A Dissertation for the Degree of Doctor

of Philosophy

Pathogenesis of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus

by

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Department of Veterinary Pathology

Graduate School of

Seoul National University

August, 2015

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獸醫學博士 學位論文

Pathogenesis of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus

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Pathogenesis of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus

By

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A dissertation submitted in partial fulfillment of
the requirements for the degree of
DOCTOR OF PHILOSOPHY

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Abstract

Pathogenesis of Highly Pathogenic Porcine Reproductive and Respiratory Syndrome Virus

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Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) is the causative agent of PRRS. A new disease syndrome known as 'high fever disease' was first reported in China in 2006, since then spreading rapidly in neighboring Asian countries such as Vietnam, Laos, Cambodia, Myanmar, Philippines, and Russia. Currently, infection of HP-PRRSV causes very huge economic loss for the Asian swine industry.

This “high fever disease” was recognized as an atypical form of PRRS and the causative virus was named as highly pathogenic PRRSV (HP-PRRSV). In Vietnam, HP-PRRSV lead to the death of thousands of pigs as the virus spread from northern through central and to southern Vietnam within 3 months of the first outbreaks in 2007.

The first study aimed to compare the virulence of northern and southern Vietnamese strains of highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) as assessed by the level of viral replication, gross and microscopical lung lesions and virus distribution in experimentally infected pigs. The northern and southern Vietnamese HP-PRRSV strains share 96.7% (non-structural protein 2) and 99.3% (open reading frame 5) nucleotide identity. On experimental challenge, approximately 50% of pigs infected with northern Vietnamese HP-PRRSV died, while death was not observed in any pigs infected with southern Vietnamese HP-PRRSV. Mean viral titres (expressed as $\log_{10}TCID_{50}$/mL) were significantly (P <0.05) higher in sera and lungs from pigs infected with the northern Vietnamese HP-PRRSV than from those infected with the southern Vietnamese strain at multiple time points. Lung lesion scores and PRRSV antigen within pulmonary and lymphoid lesions were significantly (P <0.05) higher in pigs infected with northern Vietnamese HP-PRRSV than in those receiving southern Vietnamese HP-PRRSV at multiple time points. PRRSV antigens were observed in cardiac myocytes, gastric and renal tubular epithelial cells, and astrocytes and microglia of white matter in the brain from pigs infected with the northern Vietnamese HP-PRRSV strain only. Thus, genetic similarity did not predict

the degree of virulence of these strains. Northern Vietnamese HP-PRRSV was more virulent and had extended tissue tropism when compared with southern Vietnamese HP-PRRSV.

The objective of the second study was to compare the pathogenicity of highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) infection between wild and domestic pigs based on clinical, immunological, and pathological evaluation. Upon challenge with HP-PRRSV, five wild pigs died compared to none of the domestic. Anti-PRRSV antibody titers were significantly ($P < 0.05$) higher in wild HP-PRRSV-infected pigs versus the domestic HP-PRRSV-infected pigs at 21 days post inoculation (dpi). Lung lesion scores at 7 dpi were also significantly ($P < 0.01$) higher in domestic infected pigs than wild infected pigs. The most striking difference was the viral tissue distribution between the wild and domestic HP-PRRSV-infected pigs. HP-PRRSV-positive cells were observed in bronchiolar, gastric, and renal tubular epithelial cells from wild HP-PRRSV-infected pigs only. The results in this study demonstrated a genetic difference exists between wild and domestic pigs, which could results in different clinical signs, immunological responses, and pathological outcomes to HP-PRRSV infection.

In the last study, a total of 34 highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) strains isolated from Vietnam during 2013–2014 were sequenced and analyzed. Partial sequence of nonstructural protein 2 (Nsp2) gene and

full sequence of open reading frame 5 (ORF5) gene was used for the analysis. The HP-PRRSV strains were isolated from pig herds that had never been vaccinated for PRRSV. The nucleotide homology of Nsp2 and ORF5 ranged between 96.4 to 100% and 83.2 to 100%, respectively. All of the 34 Vietnamese HP-PRRSV strains showed two discontinuous 30 amino acids deletions in the Nsp2 gene as a genetic marker of prototypic Chinese HP-PRRSV. Amino acids at position 13 and 151 in ORF5 are arginine (R) in 29 out of 34 Vietnamese HP-PRRSV isolates as those in prototypic Chinese HP-PRRSV. Genomic analysis of ORF5 from all Vietnamese HP-PRRSVs revealed six subgroups; Viet 1 to 4, JXA1-like, and VR-2332-like. Nucleotide and amino acid sequence analysis of 34 Vietnamese HP-PRRSV isolated during 2013–2014 indicate that Vietnamese HP-PRRSV has undergone rapid evolutionary changes in recent years when compared with Vietnamese HP-PRRSV isolated before 2012.

Conclusively, genetic similarity did not predict the degree of virulence of these strains. Northern Vietnamese HP-PRRSV was more virulent and had extended tissue tropism when compared with southern Vietnamese HP-PRRSV. The genetic difference exists between wild and domestic pigs, which could results in different clinical signs, immunological responses, and pathological outcomes to HP-PRRSV infection

Keywords: highly pathogenic porcine reproductive and respiratory syndrome virus; pathogenicity; respiratory disease; genetic difference; pig; wild pig.

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LIST OF ABBREVIATION

a.a	Amino acid
CPE	Cytopathic effect
Ct	Cycle threshold
DNA	Deoxyribonucleoic acid
dNTP	Deoxynucleotide triphosphates
dpc	Days post challenging
dpi	Days post infection
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
ELISA	Enzyme-linked immunosorbent assay
E	Envelope protein
EU	European
GP	Glycoprotein
HP-PRRS	Highly pathogenic porcine reproductive and respiratory syndrome
HP-PRRSV	Highly pathogenic porcine reproductive and respiratory syndrome virus
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
ISH	In-situ hybridization

Kb	Kilobase
M	Membrane protein
MARC-145	Subclones of MA104 monkey kidney cell
MLV	Modified live virus
N	Nucleocapsid protein
NSP	Non-structural protein (coding region)
ORF	Open reading frame
PAM	Porcine alveolar macrophages
PCR	Polymerase chain reaction
pp1a	Polyprotein 1a
pp1ab	Polyprotein 1ab
PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
RNA	Ribonucleoic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
$TCID_{50}$	Median tissue culture infective dose
US	United States

LITERATURE REVIEW

1. PRRS at a glance

PRRS in global settings

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease, and is characterized by reproductive failure in pregnant sows and respiratory disease in nursery and grower/finishing pigs (Zimmerman et al., 2012). The disease was recognized firstly in the United States and Europe (United Kingdom) in the late 1980s (Roberts et al., 2009; Zimmerman et al., 2012). In the early 1990s, this disease was also identified in Asia (Kweon et al., 1994; Murakami et al., 1994). The causative PRRS virus was isolated in porcine alveolar macrophage in 1991 (Wenswoort el al., 1991) and later on established in monkey cell line in the USA in 1992 (Benfield et al., 1992). Currently, porcine reproductive and respiratory syndrome virus (PRRSV) is becoming widespread throughout the world.

PRRSV causes reproductive disorders in sows and respiratory diseases in piglets. Since emerged in late 1980s, PRRSV has devastated the global swine industry, causing immense economic losses (in review by Meng, 2012). The disease lost approximately $560 million each year in the United States (Neumann et al., 2005). The novel outbreaks of PRRS continue to appear periodically worldwide. Particularly, a variant

strain of PRRSV emerged in pigs from China in 2006 and Vietnam in 2007, caused a severe, devastating disease, mentioned as 'porcine high fever syndrome-PHFS' with 20–100% mortality (Tian et al., 2007; Metwally et al., 2010; Zhou and Yang, 2010).

HP-PRRS outbreaks in Vietnam

Evidence of PRRSV in Vietnam was firstly reported in 1997 from pigs imported from the US, was later identified in southern Vietnam in 2006 (Kamakawa, 2006). The first large scale reports of PRRSV outbreaks occurred in Hai Duong province in March, 2007 (44 outbreaks in both northern and southern provinces affecting 44,000 pigs with observed mortality rates of 24%) (MARD, 2010). Genetic characterization of selected PRRSV isolates involved in the China and Vietnam 2006-2007 outbreaks identified a novel 30 aa discontinuous deletion in the Nsp2 gene (Tian et al., 2007; Feng et al., 2008), as a genetic marker for increased virulence. The Nsp2 deletion variant has remained the most prevalent lineage of PRRSV in the region at least until December 2011 (Yu et al., 2012). In 2008, total mortality due to deaths and culling was estimated at more than 300,000 in 26 of 60 provinces of Vietnam. In the truly devastating outbreaks of 2010, 53 of 63 provinces were involved, and more than 1,100,000 pigs destroyed (Figure 2, Dung et al., 2013). Although PRRSV is not transmissible to humans, potential zoonotic implications for human health have been suggested, especially in relation to *Streptococcus suis* serotype 2 (Hoa et al., 2013). Temporal and spatial analyses (Dung et al., 2013) indicated that there have been spatial clusters of

PRRS outbreaks in the red river delta, north center, west south and Mekong river delta. These are all important areas for pig production in Vietnam.

Although PRRSV was clearly the major causative agent of the observed morbidity and reproductive disorders of the 2007-2010 outbreaks, experimental studies using a Vietnamese PRRSV isolate have failed to reproduce the severe clinical syndromes seen in the field (Metwally et al., 2010), suggesting the involvement of secondary or concomitant infections. Among the agents suspected of involvement were virulent classical swine fever virus (CSF), porcine circovirus type 2 (PCV2), and bacterial agents such as *Mycoplasma hyopneumonia*, and *Streptococcus suis* serotype 2 (Hoa et al., 2012; Le et al., 2012).

The extraordinary losses associated with the outbreaks led to increased support from international organizations for diagnostic testing and strengthening of veterinary services. In 2009, selected government veterinary laboratories instituted a protocol for differential molecular screening of outbreak specimens for PRRS, CFS, and PCV2 and bacterial culture for *Pasteurella multocida, Streptococcus suis, Mycoplasma hyopneumoniae, Haemophilus parasuis*, and *Actinobaciilus pleuroneumonia.* Overall, during the period 2009-2011, approximately 60% of suspected PRRS outbreaks were confirmed positive for PRRSV, while the remaining 40% were negative for PRRSV but positive for PCV2 and/or other co-infecting bacterial agents (Dung et al., 2013). More precisely, between September 2009 and October 2011, screening on samples from

suspected PRRSV outbreaks confirmed presence of PCV2 in 18 of 19 outbreaks from 12 provinces, and an overall rate of 42% PCV2 positivity among samples tested. A retrospective study of 2007-2009 PRRS outbreak specimens also confirmed that 90% of samples were positive for PCV2.

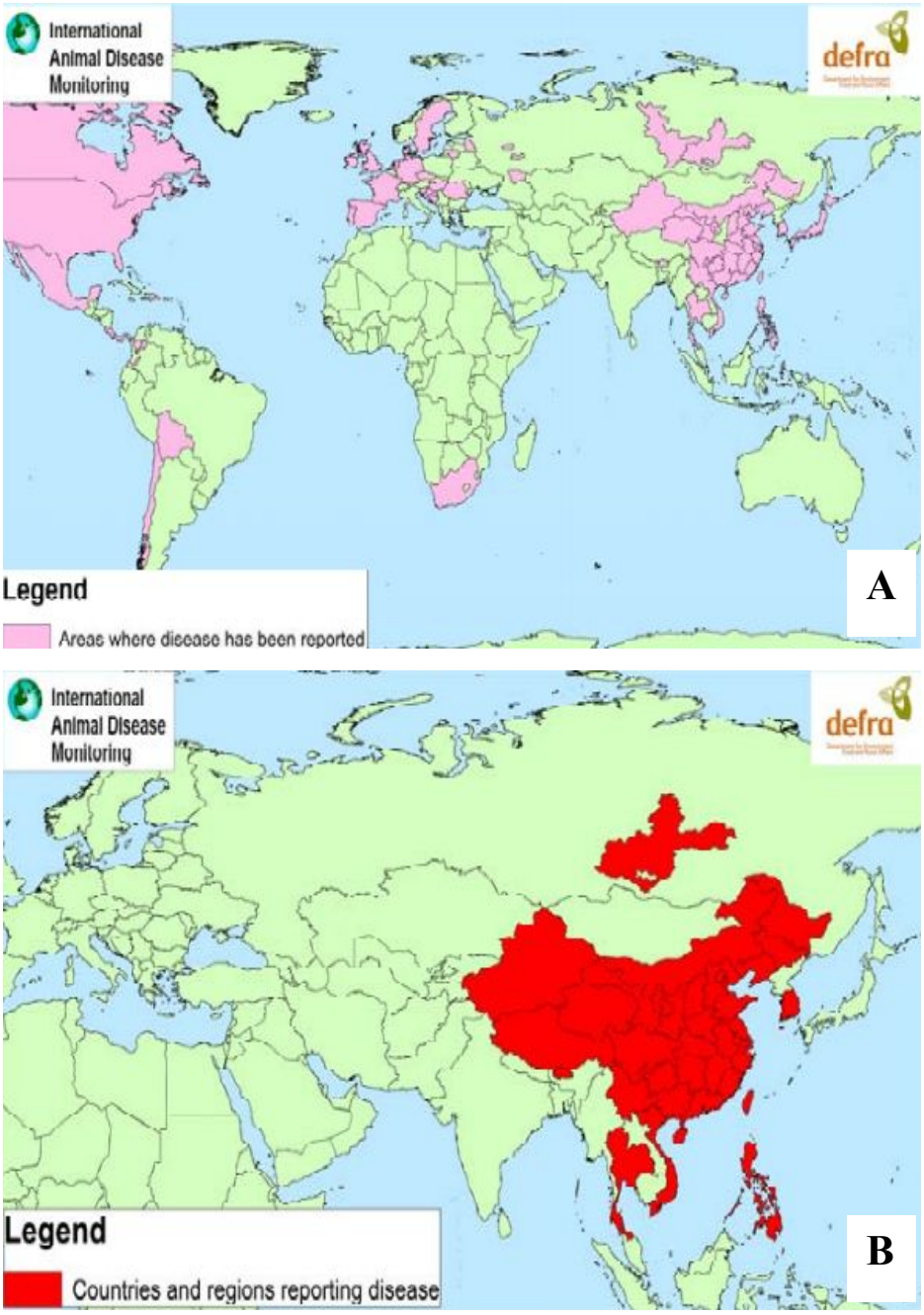

Figure 1. Outbreaks of PRRS between 2005 and 2009 (OIE, 2009). [**A**], Classical PRRS outbreaks caused by Type 1 (European) and Type 2 (North American); [**B**], Highly Pathogenic PRRS outbreaks caused by a variant strain of the Type 2 PRRS virus.

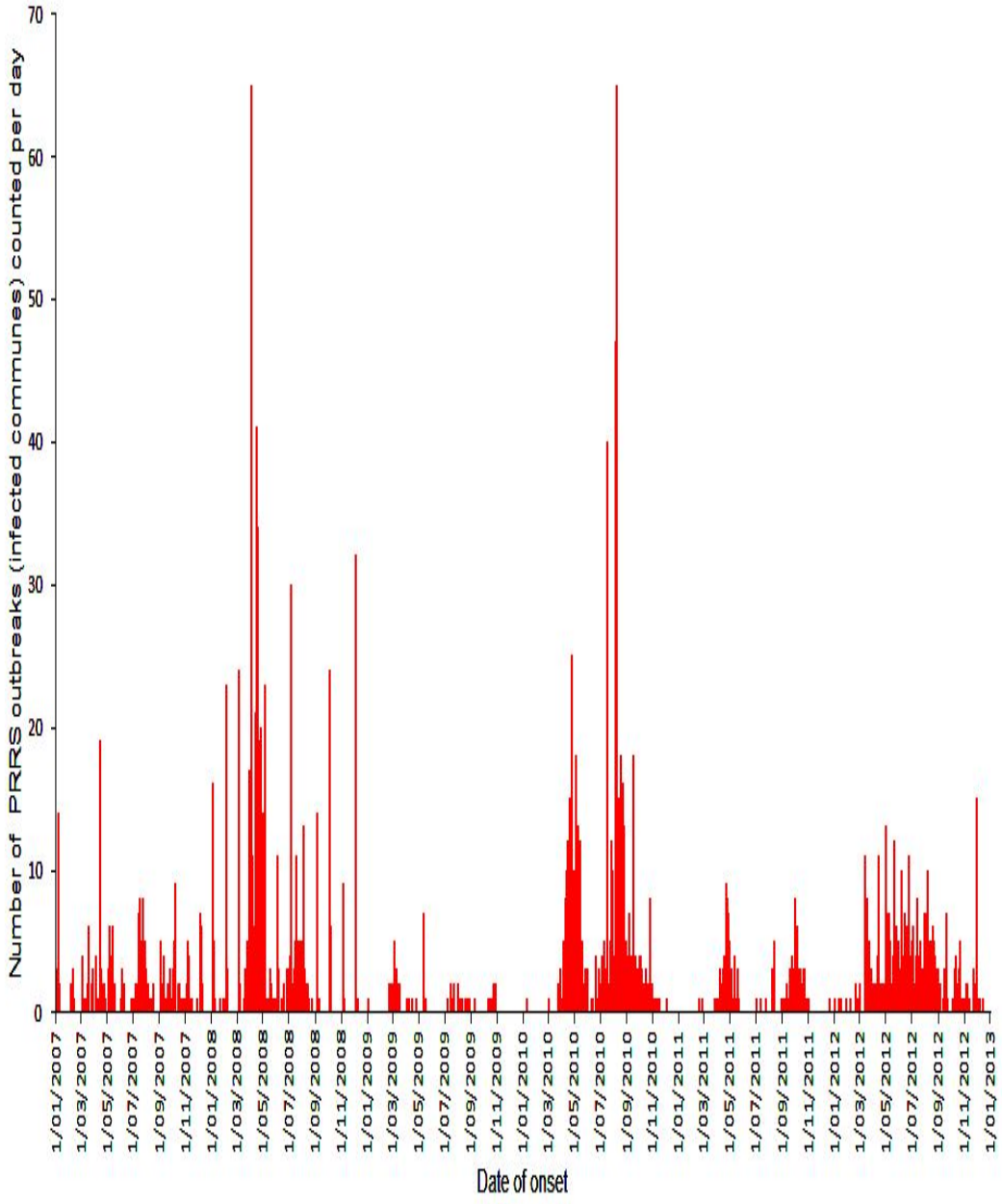

Figure 2. HP-PRRSV outbreaks in Vietnam, 2007-2013 (Dung et al., 2013)

2. The PRRS virus

The porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the family Arteriviridae in the order of the Nidovirales. The genome of virus is a single-strand, positive-sense, RNA molecule of approximately 15 kb (Meulenberg et al., 1993; Meng et al., 1994; Snijder and Meulenberg, 1998). The genome encodes at least 10 open reading frames (ORFs), including the recently discovered ORF5a (Meulenberg et al., 1993, Wu et al., 2001; Dokland, 2010; Johnson et al., 2011). ORF1a and ORF1b constitute about 75 % of the genome, and encodes two long non-structural polyproteins (pp), pp1a and pp1ab (Meulenberg et al., 1993, Snijder and Meulenberg, 1998). ORF2 to 5 encodes the membrane glycoproteins (GP: GP2-GP5), and ORF6 and ORF7 encodes a non-glycosylated membrane protein (M) and the nucleocapsid (N) protein, respectively. Two small genes, ORF2b and ORF5a, are fully embedded in ORF2 and depending on the genotype partially or fully embedded in ORF5, encodes the non-glycosylated proteins E and ORF5a protein, respectively, characterized in figure 3 and 4 (Wu et al., 2001; Music and Gagnon, 2010; Firth et al., 2011, Johnson et al., 2011). The highly polymorphic ORF5 gene encodes the major envelope protein GP5 which is the main protein that induces neutralizing antibodies. The neutralizing epitopes have also been identified in GP3, GP4 and M (Lopez and Osorio, 2004).

Currently, two distinct genotypes of PRRSV were characterized included European (Type 1) and North American (Type 2) (Meng et al., 1995). PRRSV possessed

extensive antigenic, genetic and pathogenic variations (Meng, 2012). Phylogenetic analyses showed that there was existence multiple genetically distinct clusters of PRRSV within the North American genotype and the European genotype (Stadejek et al., 2008; Murtaugh et al., 2010; Shi et al., 2010). In 2006, the HP-PRRSV an atypical strain of PRRSV with high mortality was recently emerged in China, Vietnam (Zhou and Yang, 2010), Lao (Ni et al., 2012), Thailand (Nilubol et al., 2012), Phillipines, Cambodia and Russia (Roberts et al., 2009), and underscores the importance of constantly evolving nature of emerging viruses, which can produce new variants with more deadly consequence and dramatically effects on swine population (Tian et al., 2007; Murtaugh et al., 2010; Meng, 2012).

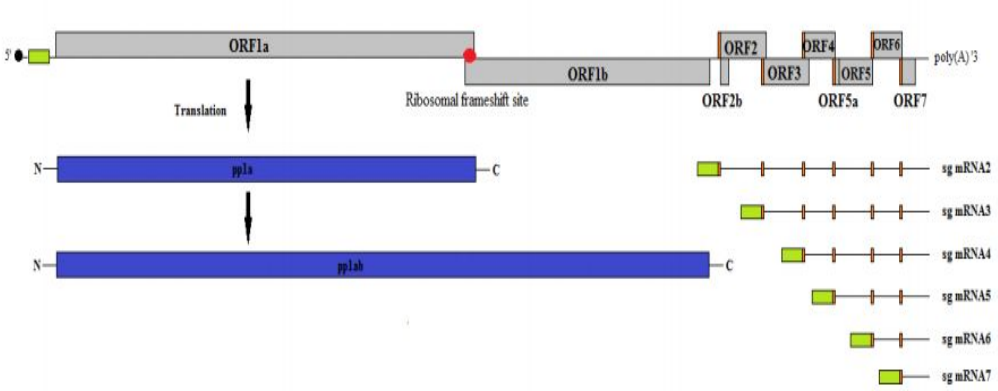

Figure 3. Schematic representation of PRRSV genome orientation. Each ORF encoded by the PRRSV genome is represented as a rectangle marked with the respective name of the gene. The 5' methylated cap structure is shown as a black sphere and the ribosomal frameshift is marked with a red sphere. The black lines at both termini represent the UTRs. The green box at the 5'-UTR represents the common leader sequence and the orange boxes located 5' to the ORF of the structural proteins represent the mRNA bodies. sg mRNA2-7 is shown to the right of the figure and the polyproteins pp1a and

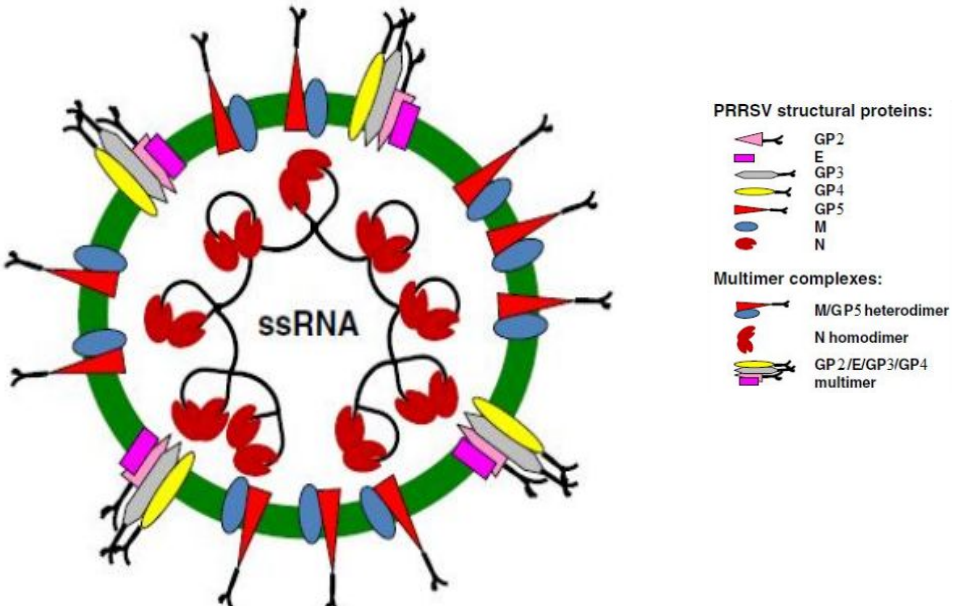

Figure 4. Schematic representation of the PRRSV virion. The orientation of the structural proteins of GP2, E, GP3, GP4, GP5, M, and N protein are shown. GP5/M forms a heterodimer and the minor glycoproteins and E forms a multimeric complex. The N protein homodimers are shown surrounding the PRRSV RNA genome (Music and Gagnon, 2010).

3. Genetic diversity of PRRSV

PRRSVs were divided into two separate genotypes. Type 1, European genotype, mainly comprised of viruses from European countries and Type 2, North American genotype, mainly comprised of viruses from North America (Mardassi et al., 1994; Murtaugh et al., 1995; in review by Shi et al., 2010).

Type 1 PRRSV epidemic was recorded in the early 1990s, as earliest record (Wensvoort et al., 1991). The important strain Lelystad which is considered prototype of Type 1 PRRSV was isolated and completely sequenced in The Netherlands. Lelystad formed highly genomic homology with strains sampled from Belgium, France, Germany, Britain and Spain (Suarez et al., 1996; Drew et al., 1997; LeGall et al., 1998; Forsberg et al., 2002). Based on entire collection of current Type 1 PRRSV ORF5 sequences, Shi et al. (2010) demonstrated that inter-country transmission of viruses was observed and as evidenced by the heterogeneous geographical components in Western Europe. The Type 1 PRRS virus diversity was also documented in Eastern Europe. The average pairwise comparison of percentage difference reached 18.2% for ORF5 and 12.0% for ORF7 (Shi et al., 2010).

Type 2 PRRSV majorly distributed in North America where VR-2332 is considered the prototype. The global phylogenetic study (Shi et al., 2010) divided Type 2 PRRSV isolates into nine well-supported lineages, including five large clusters and four small

groups, each separated with higher than 10% genetic distance. Inside there, 7 of 9 have their diversity maintained by North American sample while the remaining two are found in Asian countries (Madsen et al., 1998; Kang et al., 2004; Thanawongnuwech et al., 2004; Chen et al., 2006; An et al., 2007; Kim et al., 2009; Shi et al., 2010; Nguyen et al., 2013).

The emerged atypical outbreaks more severe PRRS caused by a virulent variant of PRRSV happened in US in 2001 (Han et al., 2006). The virus isolate characterized by three discontinuous depletions in Nsp2 compared to VR-2332. In recently, atypical outbreak with specified by prolonged high fever, red skin and very high mortality in pig all ages caused by highly pathogenic PRRSV in China/2006 (An et al., 2007; Tian et al., 2007). The virus characterized by discontinuous depletion of 30 amino acid in Nsp2, which is excluded as potential high virulent determinants in study used chimeric infectious clone model (Zhou et al., 2009). The same variant virus widely spread to some Asia neighboring countries as Vietnam, Thailand, Laos, Campuchia and Phillipines (Roberts et al., 2009; Zhou and Yang, 2010; Ni et al., 2012; Nilubol et al., 2012).

4. Pathogenicity and pathogenesis

There were huge differences in pathogenic capacity and pathogenesis among each genotypes of PRRSV (Halbur et al., 1995; Halbur et al., 1996; Han et al., 2013). The

EU genotypic PRRSV rapidly reaches level close to 10^5 to 10^6 virions per ml in blood of 3 week-old pigs (Han et al., 2013), while US genotypic PRRSV strain highly pathogenic peak at 10^8 to 10^{10} virions per ml in blood (Liu et al., 2010; Guo et al., 2013). Evenly a remarkable difference in pathological outcome as viral replication in tissues and inducing interstitial pneumonia in pigs were shown among various EU genotypic PRRSV isolates (Han et al., 2013) and among US genotypic PRRSV isolates (Halbur et al., 1995; Halbur et al., 1996). The differences were found in the severity of gross and microscopical pulmonary lesions and the distribution of virus-labelled cells in lung and lymph nodes, and significantly more PRRSV-positive cells detected in the lung and lymph nodes of pigs inoculated with the one Korean isolate than those of pigs inoculated with Lelystad virus (Han et al., 2013).

Clinical outcome post-infection of virus are reproductive failure and increasing mortality in young pigs due to severe respiratory disease and very poor growth performance. However, the subclinical infection of PRRSV participated as co-factor together with various microbial pathogens caused different polymicrobial disease syndromes like porcine respiratory disease complex and porcine circovirus associated disease in setting of production system (Chand et al., 2012).

The PRRSV possesses several properties related to viral pathogenesis including cytopathic replication in macrophages, the capacity to establish a persistent infection and cause of severe disease, described in details in review by Chand et al. (2012). The

diversity of outcomes in viral pathogenesis is due to a consequence of a complex set of the virus versus the host interaction in there the acute phase of viremia covers approximately 28 days, with primarily targets alveolar macrophages (PAMs). The acute outcome of respiratory depress is a consequence of the release of multiple inflammatory cytokines in the lung. Following the initial clearance from the blood, PRRSV viremia periodically reappears (Boddicker et al., 2012) with lymphoid tissues as the primary site of virus replication and the persistent in there for more than 100 days post-infection and easily shed to sentinel pigs evenly in the asymptomatic period (Horter et al., 2002; Rowland et al., 2003).

The persistent infection is a specific pathogenesis of PRRSV that detail described in a review by Chand et al. (2012). The mechanism of persistent depends on interaction of various factors in basis such as [1] a complex virion structure included a heavily glycosylated surface, [2] re-direction of the humoral response towards non-surface proteins, [3] antigenic and generic drift, and [4] subversion of interferon gen induction. How the virus extinct from host body is not clear probably correlates with the disappearance of permissive cells together with a partially protection by immune response that described in immunology section of this literature.

A new disease syndrome known as “high fever disease” was first reported in China and Vietnam in 2006 (Tian et al., 2007; Feng et al., 2008). Later, the “high fever disease” was recognized as an atypical form of PRRS and the causative virus was named as

highly pathogenic PRRSV (HP-PRRSV) (Tian et al., 2007). HP-PRRSV caused high morbidity (50-100%) and mortality (20-100%). Symptoms include high fever (~41^{o}C), severe dyspnea, lameness, and shivering (Tian et al., 2007; Zhou et al., 2009; Zhou and Yang, 2010). The pathogenesis of respiratory disease caused by Chinese HP-PRRSV in nursery pigs has been reported. Chinese HP-PRRSV showed acute systemic infection with high and wide tissue tropism to internal organs and induced severe interstitial pneumonia (Li et al., 2012; Han et al., 2014).

The PRRSV entries to the body and infects predominantly in alveolar macrophage of the lungs (Murtaugh et al., 2002; in review of Kimman et al., 2009). Other cells like monocyte, macrophage lineage, pulmonary intravascular macrophage, subsets of macrophages in lymphoid tissues, intravascular macrophages of the placenta and umbilical cord are considered sites of reproductive infection (Duan et al., 1997; Lawson et al., 1997).

Sialoadhesin functions as receptor for attachment and internalization of PRRSV. It functions together with CD163 probably during un-coating of the virus (Duan et al., 1998; Vanderheijden, et al., 2003; in review of Kimman et al., 2009). However, the in-activated monocytes are not a site of PRRSV infection that could be a phenomenon showed no or extremely low level of sialoadhesin expression of these cells. This suggests that sialoadhesin may play a key role in susceptibility to PRRSV infection (Delputte et al., 2005). Heparan sulfate is the first binding-site on alveolar macrophage

during PRRSV getting early infected (Delputte et al., 2002).

Apoptosis/necrosis is other characteristics considered of infective mechanism of PRRSV. There are a number of published studies identifying PRRSV infection and apoptosis/necrosis (in review by Miller and Fox, 2004). Both infected cell lines and animal cells clearly showed apoptotic signal that contributed to pathology of PRRSV infection. Apoptotic cells are found widely within infected cells included alveolar macrophages in lungs, germ cell in testes, and lymphoid tissues (Sur et al., 1997; Sur et al., 1998; Coster et al., 2008) and endometrium, fetal placentas tissues (Karniychuk et al., 2011). Furthermore, apoptosis and necrosis has seen within in vitro infected cells (Sirinarumitr et al., 1998; Kim et al., 2002; Miller and Fox, 2004), as remarkable in MARC-145. The contrary terms of apoptosis or necrosis caused by PRRSV infection still remained however it is important to noted possibility that the DNA fragmentation may have resulted from necrosis as mentioned by van Lookeren Campagne et al. (1995).

5. PRRSV infection in wild boars

Wild pigs (*Sus scrofa*) are indigenous in many Asian countries including Vietnam, where wild pigs have been extensively farmed due to continuously high demand for valuable exotic pork (Larson et al., 2005). However, wild pigs have been well established as reservoirs for several infectious pathogens that are transmissible to

domestic pigs and humans (Meng et al., 2009). Infections of PRRSV in wild boars have been reported in several countries (Bonilauri et al., 2006; Ruiz-Fons et al., 2008; Choi et al., 2012; Roic et al., 2012). The swine pathogens transmitted between wild- and domestic-pig such a seropositivity of Classical swine fever virus (Albina et al., 2000; Zupancic et al., 2002; Vengust et al., 2006), Porcine circovirus type 2 (Vicente et al., 2004; Csagola et al., 2006; Ruiz-Fons et al., 2006), Swine influenza virus (Vicente et al., 2002), and Porcine parvovirus (Vicente et al., 2002; Ruiz-Fons et al., 2006).

There is a conflict about the capacity of wild boars as PRRSV reservoirs and to retains a role in transmission of this virus to domestic pigs due to lack of convincing data (Meng et al., 2009). Whereas Plagemann (2003) hypothesized the initial source of two distinct PRRSV genotypes from European wild boars functioned as intermediated host last several decades based on several observations. The recently possible data presented in wild pigs' sera of PRRSV positivity in both Europe and America at 6.2% and 14.2%, respectively (Saliki et al., 1998; Albina et al., 2000; Zupancic et al., 2002; Plagemann, 2003; and Ruiz-Fons et al., 2006; Hammer et al., 2012). In Asia, sero-positivity (4/267) and antigen-positivity (8/246) included EU and US genotypic PRRSV were found in Korean wild boar (Choi et al., 2012). 24% prevalence of PRRSV was found in Thailand (Wiratsudakul et al., 2013). The rare experiment to confirm pathogenic ability of HP-PRRSV was conducted in six week-old hybrid wild boar (Li et al., 2007; Feng et al., 2008; Zhou et al., 2008; Wu et al., 2011). However, the pathogenesis of PRRSV or high virulent PRRSV on wild pigs is still be

questionable.

6. Immunology and vaccination

The immunity against PRRSV begins with an innate antiviral response in the cytoplasm of an infected macrophage. PRRSV elicits only a minimal interferon response at the site of infection. The weak innate response may compromise the initiation and elaboration of antigen-specific adaptive immunity (Murtaugh et al., 2002). The specific humoral immunity of IgM and IgG first appears at 5-7 days and 7-10 days respectively post infection. Peak level reach at 2-4 weeks then remain constant for a period of months and then decline to very low levels at 300 days post-infection. However, the earliest and strongest antibody response is directed against the N protein which is measureable 5-9 days post-infection (Johnson et al., 2004, Kimman et al., 2009). Antibodies against the two non-structural proteins (nsp1 and nsp2) are evident at 14 days post-infection, and reach peak levels at 28- 35 days post-infection (Oleksiewicz et al., 2001; de Lima et al., 2006; Brown et al., 2009).

Generally, the immune response in pigs against PRRSV is characterized by being delayed and defective (Beura et al., 2010). It takes at least 3 months to reach immunity peak levels in situation of natural infection, and might not appear to be solid enough to prevent reinfection, especially if the reinfection is caused by antigenically heterologous PRRSV strains (Murtaugh et al., 2002, Zuckermann et al., 2007). Most of early

produced antibodies are non-neutralizing whereas the neutralizing antibodies first appear lately around 4 weeks post-infection or even later (Lopez and Osorio, 2004). The neutralizing antibody response against the GP5 neutralizing epitope is weak and delayed, and some animals fail to make a detectable antibody response against GP5 (Yoon et al., 1994; Chand et al., 2012). The mechanism for the weak antibody response towards GP5 is linked to the N-glycosylations surrounding the neutralizing epitope, a phenomenon called N-glycan shielding (Chand et al., 2012).

The Type 2 PRRSV GP5 encodes a decoy epitope at position aa27-30 which is not neutralizing but may function to distract the humoral immune response hence delaying the induction of neutralizing antibodies against PRRSV (Ostrowski et al., 2002). Furthermore, pigs infected with PRRSV fail to generate any significant inflammatory cytokine expression in the lungs, including the type I interferons (IFN-α/β), interleukin (IL)-1, and TNF-α (Van Reeth et al., 1999; Thanawongnuwech et al., 2001). The expression of type I interferon is important for the activation of innate immune response (Kimman et al., 2009). The downregulation of IFN-α can be a crucial step in PRRSV pathogenesis as IFN-α has been shown to inhibit PRRSV replication (Albina et al., 1998, Le Bon et al., 2001).

Several vaccines have been have been developed to combat PRRSV (Murtaugh and Genzow, 2011; Charerntanakul, 2012), both attenuated live vaccines (e.g. Ingelvac® PRRS MLV and Porcilis® PRRS) and inactivated vaccines (e.g. Progressis® and

PRRomiSe®). Vaccination against PRRSV has generally not been very successful, partially because of the rapid mutation rate and evolution of the virus, lack of cross-protection, and weakness in inducing the immune response in the animal (Charerntanakul, 2012; Nauwynck et al., 2012). In comparison, protection provided by the attenuated live vaccines is general better than that from inactivated vaccines, however the protection from the attenuated live vaccine still rely on homologous situations (Murtaugh et al., 2002, Labarque et al., 2003).

MLV vaccine in general elicits weak humoral and cell-mediated immune responses. The PRRSV specific antibodies appear about 2 weeks then peak at 4 weeks after vaccination (in review by Charerntanakul, 2012). The neutralizing antibodies late appear after four weeks of vaccination but with low titer of immunization. The cell-mediated immunity responses at 2-4 week post vaccination as determined lymphocyte blastogenesis and interferon γ production.

The incomplete protection of pigs from viremia, death rate, abortion, returning to estrus was described in several experiments worldwide (Alexopoulos et al., 2005; Charerntantanakul et al., 2006; Pejsak et al., 2006; Scortti et al., 2006; Cano et al., 2007; Rowland et al., 2010; in review by Papatsiros, 2012). The reports the efficacy of a commercially available Type 2 attenuated vaccine in young pigs against heterologous challenge with a Chinese and Vietnamese HP-PRRSV isolate (Lager et al., 2014). In comparison, vaccination decreased the length of viremia and viral titer, diminished the

time of high fever and reduced macroscopic lung scores following homologous and heterologous PRRSV challenge. The new modified live PRRSV vaccine was able to reduce the level of viremia, nasal shedding, and severity of PRRSV-induced lesions in vaccinated piglets after heterologous challenge (Park et al., 2014), while in contrast findings in different studies somewhere else (Murtaugh and Genzow, 2011). However, vaccine protection seems to be rather virus genotype-specific and strain specific. The protection conferred by EU PRRS MLV vaccines is seen only after EU PRRSV challenge but not for NA PRRSV challenge and inversely. The evaluation of the efficacy of the in-place attenuated live vaccine based on highly pathogenic PRRSV against homologous HP-PRRSV in young pigs were showed in a few recent studies (Tian et al., 2009; Wang et al., 2011; Leng et al., 2012; Lu et al., 2014). The vaccinated pigs showed potential lethal protection, much milder pathological lesions and - significantly weight-gained.

The inactivated PRRS vaccines are considered less efficacious than modified live vaccines due to lack of elicit detectable antibodies by serological assay and serum virus neutralizing test (Charerntanakul, 2012). The CMI generated by this vaccine pattern was also rarely recorded. The dose boost of vaccine helps enhancing immune response and correlate with protection. However, the killed vaccine has some benefits for virus infected pigs to improve reproductive performance, like farrowing rate, number of weaning pigs, health status of born piglets from vaccinated sows (Charerntanakul, 2012; Papatsiros, 2012).

7. Diagnostic approaches

There are many diagnostic techniques to detect PRRSV included isolation of live virus (VI), detection of live or dead viral antigen (fluorescent antibody, FA; immunohistochemistry, IHC; In-situ hybridization, ISH), genetic-based testing (RT-PCR), serology testing (fluorescent antibody test, IFA; serum virus neutralization test, SVN; and immunoperoxidase monolayer assay, IPMA; ELISA, enzyme-linked immunosorbent assay) and the RFLP assay which is a crude technique for differentiating one PRRSV isolate from another (Yoon et al., 2003; PRRS Compendium Producer Edition, US.). The tentative diagnosis of PRRSV infection is suggested by clinical signs such as reproductive problems in breeding stock or/and respiratory disease (interstitial pneumonia) in pigs of any ages. The suggestive pathological lesions could be used for supportive diagnosis in case of clinically affected animals but there contains the similar pathology caused by other pathogenic viruses or bacteria therefore laboratory confirm is required.

The evidence of exist PRRS virus could be obtained by the gold standard isolation technique for virus isolating or detecting viral antigens like nucleic acid in the animals. PRRSV can be isolated on either porcine alveolar marcophages (PAMs) and African monkey kidney cell lines (Marc-145 cells) (Wensvoort et al., 1991; Dea et al., 1992; Yoon et al., 1992b; Bautista et al., 1993b; Kim et al., 1993; Zeman et al., 1993). Type 2 PRRSV cannot grow well in PAMs so other cell line for propagation is the Marc-145

cells whereas Type 1 PRRSV prefers PAMs (Yoon et al., 2003). Cytopathic effect (CPE) is visible 2-3 days following inoculation and proved PRRSV propagation in the cells.

Conventional reverse transcriptase polymerase chain reaction (RT-PCR) was wide used in detection of PRRSV. The DNA products obtained from conventional PCR can be used for sequencing, where the nucleotide composition and order will be determined. The analytical sensitivity is further improved in nested RT-PCR quantitative RT-PCR (qRT-PCR). Real-time RT-PCR is a common method for the detection of virus. Several versions and chemistries have been developed and utilized for the detection of PRRSV (Oleksiewicz et al., 1998; Kubista et al., 2006; Balka et al., 2009). The advantage using real-time PCR is that it is fast, sensitive, and can be performed in high throughput (Kubista et al., 2006). There are two common types of qRT-PCR reactions; those using Taqman® are more specific that those using SYBR® Green. In Taqman® pattern, each target is bound to a unique probe that fluorescence a different color while the SYBR® Green quantifies the amplification of a single probe and subsequently quantifies each target by the melting point distribution (Oleksiewicz et al., 1998; Lurchachaiwong et al., 2008; Balka et al., 2009).

FA, IHC and ISH are techniques used to directly detect virus or viral antigen in tissues. IFA can detect PRRS virus quickly in fresh frozen tissue section which is inexpensive and rapid. There are two disadvantage of FA test including specificity and much effect

on specimen quality. The tissue should be collected in fresh from recently dead or euthanized pigs then promptly refrigerated/frozen. The IHC has more sensitivity than direct FA for identifying PRRS virus in formalin-fixed paraffin embed tissues but it take more time and costly than the FA test. Both of techniques could be used for definitive diagnosis to confirm PRRSV infection. The detected tissues of PRRSV as described by several authors are heart, kidney, lung, lymph nodes, spleen, thymus, tonsil, adrenal gland, intestine, liver, and occasionally brain (Rossow et al., 1994; Halbur et al., 1995; Cheon and Chae, 1998; Rossow et al., 1999; Li et al., 2012; Hu et al., 2013). ISH was as well developed to detect IHC-parallel infected cells based on nucleic acid of PRRSV (Cheon et al., 1997; Hayes et al., 1997; Han et al., 2012).

There are two diagnostic ELISA-derived methods such as antigen capture ELISA and indirect ELISA tests (Rosengren et al., 2011). The antigen capture ELISA test has been developed to address the surveillance needs of identifying PRRSV infection without RT-PCR (Cai et al., 2009). The test was applied to sera and ground tissues as the specimens. The sensitivity, specificity and accuracy were 67%, 97% and 93% respectively compared with RT-PCR as reference test (Cai et al., 2009). One disadvantage of the ELISA was an unacceptable number of false positive results (Paton et al., 1992b). An indirect ELISA uses S/P ratio system based in optimal density values to interpret the positive/negative results (Albina et al., 1992; Cho et al., 1996; Takikawa et al., 1996). The most common commercial ELISA kit using to detect PRRSV antibody is HerdChek® PRRS ELISA (IDEXX Laboratories Inc., Westbrook,

Maine) which using a S/P ratio ≥0.4 is considered to be cut-off of positive. The specificity of commercial HerdChek® PRRS ELISA kit has been estimated at 99.3 to 99.5% (Nodelijk et al., 1996; O'Connor et al., 2002). The other serologic diagnosis to detect antibodies specific for PRRSV includes indirect fluorescent antibody test, serum virus neutralization test, and immunoperoxidase monolayer assay. Based on the equipped facilities in laboratory and purpose of diagnosis each technique could be applied specifically.

Several diagnostic specimens were described somewhere that could serve as alternatives to serum and visceral organs in basis obtained by venipuncture from individual pigs (in a review by Rosengren et al., 2011). Tonsil swabs, milk and neonatal tissues had been minimal detail reported. Pooled serum, blood soaked swabs and capillary tubes are wide used to detect PRRSV based on molecular techniques. Oral fluids are emerging sampling specimen being rapidly adopted (Prickett et al., 2010a). The viral concentration is lower in oral fluid sampling diagnosis by RT-PCR but time and window detection are similar or eventually longer than serum. The filter papers offer several advantages as a specimen storage media, a range of specimens could be used including tissues and blood. Meat juice sampling may be the most practical and cost effective path to monitor or control PRRS and continued disease freedom in successful areas. This sampling technique has no biosecurity concern caused by on-farm sampling and has no animal welfare implications.

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CHAPTER 1

Comparison of experimental infection with Northern and Southern Vietnamese strains of highly pathogenic porcine reproductive and respiratory syndrome virus

ABSTRACT

The aim of this study was to compare the virulence of northern and southern Vietnamese strains of highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) as assessed by the level of viral replication, gross and microscopical lung lesions and virus distribution in experimentally infected pigs. The northern and southern Vietnamese HP-PRRSV strains share 96.7% (non-structural protein 2) and 99.3% (open reading frame 5) nucleotide identity. On experimental challenge, approximately 50% of pigs infected with northern Vietnamese HP-PRRSV died, while death was not observed in any pigs infected with southern Vietnamese HP-PRRSV. Mean viral titres (expressed as $\log_{10}TCID_{50}$/ml) were significantly (P <0.05) higher in sera and lungs from pigs infected with the northern Vietnamese HP-PRRSV than from those infected with the southern Vietnamese strain at multiple time points. Lung lesion scores and PRRSV antigen within pulmonary and lymphoid lesions were significantly (P <0.05) higher in pigs infected with northern Vietnamese HP-PRRSV than in those receiving southern Vietnamese HP-PRRSV at multiple time points. PRRSV antigens were observed in cardiac myocytes, gastric and renal tubular epithelial cells, and astrocytes and microglia of white matter in the brain from pigs infected with the northern Vietnamese HP-PRRSV strain only. Thus, genetic similarity did not predict the degree of virulence of these strains. Northern Vietnamese HP-PRRSV was more virulent and had extended tissue tropism when compared with southern Vietnamese HP-PRRSV.

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) is the causative agent of PRRS and is an enveloped, single-stranded, positive-sense RNA virus belonging to the Arteriviridae family in the order Nidovirales (de Groot et al., 2011). PRRS is an economically important viral disease and is characterized by reproductive failure in pregnant sows and respiratory disease in nursing and grower/finishing pigs (Zimmerman et al., 2012). The disease was first recognized in the USA and Europe in the late 1980s (Zimmerman et al., 2012). In the early 1990s, the disease was also identified in Asia (Kweon et al., 1994; Murakami et al., 1994). Currently, PRRSV is becoming widespread throughout the world.

A new disease syndrome known as 'high fever disease' was first reported in China and Vietnam in 2006 (Tian et al., 2007; Feng et al., 2008). Later, the high fever disease was recognized as an atypical form of PRRS and the causative virus was named as highly pathogenic PRRSV (HP-PRRSV) (Tian et al., 2007). HP-PRRSV caused high morbidity (50–100%) and mortality (20–100%). Clinical signs include high fever (40–41^{o}C), severe dyspnoea, lameness and shivering (Tian et al., 2007; Zhou et al., 2009; Zhou and Yang, 2010). In Vietnam, HP-PRRSV lead to the death of thousands of pigs as the virus spread from northern through central and to southern Vietnam within 3 months of the first outbreaks in 2007 (Metwally et al., 2010; Dung et al., 2013). A

total of 44 outbreaks in both northern and southern Provinces affected 44,000 pigs with an observed mortality rate of 24% (MARD, 2010).

The pathogenesis of respiratory disease caused by Chinese HP-PRRSV in nursing pigs has been reported. Chinese HP-PRRSV showed high tissue tropism for internal organs and induced severe interstitial pneumonia (Li et al., 2012; Han et al., 2014). However, the data on microscopical lesions and virus distribution from pigs infected with Vietnamese HP-PRRSV are limited, although an infectious clone of Vietnamese HP-PRRSV was shown to induce pneumonia experimentally (Guo et al., 2013). The aim of this study was to compare the virulence of northern and southern Vietnamese HP-PRRSV in infected pigs according to the pathogenicity of experimental infection in terms of clinical signs, mortality, gross and microscopical lesions and tissue distribution of virus.

MATERIALS AND METHODS

PRRSV Isolates

Northern Vietnamese HP-PRRSV (strain MB6) was isolated from a 30-sow herd in a northern region of Vietnam in 2009. Southern Vietnamese HP-PRRSV (strain MN1) was isolated from a 250-sow herd in southern Vietnam in 2013. These two Vietnamese HP-PRRSV strains share 96.7% (non-structural protein 2 [NSP2], Genbank number KM244760 for strain MB6 and Genbank number KJ742372.1 for strain MN1) and 99.3% (open reading frame 5 [ORF5], Genbank number KM244761 for strain MB6 and Genbank number KM244763 for strain MN1) based on nucleotide sequences. The homology of deduced amino acid sequences between strain MB6 and strain MN1 is 96.2% in NSP2 and 99.5% in ORF5.

The two Vietnamese HP-PRRSV strains used in this study were analyzed phylogenetically together with a prototype of Chinese HP-PRRSVs (JXA1/2006, EF112445, JXwn06/2006 and EF641008) and some other Chinese HP-PRRSVs listed in the GenBank databases for NSP2 and ORF5 (Fig. 1).

Experimental Design

Seventy pigs purchased from a PRRSV-free herd were used at the age of 4 weeks. All animals were negative for PRRSV and porcine circovirus type 2 (PCV2) according to routine serological testing performed prior to delivery and again on arrival. In addition, PRRSV and PCV2 were not detected in serum samples from any animals used in this study by real-time polymerase chain reaction (PCR; Wasilk et al., 2004; Gagnon et al, 2008) performed prior to delivery and again on arrival.

Pigs were allocated randomly to two infected groups and one control group. Group 1 comprised of 28 pigs that were inoculated intranasally with 3 ml of tissue culture fluid containing $10^{5.5}$ tissue culture infective dose 50% ($TCID_{50}$)/ml of northern Vietnamese HP-PRRSV (strain MB6, 4^{th} passage in MARC-145 cells). Group 2 comprised of 28 pigs that were inoculated intranasally with 3 ml of tissue culture fluid containing $10^{5.5}$ $TCID_{50}$/ml of southern Vietnamese HP-PRRSV (strain MN1, 4^{th} passage in MARC-145 cells). Group 3 comprised of 14 control pigs that were inoculated with uninfected cell culture supernatants. Pigs in each group were housed separately within the facility.

Rectal temperatures were recorded daily from -2 days post inoculation (dpi) to 28 dpi. The pigs were monitored weekly for physical condition and scored at 3, 5, 7, 10, 14, 21 and 28 dpi for clinical respiratory disease severity using scores ranging from 0 (normal) to 3 (severe dyspnoea and abdominal breathing) (Halbur et al., 1995). Four infected and two control pigs from each group were sedated by an intravenous injection of sodium pentobarbital and then killed at 3, 5, 7, 10, 14, 21 and 28 dpi as previously

described (Beaver et al., 2001). Tissues were collected from each pig at the time of necropsy examination. Experimental methods were approved by the Nonglam University Institutional Animal Care and Use and Ethics Committee.

Serology

Blood samples from each pig were collected by jugular venipuncture at -3, 0, 2, 3, 5, 7, 10, 14, 21 and 28 dpi, and the sera were stored at –20°C. The serum samples were tested using the commercially available PRRSV enzyme-linked immunosorbent assay (ELISA; IDEXX PRRS X3 Ab test, IDEXX Laboratories Inc., Westbrook, Maine, USA). Serum samples were considered positive for PRRSV antibody if the sample/positive (S/P) ratio was greater than 0.4 according to the manufacturer's instructions.

Quantification of PRRSV RNA

RNA was extracted from serum and lung samples at 0, 3, 5, 7, 10, 14, 21 and 28 dpi from infected and negative control pigs and analyzed as described by Wasilk et al. (2004). Real-time PCR was designed to detect ORF7 of Vietnamese HP-PRRSV; the forward and reverse primers were 5'-CTAGTGAGCGGCAATTGTG-3' and 5'-TCATGCTGAGGGTGATGCT-3', respectively. The real-time PCR was considered positive if the cycle threshold level was $\leq$45cycles (Wasilk et al., 2004).

To construct a standard curve, real-time PCR was performed in duplicate in two different assays: (1) 10-fold serial dilutions of the PRRSV plasmid, used as the standard, with concentrations ranging from 10^{10}–10^{3} copies/ml; and (2) 10-fold serial dilutions of type 2 PRRSV cultured in MARC-145 cells from10^{6}–10^{-1} $TCID_{50}$/ml. The PRRSV plasmid was prepared as described by Han et al. (2011). Briefly, the cDNA product was cloned into the pCR2.1 plasmid (Invitrogen, Carlsbad, California, USA). The recombinant plasmid was purified using a plasmid miniprep kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions and the concentration of the purified plasmid was determined using a spectrophotometer. For serum PRRSV cDNA quantification, virus titre was calculated using a standard curve generated from serially diluted type 2 PRRSV (Han et al., 2011).

Preparation of Labelled Probe

For preparation of the probe, a 442 base pair cDNA fragment representing the 5' region of ORF5 was used. The forward and reverse primers were 5'-GTGGTGTATCGTGCCGTTC-3' and 5'-CCCTTTCTCCACAATGACG-3', respectively. Amplification was carried out in a 20 µl reaction containing 10 µl of 2× TOPsimple™ PreMIX-Forte (Enzynomics, Daejeon, Korea), 7 µl of DEPC distilled water, 2 µl of cDNA and 1 µl of each primers (10 mM). The thermal profile of PCR was 95°C for 10min, followed by 35 cycles of 95°C for 30 sec, 56°C for 30 sec and

72°C for 30 sec. The PCR products were purified with Wizard PCR Preps (Promega Biotech, Madison, Wisconsin, USA). The purified PCR product was labelled by random priming with digoxigenin-dUTP using a commercial kit (Boehringer Mannheim, Indianapolis, USA).

In-situ Hybridization

After fixation, the tissues from each pig were dehydrated through a graded series of alcohol solutions and a xylene step and embedded in paraffin wax. Three serial sections (4 μm) were then prepared from each tissue, the first was processed for in-situ hybridization (ISH) to detect in-situ viral RNA, the second was used for immunohistochemistry (IHC) to detect viral nucleocapsid protein and the third was used for routine haematoxylin and eosin (HE) staining. ISH was performed as described by Cheon et al. (1997).

Immunohistochemistry

SR30 monoclonal antibody (Rural Technologies Inc., Brookings, South Dakota, USA), specific for the nucleocapsid protein of PRRSV, was diluted 1 in 1,000 in phosphate buffered saline (PBS; 0.01 M, pH 7.4) containing 0.1% Tween 20. IHC was performed as previously described (Cheon and Chae, 1998).

Morphometric Analysis

For morphometric analysis of the gross pulmonary lesion score, each lung lobe was assigned a number to reflect the approximate percentage of the volume of the entire lung and the percentage volumes from each lobe added to the entire lung score, as previously described (Halbur et al., 1995).

For morphometric analysis of the microscopical pulmonary lesion score, lung sections were examined in a blinded fashion and the severity of the interstitial pneumonia was scored as previously described (Halbur et al., 1995), where: 0, no lesions; 1, mild interstitial pneumonia; 2, moderate multifocal interstitial pneumonia; 3, moderate diffuse interstitial pneumonia; and 4, severe interstitial pneumonia.

For morphometric analysis of ISH and IHC, two sections were cut from each of three blocks of tissue from each lung and from the tracheobronchial, mediastinal and inguinal lymph nodes and thymus of each pig. To obtain quantitative data, slides were analyzed with the NIH Image J 1.43m Program (http://imagej.nih.gov/ij/download.html). In each slide, 10 fields were selected randomly and the number of positive cells per unit area (0.95 mm^2) was determined (Halbur et al., 1996). The mean values were also calculated.

Virus Isolation

Lungs were collected for virus isolation as previously described (Halbur et al., 1995). The isolated PRRSV samples from lungs were further analyzed for the ORF5 sequence (Oleksiewicz et al., 1998).

Statistical Analysis

The normality of the distribution for the examined variables was evaluated by the Shapiro-Wilk test. Continuous data (PRRSV RNA quantification, serology and gross lung lesions) were analyzed with the Student's *t*-test. Rectal body temperatures were analyzed using one-way ANOVA followed by Tukey's multiple comparison test at each time point. Discrete data (respiratory clinical sign scores, microscopical lung lesion score and ISH and IHC scores) were analyzed by the Mann-Witney test. A value of $P < 0.05$ was considered significant.

RESULTS

Clinical Signs

Negative control pigs did not show any respiratory signs, while the HP-PRRSV-infected pigs showed severe respiratory signs (Table 1). The mean clinical respiratory scores were significantly (P <0.05) higher in pigs receiving northern Vietnamese HP-PRRSV than in those receiving southern Vietnamese HP-PRRSV from 7 to 14 dpi. Pigs infected with northern Vietnamese HP-PRRSV died at 5 (n = 1), 8 (n = 1), 9 (n = 3), 10 (n =2), 11 (n = 3), 12 (n = 2), 13 (n =1), 14 (n = 1), and 20 (n =1) dpi. Death was not observed in any pigs infected with southern Vietnamese HP-PRRSV.

The mean rectal temperatures were significantly higher in pigs infected with HP-PRRSV than in negative control pigs throughout the experiment, except at 14 and 17 dpi. The mean rectal temperatures were significantly (P <0.01) higher in pigs receiving the northern Vietnamese HP-PRRSV than in those infected with the southern Vietnamese HP-PRRSV from 3 to 6 dpi (Fig. 2). The pigs infected with northern Vietnamese HP-PRRSV had markedly increased rectal temperatures at 1 dpi, which peaked at 5 dpi and then decreased gradually until 13 dpi. Later, the mean rectal temperatures fluctuated considerably in pigs infected with northern Vietnamese HP-PRRSV between 14 and 17 dpi because most of the infected pigs had died by 14 dpi and the remaining infected pigs were moribund or comatose. Pigs infected with

southern Vietnamese HP-PRRSV had increased the rectal temperatures at 1 dpi, which peaked at 7 dpi and thereafter remained stable throughout the experiment. The temperature of negative control pigs remained normal throughout the study.

Table 1. Clinical signs of northern and southern Vietnamese HP-PRRSV

Clinical signs	*Virus*	*Number of pigs showing clinical signs/number of pigs observed*						
		3 dpi	*5 dpi*	*7 dpi*	*10 dpi*	*14 dpi*	*21 dpi*	*28 dpi*
Loss of appetite	N	12/28	14/24	11/20	15/16	7/8	0/1	0/0
	S	0/28	4/24	5/20	2/16	1/12	0/8	0/4
High fever	N	23/28	22/24	17/20	8/16	2/8	0/1	0/0
(≥40^{o}C)	S	9/28	19/24	17/20	13/16	4/12	3/8	1/4
Reluctance to	N	12/28	20/24	18/20	16/16	7/8	1/1	0/0
move	S	1/28	10/24	6/20	0/16	1/12	0/8	0/4
Diarrhoea	N	0/28	2/24	3/20	2/16	1/8	0/1	0/0
	S	0/28	2/24	2/20	1/16	2/12	2/8	0/4
Ocular discharge	N	0/28	22/24	20/20	16/16	-	1/1	0/0
	S	0/28	-	-	-	-	3/8	2/4
Cutaneous	N	0/28	6/24	9/20	8/16	4/8	0/1	0/0
erythema	S	0/28	0/24	0/20	0/16	0/12	0/8	0/4
Cyanosis	N	0/28	0/24	1/20	4/16	4/8	0/1	0/0
	S	0/28	0/24	0/20	0/16	1/12	0/8	0/4
Seizures	N	0/28	2/24	5/20	5/16	2/8	0/1	0/0
	S	0/28	0/24	0/20	0/16	0/12	0/8	0/4

N, northern Vietnamese strain; S, southern Vietnamese strain; -, missing data

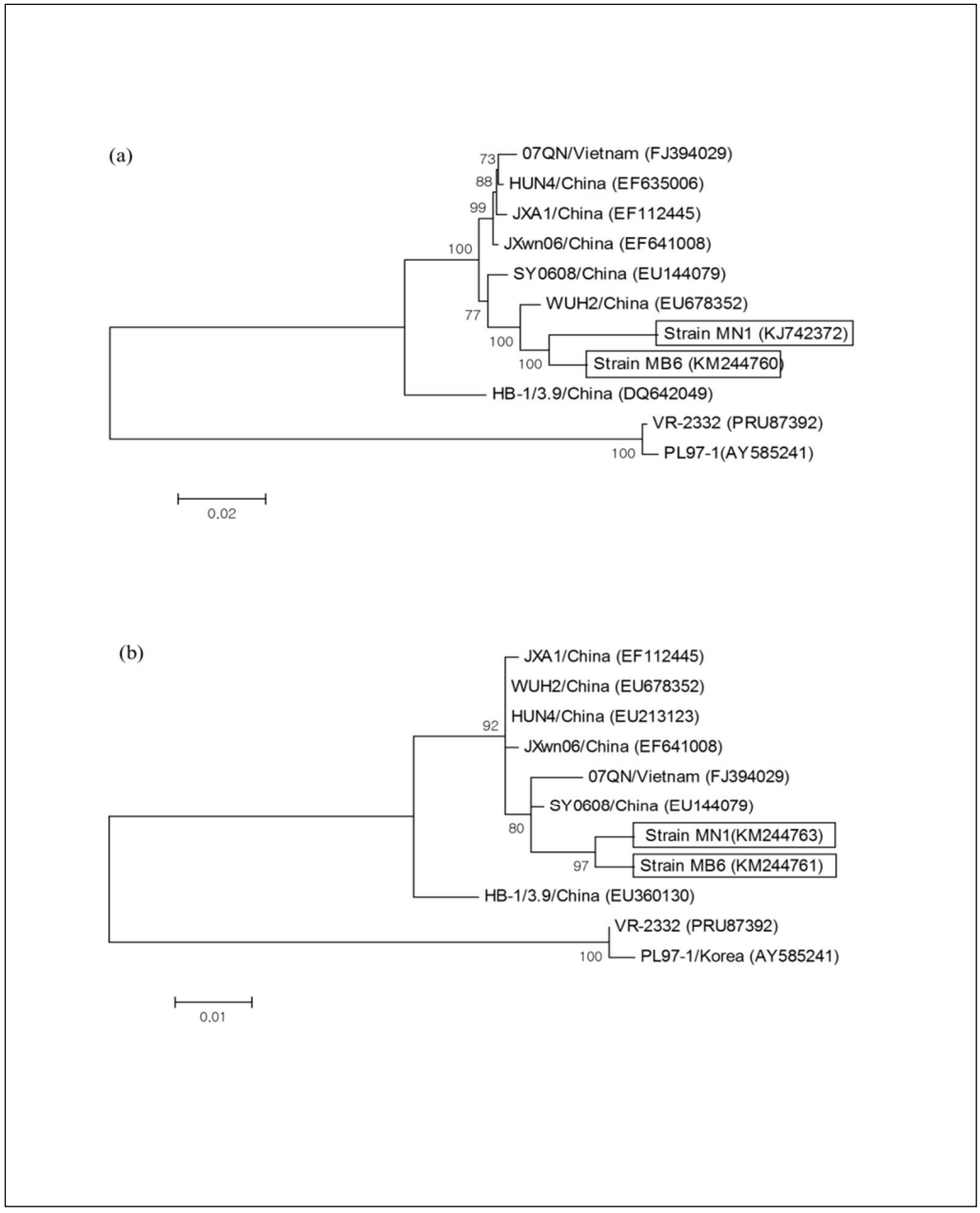

Figure 1. Phylogenetic analysis of (a) nsp2 and (b) ORF5 from Vietnamese and Chinese HP-PRRSV strains. An unrooted neighbour-joining tree was constructed from aligned nucleic acid sequences.

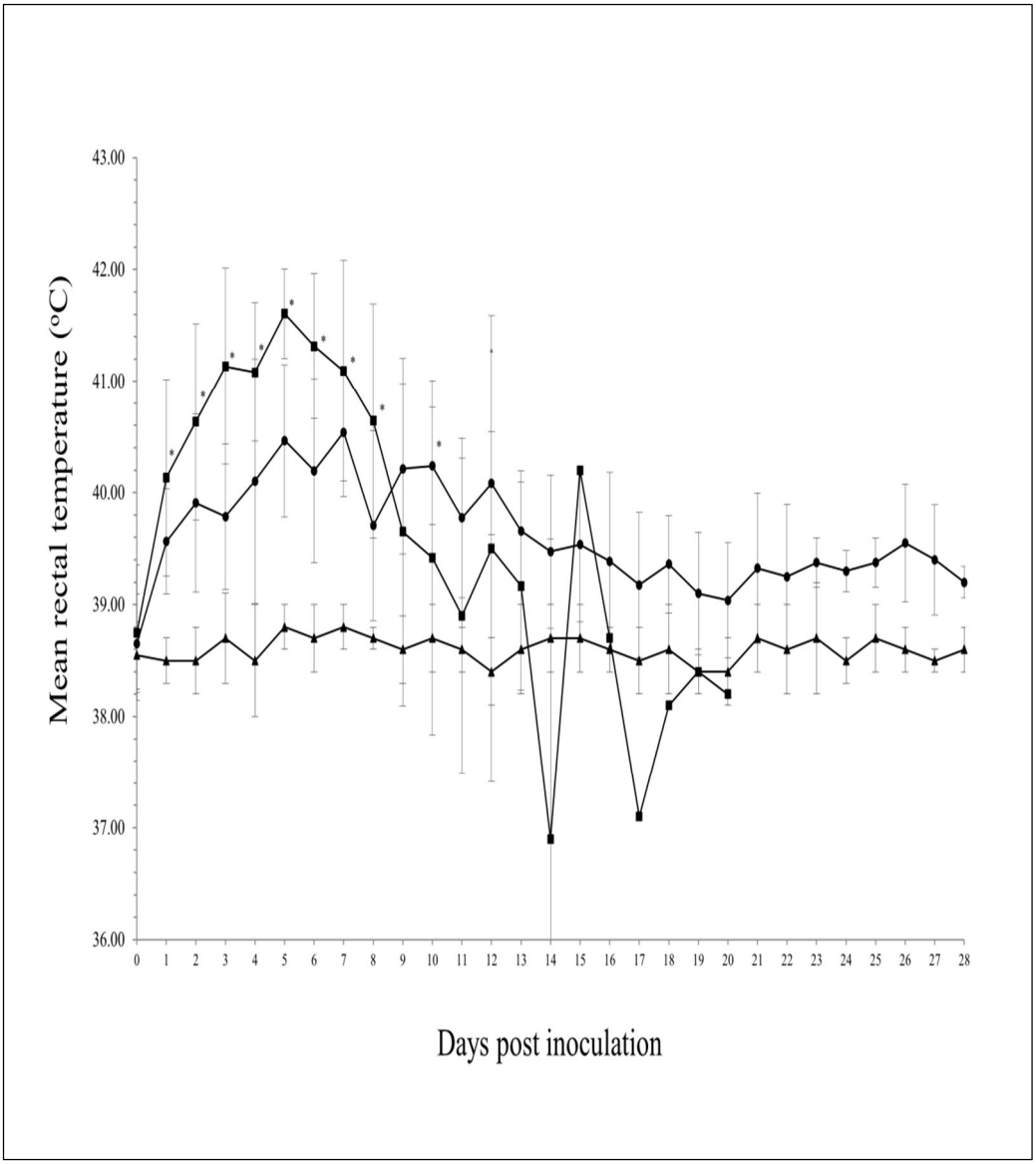

Figure 2. Mean rectal temperature in pigs infected experimentally with northern (■) or southern (●) Vietnamese HP-PRRSV and negative control pigs (▲). A significant difference between pigs receiving northern and southern Vietnamese HP-PRRSV was observed at each time point ($^{*}P$ <0.05).

Humoral Immune Response

Pigs infected with northern Vietnamese HP-PRRSV became seropositive at 7 dpi, while those receiving southern Vietnamese HP-PRRSV became seropositive at 10 dpi by ELISA. Anti-PRRSV IgG antibody titres were significantly (P <0.05) higher in pigs infected with northern Vietnamese HP-PRRSV than in those receiving southern Vietnamese HP-PRRSV from 5 to 10 dpi (Fig. 3). No antibodies specific for PRRSV were detected by ELISA in the negative control pigs at any point.

$Log_{10}TCID_{50}$/ml Quantification of PRRSV RNA in Blood and Lungs

Mean serum virus titres (expressed as $log_{10}TCID_{50}$/ml) were significantly higher in serum from pigs infected with northern Vietnamese HP-PRRSV than in serum from those receiving southern Vietnamese HP-PRRSV at 3 (P = 0.09) and 7 (P = 0.0001) dpi. In the lungs, the mean virus titres from pigs infected with northern Vietnamese HP-PRRSV were significantly higher than in those receiving southern Vietnamese HP-PRRSV at 3 (P = 0.013) and 10 (P = 0.03) dpi (Fig. 4). No viral RNA was observed in the sera or lung tissues from the negative control pigs at any time.

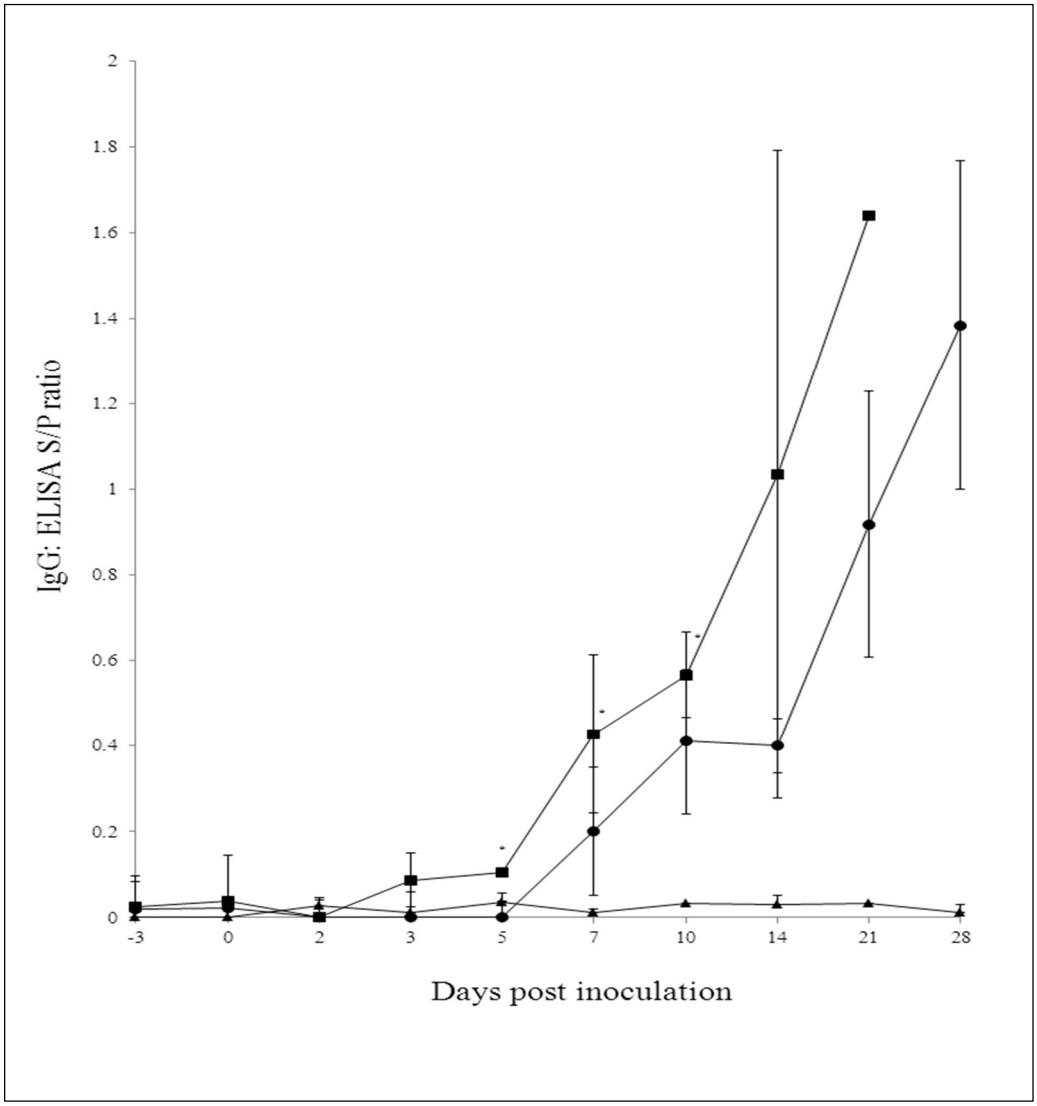

Figure 3. Antibody responses in pigs infected experimentally with northern (■) or southern (●) Vietnamese HP-PRRSV and negative control pigs (▲). A significant difference between pigs receiving northern and southern Vietnamese HP-PRRSV was observed at each time point ($^{*}P$ <0.05).

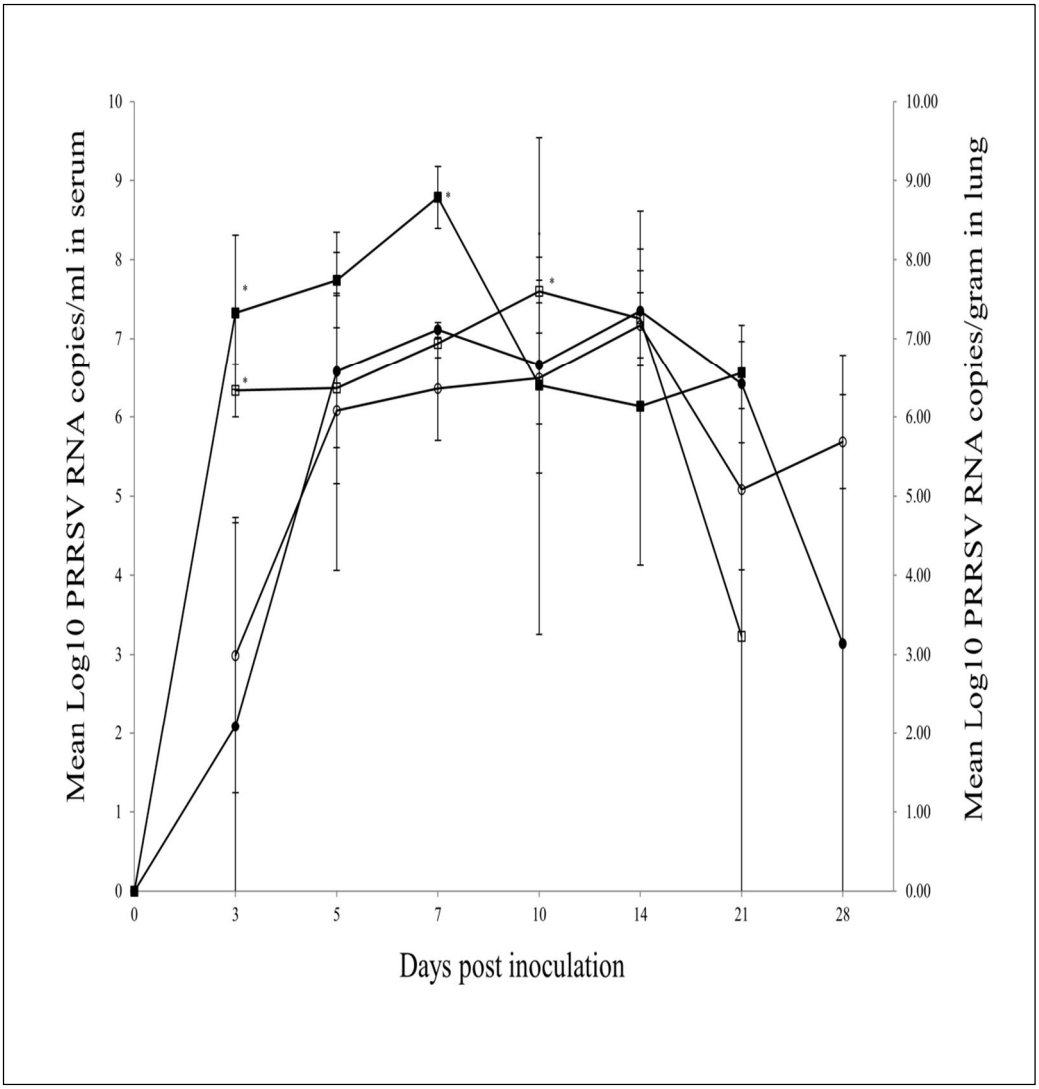

Figure 4. HP-PRRSV quantitative real time-polymerase chain reaction (expressed as $\log_{10}$ $TCID_{50}$ equivalents per ml) in blood (■ and ●) and lung (□ and ○) from pigs infected experimentally with northern (■ and □) or southern (● and ○) Vietnamese HP-PRRSV. A significant difference was observed at each time point compared with the previous time point ($^{*}P$ <0.05).

Gross Lesions

In pigs infected with northern Vietnamese HP-PRRSV, the most consistent lesion observed was severe interstitial pneumonia with congestion or petechial hemorrhages on the surface of the lungs. Interstitial pneumonia was characterized by diffuse, red-spotted or tan-mottled areas with irregular and indistinct borders and involved a majority of the lung at 7 dpi. There was generalized lymphadenomegaly with petechial haemorrhages at 5, 7, 8, 9, 10, 11, 12, 13 and 14 dpi. The petechial haemorrhages were seen in the gastric mucosa and the renal cortical surface from most of the pigs that had died at 5, 8, 9, 10, 11, 12, 13 and 14 dpi. In pigs infected with southern Vietnamese HP-PRRSV, interstitial pneumonia was characterized by multifocal, tan-mottled areas with irregular and indistinct borders and involved the craniovental portion of lung. No haemorrhages were observed in any internal organs, including the lung. Gross pulmonary lesion scores were significantly higher in pigs infected with northern Vietnamese HP-PRRSV than in those receiving southern Vietnamese HP-PRRSV at 10 (P = 0.001) and 14 (P = 0.0001) dpi (Fig. 5). No lesions were observed in negative control pigs.

Microscopical Lesions

Microscopical pulmonary lesions were characterized by thickened alveolar septa with increased numbers of interstitial macrophages and lymphocytes and by type II

pneumocyte hyperplasia. Microscopical lung lesion scores were significantly (P <0.05) higher in pigs infected with northern Vietnamese HP-PRRSV than in those receiving southern Vietnamese HP-PRRSV at 3 (P = 0.029), 5 (P = 0.013), 7 (P = 0.041), and 21 (P = 0.046) dpi (Fig 5). In pigs infected with northern Vietnamese HP-PRRSV, haemorrhages were multifocal in the lungs and other viscera from 7 to 14 dpi. Multifocal astrocytosis and microgliosis were observed in the white matter of brains from 7 to 14 dpi. No microscopical lesions were observed in lung sections from negative control pigs.

In-situ Hybridization and Immunohistochemistry

PRRSV nucleic acids and antigens were detected in the lung and other viscera from 3–28 dpi by ISH and IHC, respectively (Table 2). PRRSV nucleic acid and antigen were detected exclusively within the cytoplasm of macrophages in the lung (Fig. 6), lymph node (tracheobronchial, inguinal and mesenteric), thymus, heart, liver and Peyer's patches from pigs infected with either northern or southern Vietnamese HP-PRRSV. PRRSV expression was observed in cardiac myocytes and gastric and renal tubular epithelial cells from pigs infected with northern Vietnamese HP-PRRSV only. ISH and immunohistochemical signals were also detected in the white matter of the cerebrum and cerebellum (Figs. 7a and 7b). The labelled cells had eccentric nuclei and abundant homogeneous cytoplasm and resembled astrocytes (Fig. 8). Other positive

cells had small, hyperchromatic oval-, rod- or comma-shaped nuclei and no appreciable cytoplasm with routine HE staining, thus resembled microglial cells.

The score for the mean number of PRRSV-positive cells per unit area was significantly (P <0.05) higher in the lung and lymph nodes from pigs infected with northern Vietnamese HP-PRRSV than in those receiving southern Vietnamese HP-PRRSV at 3, 5, 7 (lung only) and 10 (lung only) dpi. The score of the mean number of PRRSV-positive cells was significantly (P <0.05) lower in the lung (14 dpi) and lymph nodes (10 dpi) from pigs receiving northern Vietnamese HP-PRRSV than in those infected with southern Vietnamese HP-PRRSV. Positive signals were not detected in sections from negative control pigs.

Table 2. Tissue distribution of northern and southern Vietnamese HP-PRRSV

Tissues	*Virus*	*Number of pigs positive by IHC/number of pigs positive by ISH*						
		3 dpi	*5 dpi*	*7 dpi*	*10 dpi*	*14 dpi*	*21 dpi*	*28 dpi*
Lung	N	2/3	3/3	3/3	4/4	3/4	1/1	-
	S	2/3	3/3	3/3	3/3	3/3	2/2	0/1
Heart	N	0/0	1/1	1/2	3/3	2/2	1/1	-
	S	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Brain	N	0/0	0/0	1/1	3/3	1/1	1/1	-
	S	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Thymus	N	3/3	3/3	3/3	3/3	3/3	2/2	-
	S	3/3	3/3	3/3	3/3	3/3	3/2	0/0
Tonsil	N	1/2	2/2	2/2	2/2	1/1	0/0	-
	S	1/1	2/2	0/1	0/0	0/0	0/0	0/0
Tracheobronchial lymph node	N	3/3	3/3	3/3	3/3	3/3	1/1	-
	S	2/2	3/3	3/3	3/3	3/3	2/2	0/0
Liver	N	0/0	1/2	2/3	2/2	1/1	0/0	-
	S	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Kidney	N	0/0	1/2	3/3	2/2	1/1	0/0	-
	S	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Spleen	N	0/1	0/2	2/2	2/2	2/2	0/0	-
	S	1/1	1/1	1/2	0/2	0/0	0/0	0/0
Stomach	N	0/0	1/1	1/1	2/3	1/1	0/0	-
	S	0/0	0/0	0/0	0/0	0/0	0/0	0/0
Ileum	N	1/1	1/1	1/2	2/2	0/1	0/0	-
	S	0/0	0/0	1/2	2/2	0/0	0/0	0/0

IHC, immunohistochemistry; ISH, in-situ hybridization; N, northern Vietnamese strain; S, southern Vietnamese strain; -, missing data

Virus Isolation

PRRSV was isolated from the lungs of 25 pigs infected with northern HP-PRRSV and 24 pigs infected with southern Vietnamese HP-PRRSV. The PRRSV isolated from the infected pigs was confirmed to be the same propagating northern and southern Vietnamese HP-PRRSV as in the challenge stock by sequence analysis of ORF5. No southern Vietnamese HP-PRRSV was isolated from the lung of the northern Vietnamese HP-PRRSV-infected pigs and vice versa. No PRRSV was isolated from the lung of the negative control pigs.

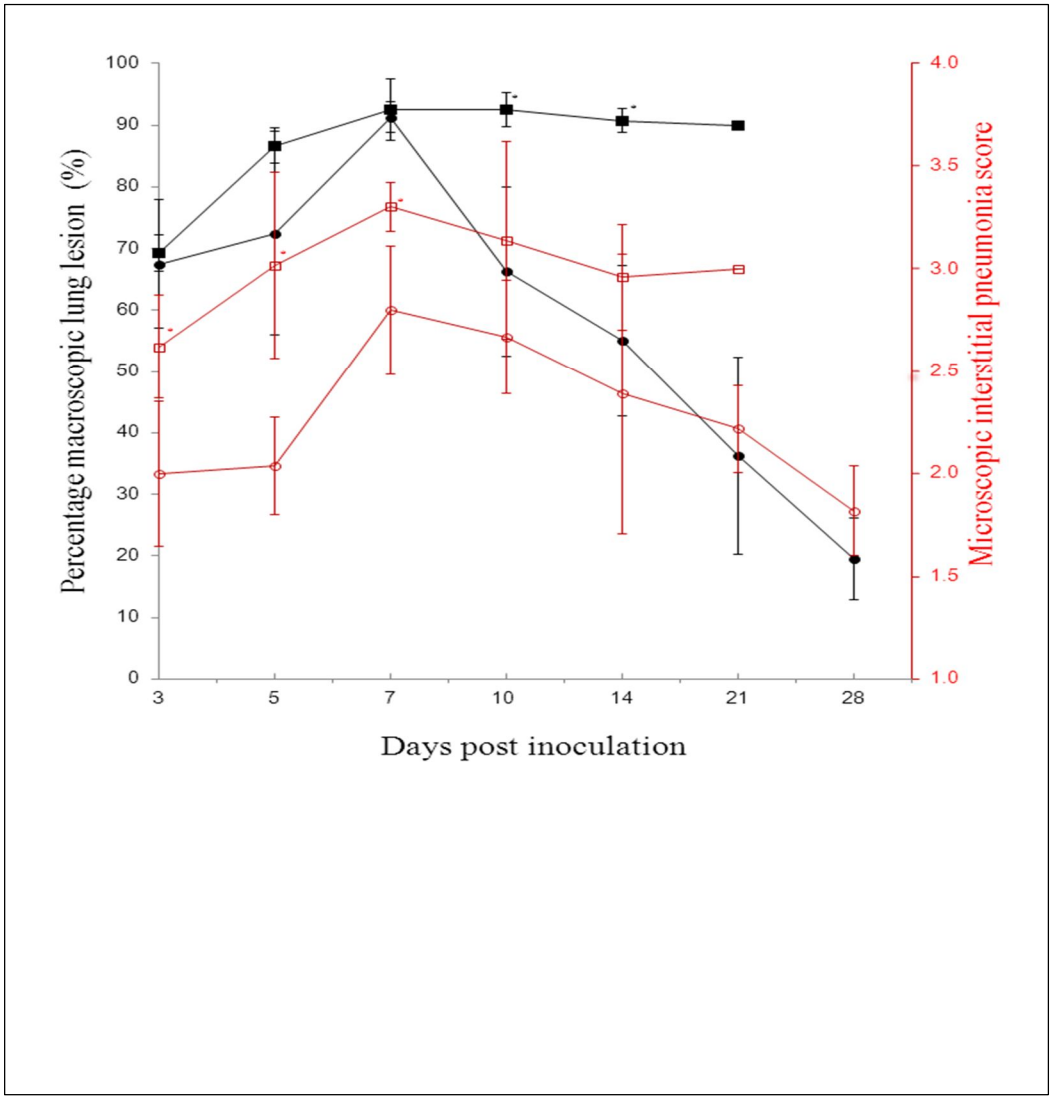

Figure 5. Mean gross (■ and ●) and microscopical (□ and ○) pulmonary lesion scores in lungs from pigs infected experimentally with northern (■ and □) and southern (● and ○) Vietnamese HP-PRRSV. A significant difference was observed at each time point compared with the previous time point ($^{*}P$ <0.05).

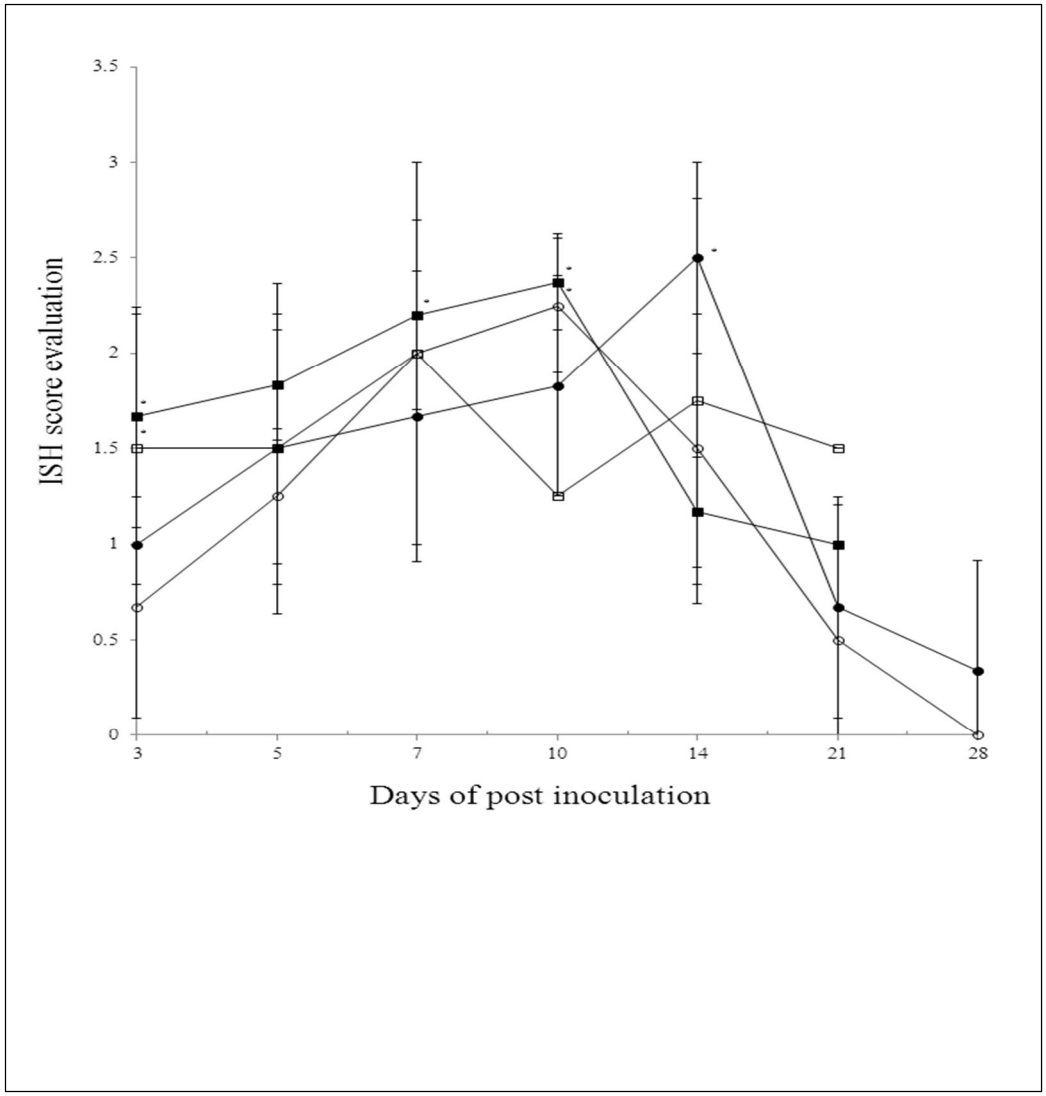

Figure 9. Mean score for ISH of the lung (■ and ●) and lymph nodes (□ and ○) in pigs infected experimentally with northern (■ and □) and southern (● and ○) Vietnamese HP-PRRSV. A significant difference was observed at each time point compared with the previous time point ($^{*}P$ <0.05).

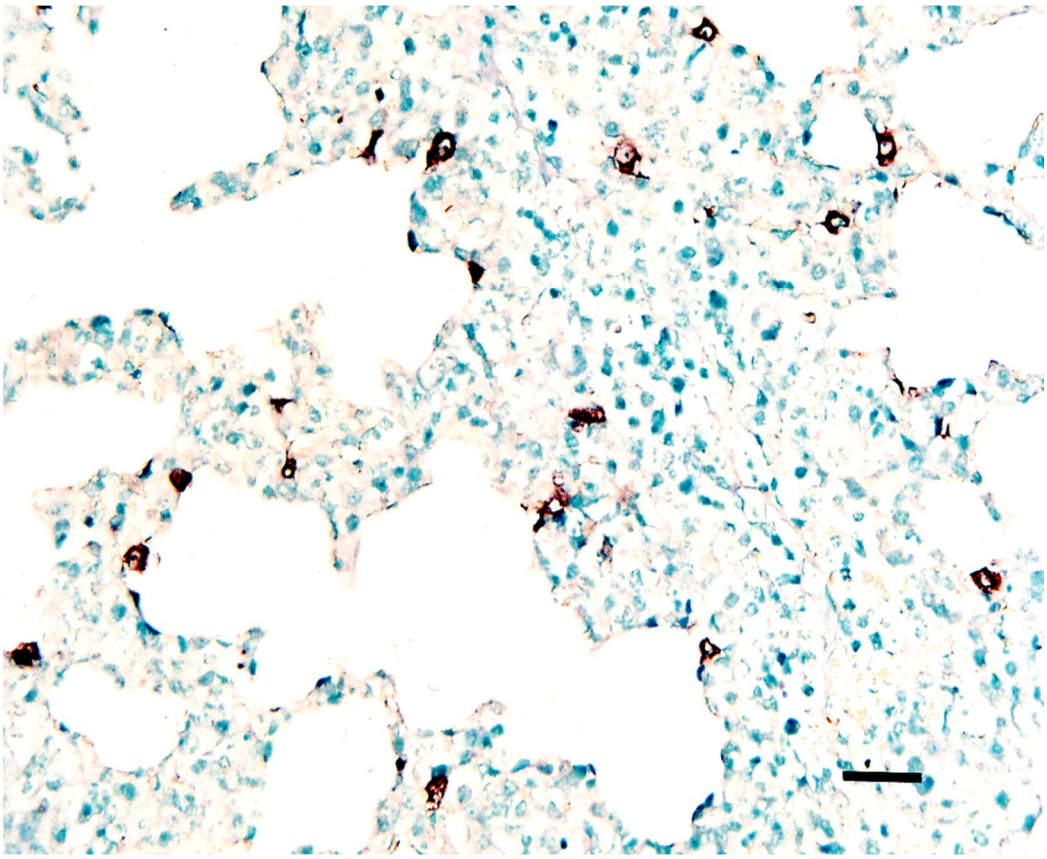

Figure 6. Lung tissue from pigs infected experimentally with northern Vietnamese HP-PRRSV taken at 7 dpi. Positive hybridization signals (black to brown grains) in macrophages. ISH. Bar, 55 μm.

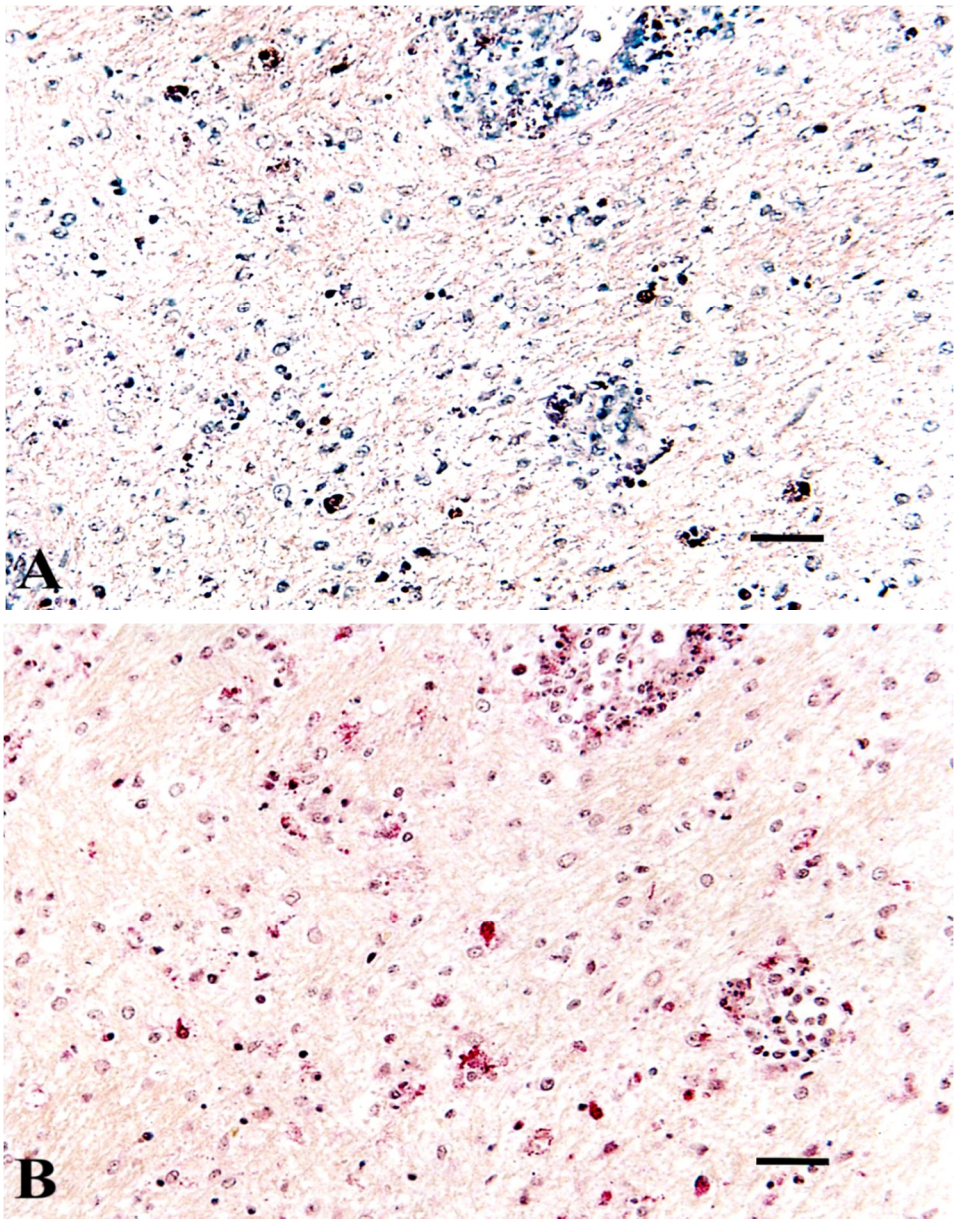

Figure 7. Cerebral tissue from pigs infected experimentally with northern Vietnamese HP-PRRSV taken at 7 dpi. (a) Positive hybridization signals (black to brown grains) in parenchymal cells. ISH. Bar, 55 μm. (b) Serial section showing that contiguous cells were also positive for immunohistochemical signals (red grains). IHC. Bar, 55 μm.

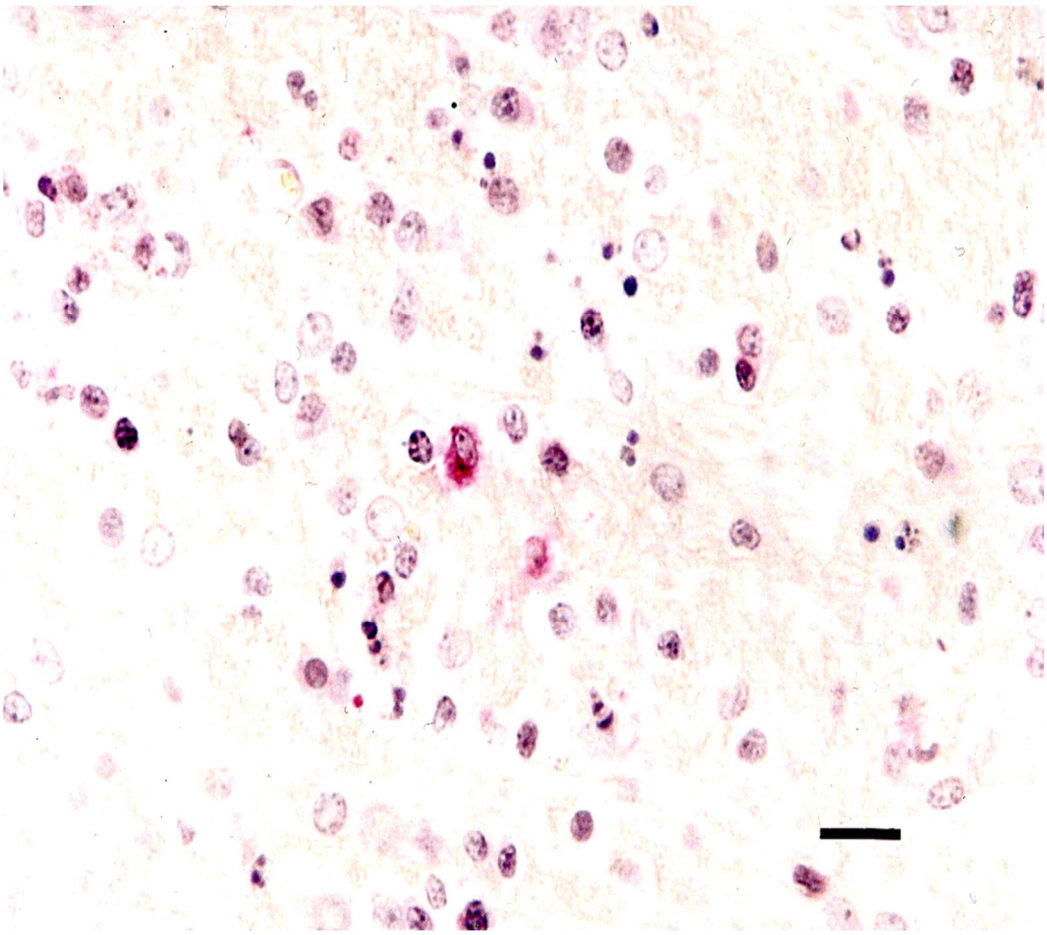

Figure 8. Cerebral tissue from pigs infected experimentally with northern Vietnamese HP-PRRSV taken at 10 dpi. Positive immunohistochemical signals (red grains) in astrocytes. IHC. Bar, 100 μm.

DISCUSSION

The results of this study show that two Vietnamese strains of HP-PRRSV cause different pathogenic outcomes in experimentally infected pigs. More virulent PRRSV strains replicate faster and induce more severe interstitial pneumonia than less virulent strains, regardless of their genotype (Halbur et al., 1995, 1996; Han et al., 2012). Therefore, viraemia, microscopical pulmonary lesion scores and PRRSV antigen within pneumonic lesions are the most important assessment criteria for determining the virulence of PRRSV. The study presented here shows that northern Vietnamese HP-PRRSV is more virulent than southern Vietnamese HP-PRRSV. This was demonstrated by a higher degree of viraemia, gross and microscopical pulmonary lesions, virus distribution and virus replication in the lungs.

Chinese and Vietnamese HP-PRRSV has been classified into sublineage 8.7 based on genetic analysis of ORF5 (Shi et al., 2010). Nevertheless, HP-PRRSV strains from the two countries differ significantly in terms of mortality rate. A high mortality rate (53.6%, 15/28 pigs) was found in pigs receiving northern Vietnamese HP-PRRSV by 20 dpi, but death was not observed in those receiving southern Vietnamese HP-PRRSV. Chinese HP-PRRSV strains JXwn06 and WUH2 caused 100 and 40% mortality rate in infected pigs, while another Chinese HP-PRRSV strain (HuN4) was not able to induce death in experimentally infected pigs (Li et al., 2012; Hu et al., 2013; Wang et al., 2014). These results show that there are differences in virulence between HP-PRRSV

strains.

Virus distribution differs between HP-PRRSV strains and between HP- and classical PRRSV. PRRSV was observed in cardiac myocytes in pigs receiving northern Vietnamese HP-PRRSV and classical PRRSV (Halbur et al., 1996), but not in those receiving Chinese (HuN4, HuN4-F112, JXwn06 and HB-1/3.9) HP-PRRSV strains (Li et al., 2012; Hu et al., 2013) or southern Vietnamese HP-PRRSV. In addition, viral antigens were observed in epithelial cells in several viscera from pigs infected with Chinese (strains JXwn06 and HB-1/3.9) HP-PRRSV (Li et al., 2012) and those receiving northern Vietnamese HP-PRRSV. These results suggest that HP-PRRSV shows diverse tissue tropism *in vivo* and this may contribute to its high pathogenicity in pigs compared with classical PRRSV.

The most striking difference in virus distribution was the presence of labelled astrocytes and microglial cells in the white matter of cerebrum and cerebellum from pigs infected with northern Vietnamese HP-PRRSV only. PRRSV nucleic acids were detected in perivascular cuffs around veins by ISH in pigs infected by Chinese HP-PRRSV (strains JXwn06 and HB-1/3.9) (Hu et al., 2013); however, no study has previously reported the detection of HP-PRRSV in parenchymal cells by either technique. In the present study, the simultaneous detection of viral nucleic acid and protein of HP-PRRSV by ISH and IHC, respectively, in parenchymal cells such as astrocytes and microglial cells indicates that these cells may be a site of replication of

HP-PRRSV. Further study is required to confirm the replication of HP-PRRSV in these cells.

Despite the fact that the incidence of fever and diarrhoea was reported to be higher in pigs infected with southern Vietnamese HP-PRRSV than in those receiving northern Vietnamese HP-PRRSV in the present study, the pigs in this experiment receiving southern Vietnamese HP-PRRSV showed fever and very mild diarrhoea, but then recovered shortly after. In contrast, pigs infected with northern Vietnamese HP-PRRSV showed diarrhoea associated with systemic signs leading to death.

The most important genetic marker of HP-PRRSV is a discontinuous deletion of 30 amino acids (90 nucleotides) in the protein product of the *NSP2* gene. All strains of Chinese HP-PRRSV contain an identical discontinuous deletion of 30 amino acids in the NSP2 protein (Li et al., 2007; Tian et al., 2007; Tong et al., 2007). Although this deletion enables characterization of HP-PRRSV, the 30 amino acid deletion in NSP2 is not related to virulence (Zhou et al., 2009). Two Vietnamese HP-PRRSVs were assigned as highly pathogenic based on generalized haemorrhage in viscera and high mortality (>30%) in the field, in addition to the presence of the same 30 amino acid deletion in NSP2. Interestingly, 99.3% sequence similarity was found in the ORF5 sequence between the two Vietnamese HP-PRRSVs. According to interpretation of sequence analysis, 97–98% sequence similarity indicates close relatedness of two viruses (Murtaugh, 2012). Nevertheless, the two Vietnamese HP-PRRSV strains have

different levels of virulence. Therefore, high genetic similarity was not predictive of virulent or pathogenic similarity between the two viruses in this study. The northern Vietnamese HP-PRRSV was more virulent than the southern Vietnamese HP-PRRSV.

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CHAPTER 2

Comparison of pathogenicity of highly pathogenic porcine reproductive and respiratory syndrome virus between wild and domestic pigs

ABSTRACT

The objective of this study was to compare the pathogenicity of highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) infection between wild and domestic pigs based on clinical, immunological, and pathological evaluation. Upon challenge with HP-PRRSV, five wild pigs died compared to none of the domestic. Anti-PRRSV antibody titers were significantly ($P < 0.05$) higher in wild HP-PRRSV-infected pigs versus the domestic HP-PRRSV-infected pigs at 21 days post inoculation (dpi). Lung lesion scores at 7 dpi were also significantly ($P < 0.01$) higher in domestic infected pigs than wild infected pigs. The most striking difference was the viral tissue distribution between the wild and domestic HP-PRRSV-infected pigs. HP-PRRSV-positive cells were observed in bronchiolar, gastric, and renal tubular epithelial cells from wild HP-PRRSV-infected pigs only. The results in this study demonstrated a genetic difference exists between wild and domestic pigs, which could results in different clinical signs, immunological responses, and pathological outcomes to HP-PRRSV infection.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) causes respiratory problems in growing pigs and reproductive failure in sows (Zimmerman et al., 2012). In 2006, a highly pathogenic PRRSV (HP-PRRSV) known as pig high fever disease was first reported in China (Tian et al., 2007; Feng et al., 2008). Since then, HP-PRRSV has spread rapidly in neighboring Asian countries such as Vietnam, Laos, Cambodia, Myanmar (Burma), Philippines, and Russia (Helen et al., 2009). Currently, infection of HP-PRRSV causes huge economic loss for the Asian swine industry (Tian et al., 2007; Zhou and Yang, 2010).

Wild pigs (*Sus scrofa*) are indigenous in many Asian countries including Vietnam, where wild pigs have been extensively farmed due to continuously high demand for valuable exotic pork. Vietnam government policies have encouraged protection of biogenetic diversity in addition to providing poor farmers with additional income options. However, wild pigs have been well established as reservoirs for several infectious pathogens that are transmissible to domestic pigs and humans (Meng et al., 2009). Infections of PRRSV in wild boars have been reported in several countries (Bonilauri et al., 2006; Ruiz-Fons et al., 2008; Choi et al., 2012; Roic et al., 2012). Since commercial wild pig farms are rapidly increasing in Vietnam, the risk of transmission of HP-PRRSV between wild and domestic pigs has increased significantly. Nevertheless, the pathogenicity of PRRSV infection in wild pigs has yet

to be demonstrated experimentally. The aim of this study was to compare the pathogenicity of HP-PRRSV infection between domestic and wild pigs based on clinical, immunological and pathological evaluation.

MATERIALS AND METHODS

PRRSV strain MN1 was isolated from lung sample of a neonatal piglet using MARC-145 cells in a 250-sow herd in southern Vietnam. Neonatal piglets in this herd had shown severe interstitial pneumonia, systemic disease and high mortality. HP-PRRSV strain MN1 was identified as type 2 HP-PRRSV on the basis of the nucleotide sequences of the nonstructural protein 2 (GenBank no. KJ742372.1) and open reading frame 5 (GenBank no. KM244763).

Thirty wild pigs purchased from wild pig reared farms and thirty domestic cross-bred pigs (25% Yorkshire × 25% Landrace × 25% Pietrain × 25% Duroc) purchased from conventional pig farms were used at the age of 5 weeks. All animals were negative for PRRSV and other viral pathogens (classical swine fever virus, foot and mouth disease virus, parvovirus and pseudorabies virus) according to routine serological testing performed prior to delivery and again on arrival. In addition, PRRSV was not detected in serum samples from any of the animals used in this study by real-time polymerase chain reaction (RT-PCR) (Wasilk et al., 2004) performed prior to delivery and again on arrival.

Pigs were allocated randomly to the infected or control group using random number generation function in Excel (Microsoft Corporation, Redmond, WA, USA). The viral inoculum contained isolate MN1 passaged twice in MARC-145 cells. Twenty of the

wild and domestic pigs were inoculated intranasally with 3 mL of tissue culture fluid containing $10^{5.5}$ of 50% tissue culture infective doses ($TCID_{50}$)/mL of HP-PRRSV. The control wild and domestic pigs (ten each) were inoculated intranasally with 3 mL of uninfected cell culture supernatants. The pigs were monitored weekly for physical conditions and scored daily for clinical respiratory disease severity using scores ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (Halbur et al., 1995). Rectal temperatures were recorded daily from 0 to 21 days post inoculation (dpi). Four infected and two control wild and domestic pigs were sedated by an intravenous injection of sodium pentobarbital and then euthanized at 5, 7, 10, 14, and 21 dpi as previously described (Beaver et al. 2001). Tissues were collected from each pig at the time of necropsy examination. Bacterial pathogens were isolated from lung tissues in wild and domestic pigs. Experimental methods were approved by the Nonglam University Institutional Animal Care and Use, and Ethics Committee.

Blood samples from each pig were collected by jugular venipuncture at -3, 0, 5, 7, 10, 14, and 21 dpi. The serum samples were tested using the commercially available PRRSV enzyme-linked immunosorbent assay (ELISA; HerdCheck PRRS X3 Ab test, IDEXX Laboratories Inc., Westbrook, Maine, USA). RT-PCR for type 2 PRRSV was used to quantify PRRSV genomic cDNA copy numbers in the serum (Wasilk et al., 2004). RT-PCR was considered positive if the cycle threshold level was $\leq$45cycles (Wasilk et al., 2004).

Immunohistochemistry (IHC) was performed to detect PRRSV antigen using SR30 monoclonal antibody (Rural Technologies Inc., Brookings, SD, USA) as previously described (Cheon and Chae, 1999). SR30 monoclonal antibody (Rural Technologies Inc.), specific for the nucleocapsid protein of PRRSV, was diluted 1: 1,000 in PBS (10 mM, pH 7.4) containing 0.1% Tween 20.

Macroscopic and microscopic lung lesions were estimated and calculated as previously described (Halbur et al., 1995). For morphometric analysis of IHC, three sections were cut from each of three blocks of tissue from one entire pulmonary lobe of each pig. To obtain quantitative data, slides were analyzed with the NIH Image J 1.43m Program (http://imagej.nih.gov/ij/download.html). In each slide, 10 fields were selected randomly and the number of positive cells per unit area (0.95 mm^2) was determined (Halbur et al., 1996). The mean values were also calculated.

The normality of the distribution for the examined variables was evaluated by the Shapiro-Wilk test. Continuous data (rectal body temperatures, PRRSV RNA quantification, serology, and macroscopic lung lesion scores) were analyzed with the Student's *t*-test. Discrete data (respiratory clinical sign scores, microscopic lung lesion score, and IHC scores) were analyzed by the Mann-Whitney *U* test. A value of $P < 0.05$ was considered significant.

RESULTS

Negative control pigs, wild or domestic, did not show any respiratory clinical manifestations whereas both wild and domestic HP-PRRSV-infected pigs showed severe respiratory symptoms (Table 1). The mean clinical respiratory scores were not significantly different between wild and domestic HP-PRRSV-infected pigs throughout the experiment. Of the wild HP-PRRSV-infected pigs 2 died at 11 dpi (n = 2) and 3 at 13 dpi (n =3). No death was observed in any of the domestic HP-PRRSV-infected pigs. No bacterial pathogens were isolated from lung tissues in wild and domestic HP-PRRSV-infected pigs.

The mean rectal temperatures were significantly ($P < 0.01$) higher in wild infected pigs than in negative control pigs from 4 to 10 dpi and from 13 to 19 dpi. In domestic pigs, the mean rectal temperatures were significantly ($P < 0.01$) higher in infected pigs versus negative control pigs from 2 to 16 dpi. When comparing wild and domestic infected pigs, the mean rectal temperatures were significantly ($P < 0.01$) higher in domestic pigs at 3, 4, 5 and 7 dpi. The negative control wild and domestic pigs' rectal temperature remained normal throughout the study (Fig. 1).

Wild HP-PRRSV-infected pigs became seropositive at 14 dpi whereas domestic HP-PRRSV-infected pigs became seropositive at 10 dpi as shown by ELISA. Anti-PRRSV antibody titers at 21 dpi were significantly ($P < 0.05$) higher in wild infected pigs

compared to domestic infected pigs (Fig. 2). No antibodies specific for PRRSV were detected by ELISA in the negative control wild and domestic pigs at any point in the experiment.

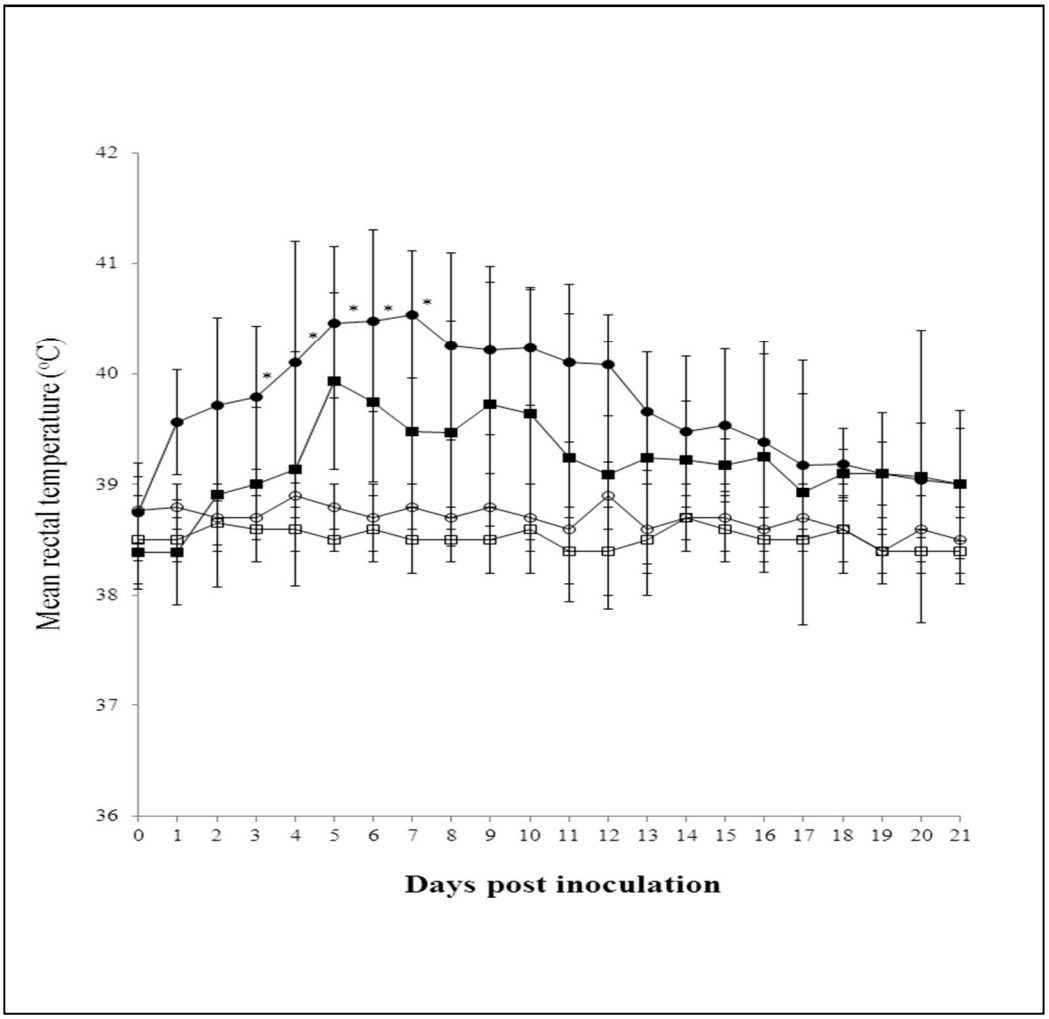

Figure 1. Mean rectal temperature in wild (■) and domestic (●) pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus, and negative control wild (□) and domestic (○) pigs. A significant difference between wild and domestic infected pigs was observed at each time point ($^{*}P < 0.01$).

Table 1. Clinical signs of wild and domestic pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus at different days post inoculation (dpi)

Clinical signs	Pigs	Number of pigs shown clinical signs/number of pigs observed				
		5 dpi	7 dpi	10 dpi	14 dpi	21 dpi
Loss appetite	Wild	7/20	13/16	5/12	0/3	0/2
	Domestic	4/20	12/16	2/12	1/8	0/4
	Control	0/10	0/8	0/6	0/4	0/2
High fever (≥ 40^{o}C)	Wild	10/20	8/16	6/12	0/3	0/2
	Domestic	15/20	13/16	9/12	1/8	0/4
	Control	0/10	0/8	0/6	0/4	0/2
Respiratory signs	Wild	2/20	4/16	7/12	2/3	0/2
	Domestic	2/20	3/16	6/12	5/8	1/4
	Control	0/10	0/8	0/6	0/4	0/2
Reluctant to move	Wild	7/20	14/16	5/12	0/3	0/2
	Domestic	8/20	6/16	0/12	0/8	0/4
	Control	0/10	0/8	0/6	0/4	0/2
Eye discharge	Wild	0/20	8/16	7/12	1/3	1/2
	Domestic	0/20	4/16	3/12	2/8	1/8
	Control	0/10	0/8	0/6	0/4	0/2
Death	Wild	0/20	0/16	0/12	5/8*	0/2
	Domestic	0/20	0/16	0/12	0/8	0/4
	Control	0/10	0/8	0/6	0/4	0/2

Notes: control pigs were wild or domestic pig; *: 5 wild pigs died on 11 dpi and 13 dpi

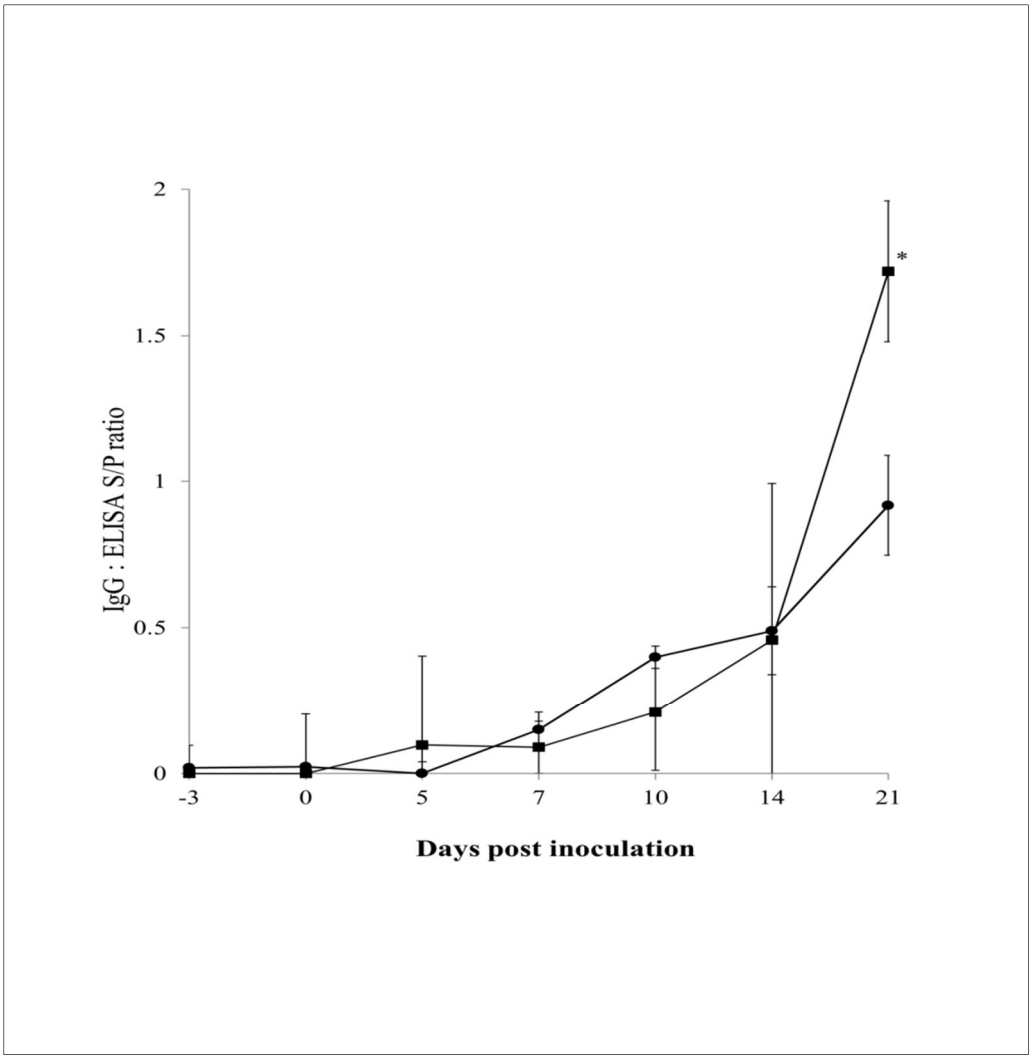

Figure 2. Antibody responses in wild (■) and domestic (●) pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus. A significant difference between wild and domestic infected pigs was observed at each time point ($^{*}P < 0.05$).

There was no significant difference in mean number of RNA copies of PRRSV in the serum of wild and domestic HP-PRRSV-infected pigs throughout the experiment (Fig. 3). No viral RNA was observed in the sera or lung tissues from the negative control wild and domestic pigs at any time during the experiment.

In infected pigs, both wild and domestic, the most consistent macroscopic lesions observed were a severe interstitial pneumonia with congestion or petechial hemorrhages on the surface of lung. Interstitial pneumonia was characterized by diffuse, red-spotted, tan-mottled areas with irregular and indistinct borders and localized throughout the majority of the lung at 7 dpi. There was a generalized lymphadenopathy with petechial hemorrhages in mesenteric lymph nodes in two of the five wild infected pigs that had died at 11 and 13 dpi. The petechial hemorrhages were seen in gastric mucosa (5/5 wild pigs) and renal cortical surface (4/5 wild pigs) from wild HP-PRRSV-infected pigs that had died at 11 and 13 dpi.

Domestic infected pigs scored significantly ($P < 0.05$) higher macroscopic lung lesions than wild infected pigs at 7 dpi whereas wild infected pigs scored significantly ($P < 0.05$) higher than domestic infected pigs at 10 dpi (Fig. 4). No lesions were observed in negative control wild and domestic pigs.

Microscopic lung lesions were characterized by thickened alveolar septa with increased numbers of interstitial macrophages and lymphocytes, and by type II pneumocyte

hyperplasia. Domestic infected pigs scored significantly ($P < 0.01$) higher microscopic lung lesions at 7 dpi whereas wild infected pigs scored significantly ($P < 0.01$) higher microscopic lung lesions at 10 dpi (Fig. 4). After 10 dpi there was no discernible difference in microscopic lung lesion scores between wild and domestic HP-PRRSV-infected pigs throughout the rest of the experiment. No microscopic lung lesions were observed in lung and other organ sections from negative control wild and domestic pigs.

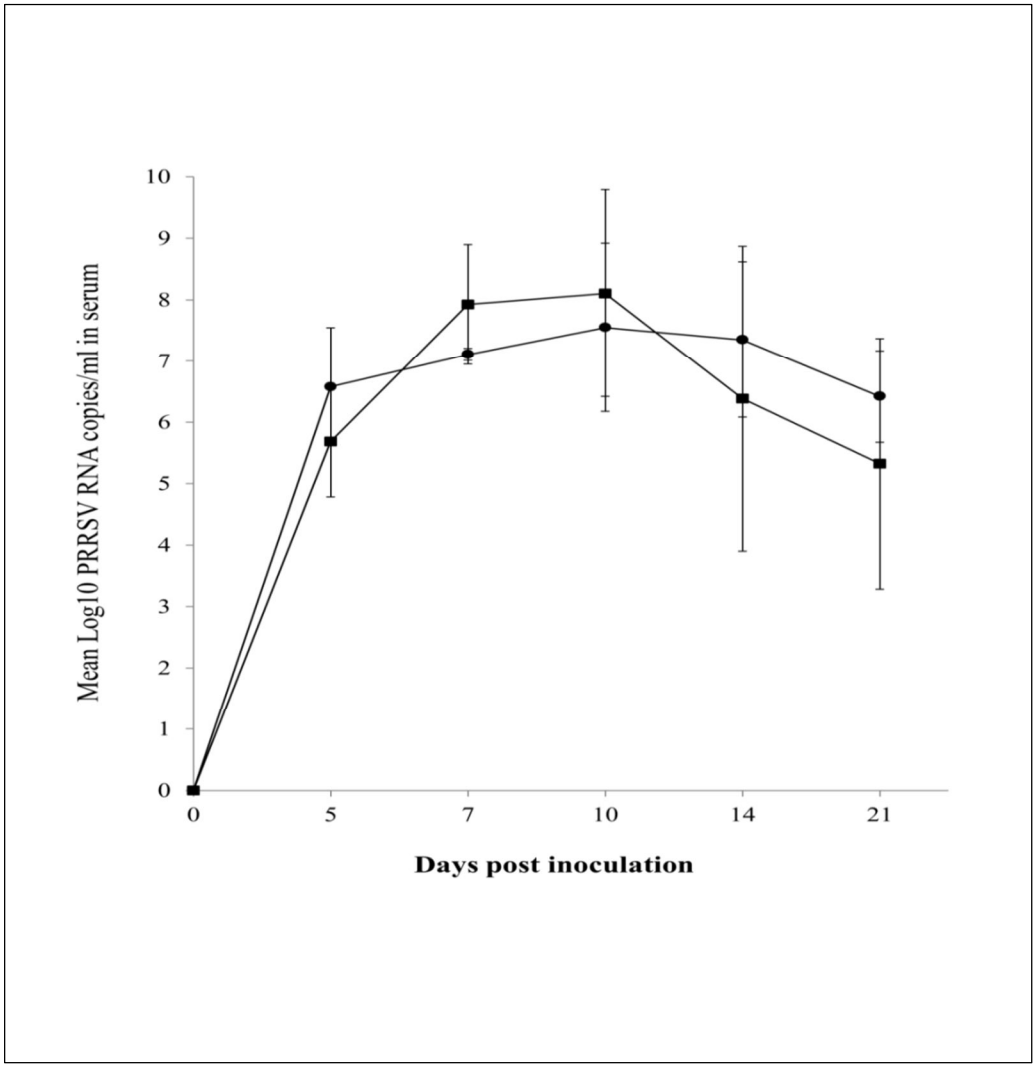

Figure 3. HP-PRRSV quantitative real time-polymerase chain reaction (expressed as $\log_{10}$ RNA copies/ml) in blood from wild (■) and domestic (●) pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus.

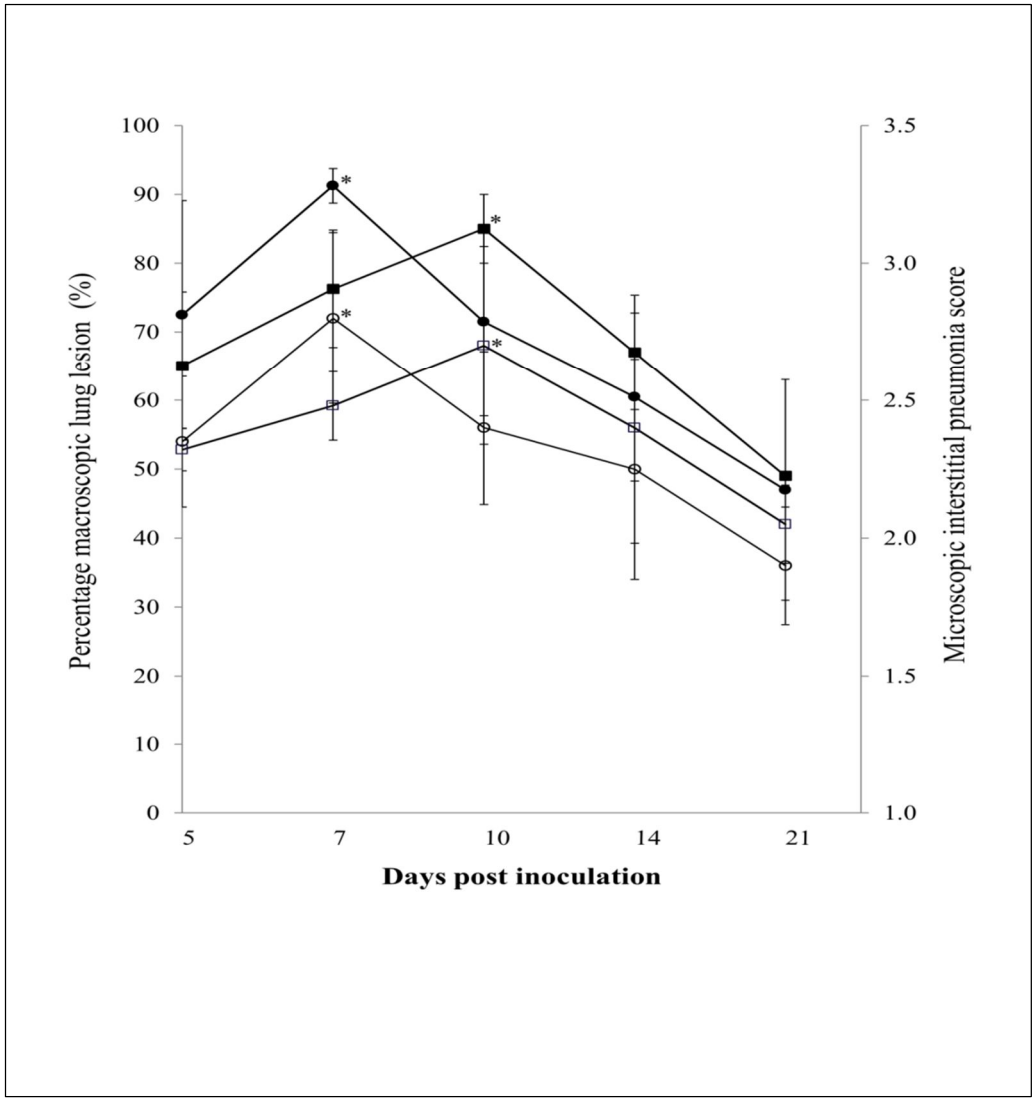

Figure 4. Mean macroscopic (■ and ●) and microscopic (□ and ○) lung lesion scores from wild (■ and □) and domestic (● and ○) pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus. A significant difference between wild and domestic infected pigs was observed at each time point ($^{*}P < 0.05$ and $^{\dagger}P < 0.01$).

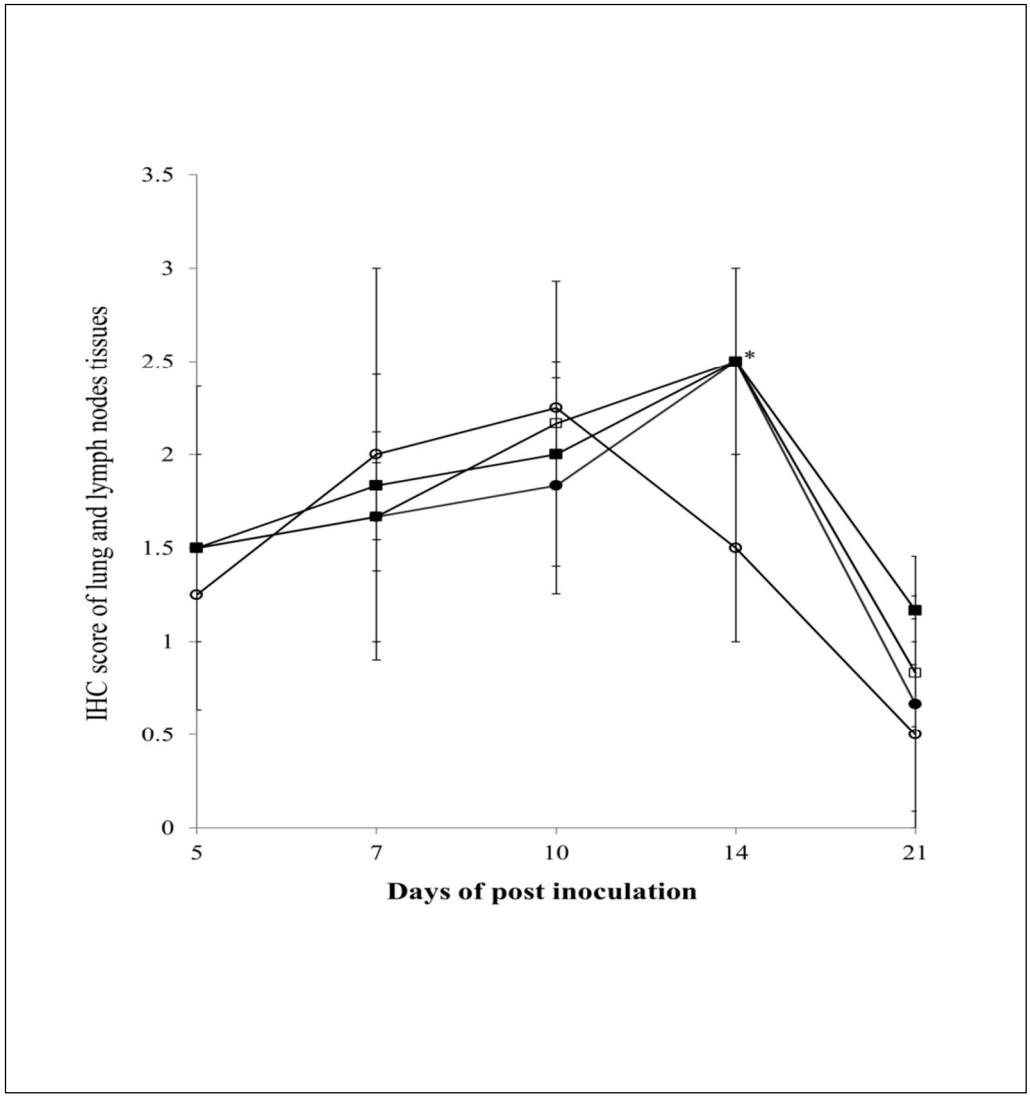

Figure 7. Mean score for IHC of the lung (■ and ●) and lymph nodes (□ and ○) from wild (■ and □) and domestic (● and ○) pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus. A significant difference between wild and domestic infected pigs was observed at each time point ($^{*}P < 0.05$).

PRRSV-specific antigens were detected by IHC in the lung and other visceral organs from 5 to 21 dpi (Table 2). PRRSV-specific antigens were detected exclusively within the cytoplasm of macrophages in lung, lymph node (tracheobronchial, inguinal, and mesentery), thymus, liver, and Peyer's patches from HP-PRRSV-infected wild and domestic pigs. PRRSV-positive cells were observed in bronchiolar epithelial cells (Fig. 5), gastric epithelial cells, and renal tubular epithelial cells (Fig. 6) from wild HP-PRRSV-infected pigs only. The score for the mean number of PRRSV-positive cells per unit area was not significantly different in lung between wild and domestic HP-PRRSV-infected pigs but significant ($P < 0.05$) higher in lymph nodes of wild HP-PRRSV-infected pigs than those of domestic HP-PRRSV-infected pigs at 14 dpi (Fig. 7). PRRSV-positive cells were not detected in sections from negative control wild and domestic pigs.

Table 2. Immunohistochemistry (IHC) of highly pathogenic porcine reproductive and respiratory syndrome virus-infected wild and domestic pigs

Tissues	Pigs	Number of pigs positive by IHC/number of pigs tested					
		5 dpi	7 dpi	10 dpi	14 dpi	21 dpi	Total
Lung	Wild	3/3	3/3	3/3	3/3	2/2	14/14
	Domestic	3/3	3/3	3/3	3/3	2/3	14/15
Heart	Wild	0/3	0/3	0/3	0/3	0/2	0/14
	Domestic	0/3	0/3	0/3	0/3	0/3	0/15
Brain	Wild	0/3	0/3	0/3	0/3	0/2	0/14
	Domestic	0/3	0/3	0/3	0/3	0/3	0/15
Thymus	Wild	3/3	3/3	3/3	3/3	2/2	14/14
	Domestic	3/3	3/3	3/3	3/3	2/3	14/15
Tonsil	Wild	2/3	2/3	3/3	3/3	1/2	11/14
	Domestic	3/3	2/3	1/3	0/3	0/3	6/15
Lymph nodes	Wild	3/3	3/3	3/3	3/3	2/2	14/14
	Domestic	3/3	3/3	3/3	3/3	2/3	14/15
Liver	Wild	0/3	1/3	2/3	2/3	0/2	5/14
	Domestic	0/3	0/3	0/3	0/3	0/3	0/15
Kidney	Wild	0/3	1/3	3/3	2/3	0/2	6/14
	Domestic	0/3	0/3	0/3	0/3	0/3	0/15
Spleen	Wild	2/3	3/3	3/3	3/3	1/2	12/14
	Domestic	2/3	2/3	1/3	0/3	0/3	5/15
Stomach	Wild	0/3	0/3	1/3	3/3	0/2	4/14
	Domestic	0/3	0/3	0/3	0/3	0/3	0/15
Ilieum	Wild	0/3	0/3	2/3	2/3	0/2	4/14
	Domestic	0/3	2/3	2/3	0/3	0/3	4/15

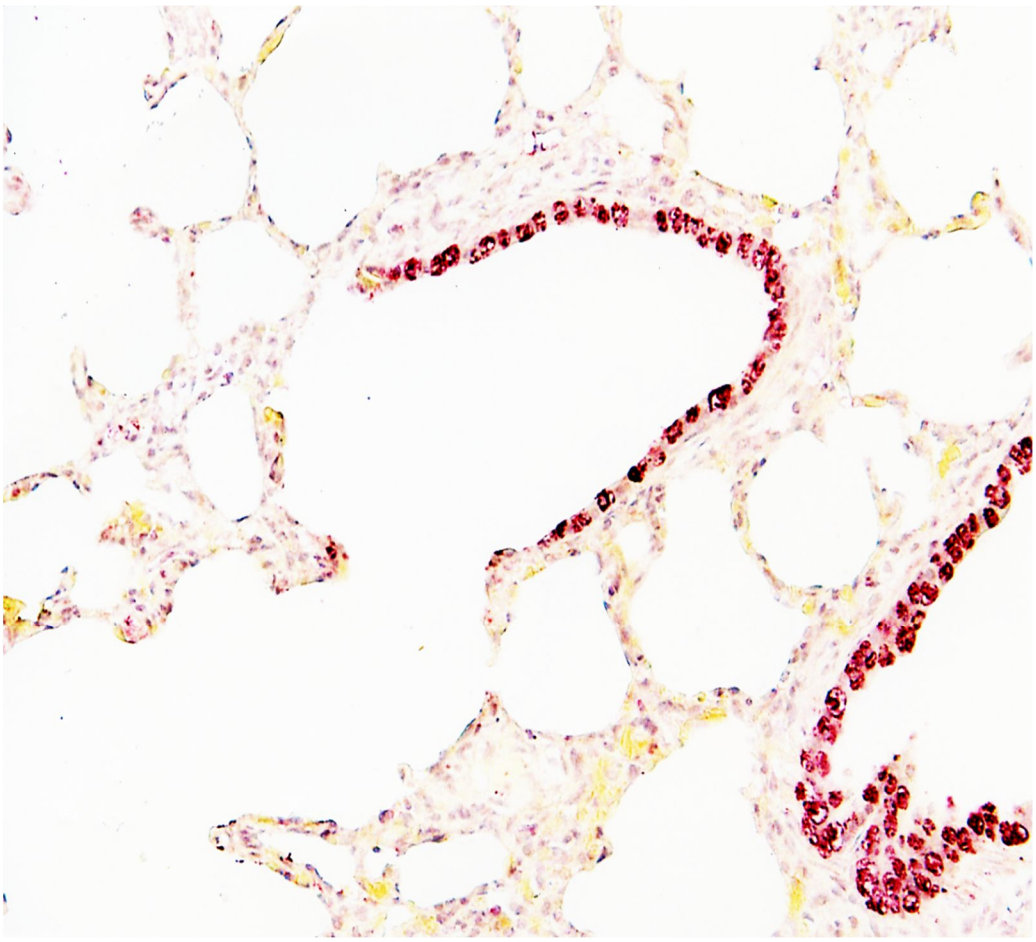

Figure 5. Lung tissue from wild pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus collected at 5 days post inoculation. Positive signals (red grains) were seen in bronchiolar epithelium.

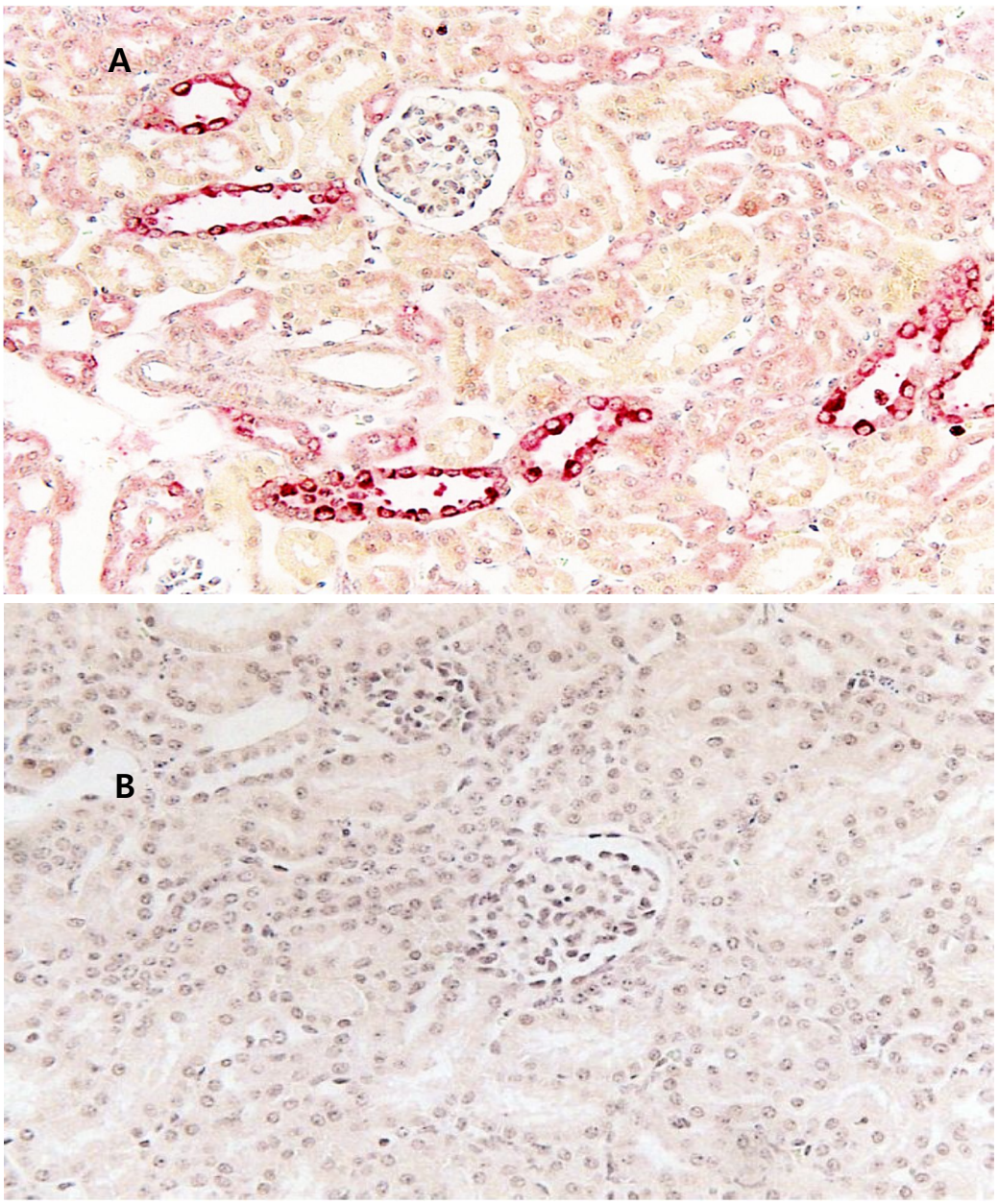

Figure 6. A, Positive signals (red grains) were seen in renal tubular epithelium from wild pigs infected with highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) collected at 14 days post inoculation (dpi). **B,** No positive signals were seen in renal tubular epithelium from domestic pigs infected with HP-PRRSV collected at 14 dpi.

DISCUSSION

This study demonstrates for the first time that a genetic difference exists between wild and domestic pigs in responses to HP-PRRSV infection. HP-PRRSV infection induced high rates (25%) of mortality and hemorrhagic lesions in internal organs from wild pigs only. In addition, HP-PRRSV induced higher levels of anti-PRRSV IgG antibody in wild pigs compared to domestic pigs. Genetic differences in response of PRRSV infection also exist among different pig breeds. Hampshire and Meishan pigs had significantly higher anti-PRRSV IgG antibody titers than Duroc pigs (Halbur et al., 1998). In addition, pigs from a cross of Hampshire and Duroc lines had significantly higher anti-PRRSV IgG antibody titers than pigs from a Large White-Landrace composite population (Petry et al., 2005).

Severity of lung lesions is an important parameter to compare virulence because the respiratory symptoms are the main clinical problem caused by PRRSV infection. Hampshire pigs had significantly greater macroscopic lung lesions induced by PRRSV when compared to Duroc and Meishan breeds (Halbur et al., 1998). In addition, pigs from a cross of Hampshire and Duroc lines had a greater incidence of interstitial pneumonia and significantly higher microscopic lung lesion scores than pigs from a Large White-Landrace composite population (Petry et al., 2005). In the present study, domestic HP-PRRSV-infected pigs exhibited higher macroscopic and microscopic lung lesion scores at 7 dpi whereas wild infected pigs displayed higher macroscopic

and microscopic lesions at 10 dpi. These results indicate that domestic HP-PRRSV-infected pigs reach the most severe lung lesions earlier than wild HP-PRRSV-infected pigs.

The most striking difference between the wild and domestic pigs is the viral tissue distribution. HP-PRRSV-positive cells were observed in bronchiolar, gastric, and renal tubular epithelial cells in wild infected pigs only. In a previous study, viral antigens were observed in gastric and renal tubular epithelial cells in stomach and kidney from Chinese HP-PRRSV (strains JXwn06)-infected pigs (Li et al., 2012). However, no study had previously reported the detection of PRRSV in the bronchiolar epithelial cells by IHC techniques. Infection of PRRSV may be dependent on the presence of viral receptors on the surface of bronchial epithelial cells in wild pigs. Four receptors of PRRSV have been identified on porcine macrophages: CD169, CD163, CD151, and heparan sulphate, (Calvert et al., 2007; Delputte et al., 2002; Vanderheijden et al., 2003). In primary macrophages, CD169 and CD163 receptors are expressed at high level, which explains why pulmonary alveolar macrophages are highly susceptible to PRRSV infection. These results suggest the presence of PRRSV receptors on the surface of bronchial epithelial cells in wild pigs. Bronchiolar epithelial cells play an essential role in pulmonary defense. Infection of HP-PRRSV adversely affects the function of bronchiolar epithelial cells, resulting in increasing susceptibility to secondary viral and bacterial infection.

This is the first study to compare the pathogenicity of HP-PRRSV between wild and domestic pigs. In addition to genetic differences between the wild and domestic pigs, other factors may have affected their morbidity and mortality following infection with PRRSV such as general health, immune status, and co-infections, etc. Significant differences in the lung lesions and humoral immune responses were observed under our experimental conditions. Further studies are needed to use several breeds of pigs to compare the pathogenesis of HP-PRRSV between wild and domestic pigs.

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CHAPTER 3

Genomic analysis of Vietnamese highly pathogenic porcine reproductive and respiratory syndrome virus from 2013 to 2014 based on NSP2 and ORF5

ABSTRACT

A total of 34 highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) strains isolated from Vietnam during 2013–2014 were sequenced and analyzed. Partial sequence of nonstructural protein 2 (Nsp2) gene and full sequence of open reading frame 5 (ORF5) gene was used for the analysis. The HP-PRRSV strains were isolated from pig herds that had never been vaccinated for PRRSV. The nucleotide homology of Nsp2 and ORF5 ranged between 96.4 to 100% and 83.2 to 100%, respectively. All of the 34 Vietnamese HP-PRRSV strains showed two discontinuous 30 amino acids deletions in the Nsp2 gene as a genetic marker of prototypic Chinese HP-PRRSV. Amino acids at position 13 and 151 in ORF5 are arginine (R) in 29 out of 34 Vietnamese HP-PRRSV isolates as those in prototypic Chinese HP-PRRSV. Genomic analysis of ORF5 from all Vietnamese HP-PRRSVs revealed six subgroups; Viet-1 to -4, JXA1-like, and VR-2332-like. Nucleotide and amino acid sequence analysis of 34 Vietnamese HP-PRRSV isolated during 2013–2014 indicate that Vietnamese HP-PRRSV has undergone rapid evolutionary changes in recent years when compared with Vietnamese HP-PRRSV isolated before 2012.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a single-stranded, positive-sense, enveloped RNA arterivirus in the family *Arteriviridae* of the order *Nidovirales* with two genotypes, type 1 and type 2 (Snijder and Meulenberg, 1998; Allende et al., 1999; Murtaugh et al., 2010). The genome of PRRSV is approximately 15 kb and consists of the 5'-UTR, non-structural proteins (NSP) coding sequence open reading frame 1a (ORF1a) and ORF1b, structural proteins coding sequence ORF2 to ORF7, and the 3'UTR with a following poly(A) tail (Snijder and Meulenberg, 1998).

In May 2006, an unusually large-scale outbreak initially called 'high fever disease' affected over 2,000,000 pigs with about 400,000 fetal cases in the Jiangxi Province of China. The causative agent was later identified as PRRSV now referred to as highly pathogenic PRRSV (HP-PRRSV) (Tian et al., 2007). HP-PRRSV is a variant of type 2 PRRSV containing a novel discontinuous 30-amino-acid (aa) deletion in NSP2 (Tian et al., 2007). In March 2007, the first outbreak of HP-PRRSV infection was reported in Hai Duong Province of Vietnam where over 65,000 pigs were affected with high mortality (24%) (Feng et al., 2008).

Among PRRSV strains, NSP2 and ORF5 regions are the most variable and have been used as markers for genetic variability (Murtaugh et al., 1995; Fang et al., 2004). Genomic analysis of ORF5 demonstrated high nucleotide similarities between

Vietnamese PRRSV and Chinese HP-PRRSV (Thuy et al., 2013); however, that study did not determine whether those Vietnamese PRRSV isolates were HP-PRRSV. HP-PRRSV now causes huge economic losses in Vietnamese swine industry (Feng et al., 2008; An et al., 2011). Although it is necessary to investigate genomic analysis for controlling of HP-PRRSV, genomic characteristics of Vietnamese HP-PRRSV have yet been elucidated. In the current study, partial Nsp2 gene sequence and the full ORF5 gene sequence of 34 Vietnamese HP-PRRSV isolates from Vietnam during 2013–2014 were analyzed to investigate the epidemiological and evolutionary characteristics.

MATERIALS AND METHODS

The virus was isolated from lung tissue samples using MARC-145 cells and confirmed as PRRSV by indirect immunofluorescence assay using a monoclonal antibody against the nucleocapsid protein of PRRSV (SDOW 17, Rural Technologies Inc., Brookings, SD, USA). A total of 34 HP-PRRSV strains were isolated from pig herds that had never been vaccinated for PRRSV.

Partial sequence of the Nsp2 gene (667 base pair, bp) was amplified with specific primers, Nsp2-F 5'-AAAGACCAGATGGAGGAGGA-3' and Nsp2-R 5'-GAGCTGAGTATTTTGGGCGTG-3'. ORF5 gene (826 bp) was amplified with specific primers, ORF5-F 5'- GGCAATGTGTCAGGCATC-3'and ORF5-R 5'-CTGGAGCCGTGCTATCAT-3'.

Total RNA was extracted from 200 µl of supernatant using a commercial reagent (TRIzol, Invitrogen). The extracted RNA was synthetized into cDNA in a master mix which included a TOPscript^{TM} Reverse Transcriptase (TOPscript^{TM} cDNA Synthesis kit, Enzynomics). The final PCR reaction was carried out with a total volume of 20 µl containing the following ingredients: 10 µl master mix 2X TOPsimple^{TM} PreMIX (aliquot)-Forte (Enzynomics, Daejeon, Korea), 1 µl primer (10 mM) each, 2 µl cDNA template and DEPC water to reach the final volume of reaction. The PCRs were run as following condition: 95^{o}C for 5 minutes and 40 cycles of 94^{o}C for 30 s, annealing at

58°C for 60 s (or 55°C for NSP2 primers only), elongation at 72°C for 60 s and final extension was at 72°C for 7 minutes.

The PCR products were purified using a commercial kit (Wizard®PCR Preps DNA Purification and PCR Clean-Up System, Promega, US), cloned with the TOPcloner Blunt kit (Enzynomics, Daejeon, Korea), and propagated in DH5α competent cells (Enzynomics, Daejeon, Korea) according to the manufacturer's instructions. Plasmid DNA was purified with a plasmid purification kit (iNtRON Biotechnology, Sungnam, Kyeonggido, Korea) and sequenced by a commercial service (Sol Gent Co. Ltd., Daejeon, Korea).

A phylogenetic tree was constructed to analyze the relationship between HP-PRRSV isolates in Vietnam and those from Southeast Asia and China using the 34 Vietnamese HP-PRRSVs from this study, and 8 Vietnamese, 12 Southeast Asian and 5 Chinese HP-PRRSVs.

The partial NSP2 gene and full ORF5 gene sequences from 34 Vietnamese HP-PRRSV isolates along with reference Chinese HP-PRRSV sequences obtained from GenBank were aligned using ClustalW (Thompson et al., 1994). Subsequently, phylogenetic trees were generated by the Mega 5 software (Tamura et al., 2011). Bootstrap values were calculated on 1,000 replicates of the alignment (Tamura et al., 2011).

Table 1. The current Vietnamese HP-PRRSVs in this study

Geographic regions	Provinces	Isolates_Years	Accession No. (NSP2/ORF5)
Northern	HaTay	D11/HT2/VN_2013	KR261750/KR261784
	HungYen	D12/HY1/VN_2013	KR261770/KR261804
Southern Central	BinhDinh	D20/BDi1/VN_2014	KR261751/KR261785
	BinhThuan	D21/BT1/VN_2014	KR261747/KR261781
		D27/BT2/VN_2014	KR261765/KR261799
		D28/BT3/VN_2014	KR261741/KR261775
		D41/BT4/VN_2014	KR261748/KR261782
Southeastern	Tp.HCM	D1/HCM1/VN_2013	KR261755/KR261789
		D16/HCM2/VN_2014	KR261760/KR261794
		D33/HCM3/VN_2014	KR261739/KR261773
	DongNai	D2/DN1/VN_2013	KR261744/KR261778
		D8/DN3/VN_2014	KR261742/KR261776
		D14/DN12/VN_2014	KR261746/KR261780
		D29/DN5/VN_2014	KR261738/KR261772
		D42/DN6/VN_2014	KR261740/KR261774
		D43/DN7/VN_2014	KR261758/KR261792
		D44/DN8/VN_2014	KR261753/KR261787
		D49/DN9/VN_2014	KR261745/KR261779
		D51/DN10/VN_2014	KR261768/KR261768
		D54/DN11/VN_2014	KR261743/KR261777
	BinhDuong	D10/BD1/VN_2013	KR261762/KR261796
		D13/BD2/VN_2014	KR261761/KR261795
		D15/BD3/VN_2014	KR261756/KR261790
		D24/BD4/VN_2014	KR261764/KR261798
		D30/BD5/VN_2014	KR261759/KR261793
		D31/BD6/VN_2014	KR261771/KR261805

		D50/BD7/VN_2014	KR261749/KR261783
	BR-VungTau	D3/BRVT1/VN_2013	KR261763/KR261797
Southwestern	BenTre	D23/BTr1/VN_2014	KR261757/KR261791
	SocTrang	D18/ST0/VN_2014	KR261769/KR261803
		D22/ST1/VN_2014	KR261766/KR261800
		D32/ST2/VN_2014	KR261752/KR261786
	AnGiang	D9/AG1/VN_2013	KR261754/KR261788
		D9/AG2/VN_2013	KR261767/KR261801

RESULTS

The sequencing analysis showed that 90 nucleotides (30 amino acids) were deleted in the Nsp2 gene of all 34 Vietnamese HP-PRRSV strains compared with the classical PRRSV strains. The phylogenetic tree constructed for the partial Nsp2 genes of the Vietnamese HP-PRRSV strains is shown in Fig 1. The 34 strains showed a nucleotide homology ranging from 96.4 to 100 %, and an amino acid homology ranging from 86.5 to 100%. The partial sequence of the Nsp2 gene from the 34 strains exhibited 97.07–99.33%, 97.44–99.67%, 92.69–94.18%, 82.91–84.58% nucleotide homology with HP-PRRSV strain 07QN (the first Vietnamese HP-PRRSV isolate, 2007), HP-PRRSV strain JXA1 (the first Chinese HP-PRRSV isolate, 2006), strain Ch-1a (the first classical type 2 PRRSV isolate, 1996), and VR-2332 strain (prototype of type 2 PRRSV, 1990), respectively. In addition, the partial sequence of the Nsp2 gene from the 34 strains exhibited 88.78 – 93.66%, 91.25–98.88%, 72.53–79.22%, 37.70–47.61% of amino acid homology with HP-PRRSV strain 07QN, HP-PRRSV strain JXA1, strain Ch-1a, and VR-2332 strain, respectively.

The sequencing analysis showed that none of the 34 Vietnamese HP-PRRSV ORF5 gene sequences contained a nucleotide deletion or insertion. The 34 strains showed a nucleotide homology ranging from 83.2 to 100 %, and an amino acid homology ranging from 82.5 to 100 %. The full ORF5 gene sequence from the 34 strains exhibited 86.8–98.3%, 87.0–99.5%, 90.0–95.1%, 84.3–98.8% of nucleotide homology

with HP-PRRSV strain 07QN, HP-PRRSV strain JXA1, strain Ch-1a, and VR-2332 strain, respectively. In addition, the full ORF5 genes from the 34 strains exhibited 86.1–97.9%, 85.5–99.5%, 88.5–93.6%, 84.3–96.8% of amino acid homology with HP-PRRSV strain 07QN, HP-PRRSV strain JXA1, strain Ch-1a, and VR-2332 strain, respectively (Fig. 1b).

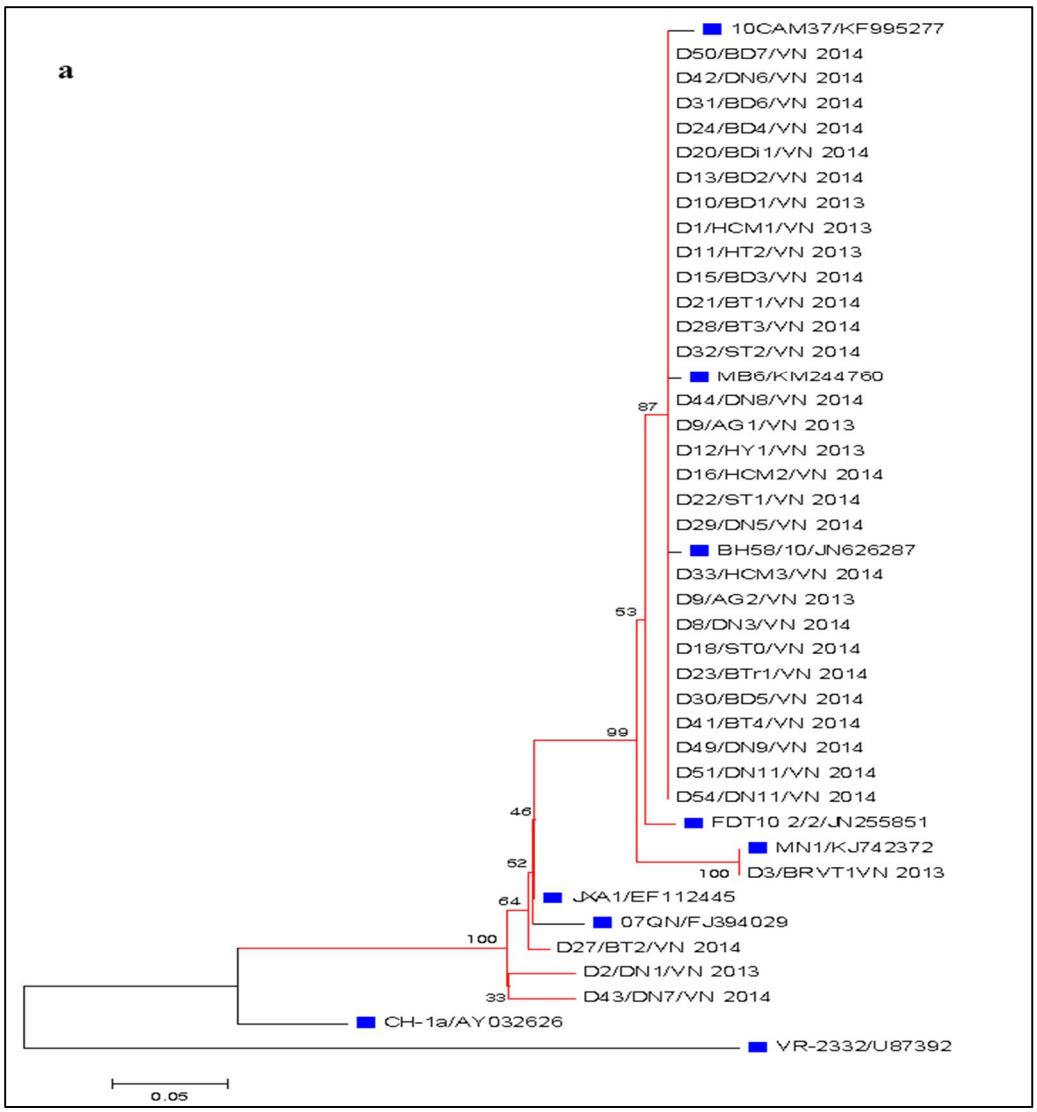

Figure 1. Phylogenetic tree of the nucleotide sequences for partial Nsp 2 genes (a) and full ORF5 genes (b) of the 34 Vietnamese HP-PRRSV strains and related reference virus. Phylogentic tree was constructed by the neighbor-joining method.

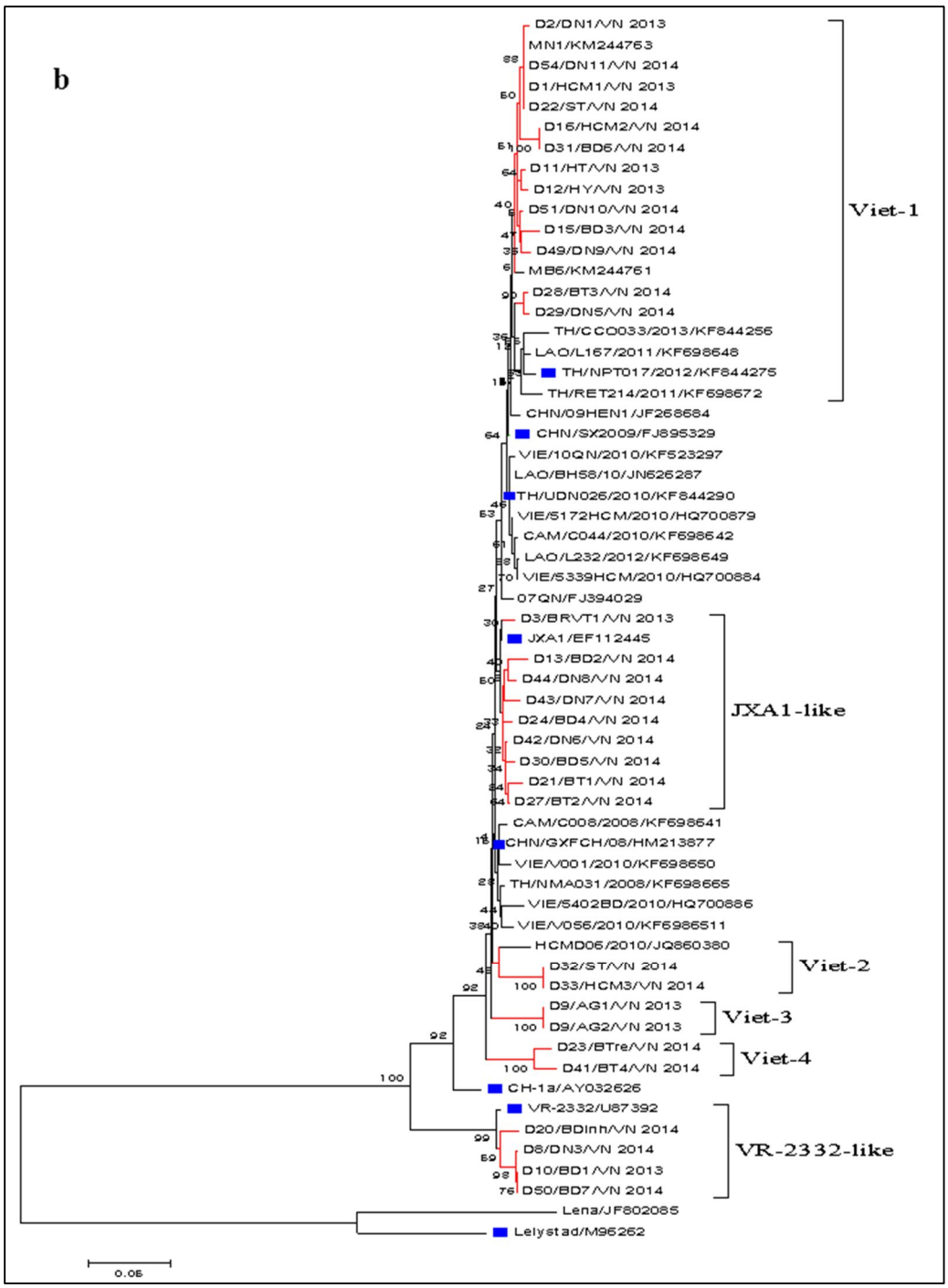

Figure 1. (cont')

DISCUSSION

The most remarkable genetic markers of HP-PRRSV are two non-contiguous amino acid (aa) deletions in the gene Nsp2 (Tian et al., 2007). All 34 Vietnamese HP-PRRSV isolates showed discontinuous 30 aa deletions (1 and 29 aa), in the gene Nsp2, at two sites corresponding to position 481 aa and 533-562 aa in PRRSV strain VR-2332 typically observed in the prototype Chinese HP-PRRSV. However, the discontinuous 30 aa deletion in Nsp2 is not associated with the virulence of HP-PRRSV (Zhou et al., 2009). On the other hand, GP5 encoded by ORF5 is one of the major viral envelope proteins and an important viral component for induction of neutralization antibodies and infectivity (Ansari et al., 2006). Amino acid residues at positions 13 and 151 of GP5 have been postulated to be associated with viral virulence. Amino acids at position 13 and 151 are glutamic acid (13Q) and glycine (151G) in low pathogenic PRRSV isolates and modified live vaccine virus (Wesley et al., 1998; Chang et al., 2002). In contrast, amino acids at both positions are replaced with arginine (R) in 29 out of 34 Vietnamese HP-PRRSV isolates as those in Chinese HP-PRRSV (Wu et al., 2009; Li et al., 2014).

A phylogenetic analysis based on the ORF5 gene indicates that the Vietnamese HP-PRRSV strains along with the reference strains can be divided into six subgroups; Viet-1 to -4, JXA1-like, and VR-2332-like. In this study, 13 and 9 of the 34 strains belonged to subgroup Viet 1 and JXA1-like respectively. Most of the Vietnamese HP-PRRSVs from 2013 to 2014 belong to subgroups Viet-1 and JXA1-like. Vietnamese HP-PRRSV

strains belonging to subgroup Viet-1 are not detected until 2012. The emergence of these strains in Vietnam is of great concern. Vietnamese HP-PRRSV strain MB6 belonging to same subgroup Viet-1 has similar high virulence as those in Chinese HP-PRRSV strains JXwn06 (Li et al., 2012). The first HP-PRRSV isolated in Vietnam in 2006 is 07QN strain, which is closely related to the JXA1 strain, a Chinese HP-PRRSV prototype isolated in 2006 (Tian et al., 2007; An et al., 2011). Subsequently, JXA1-like HP-PRRSV strains are spread widely in Thailand and the Philippines (Jantafong et al., 2015). JXA1-like HP-PRRSV is still major strains circulating in Vietnamese pigs. Interestingly, 4 Vietnamese HP-PRRSV strains are closed related to the VR-2332 strain, a type 2 PRRSV prototype isolated in 1990 (Benfield et al., 1992; Liu et al., 2013). Similarly, Chinese PRRSV strain DFLLY01, which is also closely related to the VR-2332, contained a 3 aa deletion at position 593 to 595 in the gene Nsp2 (Liu et al., 2013). These data suggest some HP-PRRSV evolution started from VR-2332-like strain with a 3 aa deletion, leading to the HP-PRRSV strain with 30 aa deletion in the gene Nsp2. Nucleotide and amino acid sequence analysis of 34 Vietnamese HP-PRRSV isolated from 2013 to 2014 indicates that Vietnamese HP-PRRSV has undergone rapid evolutionary changes in recent years when compared with Vietnamese HP-PRRSV isolated before 2012.

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