciatic nerve	(24)
Brazil	Luxol fast blue staining revealed evidence of myelin vacuolization with the formation of digestion chambers. These chambers were of different sizes, observed in the white matter of the cerebellum, brainstem, and spinal cord. Severe secondary demyelination, with either a complete absence or an inadequate amount of myelin, in areas in both the white and gray matter of the spinal cord and brainstem, with mild secondary demyelination in the cerebellum	(25)
China	No significant findings	(30)
Canada	Luxol fast blue staining revealed an important loss of myelin from the periphery of the thoracic spinal cord, more severe in the lateral and ventral funiculi	(26)
Brazil	Neuronal necrosis, gliosis, and neuronophagia with satellitosis particularly at the cerebral cortex and to a lesser extent at the spinal cord, white matter demyelination of the cerebrum and spinal cord, Wallerian degeneration of the spinal cord, and necrosis of Purkinje cells of the cerebellum. The immunohistochemistry revealed proliferation of glial fibrillary acidic protein (GFAP) cells and fibers were more severe and widespread in piglets infected by APPV	(36)

be performed in order to estimate the direct loss in affected piglets, or suggest that the indirect loss could be related to the depletion of the immune system, such as what is reported in other pestiviruses.

ATYPICAL PORCINE PESTIVIRUS GENOME

Since 2015, 20 complete APPV polyproteins from six countries (**Table 4**) have been submitted to the "GenBank" database. The virus genome of *Pestivirus* K species is ∼10.8–11.5 kb. The APPV genome has ∼25–28% pairwise identity to known pestiviruses and 68% pairwise identity to a recently partially characterized *Rhinolophus affinis* pestivirus, placing both viruses in a highly divergent lineage of pestiviruses (9).

Phylogenetic analysis has revealed that APPV sequences (complete or partial polyprotein) exhibit high genetic diversity between viral strains detected in different countries (4, 27, 29–31) and form independent clusters according to geographic location (**Figure 2**). Based on phylogenetic analysis of the Npro gene, different viral strains can be present in the same farm simultaneously and at different times (32).

A phylogenetic tree based on Bayesian analysis of 20 complete APPV polyprotein sequences and other pestiviruses demonstrated a monophyletic cluster topology for APPV. In addition, five distinct clusters were observed within the APPV clade (cluster I: North America and Asia, cluster II: Europe and Asia, cluster III: North America and Europe and clusters IV and V: Asia; **Figure 2**).

TABLE 4 | List of complete atypical porcine pestivirus genomes.

Number	Genbank accession	Country	Year	Lenght (bp)
1	KR011347	United States	2015	11.276
2	KU194229	United States	2015	11.545
3	KU041639	Germany	2015	10.908
4	LT594521	Germany	2016	11.467
5	MF167290	Germany	2017	10.908
6	MF167291	Germany	2017	10.908
7	KX929062	Netherlands	2016	11.561
8	KX778724	Austria	2016	11.535
9	KX950761	China	2016	11.043
10	KX950762	China	2016	11.043
11	KY475592	China	2017	11.304
12	KY475593	China	2017	11.464
13	KY612413	China	2017	11.043
14	KY624591	China	2017	11.466
15	KY652092	China	2017	11.475
16	MF167292	China	2017	10.815
17	MF377344	China	2017	11.556
18	MG792803	China	2018	11.526
19	MH102210	China	2018	11.534
20	MF979135	South Korea	2017	11.247

FUTURE PROSPECTS

The economic impact of some pestiviruses in swine species is manifested by devastating losses worldwide. The high genetic variability of pestiviruses is the key point triggering practical consequences in epidemiology, diagnosis, control, and economic impact on livestock, especially in pigs.

Given the wide distribution of APPV and its genetic variability in different countries, studies regarding the epidemiology, ecology, pathogenesis, pathophysiology, transmission routes, and the impact of this virus on swine farms are necessary. Recent detection of APPV in semen from commercial boar

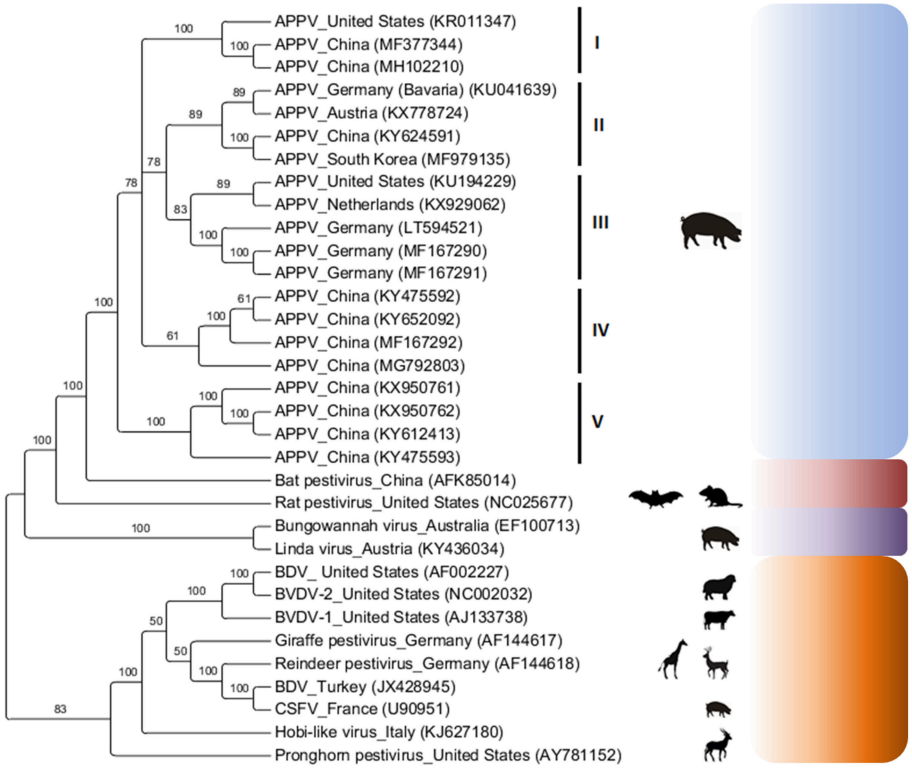

FIGURE 2 | Phylogenetic analysis of the atypical porcine pestivirus complete polyprotein (13,695 bp after alignment). Analysis was based on the Bayesian method and the GTR + I + G4 evolutionary model using MrBayes 3·1·2 (37) via CIPRES Science Gateway (38). The numbers on the nodes correspond to clade support values accessed with 10^6 bootstrap replicates. Viruses of the genus *Pestivirus* (classical and atypical) were used as an external group.

studs has sparked further investigations to clarify whether APPV can be transmitted through artificial insemination or reproductive biotechnologies, commonly used in pig breeding, which might play a significant role in the dissemination of pathogens.

Another interesting epidemiological issue is the recent detection of APPV in wild boars, demonstrating the ability of the virus to infect domestic and wild pigs. To date, the role of this wild species in the epidemiology of APPV remains unknown and further research should be conducted with this species, as the world's population of wild boars is increasing. Moreover, the impact of this ecological imbalance on the maintenance and spread of pathogens could be devastating, as in the case of classical swine fever virus spread in Europe.

Based on the available data, we highlighted some key points related to APPV infection, such as: (1) adaptive immunity of gilts/and sows; (2) possible transmission of the virus by semen; (3) absence of a commercial vaccine. For this reason, we hypothesize and suggest a few insights that may have a positive impact on the control of the infection. Since the highest prevalence of CT-piglets occurs in gilts, we suggest that the immune status of the dam and the time of infection are the key factors related to disease development. So, we recommend the use of an acclimatization strategy for replacement gilts, similarly to the protocol used for *Mycoplasma hyopneumoniae* control. In addition, to reduce the risk of APPV transmission by semen, it is advised that batches used in artificial insemination protocols are previously tested for the presence of APPV genomes. Likewise, based on the high genetic variability of APPV and recent research about the development of a subunit vaccine against APPV based on the E2 protein (39), we strongly recommend the implementation of a feedback management in farms with CT-cases, strategy that could be used until the development of an efficient commercial vaccine. Therefore, additional epidemiological information is required in order to develop strategies of control and eradication of APPV in pig production.

Finally, even though the potential for intercontinental spread of some viruses and the impact of exotic and emerging diseases on worldwide pig production is known, we strongly recommend additional epidemiological studies that will provide current essential information regarding APPV and elucidate possible routes of entry, dissemination, and genetic evolution of APPV, as well as other viral agents. This data will aid in the active surveillance of pathogens considered exotic and/or emerging around the world (i.e., porcine epidemic diarrhea virus, transmissible gastroenteritis virus, porcine reproductive and respiratory syndrome virus, and African swine fever virus).

AUTHOR CONTRIBUTIONS

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

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REVIEW
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PEDV and PDCoV Pathogenesis: The Interplay Between Host Innate Immune Responses and Porcine Enteric Coronaviruses

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Enteropathogenic porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PDCoV), members of the coronavirus family, account for the majority of lethal watery diarrhea in neonatal pigs in the past decade. These two viruses pose significant economic and public health burdens, even as both continue to emerge and reemerge worldwide. The ability to evade, circumvent or subvert the host's first line of defense, namely the innate immune system, is the key determinant for pathogen virulence, survival, and the establishment of successful infection. Unfortunately, we have only started to unravel the underlying viral mechanisms used to manipulate host innate immune responses. In this review, we gather current knowledge concerning the interplay between these viruses and components of host innate immunity, focusing on type I interferon induction and signaling in particular, and the mechanisms by which virus-encoded gene products antagonize and subvert host innate immune responses. Finally, we provide some perspectives on the advantages gained from a better understanding of host-pathogen interactions. This includes their implications for the future development of PEDV and PDCoV vaccines and how we can further our knowledge of the molecular mechanisms underlying virus pathogenesis, virulence, and host coevolution.

Keywords: PEDV, PDCoV, innate antiviral response, interferon induction and signaling, innate immune antagonism

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INTRODUCTION

Two members of swine enteric coronaviruses, porcine epidemic diarrhea virus (PEDV), and porcine deltacoronavirus (PDCoV), have recently emerged as major causative agents of lethal watery diarrhea in piglets, leading to significant losses within the swine industry worldwide. PEDV and PDCoV are classified in distinct genera in the family *Coronaviridae*, as an *Alphacoronavirus* and *Deltacoronavirus,* respectively (1, 2). The transmissible gastroenteritis virus (TGEV), also an enteropathogenic porcine alphacoronavirus, used to be responsible for severe economic losses around the globe in the 1990s. However, due to its current disappearance in many parts of the world, this review will focus mainly on PEDV and PDCoV, the two emerging swine coronaviruses.

The first PEDV outbreak occurred in Europe around 1970s (3, 4). From the 1990s onward, sporadic occurrences of PEDV infection were reported in countries such as the Czech Republic, Belgium, Hungary, South Korea, China, Italy, and Thailand (5) before emerging as a major swine

outbreak in China around 2010 (6, 7). This outbreak marked the appearance of highly pathogenic strains of PEDV associated with 80–100% morbidity and 50–90% mortality in suckling piglets (8). 2013 was another critical year, seeing the emergence of PEDV in the North American continent (9). More recently, the epidemiology of PEDV has taken a new turn, with China seeing increasing co-infection rates (up to 51%) with PDCoV (10, 11).

Compared to the discovery of PEDV, the first report of PDCoV was fairly recent, being detected in 2012 in Hong Kong during molecular surveillance of coronaviruses in avian and mammalian species (2). To date, PDCoV has been detected in many countries including the United States, Canada, South Korea, China, Thailand, Laos, and Vietnam (12, 13). Clinical severity of PDCoV infection tends to be lower than PEDV, with a mortality rate of around 40% when experimentally inoculated into gnotobiotic suckling piglets (14, 15). Nevertheless, PDCoV still causes severe disease (16). Among diarrheic pigs in the United States and China, the prevalence of PDCoV was found to be as high as 30–7%, respectively of all reported cases (10). Accordingly, PDCoV is an emerging pathogen that warrants further study because there is still little information about deltacoronavirus infection, pathogenesis, and virus-host interaction (17).

Innate immunity functions as the first line of defense against invading viruses. It identifies and alerts host cells to their presence by eliciting rapid and early cellular responses and inducing production of multiple cytokines. Lymphoid-associated tissues (including Peyer's patches, lymphoid follicles, and mesenteric lymph nodes) are the largest and the first barrier against infections of the gastrointestinal (GI) tract (18). Gut-associated lymphoid tissue (GALT)-resident professional antigen presenting cells (APCs) are therefore of particular interest in studying PEDV and PDCoV infection, with APCs such as dendritic cells (DCs) most prominently plasmacytoid DCs (pDCs) which are the major producers of types I interferons (IFNs) *in vivo* during viral infection (19) and macrophages being the first immune cells to encounter PEDV, PDCoV, and other enteric viruses (20).

Enteric coronaviruses possess pathogen-associated molecular patterns (PAMPs) such as viral glycoprotein structures and viral RNAs which can be recognized by pattern recognition receptors (PRRs) present on APCs (21). Recognition events initiate propagation of intracellular signaling, resulting in production of soluble antiviral components of innate immunity. These soluble components are primarily made up of type I and III IFNs, chemokines, and proinflammatory cytokines. Because the IFN pathway is crucial in initiating viral resistance and shaping subsequent adaptive immune responses (22), both PEDV and PDCoV need to evolve mechanisms to antagonize and suppress its induction and signaling in order to establish productive infection. Innate immune cell populations such as natural killer (NK) cells are also known to respond to porcine coronavirus infections and may play a role in disease outcome and pathogenesis (23).

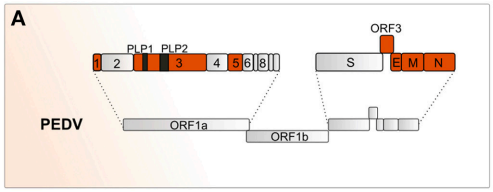
In the following sections, we will describe the relevant aspects of PEDV and PDCoV biology and pathogenesis, and review the fundamentals of antiviral innate immunity. Subsequent sections will provide an update on recent studies regarding host antiviral innate responses as well as key mechanisms and strategies that these porcine enteric coronaviruses have evolved to evade virus recognition by host PRRs, inhibit IFN induction, and block IFN signaling cascades. Finally, we will discuss the potential of harnessing innate immune machineries for the control of enteric coronavirus infection, and implications of this knowledge on development of immune modulators for effective vaccination against these two pathogens.

PEDV AND PDCoV BIOLOGY

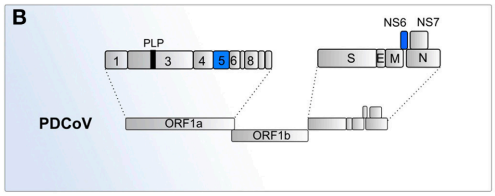
Both PEDV and PDCoV are enveloped viruses with single-stranded positive-sense RNA genomes of ∼28–26 kB in length, respectively (2, 24) Their genome organization is depicted in **Figure 1**. Open reading frame 1a (ORF1a) and ORF1b of both viruses encode two polyprotein precursors, pp1a and pp1ab, which are cleaved by the papain-like protease (PL-pro) and a serine type 3C-like protease (3CLpro) (25) to give rise to non-structural proteins (nsp) 1–16 for PEDV and nsp1–15 for PDCoV (26–28). Many of the individual nsps interact to form the replicase-transcriptase complex (RTC) responsible for viral RNA replication and transcription of sub-genomic RNAs. In addition to these replication functions, some coronavirus nsps are also involved in antagonizing host innate immune responses.

Both PEDV and PDCoV possess four structural proteins, namely spike (S), envelope (E), membrane (M), and nucleocapsid (N). Each virus has a unique set of accessory proteins, however. PEDV has only one accessory protein, ORF3 (29), whereas the PDCoV genome encodes non-structural (NS)6, NS7, and NS7a accessory protein (30, 31). Although distributed widely both within and between structural genes, the location and function of coronavirus accessory protein genes are species-specific (32). In fact, coronavirus accessory proteins possess diverse functions, including modulating viral pathogenicity (33), inducing cell death (34), or antagonizing the IFN system (35–37).

PEDV and PDCoV primarily target the GI tract of pigs, although PEDV has also been found to infect alveolar macrophages of the respiratory tract resulting in pneumonic lesions (38). While the fecal-oral route accounts for the main means of PEDV and PDCoV transmission, vomitus, and contaminated fomites such as transport trailers and feed may also be points of viral transmission (39, 40). Upon host entry via the oral route, porcine coronaviruses bind to surface receptors on susceptible cells, primarily villous epithelial cells of the small intestine brush border (38, 41, 42). In swine, porcine aminopeptidase N (pAPN) which is highly expressed in the small intestinal mucosa was implicated to play a critical role in the target cell infection of PEDV and PDCoV (43, 44). Following cell entry, porcine coronaviruses, similar to most of CoV, initially form double-membrane vesicles (DMVs) where replication/transcription probably takes place, assemble in the rough endoplasmic reticulum and the large virion containing vacuoles (LVCVs), and are transported via the Golgi apparatus for release by budding from the surface membrane of the infected cells (26, 45–48). Infected villous cells are then destroyed, leading to reduction, and shortening of the villi.



PEDV Proteins	Innate Immune Functions	References
nsp1	Antagonizes IFNs; enhances degradation of CBP; impedes nuclear translocation of NF-kB; blocks nuclear translocation of IRF-1; reduces the number of peroxisomes.	(60), (61), (74)
nsp3 (PLP2)	Antagonizes IFN by de-ubiquitination of RIG-I and STING.	(148)
nsp5 (3CLpro)	Antagonizes IFN by cleaving NEMO.	(152)
ORF3	Suppresses IFN induction *in vitro*.	(60)
S	Activates EGFR signaling leading to the impairment of type I IFN activity	(180)
E	Activates NF-kB; induces ER stress; upregulates IL-8 expression; suppresses IFN and IRF3 promoter *in vitro*.	(71)
M	Suppresses IFN and IRF3 promoter *in vitro*.	(60)
N	Activates NF-kB; induces ER stress; upregulates IL-8 expression; inhibits IFN production and ISG expression by interupting IRF and TBK1 binding.	(72), (190)



PDCoV Proteins	Innate Immune Functions	References
nsp5 (3CLpro)	Antagonizes IFN production by cleaving NEMO; inhibites IFN signaling by the cleaving STAT2.	(153), (154)
NS6	Antagonizes IFN production by blocking the recognition of dsRNA by RIG-I or MDA5.	(35)

FIGURE 1 | Both PEDV and PDCoV are enveloped viruses with single-stranded positive-sense RNA genomes of ~28 and 26 kB in length, respectively. **(A)** Within the genome of 28 kB of PEDV, so far seven encoded proteins have been shown to implicate in the innate immune modulation (highlighted here in orange). The first two of the seven open reading frames (ORFs) encode replicase 1a and 1b, respectively which are the two polyprotein precursors of 16 non-structural proteins. The rest of ORFs encode four structural proteins which constitute the virion, and one accessory protein namely ORF3. The structural protein S, E, M, and N as well as ORF3 accessory protein are implicated in the innate immune modulation and suppression (See texts for details). **(B)** Similar to PEDV, the replicase polyprotein 1a (pp1a) and pp1b of PDCoV are also cleaved by virus-encoded proteases into 16 non-structural proteins. The ensuing ORFs; however, encode four structural proteins, as well as two non-structural (NS) accessory protein NS6, NS7, and NS7a. So far, two virus-derived proteins with proposed roles as the innate immune antagonists have been reported (highlighted here in blue).

Because both PEDV and PDCoV target villous enterocytes of the porcine GI tract, establishment of a productive infection requires both penetrating the heavily guarded mucosal barriers and circumventing the host's robust and rapid innate immune response. Although many comprehensive reviews have described how other coronaviruses such as the severe acute respiratory syndrome (SARS)-CoV (49–51), and Middle Eastern respiratory syndrome (MERS)-CoV (52, 53) interact with components of innate immunity, knowledge about how PEDV, and PDCoV antagonize host innate immune responses has only started to emerge. Furthermore, due to the challenges in propagating field isolates in a biologically relevant cell culture system and difficulties in viral genome manipulation, the mechanisms behind porcine enteric virus pathogenesis remain largely unknown.

IN VITRO MODELS FOR PEDV AND PDCoV INFECTION: CELL LINES AND PRIMARY CELLS

Cell lines provide invaluable information on viral pathogenesis and its interplay with the innate immune response. The lack of suitable cell lines is therefore one of the major impediments to progress in the field. For the study of porcine enteropathogenic viruses, for instance, many of the most widely used cell lines are not even derived from natural target cells, namely enterocytes of intestinal villi. As a case in point, the staple cell line for PEDV propagation has been Vero, derived from the kidney of an African green monkey, since the process was first described by Hofmann and Wyler (54). The use of Vero cells, however, is limited to the propagation of cell-adapted PEDV strains. The success rate of expanding new variant and field-isolated PEDV in Vero cells is rather low and often comes at the cost of gradual loss of infectivity during passaging (55). While being permissive to PEDV propagation and replication, these cells have a major deletion in the type I IFN gene cluster, resulting in IFN deficiency (56–59) and thus rendering them unsuitable for studying viral modulation of innate immune responses.

Cell lines such as MARC-145 (African green monkey kidney), LLC-PK1 (porcine kidney), and ST (swine testicle) may be more appropriate for studying PEDV-mediated innate immune modulation. Zhang et al. examined various cell lines for PEDV susceptibility and discovered that the IFN-competent MARC-145 cells were also permissive for PEDV infection, exhibiting cytopathic effects (CPE) and infection foci staining comparable to infected Vero cells (60). Using these cells, they were able to demonstrate the suppression of type I IFN production and

degradation of CREB-binding protein (CBP) by PEDV. They also used LLC-PK1 and ST cells to investigate the role of PEDV nsp1 protein in the inhibition of early NF-κB activation (61).

Other immortalized cell lines permissive for PEDV include PK-15 (porcine kidney), Huh-7 (human liver), MRC-5 (human lung), and Tb1-Lu (bat lung) cells, which were used to examine PEDV receptor usage and cell entry (62). A comprehensive list of both traditional and newly established cell lines currently being tested or permissive for PEDV replication can be found in a recent review by Teeravechyan et al. (63). These cells possess a variety of phenotypes, however, and will need to be carefully vetted before use in studying innate immune responses to PEDV.

Only two immortalized cell lines of swine origin, namely ST and LLC-PK1, are known to be permissive for PDCoV and used for its isolation and propagation (64). At 2 days post-inoculation, PDCoV-infected LLC-PK1 and ST cells become enlarged and rounded, characteristics of PDCoV-associated CPE. While the presence of trypsin in maintenance media helps to improve PDCoV propagation in the LLC-PK1 cell line, its absence does not completely abrogate virus propagation, unlike for ST cells. Additionally, cell culture media supplemented with pancreatin and/or small intestine content (SIC) solution extracted from healthy uninoculated gnotobiotic pigs supported PDCoV propagation in both LLC-PK1 and ST cells. LLC-PK1 has also been used to demonstrate PDCoV antagonism of various host innate immune components (65, 66).

Although the use of these cell lines has provided invaluable information about the interaction between these two enteric coronaviruses and their hosts, it may not yield relevant biological information consistent with *in vivo* PEDV and PDCoV infection because these cells are not derived from pig intestinal epithelial cells (IEC), the known target cells of both porcine coronaviruses. In recent studies, immortalized IECs have been derived by the introduction of the human telomerase reverse transcriptase (hTERT) gene into the neonatal-derived small intestinal epithelial cells (67) and used by many groups for PEDV propagation (68–72). However, only IPEC-J2, a porcine jejunal cell line derived from a neonatal pig, has been used to study how PEDV antagonizes host cell antiviral activity (73). The use of IPEC-J2 cells could provide more biologically relevant information when investigating the pathogenesis of PEDV infection; however, others found that these cells were not always susceptible to PEDV (32). In their study, Zhang et al. claimed that IPEC-J2 cells, in addition to its relative non-permissiveness to PEDV infection, were actually heterogeneous, and that the infection rate achieved by this cell line was extremely low. A new cell line, IPEC-DQ, was thus sub-cloned and characterized for PEDV propagation (74). IPEC-DQ cells were found to support efficient and productive infection of PEDV. Furthermore, due to their ability to express type III IFNs, IPEC-DQ could potentially be used as a suitable cell model for the study of gut innate immunity and its modulation by PEDV. Nevertheless, immortalization and transformation of primary cells may affect cellular antiviral signaling, possibly resulting in misrepresentation of *in vivo* innate immune responses. In fact, a number of cellular pathways regulating IFN-stimulated genes and antiviral defense are closely linked to cellular tumor suppression activity, including anti-proliferative, pro-apoptotic, and pro-inflammatory responses (75). Accordingly, the antiviral responses observed in immortalized IEC or IPEC cells, despite being of porcine intestinal epithelial cell origin, should be further compared to those in primary IECs.

Consistent with this idea, primary porcine IECs were recently isolated and used to propagate PEDV (76). For the first time, primary porcine IECs were used as a model to study the interplay between molecular mechanisms of PEDV infection and the host innate immune response, focusing on the potential mechanism of PEDV-mediated NF-κB activation in particular. Although porcine IECs are the ideal cell type for PEDV and PDCoV research and representative of target cells *in vivo*, these cells are difficult to procure, have a short life span and, unlike immortalized cell lines, could contain a mixed population of different cell types. Ectopic or stable expression of exogenous genes in primary cells is also very difficult due to differences in doubling time and life span of each primary cell type, making clonal selection virtually impossible. Another important technical reason that limits the use of primary IECs in PEDV and PDCoV research is their hypersensitivity to trypsin required for enteric coronavirus propagation in *in vitro* culture (54).

All things considered, porcine IEC-derived immortalized cell lines remain the optimal *in vitro* models for studying the innate immune response to PEDV and PDCoV infection, balancing ease of use with a close approximation to *in vivo* target cells.

OVERVIEW OF INNATE IMMUNE RESPONSES TO VIRAL INFECTION

Mammalian hosts are equipped with innate immune mechanisms which launch immediate responses against viral infection. This first line of defense prevents the establishment of successful infection and systemic spread, and in many cases, destroys invading viruses even before the adaptive arm of host immunity is mobilized. A schematic diagram of the host innate immune signaling pathways is depicted in **Figure 2A**.

Upon viral infection, infected host cells can sense the presence of both viruses and viral products by three main classes of host PRRs (77). These are the endosomal toll-like receptors (TLRs), the cytoplasmic retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and the nucleo-oligomerization domain (NOD)-like receptors (NLRs).

TLRS are found both in the endosomal and cell surface membranes. While signaling mediated by most cell surface-localized TLRs induce only pro-inflammatory cytokine responses and not IFN expression, activation of endosomal TLRs and a plasma membrane-resident TLR4 may lead to both (78). Among the TLRs characterized to date, those localized to endo-lysosomal compartments include TLR3, TLR7, TLR8, and TLR9, with each detecting distinct forms of viral nucleic acids. On the other hand, RLRs and NLRs are cytoplasmic sensors. Three types of RLRs have been identified—retinoic acid-induced gene I (RIG-I), melanoma differentiation associated

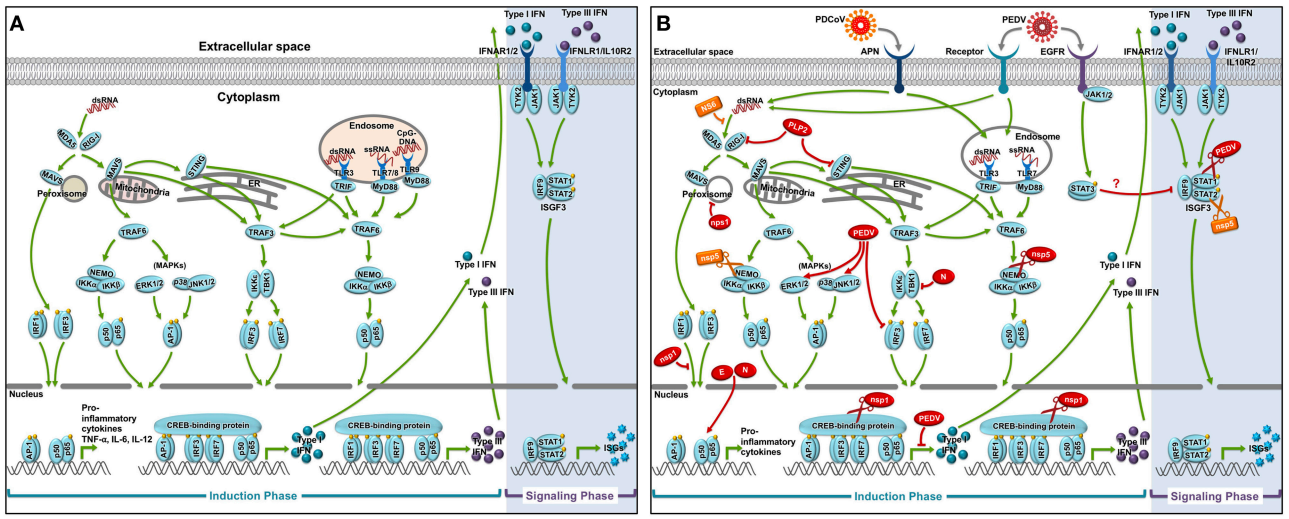


FIGURE 2 | Innate immune signaling pathways and antagonism by PEDV and PDCoV proteins. Following the cellular receptor-mediated entry into the target cells, the genomic RNAs of both PEDV and PDCoV are released into the cytosol by viral-host membrane fusion. During the cytokine induction phase, the presence of the virus-derived RNA genome as well as other replicative RNA intermediates are sensed by both the endosomal TLRs (TLR3, 7/8) and cytosolic RLRs (RIG-I and MDA5). The recognition of the virus-derived RNAs by these receptors triggers a cascade of signaling molecule activation leading to a nuclear translocation of the key transcription factors including NF-κB, IRF1, IRF3, and IRF7. Inside the nucleus, the binding of these transcription factors to their respective PRD regions drives the production of type I and type III IFNs, and pro-inflammatory cytokines which are then secreted into the extracellular space. Subsequently, in the signaling phase, the engagement of both type I and III IFNs to their cognate receptors in both autocrine and paracrine manner induces the activation of JAK/STAT pathway leading to nuclear translocation of the ISGF3 complex as well as the subsequent production of the interferon stimulating genes (ISGs) **(A)**. These ISGs confer the cells with an anti-viral state. In order to ensure the establishment of a successful infection, both PEDV and PDCoV either produce viral proteins (shown in red for those of PEDV and in yellow of PDCoV) to directly antagonize various critical steps of both IFN induction and signaling or affect indirectly the host cell anti-viral signaling cascades **(B)**.

gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP-2) (79). NLRs are mostly associated with recognition of bacterial PAMPs (80, 81) and will not be discussed further in this review.

While both TLRs and RLRs are capable of recognizing viral PAMPs, particularly double-stranded RNA (dsRNA), they utilize different adaptor proteins to initiate their signaling cascade. The TLR signal transduction pathways are dependent on either myeloid differentiation primary response 88 (MyD88) or TIR domain-containing adapter-inducing interferon-β (TRIF) (78, 82). RLRs, on the other hand, utilize the mitochondrial activator of virus signaling (MAVS/IPS-1/VISA/CARDIF) as the essential signaling adaptor protein (79, 83). TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKε) interact to relay signals to the critical transcription factors interferon regulatory factor 3 (IRF3) and NF-κB, leading to their phosphorylation and nuclear translocation (84). Activation of these transcription factors as well as AP-1 then initiate transcription of type I IFNs IFN-α and IFN-β.

The induction of IFN-α and IFN-β is one of the hallmarks of the host innate immune responses against invading viral pathogens. These secreted soluble factors represent a family of antiviral cytokines which, upon binding to their surface heterodimeric receptor (composed of the IFNAR1 and IFNAR2 subunits), leads to the activation of the receptor-associated tyrosine kinases, Janus kinase 1 (JAK1), and tyrosine kinase 2 (Tyk2). These kinases phosphorylate the signal transducer and activator of transcription 1 (STAT1) and STAT2 (85). The phosphorylated STAT1/STAT2 heterodimer then translocates into the nucleus, where it interacts with IRF9 to form IFN-stimulated gene factor 3 (ISGF3). This, in turn, binds to IFN-stimulated response elements (ISRE) in gene promoter regions, leading to the expression of antiviral effectors known as IFN-stimulated genes (ISGs) (86–88). ISGs function to restrict viral replication, modulate other aspects of innate immunity and prime the adaptive immune response (89).

Although type I IFNs and ISGs are the main host antiviral components and act as the first line of defense against viral infection, type III IFNs (such as IFN-λs) have recently been described to contribute to the host antiviral state as well as induce ISG expression (90). Type III IFNs share significant functional similarities with type I IFNs. All IFN-λs bind a heterodimeric IFN-λ receptor complex (IFNLR) for signaling (91). While previous *in vitro* studies demonstrated that types I and III IFNs are co-produced in response to viral infection or the presence of PAMPs, particularly nucleic acids which trigger both extracellular and intracellular sensors, more recent *in vivo* experiments support the observation that mucosal infections appear to trigger predominantly IFN-λ expression and a low level of IFN-β (92, 93). Consistent with this, epithelial cells which protect the GI tract mucosal lining were found to be the main source of IFN-λ production during enteric virus infections (94–96). Indeed, IFN-λ is known to be critical in controlling infection of epithelial cells by various enteric viruses, including norovirus, reovirus, rotavirus, adenovirus, and murine cytomegalovirus (97). Selectively high expression of IFNLRs on IECs in the GI tract argues for the indispensable contribution of type III IFNs to the initiation of early antiviral responses in this organ (95).

Although the induction of types I and III IFN pathways involves a great deal of overlap in the signaling cascade leading to establishment of a cellular antiviral state, there are still differences in transcription factor requirements (90, 98). While IFN regulatory factor (IRF)-3,-7, and NF-κB are essential components for induction of both types I and III IFNs, IRF1 seems to play a unique role in the type III IFN pathway (99). Additionally, unlike RLR-mediated type I IFN induction, intracellular sensors of type III IFN depend largely on peroxisomal MAVS for a rapid but rather short-lived induction of IFN expression (100, 101).

Successful establishment of viral infections generally require the ability to evade, antagonize, or subvert innate immune responses. Indeed, previous studies have shown that PEDV infection inhibits type I IFN induction in several cell types, such as MARC-145 or porcine IECs (60, 76), and exhibits relative resistance to IFN-α by inducing the proteasome dependent degradation of STAT1 (73). These observations suggest that this virus has developed strategies to prevent the biological activities of IFNs. Similarly, PDCoV has also demonstrated antagonism of IFN production in cell culture (66). The antiviral effects of IFN-λs may also play a crucial and as-yet underappreciated role in both PEDV and PDCoV infection. The mechanisms by which PEDV- and PDCoV-encoded proteins modulate components of the IFN induction pathways is summarized in **Figure 2B** and will be discussed later in this review.

CELLULAR INNATE IMMUNE RESPONSES TO PEDV AND PDCoV

In addition to type I and III IFN induction, viral infection also results in the recruitment of innate immune cells such as DCs, macrophages, and natural killer (NK) cells to the site of infection (102). These innate immune cells not only provide immediate counterattacks against invading viruses, but also present foreign antigens to T cells and prime adaptive immune responses via cytokine secretion (103).

DCs and macrophages are the two most prominent cellular components of innate immune responses. Given the essential role of both cell types in professional antigen presentation and immune cell activation, it is important to gain a better understanding of the interaction of coronaviruses with these professional APCs. A study with SARS-CoV demonstrated that although neither macrophages nor DCs were productively infected, many phenotypic changes in cell viability, expression of MHC class II, CD40, CD83, and CD86, and the ability to stimulate T cell proliferation were observed in these cells upon exposure to live virus (51). Macrophages were both refractory to such stimuli and displayed diminished phagocytic activity whereas DCs were observed to display upregulated MHC class II, CD40, CD83, and CD86 expression. As a consequence, these SARS-CoV-primed DCs were able to efficiently stimulate allogenic T cell proliferation.

For PEDV, however, the data are still conflicting regarding the susceptibility of DCs to PEDV infection (104, 105). *In vitro*, the classical PEDV strain CV777, in contrast to SARS-CoV, could productively infect both immature and mature monocyte-derived dendritic cells (Mo-DCs) leading to the enhanced ability of Mo-DCs to sample antigens and present them to T cells for T-cell activation (104). Interestingly, Gao et al. also observed that immature Mo-DCs were more susceptible than mature Mo-DCs to infection by CV777, possibly due to their higher rates of endocytosis and aminopeptidase N (CD13) expression. Furthermore, infected immature Mo-DCs up-regulated CD1a, CD80/86, and SLA-II-DR, which have been shown to enhance the cells' antigen presentation function (106, 107). Up-regulation of CD1a, CD80/86, and SLA-II-DR was observed to a lesser extent for infected mature Mo-DCs. When CV777 infection was studied *in vivo,* the virus was found to rapidly infect intestinal DCs (104). Based on these observations, Gao et al. suggested that both Mo-DCs and intestinal DCs play a role in priming and promoting an effective response during PEDV CV777 infection.

In contrast, a recent study by Wang et al. showed that PEDV failed to undergo a productive replication in porcine Mo-DCs (105). In spite of this, infection activated transcription of type I IFN and chemokine interferon-inducible protein-10 (IP-10). Unfortunately, the molecular mechanisms by which PEDV triggered type I IFN and chemokine IP-10 expression in the absence of active virus replication and the implication of these cytokines in PEDV pathogenesis and immunity remain to be determined.

DCs can also be exploited and hijacked by PEDV as vehicles for viral transmission. A recent study implicated porcine bone marrow-derived DCs in the dissemination of PEDV from the swine nasal cavity to intestinal mucosa (108), supporting the hypothesis that PEDV could be spread from infected pigs through airborne transmission. This study also verified that PEDV could enter porcine nasal epithelial cells (NECs) via their apical side adjacent to the nasal mucosa and establish transient infection within the nasal cavity. Submucosal DCs residing near infected nasal epithelial cells (NECs) then take up PEDV from the lumen across the nasal mucosa via their extended cellular processes. Despite the lack of active viral replication in these DCs, these virus-loaded DCs could subsequently transfer the viruses to T cells which then enter peripheral blood. These recirculating T cells finally shuttle the viruses to the intestinal epithelium, leading to typical PEDV symptoms. The utilization of DCs, which are widely distributed in the mucosal lining of various tissues, by PEDV as carriers to overcome mucosal barriers and disseminate throughout the body is reminiscent of how many other viruses establish a foothold upon entering the host body (109–112).

Similarly, the permissiveness of macrophages to PEDV is still unclear. Lee et al. showed that viral antigen could be detected in lamina propria-resident macrophages of infected pigs (113). There has also been one report of PEDV infection in alveolar macrophages, resulting in pneumonic lesions (38). Despite these observations, detection of viral replication in these cells has yet to be reported.

NK cells are responsible for cytotoxicity-mediated killing of virus-infected cells and are a major source of IFN-γ, TNF-α, GM-CSF, and other cytokines and chemokines (114, 115). The observation that PEDV-infected neonatal and nursing piglets with more severe symptoms possessed lower NK cell numbers

suggest for a potential role for NK cells in the host antiviral response to these pathogens (23). In their study, Annamalai et al. showed that both quantitative and qualitative variation of NK cell properties can be observed in response to PEDV infection in suckling and weaned pigs. In uninfected animals, suckling pigs, which are much more susceptible to PEDV, have drastically lower NK cell numbers than weaned pigs in both the blood and the ileum. Upon infection, significant IFN-γ production is observed from weaned pig NK cells, unlike those of suckling pigs. Strangely, frequencies of NK cells in the blood were found to be higher than in the ileum, the primary site of PEDV infection, and became even more disproportionate during the course of infection. In addition, serum levels of IFN-α, IL-12, and TNF-α peaked at an earlier time point in infected suckling pigs, indicating faster progression of disease, when compared to those of weaned pigs, and coincided with viral shedding and onset of diarrhea in both groups of pigs. Due to these disparate observations, it remains to be seen whether NK cells play a direct antiviral function during PEDV infection.

It is worth pointing out that the increase in serum pro-inflammatory cytokine and chemokine levels in both PEDV infected suckling and weaned pigs mentioned above could reflect the outcome of the simultaneous induction of the key components of the mitogen-activated protein kinase (MAPK) cascade including Erk1/2 and JNK/p38 (116, 117), and the activation of NF-κB pathway (72, 118) during the PEDV infection. As shown in the context of other pathogenic infections (119, 120), the concurrent activation of both MAPK components and the transcription factor NF-κB during PEDV infection might lead to the upregulation of both pro-inflammatory cytokines and chemokines. While the PEDV N protein was described by Xu et al. as a virus-derived intermediate responsible for triggering the NF-κB pathway activation, the exact mechanism of MAPK activation following PEDV infection is still not determined. It was demonstrated, however, that the PEDV-mediated MAPK activation enhanced the viral replication. These findings argue for the notion that PEDV, to its advantage, could manipulate host intracellular processes including the stimulation of the MAPK cascade and the NF-κB pathway.

For PDCoV infections, information remains extremely limited regarding the interplay with the cellular innate immune response. There are some reports describing infiltration of macrophages, lymphocytes, eosinophils, and neutrophils in the lamina propria of the small intestine during infection (14, 121, 122). However, there is still a lack of any evidence regarding whether these innate immune cells actually engage in anti-PDCoV responses and what such responses might be.

PEDV AND PDCoV ANTAGONISTS OF INNATE IMMUNITY

Non-structural Proteins

The coronavirus nsps have been shown to be involved mainly in viral RNA synthesis (123–127). Nevertheless, nsp1, 3, 5, 7, 14, 15, and 16 have been observed to play additional roles in host immune modulatory functions (50, 60, 128–136). Due to the early expression of these non-structural proteins, their ability to suppress innate immune responses provides invading viruses with the opportunity to replicate and establish a productive infection.

PEDV nsp1

Nsp1 is only present in alpha- and betacoronaviruses (2, 137). Despite its relatively small size at 110 amino acids in length, nsp1 shows great genetic sequence variation among alphacoronaviruses (138, 139) which may account for its functional versatility and the ability to interact with a number of host innate immune signaling molecules. Like the SARS-CoV nsp1, PEDV nsp1 was shown to be a potent IFN antagonist interfering with both IRF- and NF-κB-mediated induction of type I and III IFNs (60, 61, 74). These effects occur through either enhancing degradation of or inhibiting nuclear translocation of host key signaling molecules involved in *IFN* gene activation such as the CREB-binding protein (CBP) (60), which forms part of the promoter-binding enhanceosome complex with NF-κB, AP-1 [a complex of activating transcription factor 2 (ATF2) and JUN], and homodimers or heterodimers of IRF3 and IRF7 (140). PEDV nsp1 promotes proteasome-mediated degradation of CBP, which renders *IFNB* gene transcription induction less effective as binding of the enhanceosome to the *IFNB* gene promoter is more stable than any of its components alone (141).

PEDV nsp1 is also known to impede nuclear translocation of NF-κB, affecting not only production of IFN-β but also proinflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-15, and IL-17 (61). This occurs through inhibition of IκBα phosphorylation and its subsequent ubiquitin-mediated degradation, which are required for NF-κB transport to the nucleus where it can then bind to target sequences and initiate transcription (142–144). The activity of nsp1 against IκBα was also found to block nuclear translocation of the p65 (also named RelA) subunit of NF-κB, preventing the dimer formation between RelA and the p50 subunit of NF-κB (p50/RelA) important for NF-κB signaling. Taken together, the PEDV nsp1-mediated inhibition of type I IFNs and pro-inflammatory cytokines through suppression of NF-κB activity argue for the antiviral potential of these cytokines during the early stage of PEDV infection.

PEDV nsp1 also modulates type III IFN responses in IECs (74). In PEDV-infected IPEC-DQ, LLC-PK1, and MARC-145 cells, nsp1 was observed to block nuclear translocation of IRF1 and reduced the number of peroxisomes, where peroxisomal MAVS link RLR signaling to type III IFN induction. Furthermore, the observed reduction in the number of peroxisomes and IRF1-mediated IFN-λ suppression were dependent on the conserved amino acid residues of PEDV nsp1. This intriguing insight into PEDV interference with the type III IFN pathway should pave the way for future studies elucidating the as-yet underappreciated role for IFN-λ in the control of porcine enteritis coronavirus infection.

PEDV nsp3

In coronaviruses, the PLpro domain of nsp3 and the 3CLpro domain of nsp5 facilitate viral replication by processing pp1a

and pp1ab polyprotein precursors into nsps. It has also been demonstrated that many human and animal coronavirus-derived proteases mediate negative regulation of host antiviral innate immunity. Previous studies showed that the PLpro of the human coronaviruses SARS-CoV and NL63-CoV antagonize innate immune induction of type I IFNs via de-ubiquitination/deISGylation of NF-κB signaling molecules, inhibition of IRF3 activation and nuclear translocation, and blocking ubiquitination of STING and disrupting its dimerization (145–147). Xing et al. recently demonstrated that the PEDV PLpro domain, PLP2, also interferes with RIG-I- and STING-mediated type I IFN activation (148) through de-ubiquitinating activity, preventing the post-translational modification of RIG-I by Lys63-linked ubiquitination that is essential for RIG-I-mediated signaling (149). Similar to RIG-I, ubiquitination of STING is critical for expression of downstream antiviral genes (150, 151). Accordingly, PEDV PLP2-mediated de-ubiquitination of RIG-I and STING leads to abrogation of downstream signaling and inhibition of type I IFN expression.

PEDV and PDCoV nsp5

The 3CLpro of both PEDV and PDCoV, encoded by the nsp5 gene, have also been shown to antagonize innate immune signaling through proteolytic cleavage of host key signaling molecules (152–154). Wang et al. provided evidence that PEDV nsp5 disrupts type I IFN signaling by cleaving a critical adaptor protein, the NF-κB essential modulator (NEMO; also called IKKγ), which bridges the NF-κB and IRF signaling pathways by triggering NF-κB and IRF3 nuclear translocation and eventual induction of IFN-β production (79, 155, 156). Highly conserved histidine 41 (His41) and cysteine 144 (Cys144) of PEDV nsp5 were identified as the catalytic dyad responsible for protease activity of nsp5 and suppression of IFN-β induction. Similarly, PDCoV nsp5 also inhibits IFN-β production through the cleavage of NEMO, and its protease activity dominates its ability to antagonize IFN-β induction (154). Interestingly, nsp5 of both PEDV and PDCoV target the glutamine 231 (Q231) of NEMO, suggesting that nsp5 proteolytic cleavage of NEMO is highly conserved and specific in both coronaviruses. This is in contrast to cleavage of NEMO by 3C or 3C-like proteases of other viruses such as the foot-and-mouth disease virus (FMDV), hepatitis A virus (HAV), and porcine reproductive and respiratory syndrome virus (PRRSV) which cleave NEMO at Q383, Q304, and E349, respectively (157–159).

In addition to PDCoV nsp5 inhibition of IFN-β induction, the protein also antagonizes type I IFN signaling downstream of IFN Receptors by targeting the JAK-STAT pathway (153). Zhu et al. demonstrated that PDCoV nsp5 cleaves STAT2, one of the components of ISGF3, disrupting the function of the ISGF3 complex in initiating the ISG-mediated antiviral state. They also discovered that STAT2 cleavage activity is probably unique to PDCoV nsp5, as no other coronavirus nsp5, including those from PEDV, TGEV, SARS-CoV, MERS-CoV, HCoV-229E, HCoV-OC43, and HCoV-NL63, could catalyze STAT2 cleavage. Interestingly, while PEDV infection leads to no cleavage of STAT2, it is capable of both promoting degradation and interrupting activation of STAT1 without inhibiting STAT1 transcription (73). In their study, Guo et al. showed that PEDV infected VeroE6 and IPEC-J2 cells had diminished STAT1 levels when compared to those in uninfected cells. Unlike PDCoV-mediated STAT2 cleavage, PEDV infection-induced STAT1 degradation relies on the ubiquitin-proteasome system. Furthermore, although STAT1 degradation was confirmed in PEDV-infected cells, it remains to be seen whether any PEDV proteins are the main culprits responsible for this process.

Accessory Proteins

Although a great deal of evidence supports the notion that coronavirus accessory proteins function in host–pathogen interactions and mediate viral pathogenesis during coronavirus infection *in vivo* (33, 160), emerging studies have begun to shed light on the interplay between these accessory proteins and the host innate immune system, arguing for their possible role in the regulation of host antiviral responses (161–164). Notable immune regulation activity has been reported for coronavirus accessory proteins 3b (p3b), 6 (p6), and 9b (p9b) of SARS-CoV translated from ORF3, ORF6, and ORF9, respectively; ORF4a and ORF4b of MERS-CoV; and ns2 of mouse hepatitis virus (MHV) (36, 163–170).

The possible role of PEDV's sole accessory protein, ORF3 in host innate immune regulation remains as enigmatic as its role in pathogenesis. So far, only one study has directly implicated the PEDV ORF3 protein in suppression of type I IFN induction *in vitro*, with overexpression of ORF3 resulting in anti-IFN activity as assayed by a luciferase reporter assay (60). Many groups have, however, proposed a role for ORF3 in virus growth and replication (171–175), as some strains of cell-adapted PEDV display either internal truncation or amino acid sequence variation in the ORF3 gene (172, 175, 176). As these changes are generally seen after adaptation to IFN-deficient Vero cells, there is a possibility that future work may link ORF3 more strongly to modulation of the innate immune response.

For PDCoV, three accessory proteins have been identified, namely NS6, NS7, and NS7a (11, 30, 31). The function of these proteins in viral replication, pathogenesis, and immune regulation remain mostly unclear. NS6 has recently been shown to antagonize the host innate immune response (35). Similar to SARS-CoV accessory proteins ORF6 and ORF9b, PDCoV NS6 was identified as being virion-associated and an inhibitor of IFN-β expression (30, 163, 177, 178). NS6 acts by blocking the recognition or binding of dsRNA by RIG-I or MDA5, likely by directly binding to either the C-terminal domain (CTD) of RIG-I or the helicase domain and CTD of MDA-5, or both, as its ability to bind to viral RNA was not observed (35).

Structural Proteins

The C-terminal end of the PEDV and PDCoV genomes encode the structural proteins S, E, N, and M. Among these, ectopic expression of PEDV E, N, and M has been shown to antagonize the IFN-β and IRF3 activity (60). Recent successive publications by Xu et al. also provided details on host cell responses to the presence of PEDV E, M, and N proteins, specifically their effect on cell growth and the cell cycle, ER stress, NF-κB activation, and IL-8 and Bcl-2 expression (71, 72, 179).

PEDV S

In addition to its indispensable role in virus entry into the target cell through receptor binding and subsequent fusion of the viral and cellular membranes, a recent study by Yang et al. revealed the newly discovered role of the PEDV S protein in the impairment of the anti-PEDV activity of type I IFN (180). Yang et al. demonstrated that PEDV (both live and killed) through direct interaction between the S protein and epidermal growth factor receptor (EGFR), induced EGFR activation which, in turn, augmented PEDV infection. They further demonstrated that, by acting via one of its downstream signaling pathways, namely JAK2-STAT3, the EGFR activation helped to enhance, and facilitate PEDV replication. It is worth pointing out that while the roles of EGFR signaling in the cell to cell communication as well as the transformation of various types of cancer were well documented (181, 182), its involvement in facilitating PEDV infections through the suppression of type I IFN-mediated antiviral response is in accordance with previous findings described in studies of other viruses (183–185). Although direct binding of the PEDV S protein to EGFR is sufficient to trigger both the EGFR activation and the attenuation of type I IFN activity, further studies are still needed to identify the underlying mechanisms leading to the crosstalk between both EGFR and type I IFN signals.

PEDV E

PEDV E protein is a small 7-kDa membrane protein encoded by the *E* gene which is located downstream of PEDV ORF1a and ORF1b. The protein plays an important role during coronavirus budding (29). Xu et al. demonstrated that the E protein could induce ER stress in transfected cells through up-regulation of glucose-regulated protein 78 (GRP78), a marker of ER stress, and activation of NF-κB, coinciding with E protein localization to the ER (71). They also speculated that E protein-mediated activation of NF-κB, in turn, would up-regulate expression of the neutrophil chemotactic factor IL-8 as well as the anti-apoptotic protein Bcl2, contributing to both an inflammatory response and persistent PEDV infection. Whether, the effects above recapitulate what really happens in PEDV-infected IECs is still unknown.

PEDV M

Unlike the E protein, PEDV M is equally distributed throughout the whole cell instead of being localized mainly in the ER (179). Compared to overexpression of PEDV structural E protein, which was not found to have any effect on IEC growth and cell cycle, PEDV M altered IEC growth and induced cell cycle arrest in the S-phase via the cyclin A pathway. M protein expression neither promoted IL-8 up-regulation nor NF-κB activation in transfected IECs, probably due to the lack of ER stress-inducing effects.

PEDV N

Among the 20 mature proteins encoded in PEDV genome, the N protein is the most abundant protein in virus-infected cells and acts as a multifunctional protein involved in viral genome organization, virus assembly, cell cycle regulation, apoptosis induction, host stress response, and translational shutoff (186, 187). Similar to the E protein, PEDV N is also localized to the ER. ER subcellular localization of both E and N, but not M, might account for the ability of both proteins to cause ER stress via IL-8 up-regulation and NF-κB activation (188, 189).

Consistent with the induction of the NF-κB pathway in IECs, Cao et al. recently elucidated a possible mechanism for N protein-mediated NF-κB activation. They demonstrated that over-expressing PEDV N protein in IECs mediated NF-κB activation through TLR2, TLR3, and TLR9 pathways as siRNA silencing of these TLRs dramatically blocked PEDV-induced NF-κB activation (118). Xu et al. also discovered that PEDV N not only induced ER stress via up-regulation of IL-8, Bcl-2, and NF-κB activation, but also inhibited cell growth by prolonging the S phase stage of cell cycle and cyclin A degradation (72). Enhancement of NF-κB signaling is thought to be mediated through the immunodominant central region of N (118).

In contrast to the enhancement of NF-κB signaling observed by both Xu et al. and Cao et al. another recent study showed that PEDV N inhibits IFN-β production and ISG expression by competing with IRF3 for TBK1 binding (190). This interaction inhibited both IRF3 activation and the production of type I IFNs. In accordance with this, PEDV N, along with E and M, was shown to down-regulate both IFN-β and IRF3 promoter activity *in vitro* (60). As previously mentioned, by co-transfecting the plasmids expressing PEDV E, M, and N protein with either pIFN-β-luc or pIRF3-Luc plasmid in Hela cells, Zhang et al. observed a down-regulation of both the IFN-β promoter and IRF3-dependent luciferase activity. The results of this study suggest that the IRF3 signaling pathway is interfered in the suppression of the IFN-β production by PEDV E, M, and N protein. Interestingly, this molecular mechanism is distinct from IFN suppression mediated by the N proteins of other coronaviruses such as SARS-CoV, where N blocks an early step in IFN-β production, probably sensor recognition of viral RNA (191), and MHV, where N targets RNase L activity (192).

The discrepancies observed with the effects of PEDV N on IFN-β and NF-κB induction, as well as the distinct molecular mechanisms it uses to modulate innate immune responses point to the possibility that N protein interacts with multiple host signaling molecules involved in various host signaling pathways. Taken together, the fact that N proteins of different coronaviruses employ different mechanisms to interfere with multiple innate signaling pathways clearly demonstrates the adaptability and coevolution of each coronavirus to a specific host and its associated innate immune pressure.

HARNESSING INNATE IMMUNE ANTI-VIRAL ACTIVITY FOR PEDV AND PDCoV DEFENSE

Harnessing fast-acting antiviral mechanisms of innate immunity has shown promising results in combating a variety of pathogenic viruses. Stimulation of TLR signaling pathways via the use of TLR agonists, for example, has been shown to be an effective means for treating certain viral infections. The use

of TLR agonists as innate immune modulators was validated in a study where treatment of vaginal mucosa with a TLR-3 agonist protected mice against genital herpes simplex virus-2 challenge (193). Furthermore, triggering of another endosomal TLR, TLR-7, via systemic administration of a selective TLR7 agonist also elicited anti-hepatitis C virus activity in a clinical setting (194). Direct correlation between such antiviral status and upregulation of IFN production in response to TLR agonist treatment was demonstrated by Cervantes-Barragan et al. where type I IFNs were found to play a dominant role in TLR-mediated antiviral effects (195). In their study, pDCs were identified as the major source of type I IFN when induced through TLR-7 stimulation. While rapid type I IFN production in pDCs was observed following infection with mouse hepatitis virus (MHV), a betacoronavirus, its induction was abrogated in TLR7$^{-/-}$ or MyD88$^{-/-}$ MHV-infected mice, indicating that MHV-mediated type I IFN induction in pDCs was triggered via the TLR7/MyD88 pathway. These observations suggest that the presence of functional type I IFN-producing pDCs in swine GI tracts during exposure to PEDV and/or PDCoV may help to restrict replication of these viruses and thereby regulate the magnitude of clinical severity.

In addition to the induction of antiviral mechanisms via TLR agonists, modulation of the innate immune pathway has also been attempted with synthetic polypeptides harboring innate immune modulatory activities (196). In this study, the recombinant polypeptide N'-CARD-PTD was generated by fusing the N-terminal nuclear localization signal (NLS) of histone H2B, the caspase recruitment domain (CARD) of MAVS, and a protein transduction domain (PTD). Like the TLR agonists discussed previously, these recombinant fusion polypeptides induced strong production of type I IFNs, albeit via a pathway distinct from TLR-mediated signaling. In addition to its potent immunomodulatory function, N'-CARD PTD also augmented immune responses against influenza virus challenge in a mouse model. Whether systemic or local administration of such immunomodulatory polypeptides can restrict PEDV and PDCoV infection in swine is an intriguing question that remains to be explored.

Taking advantage of the knowledge that type I IFNs confer immediate and powerful antiviral responses, several groups of investigators have demonstrated the use of adenovirus type 5 (Ad5) vector-mediated ectopic expression of porcine IFNs or a constitutively active fusion protein of porcine IRF3 and IRF7 [poIRF7/3(5D)] for rapid cross protection against foot-and-mouth disease virus (FMDV) (197–202). While Ad5 vector-based expression of IFNs were found to be potent in the control of FMDV infection, relatively high doses of recombinant Ad5 viruses were required, restricting large-scale application as well as use in emergencies. The efficacy of Ad5 as a biotherapeutic was notably higher when expressing the poIRF7/3(5D) fusion protein, achieving prolonged systemic anti-FMDV activity and upregulation of ISGs in peripheral blood mononuclear cells (PBMC) in inoculated swine (202). The "proof of concept" use of Ad5-poIRF7/3(5D) in the protection of swine against FMDV points to the possibility that GI tract-targeted expression of type I IFNs as well as IRF7/3(5D) could be successfully used to restrict both PEDV and PDCoV. Furthermore, by utilizing similar virus vector-based platforms, regulated, and organ-specific expression of type III IFN could potentially be harnessed to protect against PEDV and PDCoV infection.

The combination of reverse genetics technology to generate recombinant infectious cDNA clones and our growing understanding of viral protein functions in modulating innate immune responses can also lead to the design of more effective candidate vaccines. IFN antagonism by non-structural, structural, and accessory proteins of PEDV and/or PDCoV such as nsp1, nsp3, nsp5, E, M, N, and NS6 can potentially be attenuated by deletion or truncation of these genes, leading to the generation of live attenuated vaccines. Consistent with this idea, disruption or mutation of the SARS-CoV E gene has been a strategy used to generate promising live attenuated SARS-CoV vaccines (203, 204). Furthermore, a TGEV strain with a deleted E gene (TGEV-ΔE) was also put forth as a potential vaccine candidate, demonstrating the ability to target mucosal tissue and induce secretory immunity (205). Despite these observations, the potential of attenuated PEDV and PDCoV carrying a disrupted E gene (or any other genes) as vaccine candidates await further investigation.

Given the critical role of many innate immune mediators, particularly the IFN system, in alleviating severe clinical symptoms, eliminating viral infection, and enhancing vaccine immunogenicity, innate immune machineries may prove powerful tools for tackling a broad range of viral diseases. Indeed, the studies described above accentuate the value and effectiveness of harnessing our knowledge regarding these machineries in antiviral strategies.

CONCLUDING REMARKS

To establish productive infection, invading viruses need to overcome their host's first line of defense, the innate immune response. Though competent and effective in protecting the host against most microorganisms, this response is still susceptible to antagonism and subversion by pathogenic viruses. Porcine enteritis coronaviruses PEDV and PDCoV, which have recently emerged as important swine pathogens, have evolved strategies to overcome host innate immunity by either avoiding being recognized by PRRs, inhibiting IFN induction, or antagonizing IFN signaling and antiviral effector machinery. While current research has provided copious amounts of invaluable data on how other coronaviruses such as SARS-CoV target molecules involved in the host innate immune response, studies dedicated to host-PEDV and PDCoV interaction have just started to gain traction.

Although both PEDV and PDCoV target pig enterocytes in the intestinal villi, the most common *in vitro* cell culture systems used to study these viruses are not derived from porcine IECs. Both newly derived porcine IEC lines and the availability of three-dimensional intestinal organoids will undoubtedly serve as alternative and more physiologically relevant models for future studies of PEDV- and PDCoV-host interaction. Furthermore,

more in-depth study of PEDV and PDCoV pathogenesis *in vivo* will provide less biased data to identify novel host innate immune modulators in the context of viral infection.

Due to their prominent early antiviral function, much attention has been dedicated to type I IFNs. While the defensive roles of type I IFNs in PEDV and PDCoV infection are indisputable, the importance of type III IFNs cannot be ignored. As type III IFNs are selectively expressed by epithelial cells of the intestinal villi in response to viral infection, its roles in anti-PEDV and PDCoV responses warrant a more thorough investigation.

The development of novel and effective mucosal adjuvants and delivery systems may be key to successful PEDV and PDCoV vaccine design for the induction of mucosal immunity, lactogenic immunity and possibly active immunity in newborn piglets. Ultimately, deeper understanding of host early anti-PEDV and PDCoV response will help pave the way to harness our understanding of innate immunity for the development of therapeutic interventions and novel antiviral compounds.

AUTHOR CONTRIBUTIONS

SK, ST, and AJ contributed conception and design of the study. SK wrote the first draft of the manuscript. ST, PF, and TC wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approve the submitted version.

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REVIEW
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Porcine Hemagglutinating Encephalomyelitis Virus: A Review

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The porcine hemagglutinating encephalomyelitis virus (PHEV) is classified as a member of genus *Betacoronavirus*, family *Coronaviridae*, sub-family *Cornavirinae*, and order *Nidovirales*. PHEV shares the same genomic organization, replication strategy, and expression of viral proteins as other nidoviruses. PHEV produces vomiting and wasting disease (VWD) and/or encephalomyelitis, being the only known neurotropic coronavirus affecting pigs. First clinical outbreak was reported in 1957 in Ontario, Canada. Although pigs are the only species susceptible to natural PHEV infections, the virus displays neurotropism in mice and Wistar rats. Clinical disease, morbidity, and mortality is age-dependent and generally reported only in piglets under 4 weeks old. The primary site of replication of PHEV in pigs is the respiratory tract, and it can be further spread to the central nervous system through the peripheral nervous system via different pathways. The diagnosis of PHEV can be made using a combination of direct and indirect detection methods. The virus can be isolated from different tissues within the acute phase of the clinical signs using primary and secondary pig-derived cell lines. PHEV agglutinates the erythrocytes of mice, rats, chickens, and several other animals. PCR-based methods are useful to identify and subsequently isolate animals that are actively shedding the virus. The ability to detect antibodies allows producers to know the status of first-litter gilts and evaluate their risk of tier offspring to infection. PHEV is highly prevalent and circulates subclinically in most swine herds worldwide. PHEV-related disease is not clinically relevant in most of the swine-producing countries, most likely because of dams are immune to PHEV which may confer passive immunity to their offspring. However, PHEV should be considered a major source of economic loss because of the high mortality on farms with high gilt replacement rates, specific pathogen-free animals, and gnotobiotic swine herds. Thus, in the absence of current PHEV vaccines, promoting virus circulation on farms with early exposure to gilts and young sows could induce maternal immunity and prevent disease in piglets.

Keywords: porcine hemagglutinating encephalomyelitis virus, coronavirus, vomiting and wasting disease, encephalomyelitis, nidovirus

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INTRODUCTION

The porcine hemagglutinating encephalomyelitis virus (PHEV) is the causative agent of neurological and/or digestive disease in pigs. PHEV was one of the first swine coronaviruses identified and isolated, and the only known neurotropic virus that affects pigs. However, PHEV remains among the least studied of the swine coronaviruses because of its low clinical prevalence reported in the swine industry worldwide. PHEV can infect naïve pigs of any age, but clinical disease is age-dependent. Clinical manifestations, including vomiting and wasting and/or neurological signs, are age-related, and generally reported only in piglets under 4 weeks old. Subclinical circulation of PHEV has been reported nearly worldwide in association with a high seroprevalence in swine herds. Protection from the disease could be provided through lactogenic immunity transferred from PHEV seropositive sows to their offspring in enzootically infected herds. However, PHEV still constitutes a potential threat to herds of high-health gilts, as evidenced by different outbreaks of vomiting and wasting syndrome and encephalomyelitis reported in neonatal pigs born from naïve sows, with mortality rates reaching 100%. In absence of effective vaccine, the best practice for preventing clinical disease in suckling piglets could be ensuring that gilts and sows are PHEV seropositive prior to farrowing.

TAXONOMY, GENOMIC STRUCTURE, AND MORPHOLOGY

Coronaviruses (CoVs) belong to the Nidovirales order, which includes the *Coronaviridae*, *Arteriviridae*, and *Roniviridae* families. The subfamily *Cornavirinae* is further divided into four genera: *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus*, and *Deltacoronavirus*. Coronaviruses are enveloped and pleomorphic positive-sense RNA viruses, characterized by club-like spikes projected from their surface, a large RNA genome, and a unique replication strategy (1). The overall diameter of CoVs can range from 60 to 160 nm as demonstrated by negative-staining electron microscopy (EM) (2). The phospholipids and glycolipids incorporated into the virus envelope are derived from the host cell cellular membranes, and therefore the envelope composition is host cell-dependent (3). Most CoVs have a single layer of club-shaped spikes (S protein) 12–25 nm in length, but PHEV and some other betacoronaviruses have a second, shorter layer of surface spikes, the hemagglutinin-esterase (HE) protein (4).

Swine CoVs present the same genomic organization, replication strategy, and expression of viral proteins as the rest of the members of the Nidovirales order (1, 3–6). Overall, the genomic RNA (25–30 kb) is large, of positive-sense polarity, and single-stranded with a large replicase gene followed by structural and non-structural or accessory genes. The genome contains a 5′ cap structure and a 3′ poly (A) tail, acting as an mRNA for translation of the replicases. The non-structural proteins encoded by the replicase gene (∼65 kDa) constitutes two-thirds of the genome, while the genes that encode the structural and accessory proteins compose approximately 10 kb of the viral genome. The 5′ end of the genome presents a leader sequence and untranslated region (UTR) required for replication and synthesis of viral RNA. Additionally, there are transcriptional regulatory sequences (TRSs) in the 3′ end of the structural and accessory genes that are required for gene expression.

Most CoVs contain four structural proteins: a large surface spike glycoprotein (S; 180–200 kDa) visible as the corona, a small membrane protein (E; 8–10 kDa), a transmembrane glycoprotein (M; 20–30 kDa), and a nucleocapsid protein (N; 50–60 kDa). The differences in the number, type, and sizes of the structural proteins are responsible for significant structural differences of the nucleocapsids and virions among Nidoviruses. However, hemagglutinating coronaviruses like PHEV also possess an envelope-associated glycoprotein, the hemagglutinin-esterase (HE; ∼140 kDa), which is made of two subunits (∼65 kDa each) linked together by disulfide bonds (7, 8).

The M protein is the most abundant structural envelope protein that contributes to the virion's shape (9). Studies on severe acute respiratory syndrome-associated coronavirus (SARS-CoV) indicated that the M protein contains three transmembrane domains, a small N-terminal glycosylated ectodomain and a much larger C-terminal endodomain (10). More recent reports suggest the M protein has a dimeric conformation and adopt two different tridimensional morphologies that contribute to membrane curvature and nucleocapsid binding (11). In transmissible gastroenteritis virus (TGEV), the hydrophilic N terminus contain a single accessible glycosylation site that is responsible for interferon induction (12). Epitopes on the protruding N- and C-terminal ends of the M protein of TGEV bind complement-dependent neutralizing monoclonal antibodies (MAbs) (13, 14).

The trimeric S protein is a class I fusion protein (15) that, in most but not all CoVs, can be structurally or functionally divided into two subunits: S1 (N-terminal globular head), which is heavily N-linked glycosylated and has binding activity to the host cell receptors, and S2 (C-terminal membrane-bound stalk), which is responsible for membrane fusion (16–20). Contrary to the conserved S2 subunit, the S1 subunit is the most heterogeneous among species of a single coronavirus, conferring host range specificity, whereas the S2 subunit is the most conserved region of the protein. The homotrimeric structure of the S protein is responsible for the distinctive "corona-like" spike structure of the virion (21). A small region of the PHEV S protein interacts with the neural cell adhesion molecule (NCAM, also known as CD56) expressed on the surface of the neurons (22), playing a role during the infection of PHEV neurons (23). Moreover, the S protein contains major antigenic and antiviral neutralizing determinants, which make it a potential target for development of vaccines and antibody-based diagnostic tools.

The E protein is the less abundant protein of the virion, and it is highly divergent among CoVs. These protein features could explain the lack of precise information related to its specific role during the infection and/or pathogenesis processes. The protein E amino acid sequence is highly conserved among swine CoVs (24). Fehr and Pelman (1) suggested that this protein is a transmembrane protein, with an N-terminal ectodomain,

a C-terminal endodomain, and ion channel activity (25). The E protein has a role in the assembling and releasing of the virions from infected cells (26). Recent studies, compiled and reviewed by Ruch et al. (26), have expanded our knowledge on the role of the E protein beyond assembling, including viral nuclear egress and induction of the host stress response. However, recombinant viruses lacking the E protein (e.g., SARS-CoV) probed not to be lethal; although, this outcome could be virus type-dependent (27).

The N protein is the most abundant coronavirus antigen produced during the course of the infection (28), and it is the only viral protein in the nucleocapsid that interacts with viral RNA to form a helical ribonucleoprotein complex. This structure, in association with the M protein, forms an internal icosahedral core within the virion helping the genome integration to the replicase-transcriptase complex during viral genome encapsidation, and subsequent formation of viral particles (29). The soluble N protein is composed of two independent N- and C-terminal RNA-binding domains (1). The N protein phosphorylation has been associated with structural changes that enhance the affinity of viral RNA compared to non-viral RNA (1, 30).

Related hemagglutinin-esterases (HEs) are also found in influenza C, toroviruses, and CoVs, likely because of relatively recent lateral gene transfer events (31). The HE protein, only present in a subset of betacoronaviruses, acts as a hemagglutinin, binds sialic acids on surface glycoproteins, and contains acetyl-esterase activity (32). The HE protein is associated with granular projections located near the base of the typical large bulbous peplomers and displays hemagglutinating (HA), acetyl-esterase (AE) or receptor-destroying enzyme (RDE) activity (7). More specifically, the isolated HE-protein from PHEV and bovine coronavirus exhibits receptor-destroying and receptor-binding activity (33). The HE protein could facilitate viral cell entry and virus spreading through the interaction with S protein (34). Interestingly, HE enhances the neurovirulence of the murine hepatitis virus (MHV) (35) but not *in vitro* (36).

GENERAL OVERVIEW OF SWINE CORONAVIRUSES

Swine CoVs are represented within three genera of the *Coronaviridae* family. Five swine CoVs have been identified, including TGEV, first described in 1946 (37); PHEV, isolated in 1962 (38); porcine epidemic diarrhea virus (PEDV), isolated in 1977 (39); porcine respiratory coronavirus (PRCV), a spike (S) gene deletion mutant of TGEV isolated in 1984 (40); and porcine deltacoronavirus, detected in 2012 (41). In addition, a TGEV/PEDV recombinant virus has been identified in swine in Europe (42–44), and a bat-HKU2-like Alphacoronavirus has been identified in swine in China (45, 46). For each swine CoV, only a single serotype is recognized (**Table 1**).

Swine coronaviruses show different tissue tropisms, including the gastrointestinal and respiratory tracts, the peripheral and central nervous systems, and the mammary glands (**Table 1**). The alphacoronaviruses TGEV and PEDV and deltacoronavirus produce mild to severe or fatal enteric disease (47). The alphacoronavirus PRCV infects the upper respiratory tract, trachea, tonsils, or lungs, with limited intestinal replication, but the asymptomatic or subclinical form occurs most frequently (48). The betacoronavirus PHEV produces vomiting and wasting disease (VWD) and/or encephalomyelitis (4).

HISTORY OF THE EMERGENCE OF PHEV

In the fall of 1957, a disease affecting nursery pigs characterized by high morbidity, vomiting, anorexia, constipation, and severe progressive emaciation was reported in Ontario, Canada (49). Subsequently, different outbreaks of a virus-like encephalomyelitis affecting neonatal pigs were systematically reported in Ontario between 1958 and 1961 (50, 51). Piglets remained clinically normal until 6 or 7 days of age, when animals started to show clinical signs, including reluctance to nurse, shivering, huddling, and squealing, followed by neurological signs including vomiting, ataxia, hyperesthesia, incoordination, and paddling. These symptoms were followed by death 2–3 days after the onset of the clinical signs. The etiologic agent of this clinical syndrome was named "hemagglutinating encephalomyelitis virus" because of its hemagglutinating properties. This virus was first isolated in primary pig kidney (PK) cells from the brains of 7–8 days old piglets showing histopathological evidence of viral polioencephalomyelitis, including perivascular cuffing with mononuclear cells, neuronal degeneration, and gliosis (38). Milder transient clinical signs such as anorexia, shivering, loss of body condition and vomiting without signs of encephalomyelitis were reported in 4 weeks old piglets from the same farms. This alternative clinical presentation was named "vomiting and wasting disease" (VWD). Shortly thereafter, it was determined that the same virus was the cause of the disease characterized by vomiting and wasting concurrently reported in Europe (52–54) and other regions in Canada (55). During the first investigations, the viral diagnosis was based on three criteria: formation of multinucleated giant cells in PK cells, hemagglutination of chicken erythrocytes in culture fluids, and inhibition of hemagglutination in hyper-immune anti-serum.

Originally, the virus was mistakenly associated with a *Myxovirus*/*Paramyxovirus* group (55). The virus was finally classified as a coronavirus in 1971 (56, 57). Specifically, PHEV belongs to the genus *Betacoronavirus* of the family *Coronaviridae* (group 2a) in the order *Nidovirales* (58). PHEV is closely related to canine, bovine, murine, human and equine coronaviruses, as well as rat sialolodacryoadenitis coronaviruses (6). The virus agglutinates the erythrocytes of mice, rats, chickens, and several other animals (59). Pigs are the only species naturally infected by PHEV, which do not constitute a hazard to human health. PHEV is the only known neurotropic coronavirus affecting pigs and is a potential threat to herds of high-health gilts. Likewise, the virus displays neurotropism in mice and Wistar rats (60, 61). Although PHEV-related diseases have different clinical manifestations, only one PHEV serotype has been described to date. PHEV can infect naïve pigs of any age, but clinical disease, morbidity, and mortality are age-dependent. Age-related susceptibility of the

TABLE 1 | Overview of clinical signs and lesions caused by different porcine coronaviruses.

Genus	Virus	Clinical signs	Lesions
Alphacoronavirus	Porcine Epidemic Diarrhea Virus (PEDV)	Enteric, diarrhea	Atrophic enteritis
	Transmissible Gastroenteritis Virus (TEGV)		
	Swine enteric coronavirus(CSeCoV)		
	Swine acute diarrhea syndrome coronavirus (SADS-CoV) (SeACoV)		
	Porcine Respiratory Coronavirus (PRCV)	Respiratory	Interstitial pneumonia and bronchiolar hyperplasia
Betacoronavirus	Porcine Hemagglutinating Encephalomyelitis Virus (PHEV)	Neurological and Digestive	Lymphoplasmacytic perivascular cuffing brain and stomach muscularis and submucosa
Deltacoronavirus	Porcine Delta Coronavirus (PDCoV)	Enteric, diarrhea	Atrophic enteritis

pigs, possible strain differences in virulence, and variation in pathogenesis may influence clinical signs (4).

GLOBAL DISTRIBUTION AND EPIDEMIOLOGY OF PHEV INFECTION

Serologic surveys (1960–1990) have demonstrated that PHEV is highly prevalent and circulates subclinically in most swine herds worldwide. Viral circulation is maintained in herd populations by continuous flow management, and pigs can be infected vertically from sows to neonates or by comingling at weaning (4). However, there have been only a few reports of clinical outbreaks of VWD or PHEV-associated mortality since the virus's 1958 discovery in Canada (49). Clinical cases have been reported in Canada (62), Belgium (59), China (63–65), Argentina (66, 67), South Korea (68), and the United States (69). Additionally, PHEV circulation in Japan was demonstrated through serological surveys (70).

The current worldwide seroprevalence of PHEV is mostly unknown. A recent seroprevalence study determined the seroprevalence of PHEV in sow herds in the US (71). A total of 2,756 serum samples of reproductive animals (>28 weeks-old) from farms with no history of neonatal VWD or outbreaks of neurological signs during 2016 were included in this study. Samples represented 104 farms from 19 swine production states. The overall seroprevalence detected was 53.34% (CI ± 1.86). The between-farm prevalence was 96.15% (CI ± 3.70). This study further demonstrated that PHEV is circulating subclinically in the U.S. swine population.

Likewise, a serological survey was performed on farms with different grades of biosecurity in Argentina (67). A total of 961 serum samples collected from 14 breeding herds and three farrow-to-finish farms were evaluated. Samples were collected from 30 randomly selected gilts, sows or growing/fattener pigs. The overall seroprevalence was 41.62% (CI ± 3.12). Among positive farms, the within herd prevalence varied from 12.5 to 86.6% for sows, 25 to 85.7% for gilts, and 3.7 to 90% for grower/fattener pigs. No statistical differences in seroprevalence as it pertained to age category or biosecurity status were observed. The presence of antibodies in grower/finisher pigs suggested that colostral antibodies may persist for more than 6 weeks or, alternatively, that the animals were subclinically infected during the grower-finisher stage. This survey demonstrated that PHEV is widespread and is undergone subclinically in Argentina.

It is generally accepted that only piglets under 3–4 weeks of age born from PHEV naïve dams are susceptible to PHEV-associated disease (72). Older pigs do not usually develop clinical disease. The presence of persistently infected subclinical carriers has not been fully demonstrated. Since PHEV is endemic in most swine populations, most dams are immune to PHEV and can confer passive immunity to their offspring. Thus, clinical outbreaks are rare and limited to litters from PHEV naive gilts or low-parity sows. In fact, there are only three major outbreaks described to date. In 2001, PHEV was isolated from newborn and early-weaned pigs with vomiting and posterior paralysis in Quebec (62), and in 2002 a 650-sow genetic nucleus in Ontario suffered an outbreak of VWD (73). In 2006 a VWD outbreak with motor disorders and high mortality, affecting a three-site herd with 6,000 sows and 55% replacement rate, was reported for the first time in Argentina (66).

CLINICAL DISEASE

PHEV can infect naïve pigs of any age, but clinical disease is variable and dependent on age, possible differences in virus virulence (74), and the course of viral pathogenesis. In growing pigs and adults, PHEV infection is subclinical, and animals develop a robust humoral immune response against the virus (66, 75). Exceptionally, transient anorexia (1–2 days) was reported in PHEV-infected sows in absence of other clinical signs (55). An experimental study performed on 7 weeks old pigs reported transient mild neuromotor signs, including tremor and generalized muscle fasciculation in 17% (2/12) of pigs between 4 and 6 days after oronasal inoculation (75). Acute outbreaks of VWD and encephalomyelitis have been reported in piglets under 3–4 weeks of age born from naïve sows, with mortality rates reaching 100%. The first signs of infection are generally non-specific and may include sneezing and/or coughing because of virus replication primarily occur in the upper respiratory tract; followed by transient fever that may last for 1–2 days. More specific clinical manifestations may appear

between 4 and 7 days after infection and are characterized by (1) VWD and (2) neurological signs including tremor, recumbency, padding opisthotonus, and finally death. Both clinical forms can be observed concurrently in the same herd during an acute outbreak. More recently, PHEV was associated with a case of influenza-like respiratory illness in a swine exhibition in Michigan, USA, in 2015 (76). Although PEHV can replicate in the respiratory epithelium, the role of PHEV as respiratory pathogen has not yet been confirmed and needs further investigation.

The VWD was experimentally reproduced and reported for the first time in 1974 (59) in colostrum-deprived (CD) pigs by oronasal and intracranial inoculation. Mengeling et al. (74) experimentally reproduced both clinical forms of the disease in neonatal pigs inoculated with a field virus isolate. Later, Andries et al. (77) evaluated the clinical and pathogenic outcomes with different routes of inoculation. In this experiment, all piglets inoculated oronasally or via the infraorbital nerve showed signs of VWD 5 days after the inoculation. However, a high percentage of animals inoculated through the stomach wall, intramuscularly, and intracerebrally showed VWD signs 3 days after inoculation. Pigs inoculated intravenously, intraperitoneal or in the stomach lumen did not show PHEV-associated VDW signs.

Suckling piglets experiencing PHEV-associated VWD show repeated retching and vomiting, which could be centrally induced (4, 49, 59, 73). The persistent vomiting and decreased food intake result in dehydration, constipation, and therefore a rapid loss of body condition. PHEV-infected neonates become severely dehydrated after few days, exhibit dyspnea, cyanosis, lapse into a coma, and die. During the acute stage of VWD outbreaks, some pigs may also display neurologic signs, including muscle tremors, hyperesthesia, excess physical sensitivity, incoordination, paddling, paralysis, and dullness (68). When the infection occurs in older pigs, there is anorexia followed by emaciation (**Figure 1**). They continue to vomit, although less frequently than in the acute stage. After the acute stage, animals start showing emaciation ("wasting disease") and often present distension of the cranial abdomen. This "wasting" state may persist for several weeks after weaning, which in most cases requires euthanasia.

Pre-weaning morbidity varies depending on the immune status of neonatal litters at the time of PHEV infection (4, 74). In piglets without lactogenic immunity against PHEV, morbidity is litter-dependent and may approach 100% when the infection occurs near birth. Overall, morbidity decreases markedly as the pig's age increases at the time of PHEV infection. Mortality is variable, reaching up to 100% in neonatal litters born from PHEV naïve dams. However, a different epidemiological picture was observed in the outbreak reported during the winter of 2006 in Argentina (66) where only suckling pigs born from an isolated pool of non-immune gilts were affected. The severity of the main clinical signs reported, including vomiting, emaciation, wasting, and death was unexpected according to previous reports in the field (73). The morbidity was 27.6% in 1 week old pigs and declined to 1.6% in 3 weeks old pigs. After weaning, 15–40% of the pigs coming from affected farrowing units showed wasting disease. An estimated 12.6% (3,683) pigs died or were euthanized (66).

FIGURE 1 | A group of pig between 5 and 7 week old severely wasted and poor body condition. Note in the same pen there is commingled litters with clinical affected and unaffected pigs (Credit Dr. Perfumo and Dr. Quiroga, College of Veterinary Medicine, Universidad Nacional de la Plata. Argentina).

The first clinical signs observed during neurological PHEV outbreaks include sneezing, coughing, and vomiting 4–7 days after birth, with a morbidity rate of approximately 100% (4, 78, 79). Mild vomiting may continue intermittently for 1–2 days. In some outbreaks, the first sign is acute depression and huddling. After 1–3 days, pigs exhibit various combinations of neurological disorders. Generalized muscle tremors and hyperesthesia are common. Pigs may have a jerky gait and walk backwards, ending in a dog-sitting position. They become weak and unable to rise, and they paddle their limbs. Blindness, opisthotonus, and nystagmus may also occur. Finally, the animals become dyspneic and lie in lateral recumbency. In most cases, coma precedes death, with a mortality rate of 100% in neonatal pigs (4). Older pigs show mild transient neurological signs, including generalized muscle fasciculation and posterior paralysis. Outbreaks described in Taiwan (65) in 30–50 days old pigs were characterized by fever, constipation, hyperesthesia, muscular tremor, progressive anterior paresis, posterior paresis, prostration, recumbency, and paddling movements with a morbidity of 4% and a mortality of 100% at 4–5 days after the onset of clinical signs.

In non-swine species, PHEV-related disease only has been induced experimentally. It was also demonstrated that suckling mice (3 days old) were susceptible in a dose- and age-dependent manner to PHEV infection through intracranial inoculation, showing neurological signs and dying (80).

PATHOGENESIS AND GROSS AND HISTOLOGICAL LESIONS

The primary site of replication of PHEV in pigs is the respiratory tract, which may result in mild or subclinical disease (76, 77, 81–83). Immunofluorescence testing revealed that epithelial cells of nasal mucosa, tonsils, lungs, and some unidentified cells in the small intestine can be infected (63). Experimental studies

using CD piglets, inoculated oronasally with PHEV, provided relevant information regarding PHEV pathogenesis (59, 77, 84). PHEV can spread from the primary sites of replication through the peripheral nervous system to the central nervous system. Primary viral replication in the nasal mucosa and tonsils allows the virus to spread to the trigeminal ganglion and brainstem trigeminal sensory nucleus. Viral spreading through the vagal nerve also allows the virus to infect the vagal sensory ganglion and brainstem vagal sensory nucleus. The virus can also spread peripherally from the intestinal myenteric plexuses to the local sensory ganglia of the spinal cord. Electron microscopy yielded the discovery of viral particles within nerve cells, moving from the periphery through the cell cytoplasm to reach the axon (85). However, viral particles could not be found in surrounding glial or in inflammatory cells (85).

After peripheral viral spreading, the virus infects well-defined nuclei of the medulla oblongata progressing to the brainstem, spinal cord, and occasionally cerebrum and cerebellum. Immunofluorescence staining in the brain revealed that the infection is always restricted to the perikaryon and processes of neurons (81). Vomiting is induced by viral replication in the vagal sensory ganglion (ganglion distale vagi) or by impulses of the vomiting center induced by vagal ganglia infected neurons (77). It has been suggested that virus-induced lesions in the myenteric plexus of the stomach that may contribute to gastric stasis and delayed stomach emptying (77).

Despite the fact that swine is the only species susceptible to PHEV natural infection, laboratory rodents such as mice (80, 86–91) and rats (92, 93) have been used as an alternative animal model for PHEV pathogenesis investigation. Ultrastructural studies in rats provided insights into neural pathogenesis, in which PHEV antigen was found in the ipsilateral dorsal root ganglions (DRGs) 3 days after peripheral inoculation into the rats' footpads (94, 95). Additional studies demonstrated that PHEV budded from endoplasmic reticulum-Golgi intermediate compartments in the cell bodies of infected neurons, and the assembled progeny viruses were vesicle-mediated, secreted, and taken up by the adjacent satellite cells (92). Cell damage surrounding satellite cells could be observed later during infection, with viral particles contained in vesicles and lysosomes. It has been demonstrated that PHEV replicates only in the cytoplasm of sensory neurons (93). Progeny virions were released through an exocytic pathway, and PHEV viral particles accumulate in dilated extracellular spaces between satellite cells. The non-neuronal cells can engulf these released virions; however, no viral particles were observed in their cytoplasm (93).

In mice, intracranial inoculation with PHEV produced multifocal cortical necrosis in the cerebral (80). Virus replication occurs in the neuron's cytoplasm (96), and specific immunofluorescence and electron microscopy the supported detection of viral particles. The virion is assembled in the rough endoplasmic reticulum and Golgi body before utilizing a membranous coating mechanism to spread through trans-synaptic communication (96). Neurological signs and fatal PHEV infection could be prevented after injection into the footpad by cutting the ipsilateral sciatic nerve 1 h after infection (93). Like the rabies virus, PHEV viral particles were found in peripheral axons and trans-synaptic spread between neurons through endo- and exocytosis, allowing PHEV to move from the periphery to the central nervous system (93). More recent studies in mice demonstrated that PHEV is involved in post-transcriptional regulation, and contributed to central nervous system dysfunction by spatiotemporal control of host microRNAs (97).

In vitro studies indicated that PHEV enters nerve cells via clathrin-mediated endocytosis in a dynamin-, cholesterol-, and pH-dependent manner that requires the GTPases Rab5 and Rab7, which are the primary regulators of the vesicular trafficking pathways (97). The cytopathic effect and mechanism-inducing cellular death in PHEV-infected pig kidney (PK)-15 cells could be attributed to a caspase-dependent pathway (98). During its replication, PHEV induces enzymatic activity of cellular proteases, cysteine-dependent proteinase, or caspase activation by enzymatic cleavage, allowing spread to neighboring cells and limiting host response. However, the specific mechanisms of caspase activation remain unknown. In addition, PHEV infection can block the phagosome and lysosomes fusion, inducing an atypical autophagy response necessary for viral replication in neurons (99). Furthermore, PHEV genome replication in PK-15 cells and; therefore, the production of infectious virus *in vitro* can be inhibited through small interfering RNAs (siRNA) that target different regions of the PHEV spike glycoprotein (100) or nucleocapsid genes (101).

A post-mortem examination of PHEV-affected animals revealed cachexia, a dilatated stomach containing abundant non-digested milk, and distension of the abdomen in some chronically affected piglets (54) (**Figure 2**). Otherwise, no other significant gross findings were normally observed.

Microscopic examination of brains of clinically affected piglets showed a non-suppurative viral-type encephalomyelitis, characterized by lymphoplasmacytic perivascular cuffing (**Figure 3**), mononuclear cells' infiltration in the gray matter of the cerebrum and neuronal degeneration, affecting the mesencephalon, pons, medulla oblongata, horns of the proximal spinal cord, and trigeminal ganglia (**Figure 4**) (69, 85, 102). These lesions were found in 70–100% or 20–60% of animals showing neurological signs or VWD, respectively (65, 103, 104). Microscopic changes in the stomach wall were found only in pigs showing VWD. The lesions were most pronounced in the pyloric gland area (54). Degeneration of the ganglia of the stomach wall and lymphoplasmacytic perivascular cuffing were present in 15–85% of affected animals (**Figure 5**). However, no pathognomonic or histologic examination of acutely affected piglets revealed epithelial degeneration and mononuclear inflammation in the tonsils and respiratory tract (69, 105).

DIAGNOSTICS

A diagnosis of PHEV can be achieved by a combination of direct and indirect detection methods. Methods for the direct detection of PHEV in the tissues of clinically affected animals include immunohistochemistry in sections of the brain, spinal cord and myenteric plexus (64, 66, 106). Tonsils and lungs dissected

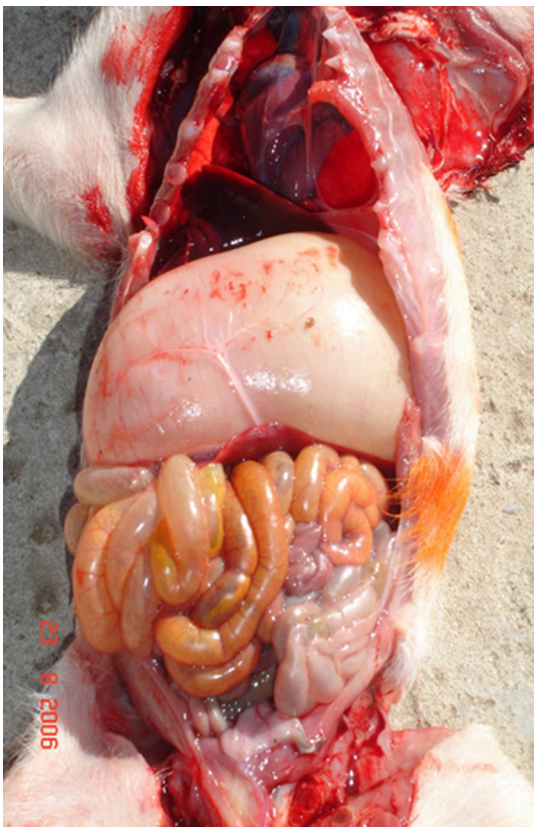

FIGURE 2 | Post mortem examination in a 5 week old pig showed severe gastric distention associated with abundant ingesta (Credit Dr. Perfumo and Dr. Quiroga, College of Veterinary Medicine, Universidad Nacional de la Plata. Argentina).

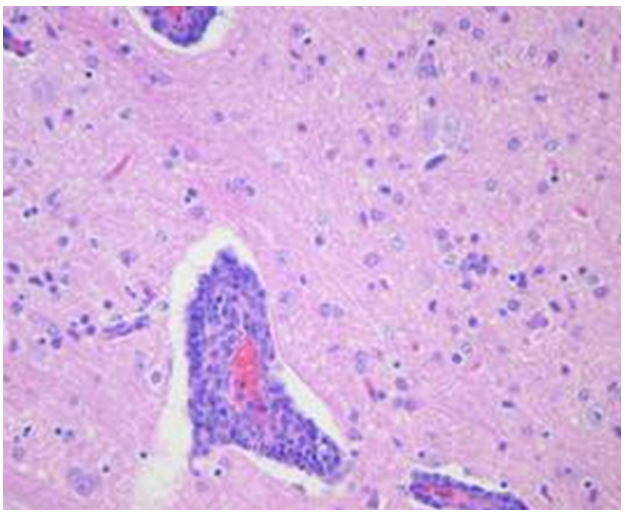

FIGURE 3 | In a section of brain there is severe cerebral vascular cuffing characterized by a large infiltration of lymphocytes and plasma cells. There is also diffuse mononuclear infiltration of the gray matter and moderate gliosis (Credit Dr. Perfumo and Dr. Quiroga, College of Veterinary Medicine, Universidad Nacional de la Plata. Argentina).

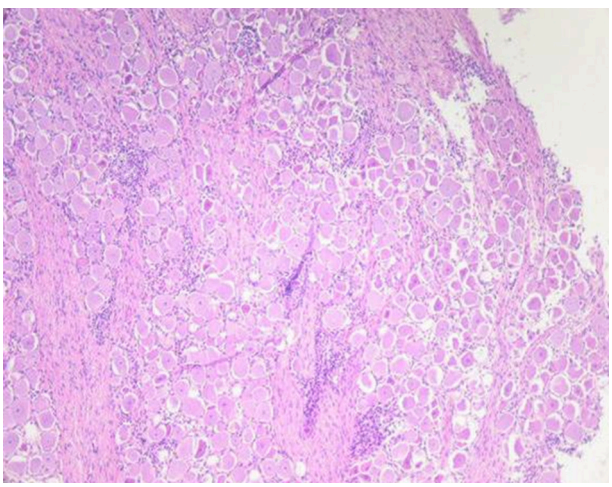

FIGURE 4 | Neuronal degeneration and necrosis in trigeminal ganglia associated with severe lymphoplasmacytic infiltration (Credit Dr. Perfumo and Dr. Quiroga, College of Veterinary Medicine, Universidad Nacional de la Plata. Argentina).

aseptically from young acutely affected piglets can be also used for testing the presence of PHEV. Detection of viral RNA by real-time reverse transcription polymerase chain reaction (RT-PCR) and/or nested PCR in different tissues including brainstem, trigeminal ganglia and spinal cord (64, 66, 107). Viral isolation is normally coupled to direct immunofluorescence and hemadsorption to detect viral growth (108).

PCR-based methods are useful to identify and subsequently isolate animals that are actively shedding the virus. Rauh et al. (109) described the development of a dry room temperature-stable real-time RT-PCR assay for the specific detection of PHEV. This RT-PCR was used to describe and compare the patterns of PHEV shedding and the dynamic of the infection in pen-based feces and oral fluid specimens collected from PHEV experimentally inoculated 7 weeks old pigs over the course of a clinical/subclinical infection. In this experiment, virus shedding was consistently detected by real-time RT-PCR in pen-based oral fluids collected from grow-finishers between 1 and 28 days post-inoculation (DPI) and feces between 1 and 10 DPI, however, viremia was not detected throughout the observation period (75). Previous reports indicated that viremia had little effect during the infection and the pathogenesis of the disease (81). Oral fluids are a suitable specimen for routine PHEV diagnosis and surveillance.

Although, the virus was first isolated in primary PK cells (38), the virus was also demonstrated to grow on other PK cell lines, including PK-15, FS-L3, SK-6, $IBRS_2$ cell lines (83, 110–112), secondary pig thyroid (SPTh) cells (113), pig embryonic pulmonary cells, and the swine testicle (ST) cell line (14, 114). It has been demonstrated that both SPTh and PK cells were most susceptible to cultivation and virus titration (115). PHEV can be consistently isolated from the tonsils and respiratory tract (nasal and pharyngeal swabs, nasal mucosal, and lungs) but irregularly from the pons and medulla, hindbrains, and stomach wall (59, 77, 82). PHEV can be also isolated from the nasal cavity of healthy

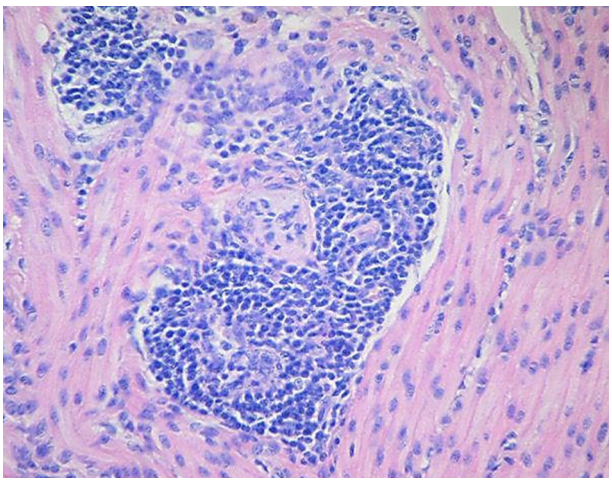

FIGURE 5 | Degeneration of the ganglia and severe lymphoplasmacytic perivascular cuffing in the tunica muscularis of the stomach wall (Credit Dr. Perfumo and Dr. Quiroga, College of Veterinary Medicine, Universidad Nacional de la Plata. Argentina).

pigs (83). Virus isolation can be difficult after 2–3 days after the onset of clinical signs or more than 8 days after experimental inoculation (59). PHEV in a culture can be detected by the formation of syncytia. Hemadsorption and/or hemagglutination tests were also used to demonstrate viral growth. One or more blind passages may be needed since specimens often contain small amounts of infectious viral particles. Although a virus grown in cell culture can still infect pigs, it can be less virulent than an isolated field strain[1].

Non-porcine cell culture has been shown to have little susceptibility to PHEV growth (112, 114). However, PHEV can grow in mice's brain cells (97, 116, 117), dorsal root ganglia cells from newborn mice (96), and in Madin-Darby canine kidney "low passage" (MDCK I) cells without prior adaptation (33).

Current indirect methods for detection of PHEV antibodies include hemagglutination (HA) and hemagglutination inhibition (HI) assays, virus neutralization (VN) tests, enzyme-linked immune-sorbent assays (ELISA), and rapid immunochromatographic strip tests (55, 71, 87, 106, 108, 113). Unlike other coronaviruses, PHEV readily agglutinates a variety of red blood cells. Specifically, PHEV attaches to N-acetyl-9-*O*-acetylneuraminic acid-containing receptors on erythrocytes (33). Girard et al. (118) originally used this feature for a differential diagnosis of PHEV-related disease from Teschen/Talfan disease and pseudorabies (Aujeszky's) disease. The HI test was adapted from the procedure suggested by the Committee on Standard Serological Procedures in Influenza Studies. Hemagglutinin-inhibiting and hemagglutinin-neutralizing antibodies can be detected in sera at 6 or 9 days, respectively, after experimental inoculation (59). Neither HI titer nor SN titers can be used for PHEV serodiagnosis or to assess degrees of antigenic relationship between isolates (55); however, the VN assay has been described as more specific than HI (110). Moreover, the ability to detect specific PHEV antibodies allows the determination of the status of first-litter gilts and evaluation of their risk of tier offspring to infection. However, serology results must be interpreted with caution as PHEV is highly prevalent, circulating subclinically in most swine herds. The development of specific monoclonal antibodies against PHEV and their utility for diagnosis and antibody-based treatment of the disease has also been reported (86).

The ability to detect antibodies allows producers to know the status of first-litter gilts and evaluate their risk of tier offspring to infection. However, in commercial swine farms, pigs are exposed to different coronaviruses with common genetic and antigenic features. The N-terminal portion (S1) of the spike protein is the only antigenic region that allows for antibody-based differential diagnosis of porcine coronaviruses, based on a complete absence of detectable cross-reactivity (24). Contrary, the N protein and especially the M protein are highly conserved among porcine coronaviruses and, therefore, should not be used for differential serodiagnosis of CoV-related diseases in pigs (24). Mora-Díaz et al. (75) developed a PHEV S1-based indirect ELISA for isotype-specific (IgG, IgA, IgM) antibody detection. Experimental data showed that PHEV infected-pigs develop detectable antibody responses by 7 days after infection, coincident with the onset of clinical signs. Specifically, the isotype-specific antibody responses in serum showed a strong IgM response at 7 DPI that declined quickly after 14 DPI. Strong IgA and IgG responses were detected by DPI 10 and declined gradually after 28 DPI.

IMMUNITY

It has been proposed but not yet fully demonstrated that the transference of lactogenic passive immunity might protect piglets from PHEV infection during the first few weeks of life. Previous *in vivo* studies demonstrated that animals with high hemagglutination inhibition (HI) antibody titers were not susceptible to PHEV infection (55). Pigs develop a detectable circulating antibody response to PHEV between 7 and 10 days after exposure. The immune response against PHEV has been recently characterized in grow-finisher pigs under experimental conditions (75). In this study, the isotype-specific antibody responses in serum showed a strong IgM response at 7 days post-inoculation (DPI) that declined after 14 DPI. A strong IgA and IgG responses were detected by 10 DPI, peaked at 28 DPI, and declined gradually thereafter. Increasing levels of systemic INF-α (DPI 3), TNF-α (DPI 10-17), and IL-8 (DPI 14) were detected by multiplex microbead-based immunoassay (Luminex®) over the course of the infection. In addition, flow cytometry analysis revealed an increase in both monocytes (DPI 10) and cytotoxic T cell (DPI 21) populations in response to PHEV infection (75). The duration of PHEV-specific antibodies has not been determined under field conditions. Sows that were exposed to PHEV rapidly developed detectable levels of antibodies (55). The duration of anti-PHEV immunity is not a critical factor as piglets become resistant to PHEV infection with age. Neonatal pigs born from immune dams, previously exposed to PHEV, are fully protected

[1]Mengeling, W. L. Ames, IA (1973), personal communication.

by maternally-derived antibodies that persist until the age of 4–18 weeks (119). More recent field studies carried out in Argentina demonstrated the presence of antibodies in grower/finisher pigs, suggesting that colostral antibodies may persist for more than 6 weeks (67).

In rats, the intravenous or intraperitoneal administration of PHEV antiserum provided partial protection against PHEV infection, evidenced by the absence of viral detection in the brain and spinal cord and the absence of PHEV-related neurological clinical signs (120).

PREVENTION AND CONTROL

Subclinical circulation of PHEV has been reported nearly worldwide. PHEV persists endemically in most breeding farms by pig-to-pig transmission and subclinical infections with colonization of the upper respiratory tract. Protection from the disease may be provided by lactogenic immunity transferred from PHEV seropositive dams to their offspring in enzootically infected herds. PHEV-related disease is a concern mainly in litters of young gilts that may not have been previously exposed to PHEV. PHEV naïve swine herds (i.e., replacement or isolated gilts and small farms) can be at risk if breaks in biosecurity allow the virus entry to the nursery in farms with low or no passive immunity (68). However, if non-immune dams are infected 2–3 weeks before farrowing they become immune, and newborn piglets are usually protected through lactogenic immunity. Moreover, it has been demonstrated that circulating anti-PHEV antibodies (hyper-immune serum), administered parentally, or intraperitoneally, protect neonatal piglets against PHEV infection. In addition, neutralizing monoclonal antibodies specifically against PHEV could be useful for antibody-based treatment of the disease (86). Despite some isolated efforts to develop a PHEV vaccine (87), overall, PHEV-related disease is not clinically relevant in most of the swine-producing countries. Thus, in the absence of current PHEV vaccines, promoting virus circulation on farms with early exposure to gilts and young sows could induce maternal immunity and prevent disease in piglets.

CONCLUSIONS

PHEV should be considered a major source of economic loss because of the high mortality on farms with high gilt replacement rates, specific pathogen-free animals, and gnotobiotic swine herds. Swine-breeding herds with low biosecurity or high pathogen loads may also be at risk of high piglet mortality because of PHEV. A better understanding of the mechanisms of viral infection and replication would assist in the development of better measures of prevention and treatment.

AUTHOR CONTRIBUTIONS

JM-D, PP, EH, JZ, and LG-L performed data analysis and conclusion and were the major contributors in writing the manuscript.

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Tracing Hepatitis E Virus in Pigs From Birth to Slaughter

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Pigs are considered the main reservoir of genotypes 3 and 4 of the human pathogen hepatitis E virus (HEV). These viruses are prevalent at a high level in swine herds globally, meaning that consumers may be exposed to HEV from the food chain if the virus is present in pigs at slaughter. The aim of this study was to determine the HEV infection dynamics from birth to slaughter using 104 pigs from 11 sows in a single production system. Serum was collected from sows at 2 weeks prior to farrowing, in addition feces and serum samples were collected from the pigs every second week, from week 1 to week 17. Feces and selected organs were also sampled from 10 pigs following slaughter at week 20. All the samples were tested for HEV RNA by real-time RT-PCR and the serum samples were tested for HEV-specific antibodies using a commercial ELISA. Maternal antibodies (MAbs) were only present in pigs from sows with high levels of antibodies and all pigs, except one, seroconverted to HEV during weeks 13–17. In total, 65.5% of the pigs tested positive for HEV RNA at least once during the study (during weeks 13, 15, and/or 17) and significantly fewer pigs with a high level of MAbs became shedders. In contrast, the level of MAbs had no impact on the time of onset and duration of virus shedding. HEV was detected in feces and organs, but not in muscle, in 3 out of 10 pigs at slaughter, indicating that detection of HEV in feces is indicative of an HEV positivity in organs. In conclusion, a high proportion of pigs in a HEV positive herd were infected and shed virus during the finisher stage and some of the pigs also contained HEV RNA in feces and organs at slaughter. The presence of MAbs reduced the prevalence of HEV shedding animals, therefore, sow vaccination may be an option to decrease the prevalence of HEV positive animals at slaughter.

Keywords: hepatitis E virus, zoonotic transmission, HEV, swine, infection dynamic, liver

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INTRODUCTION

Hepatitis E virus (HEV) can cause severe infections in humans. Four genotypes of HEV are known; genotypes 1 and 2 are exclusively found in humans whereas genotypes 3 and 4 have been found in humans and pigs. Genotype 3 is found worldwide in pigs and in humans, while genotype 4 has mainly been found in both pigs and humans in Asia, and only more recently also in Europe (1). In several European countries, there has been a dramatic increase in human cases of HEV infection caused by Genotype 3 strains. These viruses have a high sequence identity to contemporary strains circulating in pigs, indicating that swine-to-human transmission of HEV is a common event (2). Indeed, high prevalence of anti-HEV antibodies (Abs) in swine herds has been reported from several countries. Detection of the high HEV seroprevalence in older samples indicated that HEV has been present in pigs for decades. A number of studies have shown that consumers are indeed exposed to

HEV since porcine livers, bought in supermarkets, have been found to contain HEV-specific RNA (3–5). Furthermore, HEV, isolated from commercial livers, has been shown to be infectious for pigs in an experimental trial (3). In another study, a pig liver sausage, Figatellu, which is traditionally eaten raw, was found to be the cause of hepatitis in a significant number of people who consumed it (6). In addition, 2–15% of pigs have been shown to be infected with HEV at slaughter (7). Previous longitudinal studies, performed in pigs, revealed that most of the pigs became infected at 8–15 weeks of age but some of the pigs were still positive at slaughter (8–10). Maternal antibodies (MAbs) against HEV have been shown to be successfully transferred from HEV-Ab positive sows to offspring. However, in a previous study comparing a few animals in a single herd, the level of MAbs had no impact on the infection dynamic of HEV in the offspring. Thus, the protective role of MAbs in pigs is presently unclear (11). The proven zoonotic potential of HEV in pigs combined with the relatively high prevalence of HEV positive pigs in Denmark (more than 50% of the sow herds are HEV positive) (12) may have a negative impact on the safety of Danish pork products if the virus is present in Danish pigs at slaughter. Thus, it is essential to obtain a better knowledge of HEV infection dynamics in typical pig production systems. The aim of the present study was to study the HEV infection dynamics from birth to slaughter, with special focus on the impact of maternal antibody levels and the infectious status of individual pigs at slaughter. Furthermore, the distribution of HEV in different tissues of naturally infected pigs that shed virus 3 weeks prior to slaughter was examined.

MATERIALS AND METHODS

Field Study Design and the Study Herd

A longitudinal study was performed in a single farrow-to finisher herd. More than 100 crossbred pigs were sampled every second week from birth to slaughter. The pigs were kept at the breeding unit until they reached ∼30 kg after which they were moved to the finisher site situated ∼16 km from the breeding unit. Before initiating the study, the presence of HEV in the first parity sows (gilts) at the nursery site was determined by testing feces from 10 sows using an HEV specific real time RT-PCR assay (data not shown).

Selection of Sows

Two weeks prior to farrowing, serum samples were collected from 58 sows and tested for HEV Abs. Based on the measured, normalized levels of HEV Abs, the sows were divided into three groups; low ($1 \leq OD < 2$), intermediate ($2 \leq OD < 3$) and high ($OD \geq 3$) levels of HEV-specific Abs. The group of low level HEV Abs comprised 23 sows with a mean normalized OD of 1.38 (SD = 0.27). The groups with intermediate and high levels of HEV Abs each included 17 sows, with mean normalized OD values of 2.44 (SD = 0.24) and 4.50 (SD = 1.47), respectively. The farmer randomly selected four sows from each group to be included in the study. Just after farrowing, all piglets from the 12 sows were ear tagged with a unique number. If more than half of the piglets within a litter died, the sow and her piglets were excluded from the study.

Sampling of Pigs

One week after farrowing, blood sampling of all piglets was performed by a local pig health technician. Thereafter, both rectal swabs and blood samples were collected every second week until week 17 from all piglets. The pigs were restrained either manually or with a snout break and 9 mL of blood was collected by puncture of the jugular vein. The rectal swabs were collected, using a cotton swab, at the rectal surface ∼2–3 cm from the anus and then placed into a sterile container with 2 mL PBS. The samples were labeled and kept cool during transportation to the laboratory. The blood samples were stored at 4°C until further processing on the same day. The serum was extracted from whole blood by centrifugation at 3,000 RPM for 10 min at 5°C. The serum fractions were then transferred into Nunc tubes and stored at −80°C until RNA extraction. The tubes containing the cotton swabs in 2 mL PBS were shaken at 300 rpm for 1 h before the liquid was poured into 2 mL Eppendorf tubes and stored at −80°C until analysis. Individual pigs were excluded from the study if more than two sampling dates were missed.

Selection of Pigs for Tissue Sampling

Ten of the 26 pigs where shedding of HEV (as detected by the presence of HEV RNA) occurred ∼3 weeks prior to slaughter (week 17), were randomly selected for necropsy at a laboratory facility situated 100 km from the herd. At the age of 20 weeks, the pigs were transported alive to the laboratory on a vehicle with no other pigs present. On arrival, the pigs were killed by intra-cardiac injection of pentobarbiturate (50 mg/kg) and exsanguinated by cutting the arteria axillaris. At necropsy, samples of the tonsils, lungs, kidneys, spinal cord, gall bladder (intact), hepatic lymph nodes, colon with contents, small intestine with contents, mesenteric lymph nodes, heart, and the entire liver were collected. Furthermore, muscle samples (3 × 3 cm) were collected from the shoulder, neck, pork loin, tenderloin, ham, and diaphragm. Intestinal contents were collected from the colon and the small intestine. The tissue was then rinsed in cold PBS. Bile was extracted from the gall bladder with a syringe and a small piece of tissue was excised and rinsed in PBS to remove the remaining bile. All samples were transferred to labeled tubes and stored at −80°C until analysis.

RNA Extraction and PCR Analysis

Automated extraction of RNA from the rectal swab supernatant was performed on the QIAsymphony SP system (QIAGEN) using the DSP virus/pathogen mini kit version 1 (QIAGEN, Cat no. 937036). The protocol used was complex 200 V5 DSP with an elution volume of 110 μL. The HEV RNA was detected by real time RT-PCR essentially as described by Breum et al. (12) except that the concentration of the primers was changed to 500 nM for HEV2-P and HEV2-R and 100 nM for HEV2-F. Furthermore, the time settings used for the PCR cycling were changed to 15 s for denaturation and annealing and 20 s for elongation.

Serological Analysis

All serum samples were tested for the presence of anti-HEV IgG using a commercial kit (PrioCHECK® HEV Ab porcine kit; Prionics). As recommended by the vendor, only the samples

having an OD value that exceeded the OD of the cut-off control (provided in the kit) multiplied by 1.2 were regarded as positive. The OD values were normalized by dividing the OD of the sample with the OD of the cut-off control multiplied by 1.2, which eliminated plate-to-plate variations. According to the information provided by the vendor, the assay has a sensitivity of 91% and a specificity of 94%.

Statistical Analysis

The statistical analyses were performed using SAS 9.1. For the determination of the overall difference between the three groups, a mixed linear model was used. This method allowed for missing data points from individual pigs. To evaluate the differences on a weekly basis, the ANOVA was performed. Finally, to compare groups for the difference in the number of shedders, the χ^2-test was applied. For all analyses the significance level was set at $P = 0.05$.

RESULTS

Initially, a total of 12 sows and 135 piglets were included in the study, but 31 of the piglets, including one entire litter, either died or were excluded due to missing sampling points. Thus, data from a total of 104 piglets from eleven sows were included in the analysis.

Serology

Based on the levels of HEV Abs prior to farrowing, the 11 sows were allocated to one of three groups with low, intermediate or high levels of HEV Ab, designated group 1, 2, and 3, respectively. Normalized OD values, indicative of the HEV Ab levels in serum, for the included sows and the number of piglets in each litter in each group are listed in **Table 1**.

All the pigs, except for one, seroconverted during the study (**Figure 1**). The pigs in groups 1 and 2 showed similar anti-HEV Ab profiles in serum with OD values below the cut off until seroconversion that occurred between weeks 11 and 13 followed by a steady further increase in HEV IgG levels which lasted until the end of the observation period at week 17 (**Figure 1**). Group 3 showed a different profile with positive HEV IgG levels from birth until week 7 and then these group 3 pigs, like the pigs in groups 1 and 2, seroconverted between week 11 and 13 followed by a steady increase in HEV IgG levels until week 17 (**Figure 1**). No differences were seen between the pigs in groups 1 and 2 so these groups were combined in the statistical analyses. There was a clear difference in the level of HEV IgG between the pigs in group 3 compared to the pigs in group 1 and 2 from week 1 to 11, but not at week 13 to 17 (**Figure 1**).

Real Time RT-PCR

Of the 104 ear marked pigs included in the analysis, 66 pigs (63.5%) tested positive for HEV RNA in feces in at least one sample during the study period (**Table 2**). There was a significant difference in the number of viral shedders ranging from ∼73% in groups 1 and 2 to 45% for group 3 ($P = 0.032$) (**Table 2**). However, there was no significant difference in the time when the first detection of HEV shedding was observed between the groups ($P = 0.876$). None of the pigs tested positive for HEV prior to week 13 and only 9 pigs became virus positive between weeks 11 and 13 (**Figure 2**). The majority of the pigs ($n = 51$) tested positive for HEV for the first time at week 15, whereas six pigs tested positive for the first time at week 17. Of the 104 pigs, 23 (22%) tested positive for HEV in feces at two samplings and two pigs (2%) were positive at three samplings (weeks 13, 15, and 17) (**Figure 2**).

Analysis of Samples Collected From Selected Pigs at Slaughter

To analyze if the organs and tissues contained HEV at slaughter, 10 of the 26 pigs that tested positive for HEV at week 17, ∼3 weeks prior to slaughter, were randomly selected for further analysis. The 10 pigs included three, five and two pigs from groups 1, 2, and 3, respectively. The HEV IgG profiles for the 10 individual pigs from birth until slaughter are shown in **Figure 3A**. Three of the pigs (1-1, 2-1, and 3-1, one from each group denoted by the first number in the ID) were seronegative at week 17, but both pigs 2-1 and 3-1 had tested positive for HEV before week 15 (**Figure 3B**). At slaughter (week 20), three of the 10 pigs, one from each group, were still positive for HEV RNA in feces at a level similar to that observed at week 17 (**Figure 3B**). There was no significant difference in the HEV shedding pattern before week 17 for the three pigs that were positive for HEV at week 20 compared to the other seven pigs that tested negative for HEV at week 20 ($P = 0.633$). Interestingly, only the three pigs that tested positive for HEV in feces at week 20 were positive for HEV RNA in organs (**Table 3**). Only the internal organs tested positive for HEV RNA while none of the muscle samples tested positive. The liver associated samples [liver, bile, gall bladder, and hepatic lymph nodes (HLN)] were strongly positive for HEV

TABLE 1 | Grouping of piglets according to levels of HEV antibodies in sows prior to farrowing.

Group	Group 1 (Low level of HEV IgG)				Group 2 (Intermediate level of HEV IgG)			Group 3 (High level of HEV IgG)			
Sow ID	3399	3545	3485	3681	3266	3699	3548	3552	3532	3292	3145
OD (norm.)	1.3	1.4	1.4	1.4	2.5	2.8	2.9	5.8	6.1	6.9	11.6
# Piglets (in study/born)	8/8	15/19	6/10	9/10	10/11	8/10	15/18	8/10	9/10	8/10	8/10
Total # pigs	38				33			33			

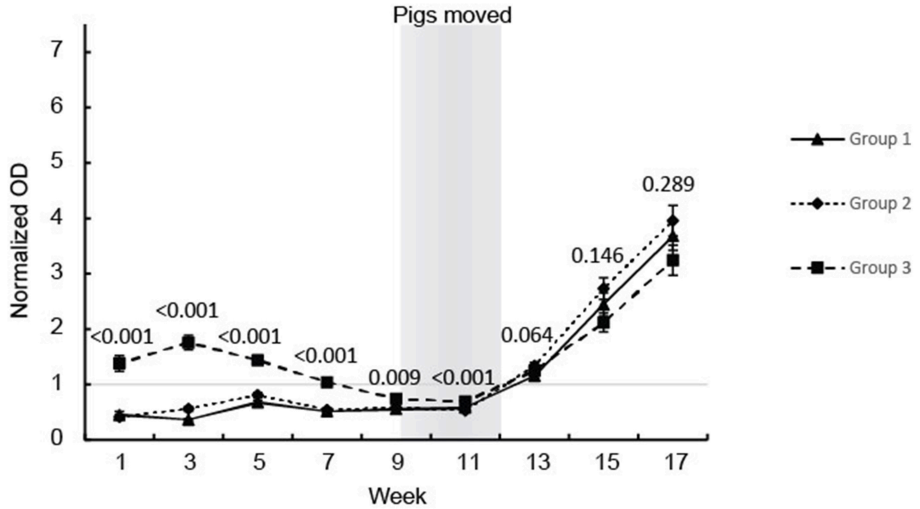

FIGURE 1 | HEV antibody (IgG) development in the eartagged pigs. The values are expressed as mean values of the normalized ODs for the serum from pigs in the three groups. The results of the statistical analysis of the differences between the pigs in group 3 compared with groups 1 and 2 (ANOVA) are indicated at each sampling point.

TABLE 2 | The number of pigs that tested positive, for the first time, in each of the three groups.

	Group 1 (low)	Group 2 (intermediate)	Group 3 (high)	Total
Week 13	2	6	1	9
Week 15	21	17	13	51
Week 17	4	1	1	6
Total shedders	27/38 (73.7%)	24/33 (72.7%)	15/33 (45.5%)	66/104 (63.5%)

Each individual pig is only included in the week when it tested positive for the first time.

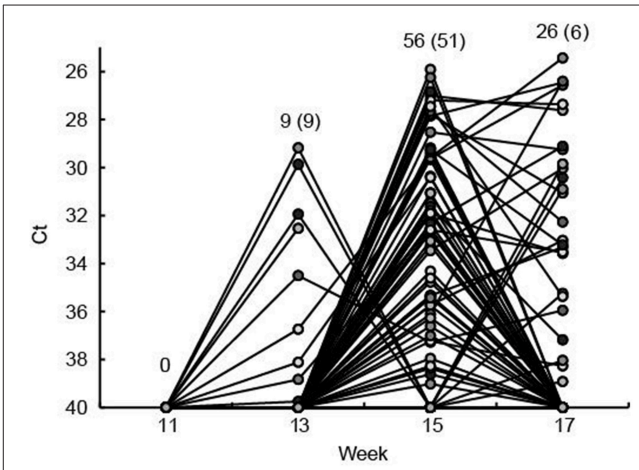

FIGURE 2 | The fecal shedding of HEV from all eartagged pigs is shown as the Ct values obtained by real time RT-PCR testing of feces. The Ct scale has been inverted and negative samples have been set at Ct 40. Plain numbers indicate the total number of pigs positive for HEV RNA at 11, 13, 15, and 17 weeks of age and the numbers in parenthesis indicate the number of pigs that tested positive for the first time in that week. Each of the eartagged pigs are marked with a circle filled with different shades of gray.

RNA (low Ct) whereas lower levels of HEV RNA were detected in extra-hepatic organs such as the lungs and tonsils.

DISCUSSION

The offspring from 11 sows with different levels of HEV specific antibodies were included in the present study. To investigate the efficacy of passive transfer of maternal antibodies on the HEV infection dynamic in the offspring, the 104 piglets were allocated to one of three groups based on the level of anti-HEV antibodies measured in the sows 2 weeks prior to farrowing. The MAbs were detected only in piglets from sows with high levels of anti-HEV Abs prior to farrowing, revealing a clear correlation between the levels of anti-HEV Abs in the sows and the maternal anti-HEV Abs in the piglets. This finding is in accordance with previous studies, which also showed that a high level of antibody is required for effective transfer from the sow (8–10). The difference in HEV MAbs levels between piglets born of sows with high level of HEV IgG (group 3) compared to the other two groups were significantly different until week 13. Previous studies have confirmed that MAbs against HEV decline at around weeks 9–13 (8, 9).

HEV RNA was detected in feces of pigs from week 13 and onwards. Thus, no viral shedding was detected in the pigs when housed in the sow herd because the pigs were moved to the finisher site at 30 kg (week 9–12). Based on the facts that anti-HEV Abs were detected in the sows prior to farrowing and that HEV RNA was detected in the gilts in the herd (data not shown), HEV was indeed present in the sow herd of this study.

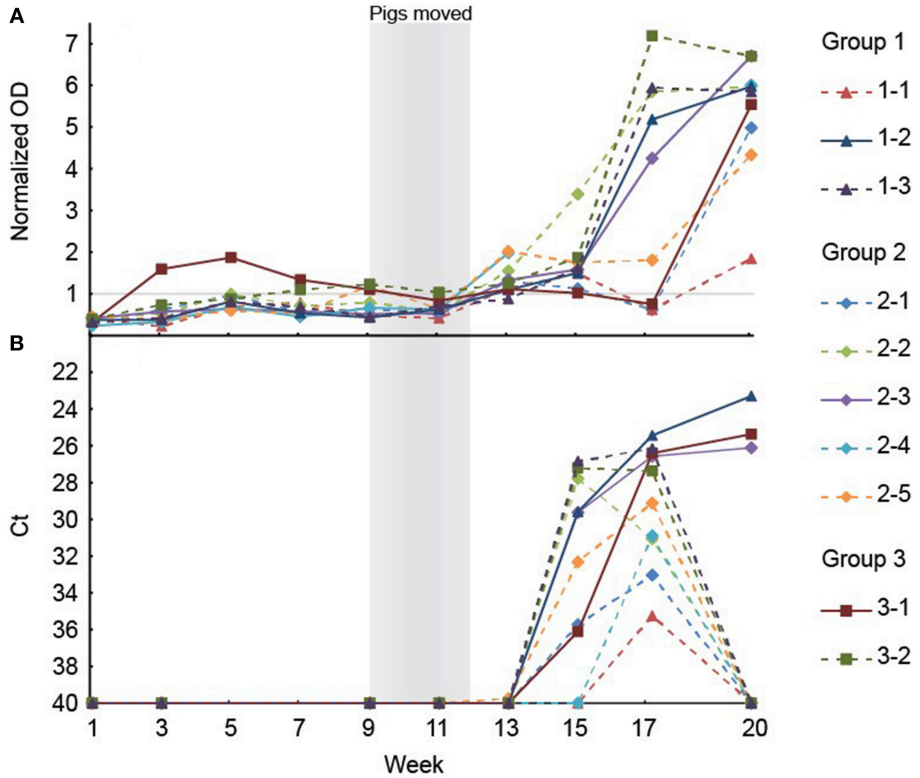

FIGURE 3 | (A) The results of the anti-HEV IgG measurements in serum from the ten pigs selected for necropsy. Note that the serological data are missing for week 15 and 17 for the pig with ID 2–4. **(B)** Positive HEV tests of feces of the ten pigs selected for necropsy. The Ct scale has been inverted and negative samples have been set at 40 Ct.

TABLE 3 | Detection of HEV RNA in samples collected from necropsied pigs.

	Pig 1–2	Pig 2–3	Pig 3–1
	C_t	C_t	C_t
Feces	23.3	26.11	25.4
Small intestine (contents)	27.0	27.9	-
Colon	–	–	–
Small intestine	–	–	–
Intestinal lymph node	–	37.2	38.3
Gall bladder	31.3	31.1	29.2
Bile	23.4	24.9	27.7
Liver	21.5	30.8	27.5
Hepatic lymph node	30.7	26.9	36.6
Kidney	–	–	–
Lung	34.7	34.1	35.3
Tonsil	–	38.8	38.3
Spinal cord	–	–	–
Muscle*	–	–	–
Heart	–	–	–

Only the three pigs with positive samples are shown.
**Muscle included six different samples of muscle collected from parts of the pig used for food products. All samples were analyzed separately.*

However, it is not clear, if the piglets were infected by HEV just prior to being moved from the breeding unit or if the pigs were infected after arrival at the finisher site. However, although there was no effect of the level of HEV MAbs on the onset or duration of viral shedding, significantly fewer pigs in the group with initially higher levels of MAbs tested positive for HEV during the study. These findings indicated that the pigs were exposed to HEV relatively late in the nursery period i.e., after the MAbs had declined in most pigs. A previous field study failed to show any effect on the level of MAbs on the risk of becoming HEV shedders, however, that study was performed on very few animals (2 litters) and the pigs were infected very early (week 3–4) indicating a high viral load in the environment (11). Another field study detected HEV RNA in feces of pigs starting in weeks 12–15 ∼3–5 weeks after the anti-HEV MAbs had waned, which is more in line with the findings in the present study (9).

Seroconversion against HEV, as measured using a commercial HEV ELISA, was observed in the present study in all pigs, except one, starting between week 11 and week 13 which is in accordance with development of IgG in previous studies (8–10). Thus, the pigs that were HEV RNA negative at all samplings in feces also seroconverted indicating that they indeed were infected or at least exposed to HEV either in a short period of time or at levels below the detection limit of the real-time RT-PCR assay. However, these animals may have been positive for HEV in other tissues or in serum. Seroconversion coincided with the first detection of viral RNA for most of the pigs. This was unexpected since IgG Abs previously have been shown to develop 2–3 weeks after onset

of viremia (9, 11). Detection of HEV in serum, in the present study, was attempted on the same sampling days as for the feces samples, but was unsuccessful even though different methods for RNA extraction were tested and the assay previously has performed very well in detecting HEV RNA in serum samples from the field (12) and in a ring trial (unpublished results). The level of HEV in serum has, however, previously been shown to be significantly lower than in feces and the viremia also seems to be of shorter duration than the fecal shedding (11). Furthermore, in an experimental trial in pigs, using intravenous inoculation of homogenates of livers with different levels of HEV, it was shown that the duration and levels of viremia were strongly correlated to the level of HEV present in the inoculum (3). Thus, a likely explanation for the finding in the present study, i.e., seroconversion coincided with positive fecal samples, could be that virus fecal excretion start days or even weeks after exposure. Another contributing factor to the early detection of anti-HEV Abs could be that the anti-porcine IgG conjugate included in the ELISA cross-reacted with IgM Abs which normally develop earlier than IgG (8, 10).

The HEV RNA was detected in internal organ samples (intestine, lymphatic tissue, bile and liver), but not in muscle, which is in accordance with previous findings (7, 11, 13, 14). Interestingly, only the pigs that tested positive in fecal samples at slaughter were also positive in organs. This indicated that testing of feces from pigs prior to slaughter could be used as an indicator of HEV presence in internal organs. However, albeit that all feces positive pigs were found to harbor HEV in tissue in one previously study (14), the predictive value of a negative feces test may be limited since HEV has been detected previously in organs from pigs that tested negative in feces (8, 14).

In conclusion, a high proportion of the pigs, in a single HEV positive herd, were infected and tested positive for HEV during the finisher stage and a fraction of these pigs also had HEV RNA in feces and organs at slaughter. High levels of MAbs reduced the prevalence of HEV positive animals and, therefore, sow vaccination may be an option to decrease the prevalence of HEV positive animals at slaughter, however, more studies are required to investigate this.

DATA AVAILABILITY

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

ETHICS STATEMENT

The study included blood sampling of animals under field conditions for diagnostic purposes and by that did not require approval from the ethic committee.

AUTHOR CONTRIBUTIONS

JK participated in the design of the study, performed the laboratory analyses on pig samples, participated in the assessment and statistical analysis and drafted the manuscript. LL participated in the design of the study and in the assessment and statistical analysis and commented and made adjustment to the manuscript. SB participated in the design of the study, in the establishment of the analytical assays and participated in the assessment and statistical analysis and commented and made adjustment to the manuscript.

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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
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Porcine Torovirus (PToV)—A Brief Review of Etiology, Diagnostic Assays and Current Epidemiology

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Porcine torovirus (PToV) is a potential enteric swine pathogen, found at especially high rates in piglets with diarrhea. It was first reported in the Netherlands in 1998 and has emerged in many countries around the world. Infections are generally asymptomatic and have not directly caused large economic losses, though co-infections with other swine pathogens and intertype recombination may lead to unpredictable outcomes. This review introduces progress in PToV research regarding its discovery, relationship with other Toroviruses, virion morphological characteristics, genetic structure and variation, recent epidemiology, diagnostic methods, and possibilities for future research.

Keywords: porcine torovirus, etiology, epidemiology, diagnostic assays, recombination

HISTORY

Toroviruses (ToV) (order *Nidovirales*; suborder *Tornidovirineae*; family *Tobaniviridae*; subfamily *Torovirinae*; genus *Torovirus;* subgenus *Renitovirus)* are responsible for infections leading to gastroenteritis in animals and humans (1–5). There are three recognized species in the *Renitovirus* subgenus: porcine torovirus (PToV); bovine torovirus (BToV), and equine torovirus (EToV) (Virus Taxonomy: 2018b Release (MSL #34); https://talk.ictvonline.org/taxonomy/). Human torovirus (HToV) is used to be the member of the *Torovirus* genus, according to the 2017 Release of Virus Taxonomy (MSL #32). Neutralizing activity against EToV has been found in the sera of other animals (cattle, goats, sheep, swine, rabbits, mice), providing serological evidence for the existence of ToVs in other animals (5).

EToV (also called Berne virus [BEV]) was the first to be discovered, and is the prototype species of the genus *Torovirus*. EToV was initially isolated in 1972 (but not reported until 1983) from a rectal swab taken from a horse in Berne, Switzerland which showed pseudomembranous enteritis, miliary granulomas and necrosis in the liver at necropsy. The isolated pathogenic agent couldn't be neutralized by antibodies against equine viruses known at the time. By electron microscopy, the virions appeared pleomorphic, mostly with smooth surfaces and spherical, though some were C-shaped, and some particles in damaged membranes had a "sausage-like" internal structure with transverse striations (1).

Another unclassified virus, BToV (also called Breda virus [BRV]) was discovered in 1982 in calves with diarrhea in Breda, Iowa, and confirmed to have antigenic differences from known diarrhea-related bovine viruses. The isolate was infectious when orally inoculated into gnotobiotic and conventionally reared calves (2).

In 1984, a similar virus was found in the feces of diarrheic patients (mainly children under 5 years-old) in England and the United States, with virions about 100 nm in diameter and a 7–9 nm capsule on the edge (3).

Previously, ToV-like agents had been detected in swine in many countries by electron microscopy or neutralization assays performed with EToV antibodies (5–8). In 1998, PToV was first detected and characterized in fecal samples from piglets in the Netherlands (4). Immunoelectron microscopy showed the elongated, 120- by 55-nm particles in fresh material. By sequence analysis, the N protein gene of PToV only has 68% sequence identity with BToV and EToV, which share 88% between them (4). PToV has since been reported in Canada, the United States, South Africa, China, Korea, and many European countries, such as the United Kingdom, Italy, Belgium, Hungary, and Spain (5–14).

ETIOLOGY

PToV Structure

ToVs are enveloped viruses with positive-sense single-stranded RNA genomes. PToV particles appear elongated in fresh samples, about 120 nm long and 55 nm wide, as observed by EM. After repeated freezing and thawing, virions have multiple forms, including round, kidney- and torus-shaped particles (4). The nucleocapsid is formed by N protein and viral RNA, surrounded by an envelope that contains the triple-spanning membrane (M) protein (10). On the surface of particles, two kinds of projections have been identified: longer protrusions (about 19 nm) with a drumstick or petal shape, considered to be spike (S) proteins; and shorter ones (6 nm in length) speculated to be the hemagglutinin-esterase (HE) (4, 15).

PToV Genome

ToV genomes are nonsegmented, polyadenylated, and 25–30 kb in size, with similar organization to other coronaviruses (16, 17). Both the 5′ and 3′ termini of the genome have a non-translated region (NTR), and the 5′ two-thirds of the ToV genome contains two large, overlapping open reading frames (ORF1a [13,254 bp] and ORF1b [6,875 bp]) connected by a frameshift (**Figure 1**), which encode two replicase polyproteins. There are four smaller ORFs downstream encoding the structural proteins (S [4,722 bp]; M [702 bp]; HE [1,284 bp]; and N [492 bp]), which are expressed through a 3′-coterminal nested set of subgenomic mRNAs (17–21). The first genomic sequence of PToV from Shanghai, China (strain SH1; GenBank accession no. JQ860350) was 28,301 bp in length, and had 79% sequence identity with BToV (21).

PToV Structural Proteins

The mature ToV S protein contains two functional domains, S1 and S2, which mediate receptor specificity and determine viral tropism; S1 can bind independently to cellular receptors whereas S2 mediates fusion of the viral and cell membranes (22). Based on their electrophoretic mobility upon endoglycosidase F treatment, it has been demonstrated that the putative cleavage site, composed of five consecutive arginine residues, plays a role in post-translational processing *in vivo*. There are structural features in common with other coronavirus S proteins, such as the N-terminal signal sequence, an assumed C-terminal transmembrane anchoring domain, two heptad-repeat domains and a probable "trypsin-like" cleavage site (23). Due to its important role in host receptor binding, the S protein is the major target of neutralizing antibody responses.

The ToV M protein, previously referred to as the envelope protein, has three transmembrane α-helices in its N-terminal part. Its C-terminal part is exposed on the cytoplasmic face of the membrane, a feature of coronavirus M proteins. This protein lacks a cleavage signal sequence, so it is suspected that one of the hydrophobic transmembrane domains acts as an internal signal sequence (24).

Observed by EM, the HE protein is distributed on the virion surface as small projections. It is a class I membrane protein, and a member of the receptor destroying enzyme (RDE) protein family. There are two main domains identified in the monomer: the enzymatic acetyl-esterase region (E); and the receptor binding or lectin domain (25). The HE protein specifically but reversibly binds to mucopolysaccharides, mediating adhesion of virions to the intestinal wall. Through binding to 9-O-acetylated receptors then cleaving and rebinding to glycosylated surfaces (26, 27), virions can theoretically migrate through the mucus layer, thus promoting infection (18).

The open torus-shaped core of ToV is formed by the viral genome binding to the phosphorylated N protein, which is the only viral RNA binding protein. This 18.7 kDa protein is the most abundant protein among the ToV virions (28). Structural predictions of N protein have revealed the presence of four *N*-glycosylation sites [associated with antigenicity and immunogenicity (29)], two protein kinase C phosphorylation sites, one casein kinase II phosphorylation site, and an N-myristoylation site.

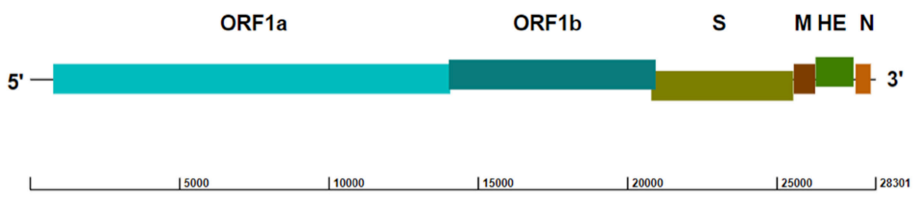

FIGURE 1 | Genome structure of PToV strain SH1 (GenBank accession no. JQ860350). The ORFs encoding the replicase polyproteins (ORF1a and ORF1b) and structural proteins spike (S), membrane (M), hemagglutinin-esterase (HE), and nucleocapsid (N) are indicated.

PToV Strain Variability

Sequencing studies around the world have shown significant variation among PToV isolates by geographic region. The genetic diversity of PToV in three Korean farms was studied in 2010, showing low nucleotide and deduced amino acid identities of partial S genes. Among the Korean PToV strains there was 73.5–94.1 and 71.4–95.0% identity in nucleotide and deduced amino acid sequences, respectively, whereas comparison with the Netherland strain Markelo revealed 74.0–93.1% and 73.2–95.5% identity, respectively. In addition, phylogenetic analysis of the N gene showed that different PToV strains were emerging in Korea, and even within the same farm (11).

The complete genome of the first PToV strain identified in the United States (PToV-NPL/2013, GenBank accession no. KM403390) contained 28,305 bp, with 92% identity to PToV-SH1. The predicted S, M, HE, and N proteins shared 94, 99, 92, and 96% amino acid identity with PToV-SH1, respectively. The gene encoding HE had 80–95% identity to other PToV strain sequences in GenBank, while the N and M genes were 95 and 93–97%, respectively, indicating that HE is more diverse among PToV strains (14).

Through phylogenetic analysis, the M gene sequences of 19 novel PToV strains from Sichuan, China and 21 ToV strains in GenBank could be classified into two genotypes (I & II). All of the novel Sichuan strains belonged to genotype I along with two Korean sequences (GU-07-56-11 and GU-07-56-22), whereas all other representative Korean, Netherlands and Canadian strains from GenBank belonged to genotype II. Putative amino acid sequence identities of the M gene were 99.1–99.6% among the 19 Sichuan strains and 97.4–99.6% between Sichuan and foreign strains, demonstrating high conservation of the M gene (30). Since the M gene sequences available were highly conserved, genotyping of PToV based on the M gene may be meaningless.

The sequence differences between the four *Torovirus* species range from 30 to 40%, which makes it easy to distinguish between them. However, evidence of intertype recombination was found in 2003, where BToV variants had emerged from recombination with PToV at the 3′ end of the HE/N genes and the 3′-NTR (31). Similar recombination events have also been found in ORF1a, ORF1b, and the 5′-NTR (32). Furthermore, some PToV and BToV variants have been isolated with chimeric HE genes, speculatively from recombination with some unknown ToVs (31). This ability for recombination underlines the potential for cross-species transmission and even the risk of zoonosis.

Inter-order Recombination Between ToV and Enterovirus in Swine

Interestingly, evidences of inter-order recombination between ToV (order *Nidovirales*) and the order of *Picornavirales* were discovered in different geographical locations most recently. The porcine enterovirus G (EV-G) genome with an insertion of ToV papain-like cysteine protease gene (PLCP) at the 2C/3A junction was detected in the feces of diarrheal pigs from farms in the United States, Belgium, Japan, China, and South Korea (33–38). The inserted PLCP sequences had lengths varied in strains from different geographical locations, ranging from 194 to 223 amino acids, which form a separate cluster distantly related to those of PToV, BToV, and EToV, suggesting that these viruses might have a common ancester. Since a mutant virus without the insertion by reverse genetics produced impaired growth and higher expression levels of innate immune genes in infected cells (36), the PLCP sequence might help the EV-G-PLCP strains establish a new host immune evasion strategy and, in some cases, determine its pathogenic potential.

DIAGNOSTIC ASSAYS

Immunoelectron microscopy was used to detect PToV in the early years. However, this method was costly, time-consuming, and not suitable for processing a large number of samples. In addition, the polymorphism of ToV particles (4) decreases the specificity and accuracy of this detection method, and the fact that viral particles are not shed in the feces for a long duration decreases the likelihood of detection.

Neutralization assays using EToV virions obtained from cell culture (39) and an indirect ELISA method using BToV virions obtained from infected gnotobiotic calves (40) have been applied to serological diagnosis of PToV by detecting cross-reacting antibodies. However, the difficulty of virion purification and lost sensitivity resulting from use of heterologous antigens make these assays inappropriate for widespread use. The lack of a PToV culture system and infection model makes it difficult to obtain a large number of virions, hampering development of diagnostic methods and epidemiology studies. The only method available for serological diagnosis of PToV is an indirect ELISA based on a recombinant His-tagged N protein expressed in a baculovirus system. Serum samples ($n = 15$) collected from 6 to 8 week-old healthy piglets from a farm located in Galicia, Spain, tested by this method obtained a positive rate of 100%, whereas the positive rates when tested by western blot and neutralization assay of EToV virions were 60% (9/15) and 93% (14/15), respectively (10). It could be seen that this ELISA method had a high sensitivity, but its specificity may be slightly worse. The indirect ELISA based on PToV-HE protein is less sensitive and may have false negative results because of the diversity of HE proteins and the antigenic differences between HE lineages (14, 25). Development of other ELISA diagnostic methods based upon the PToV S protein is needed, according to experiences from porcine coronavirus such as porcine epidemic diarrhea virus (PEDV) (41).

Currently, PCR is the most widely used diagnostic assay, with the advantages of being low-cost, convenient, and highly sensitive and specific. Apart from conventional reverse transcription PCR (RT-PCR) (4, 10, 13, 31), quantitative RT-PCR (qRT-PCR) methods with primer pairs derived from the N gene (9, 39) have also been established to detect PToV and investigate epidemiology, since the N protein is the most abundant protein in the virus and has a high sequence conservation. A one-step SYBR Green qRT-PCR based on the PToV M gene was shown to be more sensitive than conventional RT-PCR and nested PCR (42). A multiplex RT-PCR method was developed for simultaneous detection of porcine kobuvirus (PKV), porcine astrovirus (PAstV) and PToV using primers based on their M

gene sequences (43). Furthermore, an RT-LAMP method based on 4 specific primers from the N gene was developed for the quick detection of PToV (44).

EPIDEMIOLOGY

The worldwide distribution of PToV has been proven, with a high infection rate in pigs. However, due to limitations of diagnostic assays and asymptomatic infections (4, 13), there are not many reports on epidemiology. In 1998, Kroneman et al. performed a neutralization assay using EToV to detect cross-reacting antibodies and found that 81.4% (96/118) of the pig serum samples collected from farms in the Netherlands contained EToV-neutralizing antibodies (4). A qRT-PCR method was applied to detect PToV in rectal swabs collected from piglets at a farm in northeastern Spain in 2010, with a positive detection rate of 39.6% (19/48) (9). A longitudinal serological and virological study of PToV in Spain detected serum antibody levels by N protein ELISA, and fecal shedding by qRT-PCR based on the N gene. Seroprevalence in one hundred and twenty piglets at 1, 3, 7, 11, and 15 weeks-of-age was 92, 58, 91, 100, and 100% positive, respectively, and the corresponding 30 sows were all seropositive, reflecting the process of maternal antibody decline and subsequent immune response. As for fecal shedding in a 36-piglet subpopulation, 92% (33/36) of piglets had detectable PToV RNA at some age (39). Another epidemiological study in Spanish farms was done in 2012, with serum samples collected from 100 farms tested by N protein ELISA, revealing a total seroprevalence of 95.7% (2550/2664) and prevalence at different ages ranging from 59.4 to 99.6%. The lowest seroprevalence was detected in 3-week-old piglets (98/165) (45).

Shin et al. examined the prevalence of PToV in Korea in 2007, revealing 6.4% (19/295) of diarrheic pig samples were positive by RT-PCR (11). Among samples from diarrheic pigs collected in Korea during 2004–2005 and 2007, 36% (31/86) were positive by SYBR Green qRT-PCR (42). RT-PCR targeting the S gene was used to test stool and intestinal samples of diarrheic piglets from 20 farms in southwest China collected in the winter of 2011, with 45% (9/20) farms positive for PToV. In addition, 7 of those 9 farms had mixed infection with other swine viruses including PEDV, PKV, porcine rotavirus group A (PRV-A), transmissible gastroenteritis virus (TGEV), PAstV and mammalian orthoreovirus (MRV) (12). In Sichuan Province in the southwest of China, 872 fecal samples collected from diarrheic swine in 2011–2013 were tested by RT-PCR based on the conserved region of the S gene. An overall positive rate of 37.96% (331/872) was found, with positive co-infection with PEDV, TGEV or PRV-A in 4.1% (36/872) of these samples. Among the different ages tested, piglets at 1–3 weeks-of-age had the highest infection rate of 42.47% (295/697) (30).

DISCUSSION

Most of our existing knowledge about ToV is based on the study of BToV and EToV, or the members of the *Coronaviridae*. The lack of an adaptive culture system and infection model to grow the virus hampers the study of viral characteristics and development of diagnostic tools. On the other hand, as PToV has not caused great economic losses, people do not pay attention to it, resulting in a lack of research on treatment and prevention.

The limited studies of sequence diversity may impede development of accurate diagnostic assays and vaccine production. Especially, a lack of study of the variability of the S gene also limits our understanding of the serology of PToV. So far, though many test methods have been established, there are no commercially available diagnostic kits. The expression of structural proteins by various means is important in order to screen for antibodies against PToV, and monoclonal antibodies are needed for further research on important topics like the mechanism of pathogenesis. In particular, the sequence of structural genes, as well as their processing and modification, may affect host specificity of the virus.

Intertype recombination events that have occurred in Europe (31) and Japan (32), among other places, remind us not to underestimate the danger posed by PToV from the possibility of cross-species infection. The mechanism of pathogenesis of PToV is still unclear, and its role during co-infections with other swine enteric pathogens such as PRV A, PAstV, PEDV, TGEV, PKV and *Salmonella* spp. is unknown (11, 30, 46). Considering the prevalence of asymptomatic PToV infections, more research is needed to explore whether it may aggravate the diseases caused by other swine pathogens.

AUTHOR CONTRIBUTIONS

Z-MH, Y-WH wrote the manuscript. All other co-authors edited the manuscript.

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Factors Associated With Time to Elimination of Porcine Epidemic Diarrhea Virus in Individual Ontario Swine Herds Based on Surveillance Data

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Porcine epidemic diarrhea virus (PEDV) emerged into Canada in January of 2014. The virus was considered to be of high importance and the number of new cases were tracked using different mechanisms by stakeholders such as veterinary services from the provincial government and the swine industry. In addition to the initial date of infection, veterinary organizations in the swine industry maintained a disease control program (DCP) database that contained the date of declaration of freedom from PEDV in individual herds. Such data allowed for the determination of the duration of PEDV infection in individual herds based on herd type, year and season of diagnosis. Therefore, the objective of this study was to determine time to PEDV elimination in Ontario swine herds infected between 2014 and 2017, on the basis of records from the DCP database; and to identify factors associated with the likelihood of elimination. Duration of time to eliminate PEDV was estimated using Kaplan-Meier survival curves. The final Cox's proportional hazard model included herd type, season and year of diagnosis. The hazard of PEDV elimination for premises that were farrow-to-wean was 3.36 times larger (*P*-value: 0.044, 95% CI: 1.03, 10.93) than for farrow-to-feeder herds. Herds diagnosed in the summer and fall had hazard ratios of 1.40 (*P*-value: 0.044, 95% CI: 1.03, 10.93) and 7.32 (*P*-value: <0.001, 95% CI: 3.12, 17.18), respectively compared to herds diagnosed in the winter months. The hazard ratio for herds diagnosed in 2015 was 0.54 (*P*-value: 0.015, 95% CI: 0.33, 0.89) compared to herds diagnosed in 2014. Factors associated with time to elimination are likely reflective of the complexity of infection control practices applied in herds with different demographics and population structures, seasonal variability in the pathogen transmissibility, and the availability of resources to manage an emerging production-limiting disease. The median times to elimination were relatively long, which could be due to how it was measured, decisions made at the level of individual herds or delays related to reporting PEDV elimination. Design of control measures for production-limiting diseases at the regional level should take these factors into consideration.

Keywords: swine, porcine epidemic diarrhea virus, time to elimination, Ontario, surveillance

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INTRODUCTION

Porcine epidemic diarrhea virus (PEDV) emerged into Canada in January 2014, soon after the initial detection in the United States (1). The virus is highly contagious and is associated with mortality ranging between 80 and 100% in suckling pigs (2–4). Incursion of porcine epidemic diarrhea (PED) has a large impact on animal health and profitability of individual farms; which can result in high loses for the entire swine-producing sector when a large outbreak occurs (4, 5). Despite this, PED is not considered a reportable disease at the federal level in Canada, similarly to other jurisdictions. Nonetheless, it is considered as provincially reportable in several Canadian provinces including Ontario (6). Both, legislative framework in the province of Ontario and concerns about the impact of this disease in the swine-producing sector supported establishment of several mechanisms of PEDV surveillance with different surveillance coverage (7). One of the surveillance mechanisms is based on the disease control program (DCP) database, which is known as the PED Ontario Area Regional Control and Elimination program (ARC&E). The DCP is based on swine producer volunteer participation and was implemented to monitor disease trends over time. The uniqueness of the DCP database is that it tracks the dates of the initial PEDV incursion, as well as the dates the herds declare freedom from infection from PEDV on the basis of established criteria. This allowed detailed estimation of incidence and prevalence over time in this source population (7). Briefly, the estimated prevalence and 95% confidence intervals (CI) of the virus at the end of 2014, 2015, and 2016 were 4.36 (3.07, 5.99), 2.25 (1.49, 3.26), and 1.35 (0.79, 2.16), respectively (7). A decrease in prevalence, despite occurrence of new cases, has been achieved through implementation of targeted elimination programs at the individual herd level. Soon after PEDV emerged, veterinary practitioners developed approaches that allowed planned elimination of PEDV from swine herds. However, the time to elimination of the virus was premises-dependent and depended on the elimination strategy employed. For planning purposes, the time to PEDV elimination for specific herds could be projected on the basis of the herd type, its demographics, and infection control practices that are planned to be implemented. However, under field conditions, additional factors such as the demographics of the entire production system, the number of animal movements, availability of resources and the herd owners' overall willingness to eliminate a production-limiting disease could affect time to PEDV elimination for specific herds. Since the dates of disease incursion and elimination in individual herds are available, the DCP database could be an appropriate resource for evaluating the time to PEDV elimination under field conditions in the entire population (source population) participating in the DCP program. Therefore, the objective of this study was to determine time to PEDV elimination in Ontario swine herds infected between 2014 and 2017, on the basis of records from the DCP database; and to identify factors associated with the likelihood of elimination.

METHODS

Data Source

The source population for this study was the OSHAB PED Ontario Area Regional Control and Elimination program (ARC&E) database. This DCP and database was initially created for controlling porcine reproductive and respiratory syndrome virus (PRRSV) (8) and then was adapted to include PEDV when it emerged into Canada in 2014. The DCP is a voluntary program that collects diagnostic data including PEDV herd status of Ontario swine herds as outbreaks are reported, or as herds are classified as having eliminated PEDV from premises. The data collected from the participating herds include the premises identification number, herd type, herd size, date of enrollment into the database, PEDV status of premises on date of enrollment and the date(s) in which the premises changed their PEDV status to "free-from-PEDV." For a premises to be included in the current study the following inclusion criteria were fulfilled: (1) the premises participated in the DCP from January 2014 to October 2017, (2) the premises was located in Ontario, and (3) the PEDV infection status of the premises was available.

Premises PEDV Infection Status

The DCP monitors infection status of the volunteer premises over time. Thus, the database contains herd (premises) infection status information i.e., whether a herd has eliminated the virus, whether any subsequent infection has occurred or any other changes in infection status, and the dates when the changes in infection status occurred. In the database, there are 4 types of premises infection status classifications: (1) confirmed positive, (2) presumed positive, (3) presumed negative and (4) confirmed negative. Premises that were classified as PED confirmed positive were premises that had confirmed positive real-time reverse, transcriptase polymerase chain reaction (RT-PCR) test for PEDV at the Animal Health Laboratory (AHL) at the University of Guelph. A presumed positive status was declared based on pig flow and movement as identified by the premises' veterinarian and did not require any diagnostic testing. Thus, premises that housed animals that were sourced from a PED-positive premises were classified as presumed positive due to movement of presumed infected pigs. Presumed negative premises were previously positive premises (i.e., either previously confirmed or presumed positive), where the producer implemented measures to eliminate PEDV from the herd and confirmed the virus to be eliminated through animal or environmental testing. Sampling methods for classifying premises as presumed negative were based on herd type and pig flow, and considered different types of samples (i.e., individual swabs, Swiffer samples, oral fluids, etc). The basic considerations for all sampling types were: 98% individual test sensitivity, 100% individual test specificity, maximum design prevalence of 10%, and 95% confidence in detection of disease at the design prevalence level (9). Lastly, premises that were classified as confirmed PED negative were premises in which there were no clinical or diagnostic evidence of

PED for at least 6 months after the presumed negative status update.

Descriptive Analysis

Data was entered into Microsoft Excel Version 16.14.1 (Microsoft, Redmond, Washington, USA) and then imported into Stata Version 13.1 (StataCorp, College Station, Texas, USA). The proportion of premises that were confirmed PED-positive, presumed PED-positive and presumed PED-negative by herd type were documented. Also, the proportion of herds to eliminate PEDV by herd type, season and the year of PEDV diagnosis were recorded. The median time to elimination and the 25th percentile, along with 95% confidence intervals (CI) were estimated by herd type, season and the year of PEDV diagnosis.

Statistical Analysis

The DCP database consisted of 144 confirmed or presumed PED-positive case herds. Four herds reported subsequent infections, which were excluded from further analysis. In addition, one herd was excluded because the herd type was unknown and another herd was excluded because it was categorized as an isolation/acclimatization unit. Therefore, 138 confirmed or presumed PED-positive case herds were included in the study. A binary variable was created to indicate whether the case herds eliminated PEDV (censored = 1) off-site during the study duration and if the herds did not eliminate PEDV (censored = 0) during the study duration or due to loss-to-follow-up (censored = 0). For the case herds that did not report a change in the virus status over the study period of interest ($n = 8$), the herds were considered to be censored at times when their observation period ended. Similarly, there were cases ($n = 14$) that reported a change in infection status change that was >100 weeks (∼2 years) after the initial date of infection. These herds were censored at 100 weeks. Consequently, a total of 22 herds had their time censored and 116 herds had the event of interest (i.e., reported to have eliminated the virus at least 10% level with 95% confidence).

The time taken to eliminate PEDV from participating premises were estimated using Kaplan-Meier survival curves by herd type, season of diagnosis and year of diagnosis. The variable season was computed and based on northern meteorological seasons. Winter was defined as any confirmed or presumed PEDV diagnosis between December 1st and February 28th, as well as February 29th for the year of 2016 to account for the leap year (10). Any confirmed or presumed PEDV diagnosis between March 1st and May 31st, June 1st and August 31st and, September 1st and November 30th were classified into the variable season as Spring, Summer, and Fall, respectively (10). Log-ranked tests were computed for the 3 categories of Kaplan-Meier survival curves (herd type, year of diagnosis and season of diagnosis).

A Cox's proportional hazard model was constructed to investigate the effect of explanatory variables including herd type, season of diagnosis and year of diagnosis on the time to eliminate PEDV from the premises. The time to event (i.e., elimination) was identified as the time in weeks for a premises to change from confirmed or presumed PED-positive to presumed PED-negative. A failure occurred if the premises eliminated PEDV. Univariable analysis was done using the 3 predictor variables mentioned above, separately. The multivariable model was built using a manual forward selection procedure, with a $p < 0.10$, based on a partial likelihood ratio test as an inclusion criterion. The assumption of the Cox's proportional hazard model was evaluated graphically showing the logarithm of the estimated cumulative hazard function. Goodness-of-fit was evaluated using a Hosmer-Lemeshow test and a Harrell's C concordance statistic. Deviance and score residuals were evaluated.

RESULTS

Descriptive Analysis

From January 2014 to October 2017, a total of 138 PED cases were reported in the DCP database. From the participating premises in the DCP database, 60.1% were finisher sites ($n = 83$), 11.6% were nursery sites ($n = 16$), 10.2% were farrow-to-finish ($n = 14$), 10.2% were farrow-to-wean ($n = 14$), 4.3% were wean-to-finish ($n = 6$) and 3.6% were farrow-to-feeder ($n = 5$), respectively.

Ninety-four cases (65.2%, 90/138) reported that they were confirmed PED-positive. Of these 90 cases, 92.2% ($n = 83$) reported that they eliminated PEDV and therefore gained a presumed-negative status. Forty-eight cases (34.8%, 48/138) reported that they were initially presumed PED-positive, at their

TABLE 1 | Descriptive statistics[1] using a disease control program database* to determine the median and 25th percentile amounts of time it took, in weeks, to eliminate porcine epidemic diarrhea virus.

Variable	Number of premises	Median	95% CI	25th percentile	95% CI
Herd type:					
Farrow-to-wean	13	25	(16, 31)	18	(2, 25)
Wean-to-finish	4	42	(32, NA)	32	(32, 55)
Farrow-to-finish	15	32	(16, 47)	24	(10, 31)
Finisher only	84	33	(30, 38)	25	(21, 28)
Nursery only	17	23	(14, 31)	16	(1, 23)
Farrow-to-feeder	5	43	(18, NA)	27	(18, 79)
Season:					
Winter	46	34	(31, 38)	29	(25, 32)
Spring	58	37	(28, 42)	24	(20, 28)
Summer	26	24	(17, 28)	16	(3, 22)
Fall	8	11	(1, 36)	1	(1, 16)
Year:					
2014	92	30	(26, 35)	23	(20, 25)
2015	27	34	(30, 64)	24	(12, 33)
2016	16	32	(21, 44)	21	(3, 32)
2017	3	18	(13, NA)	13	(13, NA)

The Area Regional Control and Elimination program (ARC&E) database was used to collect diagnostic data on porcine epidemic diarrhea virus herd status of Ontario swine herds on a weekly basis using the Animal Health Laboratory (AHL) at the University of Guelph. Premises that volunteered to participate in the program from January 2014 to October 2017 were included in the study. Descriptive survival analysis statistics are described above.

CI, confidence interval; SE, standard error.

[1]*Kaplan-Meier survival curves by herd type, season of diagnosis and year of diagnosis were created. Kaplan-Meir estimates of the median and 25th percentile time in weeks to eliminate porcine epidemic diarrhea virus was calculated.*

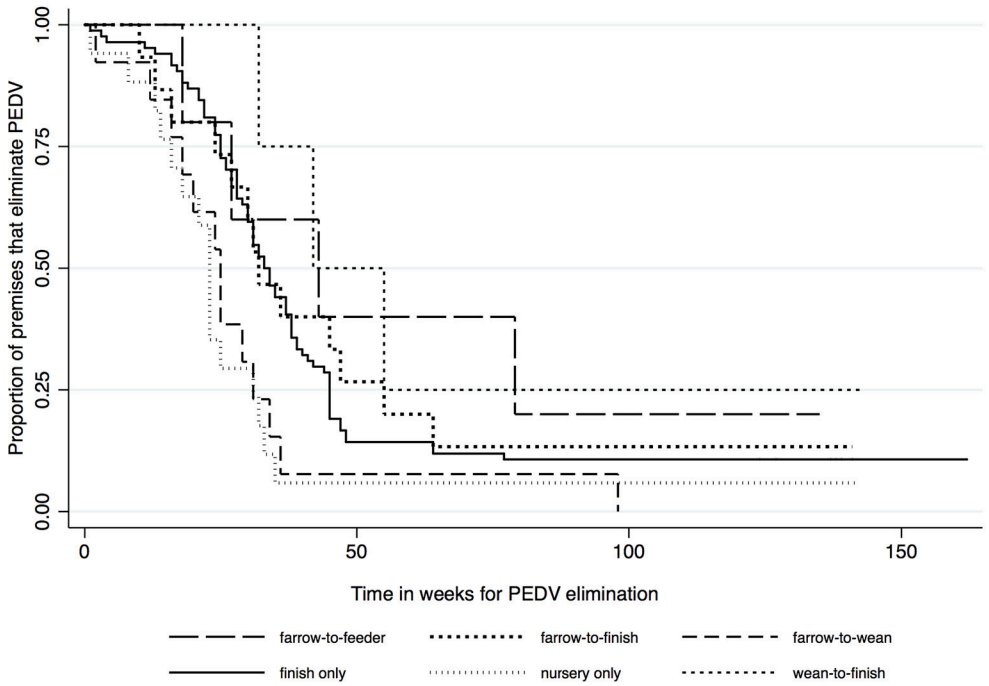

FIGURE 1 | Kaplan-Meier survival functions based on herd type using the porcine epidemic diarrhea virus disease control program database*. *The Area Regional Control and Elimination program (ARC&E) database was used to collect diagnostic data on porcine epidemic diarrhea virus herd status of Ontario swine herds on a weekly basis using the Animal Health Laboratory (AHL) at the University of Guelph. Premises that volunteered to participate in the program from January 2014 to October 2017 were included in the study.

initial date of infection. Of these 48 cases, 97.8% ($n = 47$) reported that they eliminated PEDV during the study period and achieved a presumed-negative status.

Kaplan-Meir estimates of the median and 25th percentile time in weeks to eliminate PEDV are displayed in **Table 1**. Nursery herds had the shortest median (23 weeks, 95% CI: 14, 31) and 25th percentile (16 weeks, 95% CI: 1, 23) for the duration of time it took in weeks to eliminate PEDV. Farrow-to-feeder herds had the longest median time (43 weeks 95% CI: 18, NA) and second longest 25th percentile (27 weeks, 95% CI: 18, 79) for the amount of time it took in weeks to eliminate PEDV. Cases that were diagnosed in the spring and winter seasons had higher medians and 25th percentiles for the amount of time it took in weeks to eliminate PEDV compared to cases that were diagnosed in fall and summer seasons (**Table 1**). The median time to PEDV elimination in swine herds infected in 2014, 2015, 2016, and 2017 were 30, 34, 32, and 18 weeks, respectively (**Table 1**).

Statistical Analysis

Kaplan-Meier survival functions based on herd type, season and year of diagnosis are presented in **Figures 1–3**. The log-rank test statistic evaluating the equality of survival functions between herd types was statistically significant ($p = 0.0029$). Similarly, the season a premises was declared as PEDV-positive ($p < 0.001$) and the year of initial PEDV confirmation ($p = 0.0105$) were both statistically significant.

The results of the univariable analyses conducted through Cox's proportional hazard model are reported in **Table 2**. Briefly, herd type ($p = 0.011$), season ($p < 0.001$), and year of initial diagnosis ($p = 0.019$) were all associated with the likelihood of elimination in univariable analyses. The final multivariable model also included herd type, season of diagnosis and year of diagnosis and is presented in **Table 3**.

Farrow-to-wean premises were 3.36 times more likely than farrow-to-feeder herds (referent category) to eliminate the virus throughout the study period (**Table 3**). The hazard ratio for premises diagnosed in the summer and fall months was 1.40 ($p < 0.001$, 95% CI: 2.74, 9.27) and 7.32 ($p < 0.001$, 95% CI: 3.12, 17.18), respectively. Thus, premises that were diagnosed in the summer and fall months were more likely than herds diagnosed in winter months (referent category) to eliminate PEDV. Premises that were diagnosed with PEDV in 2015, had a hazard of eliminating PEDV that was 0.54 times the hazard of eliminating PEDV in herds diagnosed with PEDV in 2014 ($p = 0.015$, 95% CI: 0.33, 0.89). This suggests that herds that were diagnosed with PEDV in 2015 were less likely to eliminate the virus compared to premises that were diagnosed in 2014 (referent category). In contrast, premises that were diagnosed in 2016 were 1.62 times more likely to eliminate the virus compared to herds diagnosed in 2014 ($p = 0.10$, 95% CI: 0.91, 2.89).

The assumption of the Cox's proportional hazard model was examined graphically showing the logarithm of the estimated cumulative hazard function. There was no indication that the

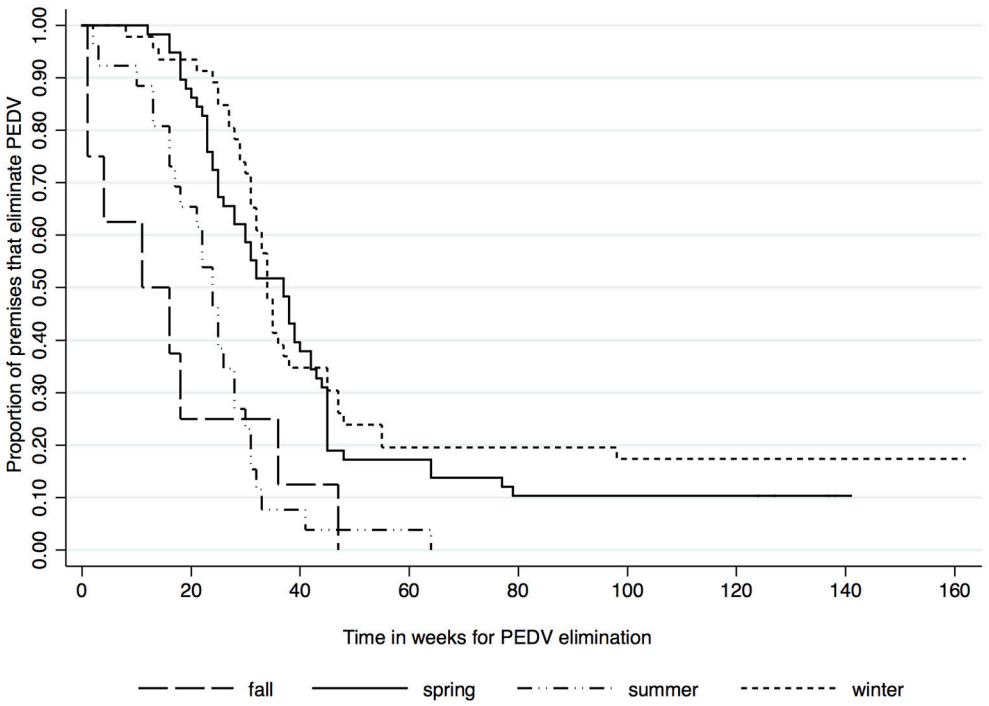

FIGURE 2 | Kaplan-Meier survival functions based on season of diagnosis using the porcine epidemic diarrhea virus disease control program database*. *The Area Regional Control and Elimination program (ARC&E) database was used to collect diagnostic data on porcine epidemic diarrhea virus herd status of Ontario swine herds on a weekly basis using the Animal Health Laboratory (AHL) at the University of Guelph. Premises that volunteered to participate in the program from January 2014 to October 2017 were included in the study.

season of diagnosis and herd type variables had a time varying effect and therefore, the assumption of proportional hazards was met. The Hosmer-Lemeshow test indicated that the model fits the data ($p = 0.46$). Also, Harrell's C concordance statistic computed (0.72) found that the model had good overall predictive ability. There were no outliers or influential observations found.

DISCUSSION

Following the emergence of PEDV into the United States in 2013, many actions were taken in Ontario in anticipation of the emergence of the virus into Ontario. Newsletters, producer meetings and advertisements were communication tools that were used to inform producers of the risk of PED entry and to elaborate on prevention strategies (11). Following the initial emergence, the outbreak in the province of Ontario was well controlled, which was achieved through quick identification of the suspected source of outbreak and implementation of biosecurity practices aimed to prevent further spread of infection. This resulted in a relatively low prevalence of infected herds (7), which could have contributed to willingness to eliminate PEDV infection. Veterinarians have implemented site-specific elimination strategies in Ontario, however the duration of time for a premises to eliminate the virus is variable based on a multitude of factors (i.e., the initial start time for the elimination process may depend on the PEDV status of the sow herd, or the season). The starting time for the time to elimination in this study was not the start date of control measures aimed at elimination, but the date of original infection. In part, due to this reason, the median time to elimination was relatively long. However, we believe that this time to elimination gives veterinary authorities reasonable overview of time to elimination for a newly emerging disease in the area, for which previous experience in elimination did not exist.

An important finding in this study is that with the exception of 2015, the estimated hazard of eliminating PEDV increased over the years examined. Although exact reasons are difficult to determine, it is possible that a combination of factors played a role. Veterinary practitioners were initially dealing with a new emerging disease into Canada, and it is possible that they developed more expertise in procedures to eliminate PED from herds as time went on. Additionally, most cases occurred during the first 2 years ($n = 92$ in 2014 and $n = 27$ in 2015) of the outbreak and it is possible that resources needed to be prioritized between actions needed to prevent further spread and actions to eliminate infection from already infected sites, particularly if such sites required substantial planning. In contrast, the number of new cases in 2016 ($n = 16$) and 2017 ($n = 3$) was substantially lower.

Another important finding in this study was that herds diagnosed in winter and spring months required more time to eliminate the virus. This was likely due to PEDV's survivability and ability to remain infectious. Typically, coronaviruses can survive temperatures from 56°C for 10–15 mins, 37°C for

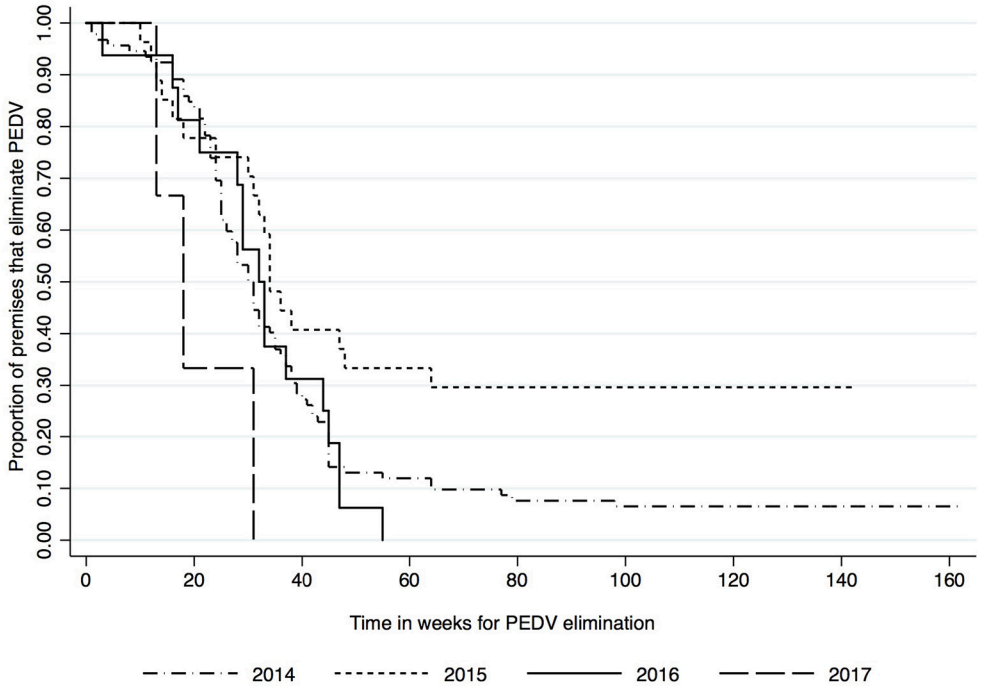

FIGURE 3 | Kaplan-Meier survival functions based on year of diagnosis using the porcine epidemic diarrhea virus disease control program database*. *The Area Regional Control and Elimination program (ARC&E) database was used to collect diagnostic data on porcine epidemic diarrhea virus herd status of Ontario swine herds on a weekly basis using the Animal Health Laboratory (AHL) at the University of Guelph. Premises that volunteered to participate in the program from January 2014 to October 2017 were included in the study.

several days, 4°C for several months, and while frozen at −60°C many years without losing infectivity (12). Thus, it was likely difficult to eliminate the virus due to its survivability in Ontario's temperatures in the spring and winter months. It is also possible that due to the lack of external pressures, producers who had positive herds waited until warmer months to start with the PEDV elimination protocol.

Farrow-to-wean herds were found to eliminate the virus in a shorter amount of time compared to farrow-to-feeder herds. This was an expected finding, since in a farrow-to-wean operation; the system is generally less complex than a farrow-to-feeder or farrow-to-finish operation. For instance, farrow-to-wean herds have fewer types of production classes than farrow-to-finish herds. The presence of nursery pigs on the same site as suckling pigs complicates infection control practices since a separate set of control measures and operating procedures need to be designed and implemented for the nursery stage of production. This requires resources, strict adherence to internal biosecurity protocols and often demographic measures, such as creation of an interruption, or gap, in pig flow. Pig flow through a production system, and more specifically, the creation of a gap in pig flow, is now recognized as an essential aspect of achieving earlier farrowing site elimination by allowing more effective cleaning and disinfection protocols required for successful elimination (13). Pig flow through a production system is the frequency of introducing new pigs into a population and the amount of opportunity these pigs have to come in contact with other pigs. A gap in pig flow however is often a one-time event to prevent the entrance of new animals to control the spread of the virus. A partial depopulation could present a gap in pig flow, where infected animals are removed from the herd, followed by cleaning and decontaminating the site. The database did not include details about specific infection control practices, such as the details of pig flow or attempts to generate a gap in pig flow. Nonetheless, it is also worth pointing out that the variability in the time to elimination was markedly higher in farrow-to-feeder than in other herd types. It is possible that this time to elimination is not only driven by herd demographics and pig flow, but also with other factors such as willingness to eliminate, which was not directly measured in this study.

An important concept for this study is that the data collected was from a large-scale industry-based surveillance program. This study does present limitations. Firstly, the DCP is based on voluntary participation. The Animal Health Act in Ontario required that all PED-positive herds report to the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA), by law, when the hazard was deemed emerging. The OMAFRA surveillance program only accounts for primary case herds, which are case herds with a positive diagnostic test (RT-PCR) for PEDV (14). Thus, secondary cases due to animal movement were not included in the OMAFRA surveillance program. Unlike the surveillance program managed by OMAFRA, where 100% coverage of primary PEDV-infected cases were included, the DCP used in the study only includes primary case herds that

TABLE 2 | Results of univariable analyses[1] using a disease control program database* to determine the hazard ratios associated with herd type, season of diagnosis, and year of diagnosis with the amount of time it took, in weeks, to eliminate porcine epidemic diarrhea virus.

Variable	Hazard ratio	*p*-value	Overall *p*-value	95% CI
Herd type:				
Farrow-to-feeder†				
Farrow-to-wean	2.98	0.058	0.011	0.97, 9.20
Wean-to-finish	0.77	0.73		0.17, 3.45
Farrow-to-finish	1.31	0.64		0.43, 4.03
Finisher only	1.46	0.46		0.53, 3.99
Nursery only	3.35	0.032		1.11, 10.10
Season:				
Winter†				
Spring	1.20	0.40	<0.001	0.79, 1.82
Summer	3.26	0.001		1.95, 5.45
Fall	3.57	<0.001		1.65, 7.69
Year:				
2014†				
2015	0.54	0.015	0.019	0.33, 0.89
2016	1.07	0.80		0.63, 1.83
2017	2.91	0.072		0.91, 9.32

The Area Regional Control and Elimination program (ARC&E) database was used to collect diagnostic data on porcine epidemic diarrhea virus herd status of Ontario swine herds on a weekly basis using the Animal Health Laboratory (AHL) at the University of Guelph. Premises that volunteered to participate in the program from January 2014 to October 2017 were included in the study. Descriptive survival analysis statistics are described above.

† Referent categories.

SE, standard error; CI, confidence interval.

[1]A Cox's proportional hazard model was constructed to investigate the effect of several predictor variables including herd type, season of diagnosis and year of diagnosis upon the time to eliminate PEDV from the premises in 3 univariable models. The time to event (i.e., elimination) was identified as the time in weeks for a premises to change from confirmed or presumed PED-positive to confirmed or presumed PED-negative. A failure occurred if the premises eliminated PEDV.

TABLE 3 | Results of multivariable analyses[1] using a disease control program database* to determine the hazard ratios associated with herd type, season of diagnosis, and year of diagnosis with the amount of time it took, in weeks, to eliminate porcine epidemic diarrhea virus.

Variable	Hazard ratio	*p*-value	95% CI	Partial likelihood ratio (*p*-value)
Herd type:				
Farrow-to-feeder†				
Farrow-to-wean	3.36	0.044	1.03, 10.93	<0.001
Wean-to-finish	0.61	0.53	0.13, 2.83	
Farrow-to-finish	0.80	0.70	0.25, 2.54	
Finisher only	1.07	0.89	0.39, 2.98	
Nursery only	2.33	0.15	0.74, 7.36	
Season:				
Winter†				
Spring	1.40	0.20	0.84, 2.31	<0.001
Summer	5.04	<0.001	2.74, 9.27	
Fall	7.32	<0.001	3.12, 17.18	
Year:				
2014†				
2015	0.42	0.002	0.25, 0.72	<0.001
2016	1.62	0.10	0.91, 2.89	
2017	2.15	0.21	0.64, 7.15	

The Area Regional Control and Elimination program (ARC&E) database was used to collect diagnostic data on porcine epidemic diarrhea virus herd status of Ontario swine herds on a weekly basis using the Animal Health Laboratory (AHL) at the University of Guelph. Premises that volunteered to participate in the program from January 2014 to October 2017 were included in the study. Descriptive survival analysis statistics are described above.

† Referent categories.

SE, standard error; CI, confidence interval.

[1]A Cox's proportional hazard model was constructed to investigate the effect of several predictor variables including herd type, season of diagnosis and year of diagnosis upon the time to eliminate PEDV from the premises in a multivariable model. The time to event (i.e., elimination) was identified as the time in weeks for a premises to change from confirmed or presumed PED-positive to confirmed or presumed PED-negative. A failure occurred if the premises eliminated PEDV. In the current study, the Hosmer-Lemeshow test indicated that the model fits the data ($p = 0.46$). Also, Harrell's C concordance statistic computed (0.72) found that the model had good overall predictive ability.

volunteered to participate in the program, and secondary cases resulting from animal movement from such cases (7).

It is also possible that some producers or veterinarians did not follow up to report that the case indeed eliminated PEDV from the premises, in which case the estimated time to elimination would be longer than in reality. There were 14 premises for which the records indicated that the time between initial infection and a change in status to presumed negative was longer than 100 weeks. The survival time of these premises was censored at 100 weeks. Since participation in this large-scale disease monitoring program is not mandatory, it is possible that some of these premises were not working toward eliminating the virus, since there was no external pressure to do so. Alternatively, it is likely that owners that had a low prevalence of PEDV on-site, may have not tested pigs to confirm PEDV status (i.e., the absence from infection). However, both of these scenarios could occur with a production-limited disease. If large-scale disease control programs are initiated at the level that is different than a premises, or production-system level; veterinary authorities should be aware of the situations where time to negativity could take a long time. In addition, populations with high replacement and/or birth rates such as swine herds could have considerable number of susceptible animals introduced into a population that is partially immune due to recent exposure. This situation could provide opportunity for infectious agents to continue circulating at low levels. Consequently, declaring freedom from infection at 10% may not be sufficient. However, making a decision about the design prevalence should be weighed against the disease epidemiology and cost to producers. Another limitation is the database was missing variables for herd size. Due to this, the authors decided not to consider this variable in the analysis. However, despite these limitations, this study provided novel insight in regards to PEDV elimination times in Ontario.

In conclusion, this study allowed estimation of time to PEDV elimination based on a large-scale disease control program database, which considered time between initial infection and confirmation of PEDV freedom at a minimum level of 10%. Under such assumptions, the median time to elimination of PEDV from Ontario swine herds varied between 23 weeks in nursery herds (standard error =1 week), and 43 weeks (standard error =17.5 weeks) in farrow-to-feeder herds. Herd type, season, and year of original diagnosis were all associated with the time to negativity ($p < 0.05$) in the multivariable model. Among the sow herds, farrow-to-wean herds had the highest hazard of PEDV elimination. These results are reflective of the complexity of the infection control practices applied in herds with different demographics and population structures. The hazard of elimination was also higher in herds that had the initial infection during summer and fall than in herds that had the initial infection during winter. This could be a reflection of seasonal variability in the pathogen transmissibility or decisions made at the level of individual herds to proceed with infection control measures when the likelihood of success is the highest. With the exception of the second year after initial emergence, the hazard of elimination increased over years, which could reflect the availability of resources to manage an emerging production-limiting disease. The median time to elimination was relatively long in all herd types. However, this could be a consequence of the way it was measured, the decisions about implementation of infection control measures which could be made at the level of individual herds, multi-site production systems or possibly delays related to reporting PEDV elimination. Nonetheless, the design of control measures for production-limiting diseases at the regional level should consider these factors.

ETHICS STATEMENT

Research Ethics Board of the University of Guelph approved the study. Data for this study were obtained from Swine Health Ontario under an appropriate data sharing agreement based on secondary data usage.

AUTHOR CONTRIBUTIONS

AP conducted data management, analysis, and interpretation of results under guidance of ZP and TO. Manuscript was written by AP, with input from ZP, TO, CD, and JH.

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Classical Swine Fever in China-An Update Minireview

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Classical swine fever (CSF) remains one of the most economically important viral diseases of domestic pigs and wild boar worldwide. The causative agent is CSF virus, it is highly contagious, with high morbidity and mortality rates; as such, it is an OIE-listed disease. Owing to a nationwide policy of vaccinations of pigs, CSF is well-controlled in China, with large-scale outbreaks rarely seen. Sporadic outbreaks are however still reported every year. In order to cope with future crises and to eradicate CSF, China should strengthen and support biosecurity measures such as the timely reporting of suspected disease, technologies for reliable diagnoses, culling infected herds, and tracing possible contacts, as well as continued vaccination and support of research into drug and genetic therapies. This mini-review summarizes the epidemiology of and control strategies for CSF in China.

Keywords: classical swine fever, CSF virus, China, epidemiology, control strategy

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INTRODUCTION

Classical swine fever virus (CSFV), is a *Pestivirus* in the Flaviviridae family. It is highly contagious and causes disease that can be acute (i.e., transient or lethal) or chronic. Disease progression is dependent on a number of factors, such as strain virulence, host factors, and secondary pathologies. Typically though the acute disease is characterized by high fever, inappetence, and general weakness followed by neurological deterioration, petechial hemorrhages of the skin, and splenic infarction (1, 2). These acute CSFV infections result in high morbidity and mortality rates can be as high as 100%. Subclinical signs such as intermittent fever and inappetence can be seen in chronically infected pigs, and although not life threatening, morbidity is still high (3, 4).

Because of its worldwide distribution and its immense economic impact on the porcine industry globally (5–7), CSF is reportable to the World Organization for Animal Health (OIE) (8). China has also classified CSF as a class A animal infectious disease (9), and according to the National Medium-Term and Long-Term Animal Disease Control Program issued in 2012, CSF, along with other the major animal diseases (Newcastle Disease, Foot-and-Mouth Disease, Highly Pathogenic Avian Influenza), is deemed "most important" and has priority status in disease prevention and control programs (10).

Domestic pigs and wild boars are the known reservoirs for CSFV (11). Since its initial identification in 1833 in the United States, CSFV spread worldwide (12). In recent decades, many countries have implemented strategies for surveillance and control (13). Essential elements of any effective strategy include early diagnosis, culling of infected pigs, formulation and implementation of appropriate veterinary regulations, environmental rehabilitation, as well as prophylactic measures. Where well-implemented these policies have proven remarkably successful in controlling CSF (14). Canada successfully eliminated CSF in 1963, followed by the USA in 1976

and Mexico in 2018 (oie.int); recent data from the World Organization for Animal Health released show that there are now approximately 34 CSFV-free countries (www.oie.int). In areas with dense wild boar populations CSF tends to become endemic whereas it is often self-limiting in small, less dense populations. There has however been a disturbing trend of recurrence in some countries that had declared CSF eliminated (France, the Netherlands, Germany, and Belgium) (6, 15). Parts of Asia and South America have also seen an uptick in cases, of note are the recent reports from Japan of a few documented cases (16).

EPIDEMIOLOGICAL CHARACTERISTICS

Current Epidemiology

China has the largest pig breeding industry in the world, accounting for more than half of global production along with ∼40 million sows and 7 billion fattening pigs (17). According to the Veterinary Bulletin of China, there were 475, 268, 115, 28, 28 cases documented in 2010, 2011, 2012, 2013, and 2014, respectively. There were only 21 cases documented in 2017. The results showed that CSF outbreaks in China has been decreasing over time in recent years (18, 19). As encouraging as this data is, challenges remain for China in the effort to eradicate CSF (20, 21). As the epidemic outbreaks of past years have largely been replaced by sporadic outbreaks, and the virulence of wild type CSFV has decreased, the course of disease has shifted from acute and sub-acute to a chronic form. In addition, there are well documented reports that CSFV may spill over directly or indirectly from wild boar to domestic pigs (6). It was proven that 60% of 92 cases were caused by direct or indirect contact with wild boar (22) in Germany. Remarkably, Japan has reported many cases of CSF in wild boars last September (16). However, there are few cases of virus transmission between wild boar and domestic pig in China (23).

A major challenge facing China is preventing the sporadic outbreaks of CSF on the smaller and medium pig farms (24, 25). Large-scale pig farms have very high immunization rates, as all pigs (boars, sows, and growing and fattening pigs) are immunized, but small and medium-sized farms are not as well-supported and face problems with immunization, these include: (1) immune tolerant gilts are not eliminated before entering the population, (2) immunization procedures are not standardized and do not follow the curve of maternal antibody, therefore, piglets may not receive sufficient immunization, (3) antibody titer is not monitored annually. In this case, even as the population receives cohort immunization, the immune effect is not ideal (26, 27). Clearly better prevention and control measures, with the support from the Veterinary Bureau, are needed to eradicate CSF in China.

Mixed Infections

Co-infection by CSFV and other pathogens complicates diagnosis, treatment, and prevention protocols; as a result morbidity and mortality rates can be quite high. In China, commonly found coinfections with CSFV are porcine reproductive and respiratory syndrome virus (PRRSV), pseudorabies virus (PRV), porcine circovirus type 2 (PCV2), swine influenza viruses (SIV), and often secondary infection such as *Haemophilus parasuis*, swine *pasteurellosis*, *Streptococcosis*, swine enzootic pneumonia, paratyphoid, *colibacillosis*, *toxoplasmosis*, and *eosporophilosis* (28). Some cases have been currently reported that PRRSV and CSFV coinfections are common in Chinese pig populations (29, 30). This combination of pathogens is particularly costly to the Chinese pig industry, because PRRSV is immunosuppressive it seriously inhibits the immune response to the CSF vaccine. Further reports have shown that two other *Pestiviruses*, BVDV and BDV, strongly inhibit the immune response of vaccine against CSFV (31, 32). Based on a coinfection model for PCV2 and CSFV, bioinformatic analyses indicated that mitochondrial dysfunction, nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated oxidative stress response and apoptosis signaling pathways might be the specific targets during PCV2-CSFV coinfection (33). These cases highlight the complexity of CSF control in China.

GEOGRAPHICAL DISTRIBUTION OF GENOTYPES

China and surrounding countries, especially countries of Southeast Asia, have long been the epidemic areas (34). Broadly speaking, molecular epidemiology seeks understand how the interaction of genetic traits and environmental factors result in disease (35). CSFV is a positive single-stranded RNA virus, with a genome approximately 12.3 kb; it comprises a single open reading frame (ORF) that is translated into a single polyprotein composed of 3,898 amino acids. The coding region is flanked by two non-coding regions at both ends (5′ UTR and 3′ UTR) (36, 37). Phylogenic typing has been based on partial sequences of 5′-UTR, E2, and the polymerase gene 5B (NS5B). CSFV isolates worldwide are divided into three genotypes and 11 subgenotypes (1.1, 1.2, 1.3,1.4, 2.1, 2.2, 2.3, 3.1, 3.2, 3.3, and 3.4) (38–41); subgenotype 2.1 is further divided into sub-subgenotypes 2.1a, 2.1b, 2.1c, and 2.1d (42, 43). While globally genotype 2 has been the most prevalent in the last few decades (44–46), all isolates from the Americas belong to genotype 1. The Cuban isolates are clustered in subgroup 1.2, the isolates from Honduras and Guatemala are clustered in subgroup 1.3, and the isolates from Argentina, Brazil, Colombia, and Mexico generated four poorly resolved clusters in subgroup 1.1. However, a present report demonstrated that the Cuban isolates are more divergent from other so far known CSFV subgenotype 1 isolates and form a novel separate subgenotype that is proposed to be designated subgenotype 1.4 (47, 48). Apart from the CSF outbreak in South Africa in 2005 and in Israel in 2009, which were caused by subtype 2.1, very little is known about CSFV in Africa and the Middle East (49). The reports in India show that there is a mixed population of subgenotypes 1.1, 2.1, and 2.2 co-circulating; historically subtype 1.1 was dominant (50–52). The global distribution of subtypes is shown in **Table 1**.

There is a high degree of variation among the prevalent strains of CSFV in China. In the 1990s, the main epidemic strains of CSFV in mainland of China belonged to subtypes 1.1, 2.1, 2.2, and 2.3 (53). Subtypes of 2.1, 2.2, and 2.3 were

TABLE 1 | Global distribution of CSFV subgenotypes.

Genotypes of CSFV	Countries
1.1	Argentina, Brazil, Colombia, Mexico, Italy, Russia, India, China
1.3	Honduras, Guatemala
1.4	Cuba
2.1	South Africa, Germany, The Netherlands, Italy, Spain, Belgium, Croatia, Lithuania, Israel, India, Korea, China, Taiwan, Laos, Mongolia, Indonesia, Vietnam
2.2	Germany, Italy, Czech Republic, Former Yugoslav Republic of Macedonia, India, Nepal, Laos, China
2.3	Italy, Croatia, France, Romania, Bulgaria, Serbia, Slovakia, Czech Republic, Russia, China
3.2	Korea
3.3	Thailand
3.4	Taiwan, Japan

The data in this table have been published in the past 20 years.

predominant in provinces with the most developed pig industry, such as Beijing, Hubei, Jilin, Sichuan, Fujian, Henan, Guangxi and Inner Mongolia. However, these 4 subgenotypes are endemic in Guangdong Province. Since the beginning of the 21th century subgenotypes 2.2 and 2.3 have become less dominant. Presently, 2.1 is the prevalent subgenotype in mainland China (41–45), and 2.1b has become the dominant epidemic subgenotype (54, 55), with 2.1c becoming dominant in southern China (42) and in Taiwan subgenotype 3.4, the dominant subgenotype before 1996, has gradually been replaced by 2.1 (41). Jiang et al. (56) analyzed sequences of 8 epidemic strains isolated in Hunan in 2011–2012, and found that 5 isolates formed a single evolutionary branch of the 2.1 subgenotype along with the isolates from Guangdong and Guangxi. Isolates forming this new branch are designated subgenotype 2.1c. Subgenotype 2.1c is also distributed in Thailand and Laos. Tu et al. (53) showed that subgenotype 2.1c appeared in Guangxi as early as 1998 and Peng et al. (57) further divided 15 isolates of subtype 2.1 from Guangdong in 2011 into three subgenotypes of 2.1b, 2.1c, and 2.1d, while the prevalence of 2.1c and 2.1d was the first reported in Guangdong (32, 58). The distribution of genetic diversity is probably related to the transportation of pigs and the level of development of the pig industries.

In order to further understand the genetic diversity of CSFV in China, 39 isolates from Guangdong and Guangxi from 2004 to 2012 were sequenced and analyzed. Based on partial E2 gene fragment (190 nt) and full-length E2 gene sequence (1119 nt), phylogenetic analysis showed that the currently prevalent subgenotype 2.1 can be further divided into 10 sub-subgenotypes (2.1a∼2.1j), and the isolates previously identified by Peng *et al* as 2.1d are now reclassified into subgenotype 2.1g (57). According to temporal and spatial distribution characteristics, the currently most prevalent subgenotype is 2.1b, the second prevalent subgenotypes are 2.1d and 2.1g, and the silent subgenotypes are 2.1a, 2.1e, and 2.1f (59).

In summary, all four subgenotypes existed before 2008; 2.1 was the most predominant, followed by 1.1, 2.2, and 2.3 which were geographically scattered. Under pressure of the C strain vaccine (1.1), the prevalence of subgenotype 1.1 gradually decreased, and subgenotypes 2.2 and 2.3 gradually withdrew from the epidemic areas, leaving subgenotype 2.1, which is the most phylogenetically distant from the vaccine strain, the dominant CSFV strain in China (60). The epidemic strains in China are genetically diverse, the most prevalent genotype 2 strains are related to those from Europe, possibly originating from the same viral ancestor. We speculate that it may be due to the long-term introduction of pig breeds from EU countries. Although, the epidemic strain of genotype 3 has not been reported in China, it is necessary to maintain surveillance to prevent its introduction from areas surrounding China, such as South Korea (61), Taiwan, and Japan (16).

EVOLUTION OF VARIANTS AND VACCINE PROTECTION

In the more than 60 years since the safe and effective attenuated vaccine was developed in 1954 and used in China (62), CSF has been effectively controlled but not eradicated. In recent years, CSF outbreaks have tended to occur sporadically. Since 2015, the abortions, stillbirths, and diarrhea have increased gradually. Whether these conditions are related to the changes of subgenotypes has not been effectively verified. However, it is certain that there are genetic differences among different subgenotypes (63).

E2 (gp55), the envelope glycoprotein is where most of the antigenic epitopes of CSFV are concentrated, it is highly immunogenic and induces neutralizing antibodies (64). The mutation rate of E2 is between 3 and 25%, it is one of the regions with the greatest mutation rates (65, 66). The percent homology between full-length E2 genes of CSFV subgenotypes is shown in **Table 2**. Note that the percent homology between subgenotype 2.1 and 1.1 (the vaccine strain) is the lowest, suggesting the reason why subgenotype 2.1 is the main epidemic in China (56).

Many studies have shown that vaccination has exerted an influence on the evolution of classical swine fever virus (67). In recent years, a number of immune escape mutant strains, those that are not neutralized by polyclonal antibodies against C strain, have been identified (68). Therefore, we asked whether genotype 2.1 has characteristics of these immune escape mutant strains, while genotype 2.2 and 2.3 gradually disappear under vaccine-induced immune pressure. Results of a cross neutralization test show that the neutralizing ability of the immune pig serum against the C strain is not significantly different from that of the 2.1 major subtype strains that were prevalent in the late 20th century, indicating that the antigenicity of genotype 2.1 has not changed significantly over time, but that its neutralization ability is lower than that of genotype 2.2 and 2.3. This suggests that genotype 2.1 may survive more easily in the natural immune environment, though of course, this speculation needs further study.

TABLE 2 | Percent nucleotide homology of full-length E2 between CSFV genotypes (56).

Genotype	2.1c(%)	2.1a(%)	2.1b(%)	2.2(%)	2.3(%)	1.1(%)	1.2(%)	3.4(%)
2.1c	94.8–100	90.2–94.9	89.9–93.8	87.3–90.1	84.5–85.4	80.8–84.5	81.9–93.0	81.6–83.1
2.1a		94.9–100	91.1–95.7	87.3–91.3	86.5–89.6	81.7–85.7	83.3–85.0	81.4–83.7
2.1b			93.3–100	87.1–89.0	86.4–89.0	81.4–84.4	81.7–83.7	81.2–82.5
2.2				93.7–100	86.5–90.7	81.6–85.0	82.9–85.5	82.3–84.0
2.3					94.6–100	80.7–85.1	82.0–83.6	80.5–81.9
1.1						91.8–100	88.2–93.1	81.6–85.7
1.2							94.2–100	84.4–84.8
3.4								98.3–100

TABLE 3 | Comparison of the homology of the isolates from different regions and laboratories from 2010 to 2015, compared with that of C strain.

Background			Between the isolates		Compared with C strain vaccine	
References	Year	Strains	Nucleotide%	Amino acid%	Nucleotide%	Amino acid %
Fu et al. (69)	2010	7	96.3–99.3	95.6%−100	80.6–81.7	78.0–80.2
Zhu et al. (70)	2011	53	90.15–100	/	79.4–83.1	81.5–85.4
Wang et al. (71)	2012	26	87.1–100.0	/	79.9–91.4	/
Huang et al. (72)	2012–2014	30	81.0–100	85.4–100	81.1–99.0	85.8–97.9
Guo et al. (73)	2012–2014	2	98.7	98.7	83.1–83.6	/
Feng et al. (74)	2015	14	/	/	81.1–82.4	88.2–89.8

To further investigate viral gene variation, we compared the amino acid and nucleotide sequences of the E2 gene of the C strain vaccine with those of epidemic strains isolated from different regions in China from 2010 to 2015. We found 79.4–99.0% nucleotide homology and 78.0–97.9% amino acid homology (**Table 3**).

In general, the homology of nucleotide and amino acid sequences between the isolates and C strain is about 80%, except for some isolates that were very similar to the C strain. Given that the % homology of E2 and other major antigenic proteins between the isolates and the C strain is quite different, does this indicate that the vaccine is failing to provide effective immune protection for pigs? Wang et al. (75) studied the immuno-protective effect of the C strain vaccine against 9 genotypically different strains epidemic in China that present with different clinical pathogenicities. The results showed that the C strain vaccine did produce protection against the tested strains, subgenotypes 1.1, 2.1, and 2.2. Importantly the immunized pigs that were challenged with the test strains did not shed virus. These results provide a scientific basis for the continued use of C strain vaccine in China, but in order to eradicate CSF, it will not be enough. It is not possible to distinguish between vaccinated and naturally infected animals, therefore, the new labeled vaccine will play an important role (76, 77). Up to very recently, only E2 subunit marker vaccines were available on the market (20, 21, 78). In 2014, a new live attenuated marker vaccine CP7_E2alf was licensed by the European Medicines Agency. The resulting data from Friedrich- Loeffler-Institut showed that "CP7_E2alf" is a new instrument in the tool-box of CSF control and can be used to revisit emergency vaccination scenarios (79). Although the vaccines are currently sufficient to provide effective immune protection, they are not omnipotent. In order to cope with future crises, China should strengthen biosafety through continued vaccination and developing alternative methodologies in order to realize the eradication of CSF.

ERADICATION STRATEGIES

There are two strategies for CSF control in the world: preventive immunization and comprehensive culling. For most countries that have no endemic CSF, such as the United States, Canada, Brazil, Chile, South Africa, and the EU countries, culling is used to control CSF. In China, large-scale culling is not feasible, for the present prophylactic vaccination is the best way to reduce the CSF disease burden. The C strain vaccine is widely used in China, but in addition to the problem of being unable to distinguish naturally infected pigs from immunized pigs, use of the C strain vaccine poses other practical problems such as immunization optimization, immunosuppression, vaccine quality, and of course availability and compliance. For example, antibody levels of sows may be above 90%, but the antibody titers of nursery pigs is uneven. Chinese scientists have been working hard to develop new gene-labeled and E2 subunit vaccines for many years and these will be powerful tools for CSF eradication (80, 81). A recombinant E2 subunit vaccine, Rb-03 strain, was developed by Xinjiang Tiankang Animal Husbandry Biotechnology Co., Ltd. in 2016. After vaccination, with this engineered strain, pigs were challenged with CSFV Shimen strain. Challenged pigs did not show clinical signs of CSF and cleared the virus quickly. If such vaccines can produce reliable clinical protection, Chinese pigs may be no longer be diseased by the Shimen strain. The protective efficacy of the subunit vaccine was not different from that of the C strain. The latest unpublished data showed that E2 subunit

vaccine can induce 100% protection against subgenotypes 2.1b, 2.1c, 2.1h, and 2.2.

The biosecurity levels in large-scale pig farms are constantly improving as the Chinese government gives more priority to CSF eradication policies. Listed below are some specific conditions that need to be pursued if the goal of CSF eradication by the end of 2020 is to be met. The conditions are: (1) Cooperative prevention and control. In addition to monitoring and documenting CSFV infection rates and antibody levels, we should also closely monitor the other important swine diseases that are often coinfections with CSFV (such as PRRS, PCAD, PR, etc.) (29, 82, 83); (2) Technical support. An eradication program needs skilled veterinarians, up-to-date diagnostic and monitoring technologies; (3) High quality vaccines must be widely available; (4) Maintain, or pursue where needed, high quality biosafety. Twice yearly etiological investigations should be conducted and where possible, pigs testing positive for pathogens should be culled.

The development of CSFV antigen and antibody detection technologies are important for the prevention and control of CSF. For example, epidemiological investigation and real-time monitoring of antibody levels in immunized pigs are indispensable steps in the process of eradication. Currently there are many diagnostic methods, among which the diagnosis of clinical symptoms is the most direct. But even professional veterinarians are prone to misjudgment in the diagnosis of clinical symptoms and pathological changes. Therefore, to get more reliable results, immunology and molecular biology methods are commonly used to determine levels of CSFV infection as follows (84). The most common immunological detection methods in China are immunofluorescence technology (IFA), virus neutralization tests (VNT), immune colloidal gold technology (GICT), and enzyme linked immunosorbent assay (ELISA), which is the most widely used. Commercial test kits provide rapid reliable detection, greatly improving detection efficiency by allowing for early diagnosis and efficient immune surveillance. It is however still impossible to distinguish between vaccinated and infected animals, and further research is needed. Xu et al. (85) used eukaryotic expression methods to express CSFV E2 protein then purify it from an inclusion. They then developed an indirect ELISA, thereby laying a solid foundation for the development of a diagnostic kit. In recent years, more and more research to detect antibodies and pathogens in the oral fluids of swine has been reported. With the rapid development of molecular biology technologies, their role in the diagnosis of animal diseases have become prominent. Presently, the most widely used CSFV nucleic acid detection technologies, RT-PCR, RT-nested PCR, RT-nested PCR based restriction fragment length polymorphism (RFLP), real-time RT-PCR, and RT-LAMP, have been developed in China to detect CSFV and/or differentiate wild-type CSFV and C-strain. Due to co-infections of CSFV with other viruses, several multiplex PCR assays have been developed in China, allowing simultaneous detection of CSFV and other porcine viruses.

Depending on vaccination alone though, may not be sufficient to eradicate CSF and the development supplemental antiviral strategies are needed. Anti-CSFV therapies such as capsid-targeted virus inactivation (86), RNA-hydrolyzing recombinant antibody (87), RNA interference (88), Imidazo[4,5-c]pyridines (89), and uridine derivatives of 2-deoxy sugars (90) have been reported but their clinical effect and practical application for CSF control needs further study and development. Our lab has found porcine Mx1 has anti-CSFV activity (91) and continue to dissect the mechanism of poMx1 against CSFV (92). Our findings will provided significant information for the potential development of a novel antiviral therapy. In addition, our research clarified the pathway of CSFV internalization (93, 94), which will promote our current understanding of pestivirus cellular entry pathways and provide novel targets for antiviral drug development. Finally, anti-CSFV transgenic pigs have been produced by somatic nuclear transfer and *in vitro* and *in vivo* viral challenge assays have demonstrated that replication of CSFV and CSFV-associated pathologies and mortality in these pigs is effectively limited (95), and a recent report that transgenic pigs refractory to CSFV have been successfully developed using a CRISPR/Cas9-mediated knock-in strategy, offers exciting promise (96). Interestingly, we know that the host factor JIV can promote viral replication (97, 98). If the researchers use the CRISPR/Cas9 technology to knock out the JIV gene and breed another pig that is resistant to CSFV, it is possible in the future.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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MINI REVIEW
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Next-Generation Sequencing Coupled With *in situ* Hybridization: A Novel Diagnostic Platform to Investigate Swine Emerging Pathogens and New Variants of Endemic Viruses

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Next generation sequencing (NGS) can be applied to identify and characterize the entire set of microbes within a sample. However, this platform does not provide a morphological context or specific association between the viral or bacterial sequences detected and the histological lesions. This limitation has generated uncertainty whether the sequences identified by NGS are actually contributing or not for the clinical outcome. Although *in situ* hybridization (ISH) and immunohistochemistry (IHC) can be used to detect pathogens in tissue samples, only ISH has the advantage of being rapidly developed in a context of an emerging disease, especially because it does not require development of specific primary antibodies against the target pathogen. Based on the sequence information provided by NGS, ISH is able to check the presence of a certain pathogen within histological lesions, by targeting its specific messenger RNA, helping to build the relationship between the pathogen and the clinical outcome. In this mini review we have compiled results of the application of NGS-ISH to the investigation of challenging diagnostic cases or emerging pathogens in pigs, that resulted in the detection of porcine circovirus type 3, porcine parvovirus type 2, Senecavirus A, and *Mycoplasma hyorhinis*.

Keywords: pig, diagnosis, emerging infectious diseases, NGS-ISH, PCV3, PPV2, SVA

INTRODUCTION

The U.S. swine industry has evolved from small independent farming operations to integrated large systems (1). Along with this process, the production systems have faced emerging health challenges, particularly regarding virus diseases (2). In addition, virus-associated syndromes and disease complexes have become more common due to the involvement of multiple pathogens or virus subtypes in the same tissue (2). The early identification of pathogens in pig herds is crucial for the decision-making process in regards of disease control, prevention, strategy of treatment and, therefore, mitigation of the impact of a particular disease (3). Next-generation sequencing has been recently used to detect nucleotide sequences in challenging diagnostic cases,

but it does not provide morphological context that would allow the association of a specific viral sequences with the histological lesions. This scenario generates uncertainty whether the sequences identified by NGS are actually contributing for the clinical signs. While immunohistochemistry may overcome this limitation by detecting antigens in association with histological lesions, it requires specific antibodies that are not always promptly available commercially (4). Alternatively, *in situ* hybridization assay allows the detection of nucleotide sequences in histological sections without requiring the development of such antibodies.

Traditional diagnostic methods are still extremely important to veterinary diagnostics medicine. However, the turnaround time for results that are crucial during diagnostic investigations of unsolved cases can be much to slow. Q-PCR is an assay with a short turnaround that offers an indirect quantification of the amount of a microorganism in the sample, but it is not able to distinguish whether a given pathogen was viable in the sample, whether its presence is associated with histological lesions of if the pathogen/lesions association correlates with the clinical signs. Bacterial and viral isolation is considered the gold standard method for the definitive diagnostics of numerous infectious diseases, but these classic approaches can be time consuming. Also, isolation of an emerging pathogen faces obstacles, such as what is the susceptible cell line or media for isolation, if it is a caused by a non-culturable agent and are there multiple agents involved in the disease syndrome (5).

Tissue-based diagnosis is undeniably one of the most important approaches for the identification of a potential role of the pathogen as the etiologic agent of that disease. The histological lesions recognized by the pathologist can be associated with the presence of a given pathogen detected by immunohistochemistry within the lesions (4). Nevertheless, when dealing with an emerging disease, production of a sensible and specific antibody and the optimization of the immunohistochemistry protocol can take several months, delaying effective actions to control a particular emerging disease. This mini-review focuses on the development and application of a new platform of diagnostics that combine next-generation sequencing and *in situ* hybridization for investigating unsolved diagnostic cases in swine.

NEXT-GENERATION SEQUENCING AND *IN SITU* HYBRIDIZATION

Sequence technologies have risen in the past few years as a meaningful ancillary tool for diagnostic investigation of infectious diseases. Next generation sequencing (NGS) is one of the most recent sequencing approaches included in the range of diagnostic methods for veterinary diagnostic investigations (6–8). The NGS advantage in relation to simple sequencing relies on a *de novo* or mapping assembly of sequenced regions, dismissing the use of specific targets to build a complete genome. With NGS, genome fragments of virtually all the microorganisms present in the clinical samples are sequenced and then, re-assembled based on genome data bases publicly available. These characteristics of NGS make it very convenient for investigation of infectious disease outbreaks in which the etiologic agent is unknown. Although extremely sensitive, NGS lacks the association of the presence of a microorganism and the type of histological lesion. Since the development of specific antibodies for immunohistochemistry requires a long period of time, there was a demand for a diagnostic method that would allow the association of the genomes detected by NGS with a tissue lesion.

The usefulness of *in situ* hybridization (ISH) to detect pathogens within histological lesions in pigs has been recognized since the 1990's (9–12). At first, ISH probes were radioactively labeled, but due to the risk of manipulation and low sensitivity of the test, alternative labeling systems were developed (4), such as digoxigenin, biotin, dinitrophenol, and fluorescence (also named as FISH). Although the traditional ISH assays have satisfying specificity, their sensitivity has also been a target of discussion even with non-radioactively labeled probes, especially when the target was a short nucleotide sequence (4, 10, 13, 14). Recently, a new approach for increased ISH sensitivity has been developed (15). This improved method is based on a signal amplification system that allows visualization of a single molecule in paraffin embedded sections. Since then, various publications have proven the applicability of this new ISH method for research studies in veterinary medicine (16–21). In those cases, the design of ISH probes was based on the nucleotide sequences of endemic or well-described microorganisms. Withal, in cases in which the involvement of known pathogens has been ruled out by other methods, there is a need of an auxiliary method for the detection of possible new emergent pathogens or variant of known pathogens in clinical samples. Therefore, the association of NGS and ISH has been extremely advantageous to overcome those limitations (**Figure 1**).

APPLICATION

Vesicular Lesions Caused by Senecavirus A

The years of 2014 and 2015 were marked in the swine industry of the USA, Canada, China, and Brazil by outbreaks of diseases characterized by vesicular lesions on the coronary band and snouts of sows and growing pigs and acute neonatal pig deaths (22–25). In addition to the impact of the disease in pig herds itself, the vesicular lesions are clinically indistinguishable from foot-and-mouth disease (FMD) and other vesicular diseases.

Diagnostic investigations ruled out the most common vesicular diseases of pigs (swine vesicular disease, vesicular stomatitis and vesicular exanthema), as well as FMD. Then, samples were submitted for NGS. NGS revealed genome sequences with high similarity to Senecavirus A (SVA) available in GenBank (22). Since then, SVA has been confirmed as the etiologic agent of those outbreaks of vesicular diseases in pig herds in North America and South America (26, 27), and a commercial kit for SVA qRT-PCR was developed.

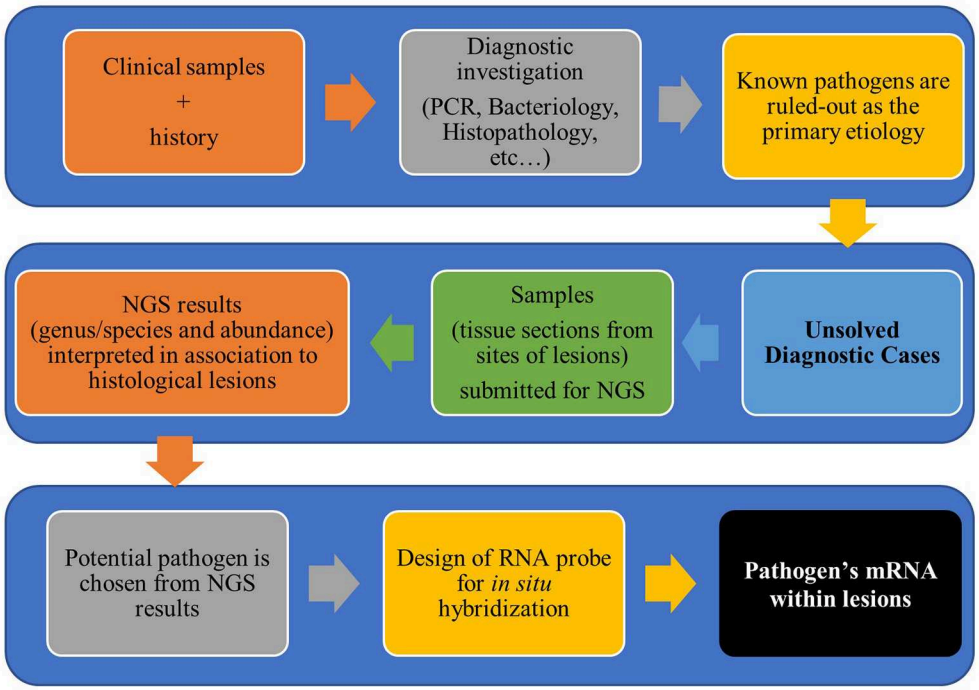

FIGURE 1 | Workflow for diagnostic investigation of unsolved cases using next-generation sequencing and *in situ* hybridization.

Although SVA has been associated with swine idiopathic vesicular disease in Canada and the USA since 2008, anti-SVA antibodies were not commercially available for IHC for investigation of SVA outbreaks in 2014. Based on the genome sequences identified by NGS, ISH probes were designed to investigate the presence of SVA within the histological lesions from RT-qPCR positive samples, including tissue samples from piglets affected by acute neonatal losses (17). Lesions in the skin of sows with snout and coronary band vesicles consistently associated with low Ct values for SVA in the RT-qPCR and a strong positive label in ISH (17). Samples from piglets affected by the acute neonatal losses did not showed histological lesions, with exception of erosive lesions of tongues from piglets (17, 23), which were also ISH positive for SVA-mRNA.

ISH has also been valuable for investigation of the pathogenesis of SVA infection in pigs. Vesicular lesions were reproduced by the experimental infection of 15-weeks-old pigs with a contemporary SVA isolate obtained from a lesion swab of a finishing pig with vesicular disease (18). Histological lesions were observed in skin (vesicles) and associated with ISH positive signals as previously reported. All other tissues were histologically normal, except lymphoid tissues, in which lymphoid hyperplasia was observed. ISH positive signals for SVA were observed in tonsils, which had higher amounts of nucleic acids determined by RT-qPCR (18).

PCV2- and PCV3-Associated Diseases

Pigs from many countries have been suffering from clinical syndromes caused by porcine circovirus type 2 (PCV2). PCV2 associated disease was first described in the early 1990's (28). The clinical signs of PCV2 are associated with the well-recognized manifestations of the infection [post-weaning multisystemic wasting syndrome (PMWS), Porcine dermatitis and nephropathy syndrome (PDNS), reproductive disease, enteric disease, lung disease] although subclinical infections are also common (29). Since the development of a vaccine against PCV2, the clinical signs of PCV2-associated diseases has been diminished (29). It was in 2016 when the new porcine circovirus was found in pigs exhibiting clinical signs similar to the PCV2-associated diseases.

Cases of post-weaned pigs with unspecific clinical signs, mainly characterized by weight loss, failure to thrive and occasionally respiratory distress were presented at the Veterinary Diagnostic Laboratory at the University of Minnesota (UMN-VDL). Although the majority of the clinical signs and histological lesions were attributed to the presence of known pathogens identified by traditional diagnostic tests, lymphoplasmacytic and histiocytic myocarditis, vasculitis and interstitial pneumonia observed in affected pigs were still lacking an etiologic explanation. Tissue samples were submitted to NGS and a "porcine circovirus-like" sequence was consistently identified. From the sequences identified by NGS, a sequence of 200 bases with high similarity to the genus *Circovirus*, was used to design a ISH probe, in order to confirm the presence of the proposed virus within histological lesions. PCV3 mRNA demonstrated by hybridization signals was observed in cardiomyocytes and in wall of arteries with inflammation (30). PCV3 has then been recognized as a new *Circovirus* species by the International Committee on Taxonomy of Viruses (ICTV) (31).

Since then, primers for q-PCR were developed based on the PCV3 genome sequence and have been used in countries from Asia (32–34), Europe (35, 36), and South America (37, 38). PCR positive results and ISH positive signals were detected in tissues sections from sows with PDNS and reproductive failure, in tissues from aborted fetuses, and in diverse samples from pigs with PMWS, especially in myocardium and arteries (39, 40). Although viral isolation is still lacking, PCV3 has been proposed as the etiologic agent of clinical syndrome associated with the histological lesions described above, based on the molecular guidelines for microbiological etiologic causation as suggested by Fredricks and Relman (41).

Although PCV2 could be ruled out as a potential etiologic agent in cases of PMWS, PNDS, and reproductive failure, there was still a possibility of a co-infection of PCV2 and PCV3, especially due to the endemic distribution of both viruses within the pig population. Hence, a duplex-ISH was developed to allow the simultaneous detection of both viruses in pig samples (37). From a total of 477 tissue samples recovered from the UMN-VDL historical cases, 9% ($n = 43$) were positive for both viruses, PCV2 and PCV3 by ISH (37). Both viruses were predominantly observed in germinal centers in lymph nodes, in peritarteriolar lymphoid tissue in the spleen, in lymphohistiocytic infiltrates of heart arterioles and also in peri-bronchiolar lymphoid cuffs (37). However, it was noted that lymphoid depletion is not a characteristic of PCV3 infection, as it is for PCV2. These results highlight the challenges for interpreting PCR results when animals are positive for PCV2 and PCV3 and reinforce the need of qualified pathologists to interpret the histological lesions and the possible association with the agent identified by NGS.

Mycoplasma hyorhinis-Associated Conjunctivitis

Recent outbreaks of swine conjunctivitis have been reported to the University of Minnesota Veterinary Diagnostic lab. After ruling out the most common causes of infectious conjunctivitis in pigs (pseudorabies, swine influenza, porcine cytomegalovirus, and Chlamydia) and discarding the possibility of a non-infectious cause palpebral conjunctiva from affected pigs were submitted for NGS. NGS results indicated a high proportion of *M. hyohrinis* genome in the samples, which were confirmed by qPCR. In order to verify whether *M. hyorhinis* was present within the lesions, and due to a lack of antibodies anti-*M. hyorhinis* for immunohistochemistry, ISH probes were designed based on the 16S sequence of *M. hyorhinis*. Hybridization signals were observed in samples from affected pigs, but not in samples from non-affected animals from unrelated non-affected herds (42). These results corroborate past investigations that indicated *M. hyorhinis* as the etiologic agent of swine conjunctivitis (43, 44).

Porcine Parvovirus Type 2-associated With Perivasculitis

A novel porcine parvovirus, parvovirus type 2 (PPV2) was originally identified in Myanmar in 2001 in a serum sample (45), and since then, PPV2 has been detected in various pig samples (46–48). There has been reports of a positive correlation of PPV2 detection and poor performance in affected pigs (47) and of presence of PPV2 in lung tissues from pigs with respiratory clinical signs and PCR positive for PCV2. PPV2 was identified by direct in *in situ* PCR in pulmonary lesions described as vascular thickness caused by lymphocytic infiltration, reduced alveolar spaces and epithelial damage, without a direct correlation with PCV2 detection in the same tissues (49). Cases of poor growth performance in nursery pigs associated with systemic perivascular inflammation were studied for potential causative agent at the UMN-VDL. Due to the lack of detection of known pathogens in samples from the affected pigs, tissue samples were submitted for NGS. The high proportion of PPV2 sequences identified by NGS along with compatible histological findings suggested the involvement of PPV2 in the cases. Samples were then tested by RT-qPCR and positive tissues were submitted to ISH. PPV2 ORF mRNA was chosen as the target for ISH probes. ISH PPV2 signals were observed in an association with the histological lesions in various tissues (lung, joint and subcutaneous tissues) within the cytoplasm of endothelial cells and in lymphoid follicles of the lymph nodes and broncho-associated lymphoid tissues (50). These results represent an important advancement for understanding the potential role of PPV2 in emerging systemic syndromes in nursery and finishing pigs.

Porcine Sapelovirus in Pigs With Polioencephlomielitis

Outbreaks of atypical neurological disease were reported in swine herds of the United States in the past few years. Clinical signs of anorexia, compromised movement, decreased responso se stimuli and mental dullness were associated with severe lymphoplasmacytic and necrotizing polioencephalomyelitis with multifocal areas of gliosis and neuron satellitosis, suggestive of a neurotropic viral infection. Due to the lack of detection of the most commonly viruses associated to neurological diseases in pigs, such as pseudorabies virus and atypical porcine pestivirus, porcine reproductive and respiratory syndrome virus and porcine circovirus, samples of brainstem and spinal cords from affected pigs were used NGS. NGS results identified porcine sapelovirus and absence of other or novel pathogens. By ISH, Sapelovirus A mRNA was detected in neurons and nerve roots of the spinal cord of affected pigs (51).

FINAL CONSIDERATIONS

The incidence of swine emerging, and reemerging diseases have increased in the past few years. Traditional ancillary tests are routinely used to investigate the involvement of known pathogens when samples from outbreaks are sent for diagnostic investigation. However, one of the characteristics of traditional ancillary tests is that they are designed to detect known pathogens. The association of NGS-ISH has been instrumental on the investigation of etiologic causes of cases in which the traditional ancillary tests did not identified involvement of known pathogens. The UMN-VDL, one of the most important diagnostic laboratories for swine infectious

diseases in the US, is a pioneer in using NGS-ISH as a tool investigate possible emerging diseases. Nevertheless, as happens to new technologies are being implemented and optimized, NSG-ISH has its drawbacks. Although NGS is currently cheaper, faster and more assessible to non-research purposes, it is still a technology that requires structured laboratories, equipment and, most importantly, qualified professionals to interpret the relevance of the several microorganisms' sequences identified by the sequencing in the swine samples. ISH also has its own limitations. By targeting mRNA, ISH helps to determine whether the virus is metabolically active within lesions. However, the approach relies on targeting a mRNA that corresponds to the DNA sequence identified by the NGS. If the chosen DNA sequence is not been translated in mRNA, the absence of positive signals can mean a false negative. In addition, ISH method used in combination with NGS it is still relatively expensive due to dependency on a single manufacturer that detains the intellectual property of the ISH technique. Nevertheless, we anticipate that the in the next few years both the demand for best diagnostic approaches for emerging diseases will stimulate both methodologies to evolve in regards of their feasibility and costs, making the NGS-ISH an important tool for identifying pathogens in swine emerging diseases.

In conclusion, the NGS-ISH diagnostic platform presented here combines the comprehensive unbiased detection of nucleic acid sequences with the morphological context shown in the histological lesions. This characteristic has been specifically important to diagnose infectious diseases in which the clinical and laboratory findings are not able to specific determine the primary pathogen involved in the clinical outcome. Additionally, the ability to detect sequences from previously unknown agents through NGS allows the design and subsequent identification of emerging pathogens within tissue sections, by targeting mRNA using ISH assay. This rapid diagnostic response is critical to implement control measurements and mitigate economic losses in the swine industry.

AUTHOR CONTRIBUTIONS

TR conducted the literature review and wrote the manuscript. LM and SR helped revising the manuscript, adding important scientific content, and refining the interpretation of the results. FV conceived of the idea of the review and helped revising the manuscript to add important scientific content and refine the interpretation of the results. All the authors reviewed the final version of the manuscript and agreed to its submission.

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