

# **A vaccine against porcine reproductive and respiratory syndrome virus**

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# **A vaccine against porcine reproductive and respiratory syndrome virus**

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Dissertation originale présentée en vue de l'obtention du grade de docteur en sciences  
agronomiques et ingénierie biologique

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# Résumé

**Xiukun SUI. (2019). Un vaccin contre le virus du syndrome reproducteur et respiratoire porcin (Thèse de doctorat en anglais).** Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège. 151 pages, 7 tableaux, 32 figures.

**Résumé** Le syndrome reproducteur et respiratoire porcin (PRRS) est causé par le virus du syndrome reproducteur et respiratoire du porc (PRRSV). Dans les principaux pays producteurs de porc, c'est une maladie importante sur le plan économique, qui provoque une défaillance de la reproduction chez les truies et des maladies respiratoires chez les jeunes porcs. Pour la prévention et le contrôle du PRRS, la vaccination est le choix principal pour la majorité des producteurs de porcs. Il existe actuellement deux types de vaccins commerciaux, les vaccins atténués vivants atténués et les vaccins inactivés, actuellement utilisés dans les élevages porcins. Ces deux vaccins présentent chacun des avantages et des inconvénients, mais ils ne permettent pas de remplir l'objectif de prévention et de contrôle de ce virus. Ainsi, un vaccin doit être efficace (i.e. induire une réponse immunitaire suffisante) et sûr (e.g. pas de réversion de la virulence). Actuellement, la plupart des recherches sur les vaccins inactivés contre le PRRSV sont axées sur leur efficacité de protection. Il manque des informations au niveau de la prévalence des souches virales, de l'état d'inactivation des vaccins et de leur qualité.

Dans notre étude, nous avons isolé une souche prévalente du virus du PRRS dans le but de développer un vaccin. Nous avons prélevé des échantillons de tissus et de sang dans la ferme suspecte, puis nous avons isolé et identifié le virus PRRS. Après cela, nous avons comparé le génome et la virulence de différentes souches. Ensuite, une souche semblable à HP-PRRSV, qui a une virulence élevée, a été choisie comme souche vaccinale candidate pour une étude ultérieure.

La meilleure procédure d'inactivation du virus du PRRS a été déterminée au niveau du type de composés (la  $\beta$ -propiolactone, la  $\epsilon$ -chlorure d'imine binaire, ou le formaldéhyde), de la durée et de la température. La  $\beta$ -propiolactone diluée à 1:2000 pendant 24 h à 4 °C permet d'inactiver rapidement et complètement le PRRS tout en conservant la réactivité du vaccin.

Deux méthodes de purification, basées respectivement sur l'ultracentrifugation à gradient de densité de saccharose et la chromatographie en phase liquide, ont été réalisées. Lorsque nous avons comparé la capacité de purification de ces méthodes, nous avons constaté qu'avec la méthode de chromatographie en phase liquide, il était possible d'obtenir des particules virales très pures ainsi que des antigènes de meilleure immunogénicité et de haute spécificité. Cette méthode décrite ici devrait être utile pour la production à grande échelle de virus PRRS hautement purifiés.

Enfin, nous présentons une évaluation préliminaire du vaccin purifié et inactivé avec la  $\beta$ -propiolactone. Après immunisation et injection d'épreuve, il y avait des signes cliniques significatifs dans le groupe contrôle. Les titres moyens d'anticorps

neutralisant anti-PRRSV étaient plus élevés et le nombre moyen de copies de virus était significativement plus bas dans le groupe vaccin inactivé. Par ailleurs, les lésions macro-et microscopiques étaient moins graves chez les porcs vaccinés. Ces résultats ont donc indiqué que le vaccin anti-PRRSV inactivé et purifié possédait une capacité à éliminer les signes cliniques induits par le virus.

En conclusion, cette thèse a contribué à la mise en œuvre d'un nouveau vaccin contre le PRRSV destiné à améliorer le degré de réponse des anticorps anti-VN et à protéger les porcs contaminés contre l'infection homologue par le PRRSV.

**Mots clés:** PRRSV, inactivant, purification, vaccin inactivé

# Abstract

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**Xiukun SUI. (2019). A vaccine against porcine reproductive and respiratory syndrome virus. (PhD Dissertation in English).** Gembloux, Belgium, Gembloux Agro-Bio Tech, Université de Liège. 151 pages, 7 tables, 32 figures.

**Abstract-** Porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV). It is an economically important disease responsible for reproductive failure in sows and respiratory disease in young pigs. For prevention and control of PRRS, vaccination is the primary choice for the majority of pig producers. There are two kinds of commercial vaccines: modified live-attenuated vaccines (MLVs) and inactivated vaccines. These two types of vaccines cannot prevent and control of PRRSV. Thus, efficient (i.e. induce protective immunity) and safe (e.g. cannot revert to virulence) vaccines are required. At present, most researches on PRRSV inactivated vaccines are focused on the protection efficiency. Information on strain prevalence, inactivation and quality of the vaccine is lacking.

In our study, a prevalent PRRSV strain was chosen for vaccine development. We collected tissue and blood samples from a suspect farm, then isolated and identified the virus. After that, we compared the genome and the virulence of the different prevalent strains. Then, a strain similar to the virulent HP-PRRSV was selected for further vaccine development.

To determine the best inactivation procedure of PRRSV, different concentrations of  $\beta$ -propiolactone (BPL), binary ethylenimine (BEI) and formaldehyde (F) were tested at different times and temperatures. BPL diluted at 1:2000 for 24 h at 4 °C completely inactivates PRRSV while maintaining adequate reactivity. This study thus provides a detailed inactivation procedure for PRRSV.

Two purification methods, based on sucrose density gradients ultracentrifugation or liquid chromatography were conducted. We found that the liquid chromatography method yields highly pure and immunogenic viral particles. The purification method described here should thus be useful in large-scale production of highly pure PRRS virus.

Finally, we describe a preliminary evaluation of the purified PRRSV vaccine inactivated with BPL. After immunization and challenge, significant clinical signs were observed in the mock group. Mean anti-PRRSV neutralizing antibody titers were higher in the inactivated vaccine group while the mean copy number of virus was significantly lower. There were less severe macroscopic and microscopic lesions in vaccinated pigs. These results indicate that the purified inactivated vaccine has the ability to reduce clinical signs of PRRSV infected pigs.

In conclusion, this thesis has contributed to the implementation of a novel experimental inactivated PRRSV vaccine aiming at priming antibody response and protecting pigs from PRRSV infection.

**Keywords:** PRRSV, inactivant, purification, inactivated vaccine



# Acknowledgements

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# List of Abbreviations

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AA	Amino acid
ADE	Antibody-dependent enhancement
APC	Antigen-presenting cells
BEI	Binary ethylenimine
bp	Base pair
BPL	$\beta$ -propiolactone
BSA	Bovine serum albumin
conc	Concentration
CPE	Cytopathic effect
CpG ODN	CpG Oligodeoxynucleotides
CSFV	Classical swine virus
CTL	Cytotoxic T-cell
d	Day
DC	Dendritic cells
DIVA	Differentiating Infected from Vaccinated Animals
DMEM	Dulbecco's modified Eagle's medium
dpi	Days post-infection
E	Envelope
EAV	Equine arteritis virus
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot assay
F	Formaldehyde
FBS	Fetal bovine serum
FMD	Foot and mouth disease
g	Gravity
h	Hour
HIV	Human immunodeficiency virus
HP-PRRSV	Highly pathogenic porcine reproductive and respiratory syndrome virus
HVJ-E	Hemagglutinating Virus of Japan Envelope
IEC	Ion exchange chromatography
IFA	Indirect fluorescent antibody
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
i.n.	Intranasal
IPMA	Immunoperoxidase monolayer assay
kb	Kilo base
KD	Kilo dalton
LDV	Lactate dehydrogenase-elevating virus
LV	Lelystad virus
M	Matrix

min	Minute
mL	Milliliter
MLV	Modified live-attenuated vaccine
N	Nucleocapsid
NGS	N-glycosylation site
NK	Natural killer
nm	Nano meter
NSP	Non-structural protein
nt	Nucleotide
OD	Optical density
ORFs	Open reading frames
OTU	Ovarian tumor
PAM	Porcine alveolar macrophage
PBL	Peripheral blood lymphocytes
PBS	Phosphate-buffered saline
PCV-2	Porcine circovirus type 2
PLGA	Poly(d,l-lactide-co-glycolide)
PLP2	Papain-like protease domain 2
PNE	Primary neutralizing epitope
PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
PRV	Pseudorabies virus
q-PCR	Quantitative polymerase chain reaction
RT-PCR	Reverse transcription polymerase chain reaction
sec	Second
SHFV	Simian hemorrhagic fever virus
S/P	Sample-to-positive
SPF	Specific-pathogen-free
TCID <sub>50</sub>	50% tissue culture infective doses
TEM	Transmission electron microscopy
TGF	Transforming growth factor
TM	Transmembrane region
TNF	Tumor necrosis factor
TRS	Transcription-regulating sequence
UTR	Untranslated region
UV	Ultraviolet
V	Voltage
VN	Virus neutralizing
μ	Micro
°C	Degree celsius

## Supplementary Data

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# 1

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## General Introduction



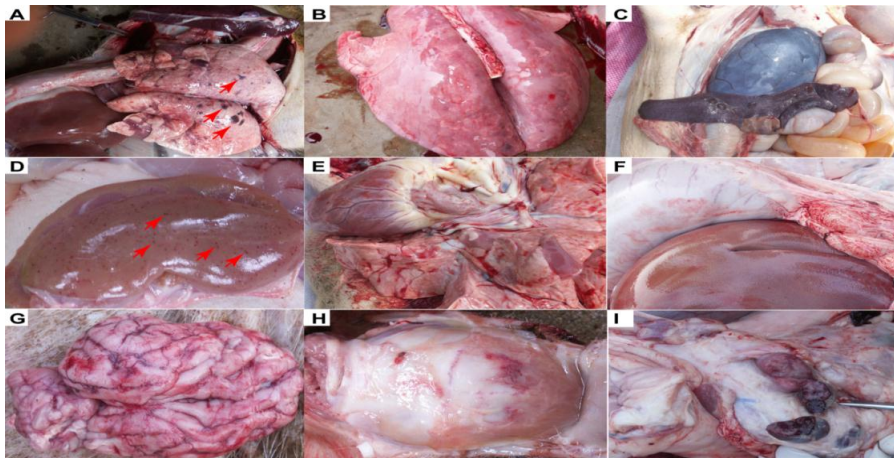
# 1 The origin, transmission route and classification of PRRS

Porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV). It is also known as high fever pig disease, swine plague and blue ear disease (Paton et al., 1991). In the late of 1980s, PRRS was first discovered in North Carolina in the United States and later in European countries and other areas of the world (Collins et al., 1992; Wensvoort et al., 1992). At the end of 1995, the first case report on PRRS occurred in northern China. In 1996, China's first PRRSV epidemic strain CH-1a was isolated from miscarried fetuses (Guo et al., 1996). After that, other epidemic strains such as BJ-4, HB-1(sh)/2002 and HB-2(sh)/2002 were successively isolated (Zhou et al., 2010). In 2006, a new PRRSV variant strain, also known as highly pathogenic PRRSV (HP-PRRSV), was identified in Jiangxi, China. It led to high morbidity (50 %-100 %) and mortality (20 %-100 %) in pigs (Tian et al., 2007). The outbreak of HP-PRRSV resulted in a dramatic decline of pig stock and high prices of pork meat in China (Feng et al., 2008). Since 2013, PRRS became prevalent again in China caused by new PRRSV variants, NADC30-like strain, which was considered to be imported from North American and went through extensive variation in China (Zhao et al., 2015; Zhou et al., 2015). Now, HP-PRRSV and NADC30-like strain became the dominant circulating PRRSV strain in China.

Currently, the World Animal Health Organization has listed highly pathogenic PRRS as one of the animal diseases that must be promptly reported. Pigs are the only natural hosts of PRRSV. Piglets or adult pigs of different breeds and genders can be infected by PRRSV. After being infected, pigs often have the following common clinical symptoms (Figure 1): respiratory infections, lameness, shivering, diarrhea, and secondary infections caused by other viruses or bacteria (Tian et al., 2007). Severe damages in multiple organs include lung haemorrhage, interstitial pneumonia, and less often mild vasculitis (Pol et al., 1991; Michel Morin & Yves Robinson 1992; Tian et al., 2007). PRRSV can be transmitted by pig-to-pig infection and transplacental infection to fetuses during mild-to-late gestation (Nathues et al., 2016; Thanawongnuwech et al., 2010). Wild boars and domestic pigs have the same susceptibility to PRRSV (Albina et al., 2000). Wild boars can act as a reservoir for infectious diseases of domestic pigs (Al Dahouk et al., 2005). The potential role of wild boars as a reservoir for PRRSV has been reported in France, Germany, and the USA with serological evidence of infection (Reiner et al., 2009). The outlined ways of spread and infection between domestic pig herds might also potentially affect wild boars, and vice versa. Rates of PRRSV infected wild boars vary drastically with location, season, and PRRSV-strain. Exchange of the virus between domestic pigs and wild boars can be expected because of high prevalences of PRRSV in domestic pigs in China. PRRSV persistence in pigs plays an important role in viral transmission because the virus is present at low levels in the infected animals (Rochon et al., 2015; Horter et al., 2001).

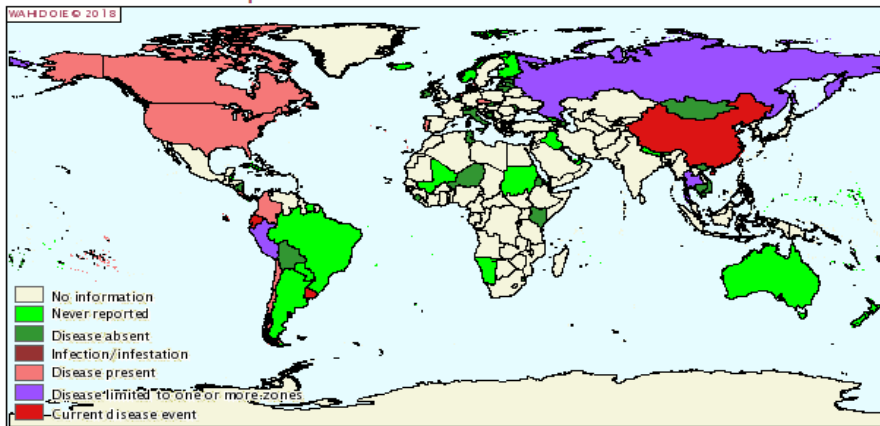


**Figure 1.** Clinical presentation of pigs with “high fever” disease (Tian et al., 2007). (A) Sick pigs with a fat and healthy appearance. (B) Sick pigs with thin and debilitated feature. (C) The shivering piglet. (D) The limping pig with erythematous blanching rash in its ears. (E) & (F) Pimples observed on the back of an infected pig. (G) & (H) Grown pigs killed during this epidemic.



**Figure 2.** Severe damage to multiple organs in dead pigs (Tian et al., 2007). Lung haemorrhage (indicated at the arrow). (B) Lung edema. (C) Spleen infarct and bladder dilatation filled with mahogany urine. (D) Kidney with many blood spots (at the arrows). (E) Heart with disorders. (F) Liver with yellow-white necrosis or haemorrhage. (G) Encephala softened slightly. (H) Brain putamen with blood egression emission. (I) Lymph node with haemorrhagic spots.

Depending on the genetic diversity and geographic distribution, PRRSV can be divided into European type (I type) and North American type (II type) (Benfield et al., 1992; Collins et al., 1992). Representative strain of European type is LV (Lelystad virus), which was named after its first isolation by Wensvoort et al (Wensvoort et al., 1991) at the Lelystad Institute in the Netherlands. The representative strain of North America is VR-2332. These two types of strains are widespread in North America, Europe, and Asia, but a few countries (Sweden, Switzerland, New Zealand, and Australia) have claimed that they have not yet detected PRRSV (Carlsson et al., 2009; Cannon et al., 1998; Cobb et al., 2015) (Figure 3).



**Figure 3.** Worldwide distribution of PRRS by 2018.

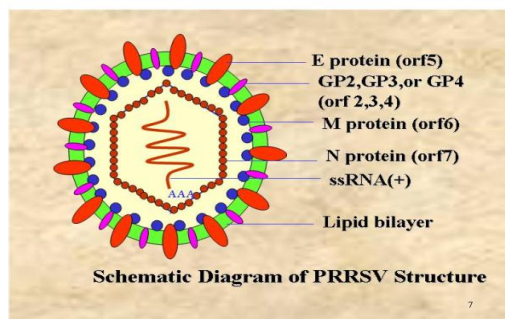
These two genotypes are associated with a similar clinical symptoms and concurrent emergence (Halbur et al., 1995). They show about 40 % genetic divergence, with a high degree of antigenic variation (Forsberg et al., 2002; Frossard et al., 2012; Le et al., 1997). At present, a number of genetically heterogeneous PRRSV widely co-circulate throughout the world and cause huge economic losses (Van et al., 2011; Stadejek et al., 2013; Zhang et al., 2018). However, there is still a lack of effective vaccines that can prevent PRRSV infection. Therefore, in-depth study of PRRSV gene structure, protein function, virus biological activity, pathogenic mechanism and development of safe and effective vaccines is mandatory.

## **2 PRRSV genome structure, protein function and biological characteristics**

### ***2.1 PRRSV genome structure***

PRRSV is a single-stranded, positive-sense RNA virus. It belongs to the family *Arteriviridae* of the order *Nidovirales*, which includes viruses with epidemiological

relevance such as lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) (Cavanagh et al., 1997; Dunowska et al., 2012). The mature virus particle has a diameter of 50-72 nm and has an icosahedral viral capsid. The capsid contains the viral RNA genome (Kim et al., 1993). The PRRSV genome is about 15 kb in length. It has a polycistronic structure, and contains at least 10 open reading frames (ORFs) flanked by two untranslated regions 5'-untranslated region (5'UTR) and 3'-untranslated region (3'UTR) (5'UTR-ORF1a-ORF1b-ORF2a-ORF2b-ORF3-ORF4- ORF5/ORF5a-ORF 6-ORF7-3'UTR). The PRRSV genome encodes a 5'UTR of 217-222 nucleotides (nt; Type 1) and 188-191 nt (Type 2) in length (Yun & Lee, 2013). Type 1 and 2 strains share approximately 50 % genetic homology (Nelsen et al., 1999; Tan et al., 2001). The 3'UTR of 114 nt (Type 1) and 148 nt (Type 2) in length. They also share approximately 60 % nucleotide identity between Type 1 and Type 2 (Choi et al., 2006; Yin et al., 2013). ORF1a and ORF1b encode the non-structural proteins (NSPs), and account for about 80 % of the total length of the viral genome. These 2 ORFs produce 14 non-structural proteins upon enzymatic cleavage. ORF2 through ORF7 encode eight structural proteins including GP2, small envelope (E), GP3, GP4, GP5a, GP5, matrix (M) and nucleocapsid (N) protein (Figure 4) (Allende et al., 1999; Firth et al., 2011; Johnson et al., 2011). After PRRSV infections, viral genomic RNA and subgenomic RNAs are generated. As a template for the viral genome, the full-length RNA also encodes non-structural proteins (NSPs). The subgenomic RNAs encodes the viral structural protein (Yoo et al., 2004).



**Figure 4.** The genome organisation and structure of PRRSV.

One of the characteristics of the ORFs encoding PRRSV structural proteins is that an antisense transcription-regulating sequence (TRS) at or near the 5' end of each structural protein coding region (ORF2-7) can form a kissing-loop interaction with a conserved TRS sequence (UUAACC). This sequence located at the 3' terminus of

the 5' UTR, and it acts similarly to a eukaryotic gene promoter (Kimman et al., 2009; Kappes & Faaberg, 2015). There is a marked difference in the ORFs and TRS sequence of European and North American PRRSV strains (Allende et al. 1999; Nelsen et al., 1999). In addition, the amino acid sequence of the Nsp2 protein of these two viruses only shares 32 % similarity (Allende et al., 1999). Because there are large differences in nucleotide and amino acid sequences between European and North American strains, it has been speculated that these two types of virus may have originated from the same ancestor and then spread and evolved in Europe and North America respectively. Although possible, it should be taken into account that the evolution of the virus in nature requires a long period and other factors. Some researchers also doubt this evolution. Nucleotide alignment of European and North American PRRSV assigned, the epidemic virus isolated in China to North American strains (Zhou et al., 2010; Guo et al., 2018).

## ***2.2 PRRSV Gene-encoded proteins and their function***

The PRRSV genome can encode 21 viral proteins. ORF2-ORF7 encodes eight structural proteins of the virus (Figure 4). One of which is the nucleocapsid N protein and the other is envelope proteins. These eight structural proteins are all essential for the production of infectious PRRSV (Terje 2010). The GP5 and M proteins are the major envelope proteins of PRRSV. They are disulfide-linked, exist as heterodimers and interact with PRRSV cell receptors (Hicks et al., 2018). GP5 contains the virus major neutralizing epitope, which has the highest degree of variation in all PRRSV structural proteins (Mu et al., 2015). The M protein is involved in the assembly and release of the virus, and its amino acid sequence is most conserved among PRRSV structural proteins (Wang et al., 2017). The M protein also contains T cell epitopes and neutralizing epitopes (Bautista et al., 1999).

The minor envelope proteins of PRRSV include GP2, GP3, GP4 and E proteins, which are encoded by ORF2a, ORF3, ORF4 and ORF2b, respectively (Matthew et al., 2015). GP2, GP3 and GP4 proteins contain weaker neutralizing epitopes. Gp3 is highly glycosylated among PRRSV structural proteins (Kim et al., 1993; Chen et al., 2014). The ORF7-encoded viral nucleocapsid N protein is the most abundant and most immunogenic protein in viral particles. It accounts for approximately 30 % of the total viral protein (Olasz et al., 2016; LeGall et al., 1998). The N protein is rich in basic amino acids and exists as a homodimer through cysteine interactions. This dimer is good for binding of the N protein to the viral RNA genome. The N protein mainly produces non-neutralization antibodies (Rahe et al., 2017; Li et al., 2015).

Non-structural proteins are encoded by ORF1a and ORF1b. ORF1a expresses 1a protein, while 1a-1b fusion protein is produced by translation frame shifting (Yoo et al., 2004). 1a protein and 1a-1b fusion protein are cleaved by cellular proteases, producing 14 non-structural proteins of PRRSV (Charerntantanakul 2012) (Figure 4). Many of these NSPs have enzymatic activities, such as Nsp2 with cysteine protease activity, Nsp4 with serine protease activity, Nsp11 with endonuclease activity, and Nsp9 with RNA-dependent RNA polymerase activity (Charerntantanakul 2012; Li et al., 2015). Nsp1 $\alpha$ , Nsp1 $\beta$ , Nsp2, and Nsp11 can inhibit IFN-mediated signaling

pathways, and they also can block the action of IFN-induced genes with antiviral activity (Chareerntantakul 2012; Li et al., 2015).

### ***2.3 Biological characteristics of PRRSV***

The host cells of PRRSV are porcine alveolar macrophages (PAMs: porcine alveolar macrophages) and blood mononuclear cells. Macrophages and testicular germ cells derived from other tissues are also susceptible to PRRSV (Sang et al., 2012; Sur et al., 1997). PRRSV was first isolated from primary cultured PAMs (Wensvoort et al., 1991). PRRSV infects PAMs causing apoptosis (Wensvoort et al., 1992; Karniychuk et al., 2011). The virulence of PRRSV is closely related to structural protein GP5 and non-structural protein Nsp3-Nsp8 (Robinson et al., 2013; Rascón-Castelo et al., 2015). The 118 amino acids at the N-terminus of GP5 protein can induce apoptosis (Roques et al., 2013). The common cellular receptors for PRRSV are pCD163 and pCD169, which are associated with the virus husking and entry into the cytosol, respectively (Li et al., 2015; Van et al., 2013). After infection, PRRSV replicates in the cytoplasm (Jourdan et al., 2012).

European PRRSV strains can easily multiply in PAMs. North American strains can infect primary PAMs or replicate in cell lines such as CL2621, Marc-145, and CRL11171. CL2621 and Marc-145 are derived from Rhesus monkey kidney cell lines (Wang et al., 2014; Faaberg et al., 1998). Until now, most of the North American PRRSV strains are isolated using in vitro culture of monkey kidney cell lines. PRRSV can cause viremia after infection in pigs. But due to the delay in the body's protective immune response, the virus is often difficult to be cleared and becomes a persistent infection. Persistent infection plays an important role in maintaining the replication and spread of PRRSV in pigs. Therefore, it is necessary to further study the mechanism of how PRRSV interacts with the host to cause persistent infection. Although the virulence of different PRRSV strains varies greatly, there is a similar organizational tropism of the strong and attenuated strains (Shang et al., 2013).

Control and eventually eradication of PRRSV is an important issue for swine production. OIE manual of diagnostic tests and vaccines for terrestrial animals provides guidelines for PRRS management: (<http://www.oie.int/en/animal-health-in-the-world/animal-diseases/porcine-reproductive-and-respiratory-syndrome/>). Upon PRRS outbreak, control measures should be applied at the individual farm level to prevent the spread of the disease. Available vaccines are effective in controlling outbreaks and preventing economic losses.

## **3 Vaccines**

### ***3.1 Inactivated vaccine***

PRRSV inactivated vaccine is safe, does not interfere with maternal antibodies, and is easy to store and transport. Its disadvantage is that it requires a high immunization dose, needs multiple immunizations and insufficiently protects against



heterologous strains. Now the existing commercial inactivated vaccines have been widely used in the European market, including the Ingelvac<sup>®</sup> PRRS (P120 strain) vaccine from Boehringer AG in sows and piglets, and Suipravac-PRRS (5710 strain) vaccine from Hipra company, Spain, and Progressis<sup>®</sup> vaccine from Merial company, France, and the SuivacPRRS-IN (VD-E1/E2 and VD-A1 strain) vaccines that can be applied simultaneously to boars, sows and piglets from Dyntec company, Czech; In USA, only briefly appeared in the PRRomiSe<sup>™</sup> vaccine of Intervet, which produced from Netherlands. In Asia, South Korea mainly uses the SuiShot<sup>®</sup> PRRS vaccine, which is produced by its local company. In addition to several European brands, the common PRRSV inactivated vaccine in China mainly include the inactivated vaccine PRRSV-SD1 produced by Shandong Qilu animal health products co., LTD; inactivated NVDC-JXA1 strain produced by Chinese animal husbandry group and Guangdong Winsun Bio-pharmaceutical Co., Ltd..

Dai et al used Hemagglutinating Virus of Japan Envelope (HVJ-E) as an immune adjuvant to add JXA1-R as inactivated vaccine, and the inactivated vaccine had better immune protection for 28-day-old piglets than that without adjuvant (Dai et al., 2011). Inactivated vaccine with HVJ-E as an adjuvant induced greater lymphocyte proliferation, up-regulation of  $\gamma$ -interferon and interleukin-2 (IL-2), and down-regulation of interleukin-10 (IL-10) than the inactivated vaccine without adjuvant. What's more, after challenge with this inactivated vaccine containing HVJ-E adjuvant can significantly reduce the clinical signs of piglets. Geldhof et al compared the efficacy of three European commercial vaccines and two self-made inactivated vaccines (07V063 and LV strains) (Geldhof et al., 2012). They found that three commercial vaccines could reduce viremia for at least one week. But neither of the two self-made inactivated vaccines affected the duration of viremia. Interestingly, compared to the three commercial inactivated vaccines and attenuated vaccines, two self-made inactivated vaccines induced the production of neutralizing antibodies against 07V063. Then, Geldhof et al performed a second experiment to compare the immunoprotective effect of inactivated vaccines by LV strain, 08V194 strain, and 07V064 strain with commercial attenuated vaccine (Porcilis<sup>®</sup> PRRS) against the wild type 08V194 strain. The inactivated vaccine against 07V064 strain and LV strain had no significant effect on the duration of viremia. Specific neutralizing antibodies against strain 08V194 were detected in all immunized animals. The attenuated vaccine could quickly induce the production of neutralizing antibodies. These two results suggest that when the field-mutant strains escape the immunity provided by the existing attenuated vaccines, the same type of inactivated vaccine may provide some protection. Karniychuk et al also used PRRSV-07V063 strain to prepare inactivated vaccines (Karniychuk et al., 2012). Three immunizations were performed on the 27th, 55th, and 83th days of pregnancy in the pregnant sow, then the 07V063 strain was used to challenge on the 90th day. This inactivated vaccine did not provide complete protection for sows and fetuses, but it could slightly reduce the viremia of the sows, and improve the survival rate of fetal pigs. Dwived et al use the biodegradable nanoparticle poly (D, L-Lactide-co-glycolide) (PLGA) encapsulated VR-2332 strain inactivated vaccine to intranasally immunize piglets

from 3 to 4 weeks of age, and challenge at 21 days (Dwived et al., 2013). They found that the duration of viremia in piglets immunized with nanoparticles-inactivated vaccine was reduced to two weeks.  $\gamma$ -interferon (IFN- $\gamma$ ) was up-regulated in lung homogenate while transforming growth factor- $\beta$  (TGF- $\beta$ ) was down-regulated. The titer of the lung homogenate in the neutralization test was significantly higher than that in the control and the common inactivated vaccine group. The nanoparticle PLGA encapsulated inactivated vaccine can be used as an effective means to immunize piglets by the nasal route. The immunization route can rapidly clear viremia and defend the PRRSV infection.

### ***3.2 Attenuated vaccine***

At present, PRRSV attenuated vaccine is the most widely used in the field. Compare to the inactivated vaccine, attenuated vaccine induce a strong humoral immunity. It also has the ability to replicate in vivo and maintain long-lasting immunity. But a full protection is only achieved against homologous strains. Moreover, it has been shown that an attenuated vaccine virus can return to virulence and cause disease (Bøtner et al., 1997; Nielsen et al. 2001). In 1995, Boehringer AG developed the first commercially North American PRRSV attenuated vaccine-Ingelvac<sup>®</sup> PRRSV MLV (VR-2332 strain, entered the Chinese market in 2005), which mainly used for pigs aged 3 to 15 weeks. After that, the company developed another North American PRRSV attenuated vaccine-Ingelvac<sup>®</sup> PRRS ATP (JA-142 strain), which was used for piglets and finisher pigs. There are also attenuated vaccines only used for Europe: Merck's Porcilis PRRS<sup>®</sup> (DV strain) attenuated vaccines for sows and gilts, and Amervac-PRRS<sup>®</sup> (VP046 strain) attenuated vaccine for piglets and gilts from Hipra of Spanish and Spanish Syva company's Pyrsvac-183<sup>®</sup> (All-183 strains) attenuated vaccine for pigs at each stage. The CH-1R attenuated vaccine, which is produced by Harbin Veterinary Research Institute of Chinese Academy of Agricultural Sciences, was the first attenuated vaccine in China. This vaccine was officially put into production in 2007. After that, other attenuated vaccines were developed: attenuated vaccine JXA1-R strain, the HUN4-F112 strain and the TJM-F92 strain for the HP-PRRSV. When immunized with these three attenuated vaccines, piglets were able to resist well homologous and heterologous strains infection (Tian et al., 2009). In 2009, the attenuated strain R98 was approved as a new veterinary vaccine. The GDr180 attenuated strain produced by Guangdong Winsun bio, also had shown excellent immunity and safety characteristics in clinical trials.

Martelli et al immunized 4-week-old piglets with the attenuated Porcilis PRRS<sup>®</sup> (DV strain, European type) vaccine by intramuscular injection and intradermal needle-free injection, respectively (Martelli et al., 2009). After a boost at day 45, immunized pigs and the control pigs were maintained in a farm contaminated with the Italian European subtype sharing 84 % nucleotide identity with the attenuated vaccine DV strain. It was observed that the immunized piglets felt better than the control group. Clinical symptoms were reduced by 68 % to 72 %, and respiratory symptoms were reduced by 72 % to 80 %. The clinical protective effect was closely

related to significantly elevated cellular immune responses, indicating that injection of the vaccine in two different ways can effectively resist infection of virus having only 84 % nucleotide identity. In addition, experiments also suggest that among the factors affecting the protective effect of vaccines, the ability of the vaccine strain to induce cellular immunity is more important than its homologous relationship with field strains. Wang et al used A2MC2 strain, attenuated vaccine Ingelvac<sup>®</sup> PRRSV MLV, VR-2332 strain and VR-2385 strain (middle virulent strains) to inoculate animals (Wang et al., 2013). The results showed that A2MC2 strains could induce animals to produce neutralizing antibodies earlier and produce higher levels of neutralizing antibodies and IFN- $\gamma$  levels than MLV strain. In addition, after immunization with this strain, animals could resist infection of the same strains or atypical strains. It also caused similar pathological damage to the VR-2385 strain, indicating that it could be a better candidate strain for the vaccine. Li et al divided 15 pigs into 3 groups and inoculated with HP-PRRSV BB0907 strain, the Invirvac<sup>®</sup> PRRSV MLV attenuated vaccine and phosphate-buffered saline (PBS), respectively (Li et al., 2013). Then they placed these three groups in the same pig rearing. When immunized with Ingelvac attenuated vaccine, the infected pigs showed low clinical morbidity, mild viremia, mild fever and lung injury, and a higher level of IFN- $\gamma$  secretion. It was speculated that the emergency immunization with attenuated vaccine could reduce the risk of exposure to HP-PRRSV in animals.

### ***3.3 Genetic engineering vaccine***

Genetic engineering vaccine refers to the use of DNA recombinant biotechnology to insert natural or synthetic genetic material into bacterial, yeast or mammalian cells, so that it can be fully expressed and purified. These types of vaccines include Differentiating Infected from Vaccinated Animals (DIVA) vaccine, vector vaccine, nucleic acid vaccine and subunit vaccine.

DIVA vaccine is a new generation of recombinant live vaccines that use molecular engineering techniques to introduce molecular markers into viral genomes to distinguish them from wild strains. Marked vaccine can be used to effectively distinguish between immunized and wild-infected pigs. This plays a vital role in the prevention and control of PRRSV. Lin et al inserted a marker gene into the N protein of the PRRSV vAPRRS strain to obtain the v7APMa strain, which can be used stably to distinguish between immunized and wild-infected pigs (Lin et al., 2012). Wang et al passed the HP-PRRSV JX143 strain in vitro with 100 times to obtain the attenuated strain JXM100 strain (Wang et al., 2013). Then the original and attenuated strains were sequenced. They found there were a continuous 88 amino acids (AA) deletion of Nsp2 and a total of 75 scattered bases mutation in the whole genome. The 88 AA deletion of JXM100 strain can be used as a marker vaccine to diagnose the vaccine immunized pigs and wild-type naturally infected pigs.

In vector vaccine, the sequence encoding the immunogen is inserted into the genome of the vector. After inoculation, the antigen is expressed in large amounts as the vaccine strain proliferates in vivo. Baculovirus is widely used in recent years as a carrier system for the efficient expression of foreign proteins. Wang et al, Nam et al,

and Wu et al all use baculovirus as a vector to express GP5 and M genes of PRRSV (Wang et al., 2007; Nam et al., 2013; Wu et al., 2013). The results showed that the new vaccine had a greater improvement in immunogenicity than ordinary nucleic acid vaccines. The ability to stimulate the body to produce IFN- $\gamma$  was positively correlated with the vaccine dose.

In nucleic acid vaccines, foreign DNA or RNA encoding certain antigenic proteins is injected directly into animal cells and synthesizes antigenic proteins through the host cell's expression system, thereby stimulating an immune response to the antigenic protein. Zhang et al mixed the molecular complement protein adjuvant with GP5 protein of PRRSV, and prepared the pcDNA3.1-C3d-p28. n-GP5 nucleic acid vaccine, which containing multiple mC3d-p28 genes (Zhang et al., 2011). Anti-GP5 antibodies, GP5 neutralizing antibodies, IFN- $\gamma$  and IL-4 on pcDNA3.1-C3d-p28.n-GP5 were significantly higher in inoculated mice compared to the pcDNA3.1-GP5 immunized group. It indicated that the adjuvant protein could effectively enhance the specific immune response of the antigen.

Subunit vaccines use surface structure component (antigen) of a microorganism. Prieto et al inoculated piglets with the purified GP5 subunit vaccine expressed in *E.coli* (Prieto et al., 2011). The immunized group showed more severe clinical symptoms such as dyspnea and progressive weight loss than the blank-injection group. This indicated that the subunit vaccine not only fails to provide immune protection, but promoted PRRSV infection in piglets. Its specific mechanism remains to be further studied. Yang et al fused ORF1b, ORF7, M and GP5 gene of PRRSV into a vector based on *Pseudomonas* exotoxin, and successfully made a subunit vaccine inducing a cytotoxic response (Yang et al., 2013). Clinical trials showed that this subunit vaccine could effectively protect piglets and sows from being infected by PRRSV. It also could reduce clinical symptoms and viremia in sows.

### ***3.4 Factors affecting the efficacy of PRRS vaccine***

Although several commercial attenuated and inactivated PRRS vaccines have been widely used to control PRRSV, there also remain concerns about the safety of attenuated vaccines and the efficacy of inactivated vaccines. First, it is still unclear which PRRSV antigen stimulates the protective immunity. So we cannot identify the best immunogen to produce the vaccine; secondly, because PRRSV is a RNA virus, the viral gene has a high frequency of mutation associated with antigenic variability. So, there is a risk to produce a mutant strain that escapes immune recognition. Thirdly, PRRSV can suppress the immune response and use other mechanisms to escape the body's immune surveillance system. PRRSV can inhibit expression of IFN- $\alpha$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the infected cells (Albina et al., 1998; Murtaugh and Foss, 2002; Van Reeth et al., 1999); fourthly, it remains to be elucidated what factors determine virulence among different PRRSV strains (Guo et al., 2018).

Antibody-dependent enhancement (ADE) is another feature of PRRSV infection

(Yoon et al., 1996). The ADE of PRRSV infection is a phenomenon in which PRRSV-specific antibodies enhance the entry of virus, and in some cases the replication of virus, into monocytes or macrophages through interaction with Fc and/or complement receptors (Tirado & Yoon, 2003). In the early stage of infection by PRRSV, pigs mainly produce non-neutralizing antibodies against viral proteins. Neutralizing antibodies are generally produced three weeks later PRRSV infection, and the titer is very low (Lopez-Fuertes et al., 2000; Kimman et al., 2009). The M and N proteins of PRRSV stimulate the production of non-neutralizing antibodies (Choi et al., 2016). In addition, GP5 neutralizing epitopes are masked by surrounding non-neutralizing epitopes (Popescu et al., 2017). In addition, there are multiple glycosylation sites near the non-neutralizing GP5 epitopes. When these sites are glycosylated, they may mask adjacent neutralizing epitopes. This mechanism impairs the production of neutralizing antibodies (Lopez et al., 2004).

Non-specific immunity in pigs also affects the production of specific immunity. In the early stage of PRRSV infection, the changes in cytokine expression profiles may affect the type of immune response. High expression of IFN- $\gamma$  may be beneficial for stimulation cellular immune response (Bautista & Molitor, 1999; Rowland et al., 2001; Binjawadagi et al., 2016). Therefore, it is important to study the role of different cytokines in protective immunity. In addition, the route, the dose and the boosting of the vaccine may affect the vaccination effect of the vaccine.

### ***3.5 Future perspective***

The ideal PRRS vaccine should enhance the innate and adaptive immunity against PRRSV, while blocking the immunosuppression of PRRSV (Renukaradhya et al., 2015; Renukaradhya et al., 2015). Following the legislation of the ministry of agriculture and rural affairs of the People's Republic of China, a PRRS vaccine must be safe. This means that, after immunization, the pigs should not have any adverse reaction, allergy symptom or stress response. The pregnant pigs should give birth normally to healthy piglets. There should be no transmission of the virus through the placenta and between pigs. The vaccine should also contain enough viral antigens able to elicit an optimal immunity. After immunization, pigs must resist the viral infection, reduce the viremia and/or attenuate clinical symptoms. In addition, the new PRRS vaccine should provide protection to different PRRSV lineage. Wild-type PRRSV infection and vaccinated animals should be clearly identified. The important sign of an effective PRRS vaccine is that when a vaccinated pig is exposed to PRRSV again, the pig should be able to rapidly produce a strong humoral and cellular immune recall response, and quickly clear the virus from the body.

Because there are different kinds of PRRSV genotypes, it may be considered to develop a multivalent PRRS live vaccine, which includes several types or different antigenic PRRSVs in order to achieve broad-spectrum immune effects against different PRRSV genotypes. The closer the antigenicity of the PRRS vaccine strain to its epidemic strain, the better the protective effect of the vaccine. It is worthwhile to consider the virus strain closest to the epidemic strain when preparing the vaccine.

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# 2

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## Objective and dissertation structure





Vaccine immunization is an effective way to prevent PRRSV infection. Two types of commercial vaccines, modified live-attenuated vaccines (MLVs) and inactivated vaccines, are currently used in many countries. Inoculation with modified live-attenuated vaccines can provide some degree of protection against PRRSV infections. However, these vaccines not induce complete protection against heterologous PRRSV strains. There is risk of reversion of MLV strains to virulent phenotypes. Compared with the MLV vaccines, commercially available inactivated PRRSV vaccines are safer but do not sufficiently protect against viremia. Furthermore, a good vaccine is also dependent on the strain, the antigen concentration and the antigen integrity after inactivation. In this context, my thesis aims at contributing to the development of high quality inactivated vaccines that are capable of adapting to new circulating virus variants and inducing neutralizing antibodies.

The first objective of this dissertation is to get a prevalent PRRSV strain. To achieve this aim, we will collect tissues samples from different farms during different years. We will then isolate, identify and compare the virulence of different isolates. One popular HP-PRRSV strain, which also had high virulence will be chosen for further study. This works is described in Chapters III and IV.

In Chapter V, we compare different protocols to efficiency inactivate PRRSV and evaluate two virus purification methods based on ultrafiltration and liquid chromatography. After this, we present the preliminary evaluation of a purified inactivated PRRS vaccine.



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## **Genomic characterization and pathogenic study of two porcine reproductive and respiratory syndrome viruses with different virulence in Fujian, China**

From Sui X., Xin T., Willems L., Zhu H., Hou S\*. 2018. Genomic characterization and pathogenic study of two porcine reproductive and respiratory syndrome viruses with different virulence in Fujian, China. *Journal of Veterinary Science*. 19(3):339-349.



**Abstract:** Two strains of porcine reproductive and respiratory syndrome virus (PRRSV) were isolated in 2006 and 2016 and designated as FZ06A and FZ16A, respectively. Inoculation experiments showed that FZ06A caused 100 % morbidity and 60 % mortality, while FZ16A caused 100 % morbidity without death. By using genomic sequence and phylogenetic analyses, close relationships between a Chinese highly pathogenic PRRSV strain and the FZ06A and FZ16A strains were observed. Based on the achieved results, multiple genomic variations in Nsp2, a unique N-glycosylation site (N<sup>33</sup>→K<sup>33</sup>), and a K151 amino acid (AA) substitution for virulence in the GP5 of FZ16A were detected; except the 30 AA deletions in the Nsp2-coding region. Inoculation experiments were conducted and weaker virulence of FZ16A than FZ06A was observed. Based on our results, a 30 AA deletion in the Nsp2-coding region is an unreliable genomic indicator of a high virulence PRRSV strain. The Nsp2 and GP5 differences, in addition to the virulence difference between these two highly pathogenic PRRSV strains, have the potential to be used to establish a basis for further study of PRRSV virulence determinants and to provide data useful in the development of vaccines against this economically devastating disease.

**Keywords:** genomics, phylogeny, porcine reproductive and respiratory syndrome virus, virulence

# 1 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the *Arteriviridae* family and the genus *Nidovirales*, which includes viruses with epidemiological relevance such as lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV), and simian hemorrhagic fever virus (SHFV) (Dea et al., 2000; Snijder & Meulenberg, 1998). PRRSV was first reported in 1987 in North America (Collins et al., 1992; Jiang et al., 2000), and later in the Netherlands and other parts of the world (Paton et al., 1991). PRRSV has strong pathogenicity with a high transmissible effect. The virus causes morbidity and mortality rates in the range of 50 % to 100 % and 20 % to 100 %, respectively (Tong et al., 2007). PRRS has resulted in huge economic losses in the livestock industry worldwide. In 2006, highly pathogenic PRRSV (HP-PRRSV) was first identified in China, where it affected vaccinated pigs (in all ages) by producing a high fever and resulted in a high mortality rate. Afterward, it rapidly spread to neighboring countries, in which their swine industries experienced widespread losses (An et al., 2011; Ni et al., 2012).

The genome of PRRSV is a single-stranded, positive-sense RNA of approximately 15 kb length that contains a 5'-capped structure and a 3'-polyadenylated tail. At least nine open reading frames (ORF1a, ORF1b, ORF2, ORF3, ORF4, ORF5a, ORF5, ORF6, and ORF7) that are flanked by 5' and 3' untranslated regions (UTR) have been identified (Firth et al., 2011; Johnson et al., 2011; Meulenberg, 2000). ORF1a and ORF1b encoded the non-structural proteins (NSPs). ORFs 2 to 7 encode PRRSV structural proteins, including small envelope (E), GP2, GP3, GP4, GP5a, GP5, matrix (M) and nucleocapsid (N) proteins (Huang et al., 2015). Based on their genetic diversity, PRRSVs are classified into two genotypes: genotype I (European type), and Genotype II (North American type). These two genotypes share only about 55 % to 70 % nucleotide identity and 50 % to 80 % amino acid (AA) sequences (Benfield et al. 1992). Within each genotype, the PRRSV presents extensive genetic variations that reflect the diversity of the strains (Forsberg et al., 2002; Murtaugh et al., 1998). Moreover, the Nsp2 and ORF5 regions of the PRRSV genome display substantial genetic variation. These regions have been used as markers for genetic variability because they are the most variable regions (Fan et al., 2014; Fang et al., 2004). In recent years, continuing evolution of PRRSV has resulted in the emergence of novel strains with different levels of pathogenicity and virulence (Karniyuchuk et al., 2010; Wang et al., 2015; Zhang et al., 2012; Zhou et al., 2009; Zhou et al., 2009; Zhou et al., 2012). That persistent variation and evolution of PRRSV is an intractable issue in the effective control of PRRS.

In this study, we sequenced two PRRSV strains isolated in 2006 and 2016 and designated them as FZ06A and FZ16A, respectively. Clinical signs, pathological changes, and humoral immune responses of the isolated strains were analysed to evaluate the difference between the two HP-PRRSV strains and to provide an improved description of the persistent variation and evolution of PRRSV, The aim of this research was to contribute to the development of a constructive and effective

database that would be useful in the development of medical vaccines against this economically devastating disease.

## **2 Materials and Methods**

### ***2.1 Ethical approval***

All animals used in this research were treated with care and with the approval of the Animal Care and Use Committee of the Chinese Academy of Agricultural Sciences, China (IACUC No.PJ2011-012-03).

### ***2.2 Clinical samples***

In 2006 and 2016, two strains of PRRSV (FZ06A and FZ16A) were isolated from pigs having high fever, labored breathing, lethargy, and anorexia in Fujian province, South China. In 2006, a pig farm had an outbreak of PRRSV from which a high death rate was observed. Likewise, in 2016, the same pig farm had a high morbidity related to PRRSV but there was no large-scale mortality. Isolated strains of the PRRSVs, as clinical samples, were immersed in sterile phosphate-buffered saline (PBS) with antibiotics (100 U/mL penicillin and 100 mg/mL streptomycin) and the suspension was centrifuged at 10,000 ×g for 10 min to extract RNA and isolate the virus. The remaining samples were filtered and kept at -80 °C until needed.

### ***2.3 Virus isolation***

Marc-145 cells were used for virus isolation. The virus sample was filtered (through 0.22 µm filter) and then inoculated to Marc-145 cells. After adsorption for 1h at 37 °C, the supernatant was removed and Dulbecco's modified Eagle's medium (Gibco, USA) with 2 % fetal bovine serum (Gibco) was added. Then, the cells were incubated at 37 °C with 5 % CO<sub>2</sub> and monitored daily for cytopathic effects (CPEs). When CPEs appeared in 80 % of the cells, the culture supernatants were harvested and then stored at -80 °C until needed.

### ***2.4 RNA extraction and RT-PCR***

Viral RNA was extracted by using AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Biosciences, U.S.A.) from the original tissue homogenates and cell culture supernatants, and reverse transcription PCR (RT-PCR) was carried out with a PrimeScript one-step RT-PCR Kit (Takara, China) according to the manufacturer's instructions. Fourteen pairs of specific primers (Table 1) were designed for the amplification and were based on the complete genomic sequences of PRRSV in the GenBank database (National Center for Biotechnology Information, USA). Fourteen overlapped fragments that covered the entire viral genome were amplified by RT-PCR. The PCR conditions involved an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec, 52 °C to 55 °C for 30 sec, 72 °C for 2 min, and final extension at 72 °C for 10 min. The amplified products were analyzed by electrophoresis in a 1 % agarose gel.

**Table 1.** Primers used for amplification and sequencing of gene fragments of PRRSV strains FZ06A and FZ16A.

Primer designation	Primer sequence (5'-3')	Position (range)	Amplified fragment (bp)
PRRSV1F	ATGACGTATAGGTGTT GG	1-1203	1203
PRRSV1R	GCGGATCCAACCTCTCC TTAACAG		
PRRSV2F	ATGGACCTATCGTCAT ACAGT	1154-2379	1226
PRRSV2R	ATGAACCTCGTCACCT TGT		
PRRSV3F	ATCAGTACCTCCGTGG CG	2108-2832	725
PRRSV3R	CATAGGTGTCATCGGC TC		
PRRSV4F	CGAGCCGATGACACC TAT	2814-4238	1425
PRRSV4R	TCTGTAACAATGCCAA GC		
PRRSV5F	CGCCAGAGTGTAGGA ACG	4070-5373	1304
PRRSV5R	GGTGACGAGACCAGC AAT		
PRRSV6F	TACTAACATTGCTGGT CTCG	5349-6663	1315
PRRSV6-R	TTCCCTCAACTTTCCC TC		
PRRSV7F	TACCGCTGCCTTCACA AT	6574-7916	1343
PRRSV7R	CGTGCCATCAATCCCA		



	GT		
PRRSV8F	GAAACACTGGGATTG ATGG		
PRRSV8R	ATGGTTGTCTTCTTTG GGT	7894-9232	1339
PRRSV9F	CGACGACCTTGTGCT GTA		
PRRSV9R	GGTGAGGACCTGCCC ATA	9122-10481	1360
PRRSV10F	GTGATGCCATTCAACC AG		
PRRSV10R	CGGATGTATGAGGCGT AG	10378-11569	1192
PRRSV11F	GAATCAGTTGCGGTG GTC		
PRRSV11R	CAGGGTGAACGGTAG AGC	11322-12186	865
PRRSV12F	GGAGTTCTCGCTTGAT GAT		
PRRSV12R	TTGGCAGTGATGGTGA TG	11759-13401	1642
PRRSV13F	GGTTTCACCTGGAATG GC		
PRRSV13R	CACTGGCGTGTAGGT AATG	13138-14371	1234
PRRSV14F	AGTTGTGCTTGATGGT TCC		
PRRSV14R	TTTTTTTTTTTTTTTTTT TTTTTTTTTTTT	14231-15360	1130

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## 2.5 Electron microscopy

The presence and morphology of the purified virus were evaluated by transmission electron microscopy (TEM) of negatively stained preparations. Briefly, carbon-

coated grids were set on a 20  $\mu\text{L}$  sample drop for 10 min and then washed with deionized water. The washed grids were then negatively stained with 1 % uranyl acetate at pH 4.5. After removal of the excess stain by using filter paper, the grids were observed under a TEM (Ma et al., 2012).

## 2.6 Sequence alignments and phylogenetic analyses

The amplified PCR products were purified by using an E.Z.N.A. gel extraction kit (OMEGA; Bio-Tek, USA). The PCR amplicons were then cloned into a pEAZY-blunt-zero vector according to the manufacturer's instructions (TransGen, China) and sequenced. The full-length genomes of the new isolates were assembled according to the sequencing data by using Lasergene7.2 software (DNASTAR, USA). By utilizing the MEGA software (ver. 5.1) (Tamura et al., 2011), the assembled genomes were aligned with reference PRRSV strains in the GenBank database (Table 2). The sequences of the complete genome, as well as those of Nsp2 and ORF5, were compared with other PRRSV isolates. Phylogenetic trees were constructed with MEGA (ver. 5.1) by using the neighbor-joining method. Bootstrap values were calculated on 1,000 replicates of the alignment.

**Table 2.** Representative PRRSV strains used in this study.

NO.	Name	Country	Year	Accession Number	Note
1	VR-2332	USA	1992	AY150564	North American type
2	Lelystad virus	Europe	1991	M96262	European type
3	Euro PRRSV	USA	1999	AY366525 4	-
4	Resp PRRS MLV	USA	1994	AF066183	Attenuated vaccine strains
5	NADC 30	USA	2008	JN654459	-
6	MN 184A	USA	2006	DQ176019	-
7	Amervac PRRS	Spain	2009	GU067771	-

8	PA8	Canada	2002	AF176348	-
9	CH-1a	China	1996	AY032626	Vaccine strain
10	BJ-4	China	2000	AF331831	/
11	JXA1	China	2006	EF112445	First HP-PRRS V in China
12	HuN4	China	2007	EF635006	-
13	HNyc15	China	2015	KT945018	NADC30-like
14	SD16	China	2012	JX087437	-
15	HENAN-HEB	China	2012	KJ143621	NADC30-like
16	CHsx1401	China	2014	KP861625	NADC30-like
17	TJ	China	2006	EU860248	-
18	WuH1	China	2007	EU187484	-
19	HUB1	China	2006	EF075945	-
20	SY0608	China	2007	EU144079	-
21	HB-1(sh)/2002	China	2002	AY150312	-
22	CH-1R	China	2007	EU807840	Attenuated vaccine strains
23	JX143	China	2008	EU708726	-
24	JXA1-P80	China	2008	FJ548853	Attenuated vaccine strains

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## ***2.7 Amino acid analysis***

By using the BioEdit sequence alignment editor software (ver. 7.2.5; Ibis Therapeutics, USA), the nucleotide sequences obtained after completion of genomic

sequencing analysis were translated into the predicted AA sequences. For the comparative analysis, representative sequences of PRRSV were selected from GenBank (GenBank accession numbers: AY150564, AY032626, AY150312, EF075945, EF635006, EF112445, JX087437, EU144079, EU860248, EU187484, and AF331831).

## ***2.8 Animals and experimental design***

Fifteen 9-week-old, specific-pathogen-free (SPF) pigs, which were negative for antibodies of porcine circovirus type 2 (PCV2) and PRRSV, were randomly divided into three groups (five pigs per group): two challenge groups (groups 1 and 2) and a control group (group 3). The third virus passage (P3) from Marc-145 cells was used in these experiments. The selected pigs in groups 1 and 2 were inoculated intranasally (i.n.) with 2 mL inocula that contained  $10^5$  50 % tissue culture infective doses (TCID<sub>50</sub>) of the PRRSV strains FZ06A and FZ16A. The group 3 animals were inoculated i.n. with 2 mL Marc-145 cell culture supernatant.

## ***2.9 Clinical observation***

After infection of the pigs, clinical signs, including depression, appetite, diarrhea, skin discoloration, coughing, labored and abdominal breathing, and respiratory rate of the animals were monitored twice a day. Rectal temperatures were recorded every day throughout the experiment until completion at 21 days post-inoculation (dpi).

## ***2.10 Detection of viral loads***

Blood samples were collected at 1, 3, 7, 10, 14 and 21 dpi followed by culturing at 37 °C for 1 h. Subsequently, the blood was centrifuged at  $3,500 \times g$  for 5 min and the serum was frozen at -80 °C. Serum samples underwent 10-fold serial dilution and were then incubated with Marc-145 cells in 96-well cell culture plates at 37 °C for 2 h. After incubation for 48 h, cells were fixed with 4 % paraformaldehyde. The viral titers were determined by an indirect fluorescent antibody (IFA) technique with anti-N protein monoclonal antibody against PRRSV N protein and FITC-labelled goat-anti-mouse IgG (ZSGB-BIO, China). Viral titers were calculated as TCID<sub>50</sub> per mL by using the Reed-Muench method.

## ***2.11 Measurement of PRRSV-specific antibody***

Serum was collected at 1, 3, 7, 10, 14 and 21 dpi, and presence of PRRSV-specific antibodies was measured by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (IDEXX Laboratories, USA), according to the manufacturer's instructions. The positive and negative cut-offs of the IDEXX ELISA were set at a sample-to-positive (S/P) ratio of 0.4.

## ***2.12 Detection of viruses in tissue samples***

At 21 dpi, all infected and control animals were euthanized and tissue samples of lung, spleen, kidney, liver and lymph nodes were collected for detection of virus by

means of RT-PCR assays. A similar analysis method (RT-PCR) was used to determine the presence of pseudorabies virus (PRV), PCV, and classical swine fever virus (CSFV) in all tissue samples.

### ***2.13 Gross pathology and histological evaluations of lungs***

At necropsy, lungs of the investigated animals were fixed in 10 % neutral buffered formalin and then analyzed for macroscopic (gross) pathologies. In addition, some of the lungs tissues collected were processed for histopathological examination followed by H&E staining, as described previously (Halbur et al., 1995).

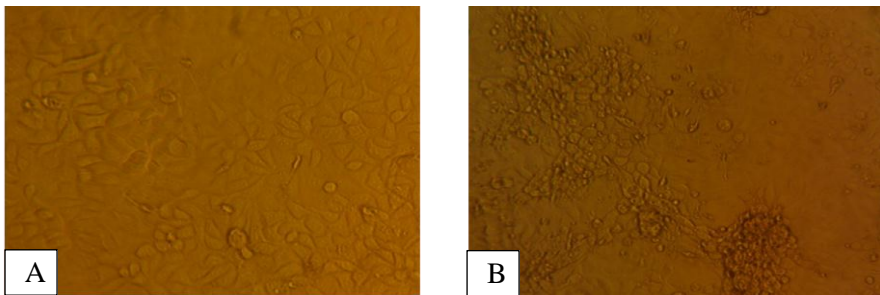
### ***2.14 Statistical analyses***

GraphPad Prism 5 software (GraphPad, USA) was used for analysis of the variability among the groups, and data were expressed as mean  $\pm$  SD values. By using one-way ANOVA and Tukey's t-test, the results were statistically evaluated. Differences were considered statistically significant when  $p < 0.05$ .

## **3 Results**

### ***3.1 Genomic Characteristics of the FZ06A and FZ16A isolate strain***

The two isolated viruses were tested as PRRSV positive by RT-PCR assays. After three passages on Marc-145 cells, a distinct CPE was observed (Figure 5). These isolates were designated as FZ06A and FZ16A. Electron microscopy of negatively stained samples revealed the presence of a viral structure with a diameter of 60 to 70 nm in isolated viruses (Supplementary Figure 1).



**Figure 5.** Cytopathic effects of isolated PRRSV cultured in Marc-145 cells. Infected Marc-145 cells became round and aggregated into clusters. A, Non-infected Marc-145; B, Virus infected Marc-145 cells. Scale bars—200  $\mu$ m (A and B)

### ***3.2 Analysis of full-length genomic sequence***

The genome sequence of FZ06A and FZ16A strains has been deposited in

GenBank under accession NO.MF370557 and NO.KY761966, respectively. The genomes of FZ06A and FZ16A strains were comprised of 15,319 and 15,320 nucleotides in length. Likewise, FZ06A and FZ16A strains possessed the genetic marker of 1+29AA (1 and 29 non-contiguous AA deletions at positions 481 and 533-561, respectively) deletions in the Nsp2. It is highly similar to a group of highly pathogenic strains of PRRSV previously isolated in China.

Following comparison of the FZ06A and FZ16A strains with 8 other PRRSV isolates deposited in GenBank (Table 3), it was observed that both FZ06A and FZ16A shared the highest nucleotide identity (range, 97.4 %-100 %) with the JXA1 isolates, 79.8 % to 93.7 % identity with NADC30- or NADC30-like (NADC30, HNyc15) strains. An 87.4 % to 95.4 % sequence identity with VR-2332 (North American type), whereas only 55.8 % to 71.7 % identity with the Lelystad virus (LV, European type), indicating that these two isolate strains belonged to PRRSV type 2. The 5'UTR and 3'UTR of FZ06A and FZ16A shared 93.6 % to 99.5 % and 92.6 % to 99.3 % nucleotide identity, respectively, with the HP-PRRSVs (BJ-4, HB-1(sh)/2002, and JXA1), and 92.6 % to 93.7 % and 87.9 % to 89.3 % nucleotide identities, respectively with the NADC30- or NADC30-like PRRSVs (NADC30 and HNyc15), as well as 62.8 % to 97.9 % and 71.4 % to 96 % nucleotide identity, respectively with VR-2332, CH-1a and LV strains. The ORF1a and ORF1b of FZ06A and FZ16A shared 87.3 % to 99.8 % identity with the HP-PRRSVs (BJ-4, HB-1(sh)/2002, and JXA1), 79.8 % to 88 % nucleotide identity with the NADC30 or NADC30-like PRRSV (NADC30 and HNyc15), and 55.8 % to 96.4 % nucleotide identity with VR-2332, CH-1a and LV. Whereas, ORF2 to ORF7 were comparatively conserved with identities of 87.4 % to 100 % with the HP-PRRSVs BJ-4, HB-1(sh)/2002, and JXA1, and 83.3 % to 90.9 % nucleotide identity with the NADC30- or NADC30-like PRRSVs (NADC30 and HNyc15). In contrast, they only showed 63.3 % to 69.5 % identity with LV (Table 3).

By comparing the Nsp2 AA sequences of other type 2 PRRSV strains, we found 65.3 % to 98 % and 65.3 % to 92 % identities between FZ06A/type 2 PRRSV and FZ16A/Type 2 PRRSV, respectively (Table 3). In addition, when compared to other PRRSV strains, the AA mutations in position 307, 361, 373, 482, 486, 514, 677, 712 and 771 were found in Nsp2 of the FZ16A, whereas there was no mutation in Nsp2 of FZ06A strain.

The AA substitutions R13 and R151 associated with high virulence ability were identified in the predicted GP5 protein of FZ06A strain. The FZ16A strain also shared the AA substitutions R13, but it also had a K151 AA substitution. Changes in the AA sequence of potential N-glycosylation sites (NGSs) in GP5 significantly influenced the susceptibility of mutant viruses to virus-neutralizing antibodies. To gain further insight into the genetic evolution of FZ06A and FZ16A, we also analyzed variation of GP5 in potential NGSs by using NetOGlyc1.0 Server. The potential NGSs were detected at six different positions: N30, N33, N34, N35, N44 and N51. The GP5 of the FZ16A strain shared five common NGSs at AA positions 30, 34, 35, 44, and 51; however, the deletion of one NGS at position 33 was not detected in any other PRRSV strains. The FZ06A strain shared six common NGSs

(Table 4).

**Table 3.** Nucleotide and deduced amino acid identities of the full-length genomes of FZ06A and FZ16A with 8 reference strains of PRRSV.

FZ06A/ FZ16A (%)	Strain identify to FZ06A/FZ16A							
	LV	VR- 2332	CH- 1a	JXA1	HB-1(sh )2002	BJ-4	NADC 30	HNyc 15
Nucleotides(length)								
5'UTR (189bp)	62.8/ 64.4	93.6/ 93.6	97.9/ 97.4	99.5/ 99.5	93.7/ 96.8	93.6/ 93.6	93.1/ 92.6	93.7/ 93.1
ORF1a (7422bp)	56.1/ 55.8	87.8/ 87.4	94.4/ 94.1	99.5/ 99	97.1/ 96.5	87.7/ 87.3	81.5/ 81.5	79.9/ 79.8
ORF1b (4383bp)	63.4/ 63.3	91.4/ 91.1	96.4/ 96	99.8/ 99.1	97.9/ 97.4	91.4/ 91	88/ 87.9	86.4/ 86.3
ORF2 (771bp)	65.6/ 65.3	92.9/ 92.9	96.6/ 96.1	99.7/ 99.2	97.3/ 96.8	93.1/ 93.1	86.5/ 86.1	90.1/89 .8
ORF3 (765bp)	65.1/ 65.1	88.9/ 89.3	96.2/ 95.4	99.6/ 98.6	96.2/ 95.4	89.2/ 89.5	83.5/ 83.5	86.1/ 85.8
ORF4 (537bp)	66.7/ 66.1	89.8/ 89.8	97/ 96.6	98.1/ 97.4	97/ 96.6	89.8/ 89.8	87.3/ 87.5	87.3/ 86.8
ORF5 (603bp)	64.1/ 63.3	88.6/ 87.9	94.7/ 94.4	98.7/ 98.8	96.5/ 96.2	88.1/ 87.4	85.7/ 86.1	83.3/ 83.6
ORF6 (525bp)	69.5/ 69.5	95.4/ 95.4	97.5/ 97.5	100/ 100	99.2/ 99.2	95.2/ 95.2	89/ 89	88.4/ 88.4
ORF7 (372bp)	68.1/ 66.4	93.3/ 93	96/ 95.7	99.7/ 99.5	97.8/ 97.6	93.8/ 93.5	90.9/ 90.6	90.3/ 90.1
3'UTR (150bp)	71.4/ 71.7	91.3/ 91.3	96/ 96	99.3/ 99.3	97.3/ 97.3	92.6/ 92.7	89.3/ 89.3	87.9/ 88
Comple te(15320 bp)	60.6/ 60.6	89.8/ 89.5	95.5/ 95	99.6/ 99	97.4/ 96.8	89.7/ 89.4	84.8/ 84.6	83.6/ 83.5
Amino acid(length)								
Nsp2	31.3/ 31.2	78/ 77.8	88.6/ 88.5	99.2/ 98	94.9/ 93.8	77.2/ 77	68.9/ 68.9	65.3/ 65.3
GP2	62.4/ 62.4	91.4/ 91.8	96.5/ 95.3	100/ 98.8	96.5/ 95.3	92.2/ 92.6	86.4/ 86.4	89.1/ 88.7
GP3	57.5/ 57.1	86.7/ 87.8	93.3/ 92.9	99.2/ 98	93.3/ 92.9	86.7/ 87.8	81.2/ 81.6	84.3/ 83.9
GP4	71.5/ 71.5	90.5/ 90.5	98.3/ 97.8	96.6/ 95	98.3/ 97.8	89.9/ 90.5	89.4/ 88.8	88.8/ 88.3
GP5	58.9/ 56.9	87.6/ 85.1	92/ 91	98/ 97	94/ 92.5	86.1/ 84.1	86.1/ 86.1	82.6/ 83.6

M	80.5/ 80.5	97.7/ 97.7	97.7/ 97.7	100/ 100	99.4/ 99.4	97.1/97 .1	93.1/ 93.1	93.7/ 93.7
N	62.8/ 63.6	94.4/ 94.4	95.2/ 95.2	100/ 100	96.8/ 96.8	95.2/ 95.2	91.1/ 91.1	91.1/ 91.1

**Table 4.** The potential N-glycosylation sites in various PRRSV strains.

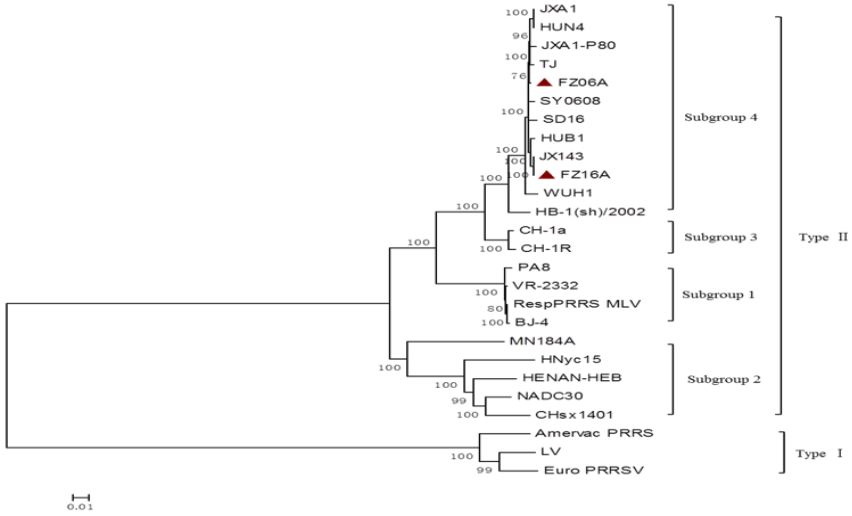
Strain name	N-glycosylation sites					
	30	33	34	35	44	51
VR-2332	N	N	D	S	N	N
JXA1	N	N	N	N	N	N
BJ-4	N	N	D	S	N	N
HuN4	N	N	N	N	N	N
SD16	N	N	N	N	N	N
WUH1	N	N	N	N	N	N
CH-1a	N	S	N	S	N	N
HUB1	N	N	N	N	N	N
HB-1(sh)/ 2002	N	S	N	S	N	N
HNyc15	N	G	N	S	N	N
FZ16A	N	K	N	N	N	N
FZ06A	N	N	N	N	N	N

### 3.3 Phylogenetic analysis

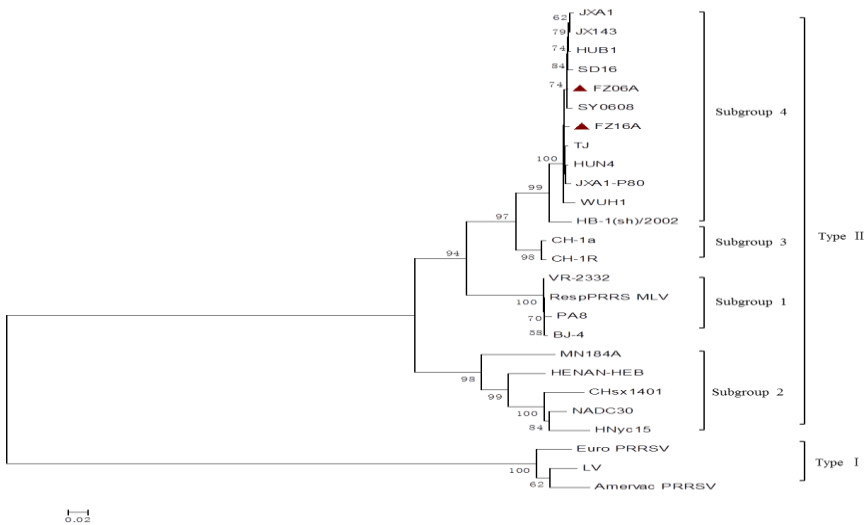
In order to determine the phylogenetic relationship between FZ06A and FZ16A strains and other characterized PRRSV isolates, phylogenetic trees were constructed based on the nucleotide sequences of the complete genome, Nsp2, and ORF5 of FZ06A and FZ16A strains by using MEGA5 software (ver. 5.1) (Figure 6). Phylogenetic analysis demonstrated that all PRRSV isolates could be divided into two types. Moreover, the Type II isolates could be divided into four main groups. Group 1 contained four North American strains (VR-2332, RespPRRS MLV, and BJ-4) and group 2 contained some recombination strains (e.g., HNyz15, HENAN-HEB, and CHsx1401). Likewise, group 3 contained several classical Chinese strains (CH-1a and Ch-1R) and group 4 contained some Chinese



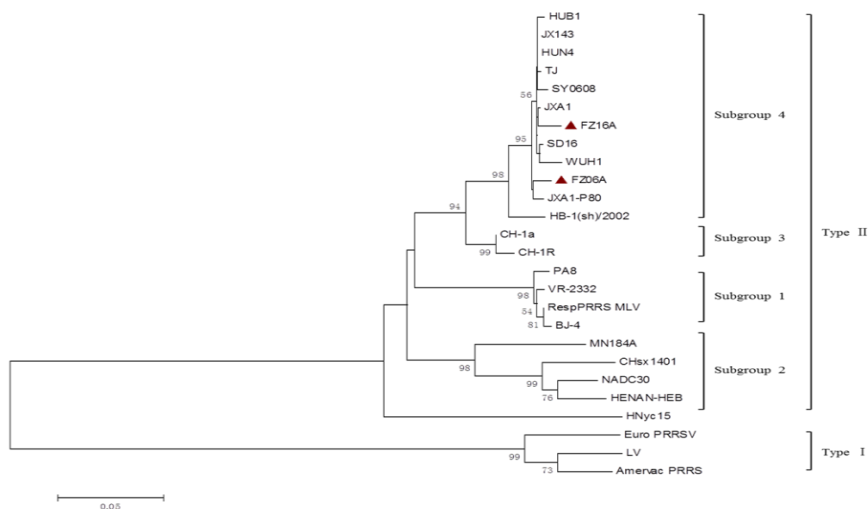
HP-PRRSV strains (e.g., JXA1, JX143, and SY0608). The phylogenetic trees showed that the FZ16A strain was similar to the Chinese isolate JX143 strain and the FZ06A strain was similar to the SY0608 strain, which belonged to the group 4.



A



B

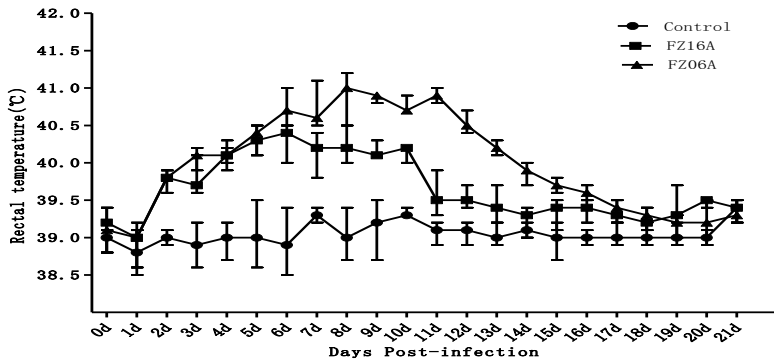


C

**Figure 6.** Phylogenetic tree of 24 PRRSV isolates based on analysis of nucleotide sequences of the complete genomic sequences (A), Nsp2 (B), and ORF5 (C) of PRRSV strains by applying the distance-based neighbor-joining method using the MEGA software (ver. 5.1) (Tamura et al., 2011). Bootstrap values were calculated on 1,000 replicates of the alignment.

### 3.4 Observation of clinical signs post infection

The rectal temperature and clinical signs of infected animals were recorded daily throughout the experiment, and there were no typical clinical signs in animals from the negative-control group and no remarkable clinical symptoms detected in the inoculated animals until 2 dpi. From 3 dpi onward, almost all inoculated animals presented with depression, low appetite, coughing, sneezing and diarrhea. The rectal temperature of FZ16A-challenged animals was mildly elevated to 40 °C at 4 or 5 dpi and this persisted for 2 to 5 days; however, rectal temperature did not exceed 40.5 °C throughout the observation period. In comparison with the FZ16A-challenged group, the rectal temperature of FZ06A-challenged animals also had a high temperature. After 4 days, the rectal temperature elevated to 40 °C and persisted at that level for 11 to 12 days, and, in some challenged animals, the temperature exceeded 41 °C. The rectal temperature in FZ06A group was significantly higher than that of the FZ16A group and control group (Figure 7).



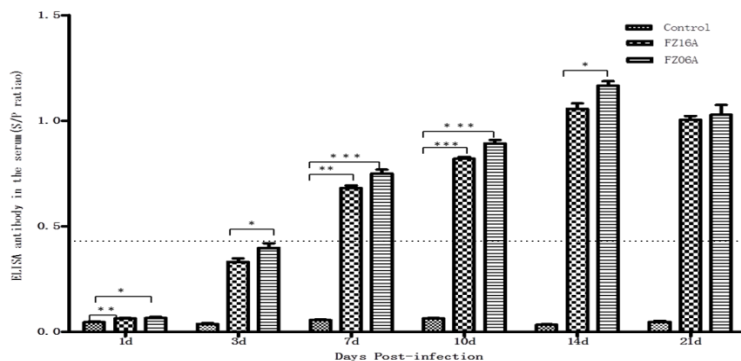
**Figure 7.** Mean rectal temperature in negative-control pigs and pigs infected experimentally with FZ06A and FZ16A PRRSV. Significant differences between FZ06A-infected, FZ16A-infected and negative-control pigs were observed at most of the time point.

### ***3.5 Viral loads in the serum samples***

The viral loads in the sera of infected pigs were measured using an IFA-microtitration infectivity assay. The sera from the FZ06A-infected group showed increased viral titers at 4 dpi, which peaked at 11 dpi. The FZ16A-infected group also showed increased viral titers, and increased viral titers were present for a longer period in the FZ16A-infected than in the FZ06A-infected group (data not shown).

### ***3.6 Antibody detection post infection***

Sera were collected at 1, 3, 7, 10, 14 and 21 dpi to monitor PRRSV-specific antibodies. The effect of antibodies against PRRSV was analyzed using a commercially available IDEXX ELISA. All inoculated pigs became seropositive (S/P ratio > 0.4) at 7 dpi. The S/P ratio then gradually increased until the end of the examination; no PRRSV-specific antibodies were detected in the control animals (Figure 8).



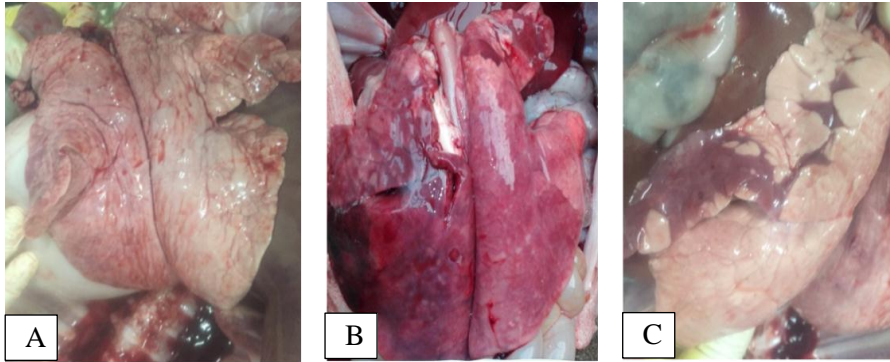
**Figure 8.** Serum antibody levels over time. Values represent average titers from five animals. Dotted line at 0.4 sample-to-positive (S/P) ratio designates threshold value above which titers were considered positive for anti-porcine reproductive and respiratory syndrome virus antibodies. Data from groups of five pigs assessed by using one-way ANOVA ( $*p<0.05$ ) ( $**p<0.01$ ) ( $***p<0.001$ ).

### 3.7 Virus detection in tissue samples

Virus detection in tissue samples from negative controls was negative for the presence of PRRSV, whereas viruses were detected in all evaluated organs of inoculated pigs. All tissue samples from the control and inoculated animals were found to be negative for the presence of PRV, PCV or CSFV.

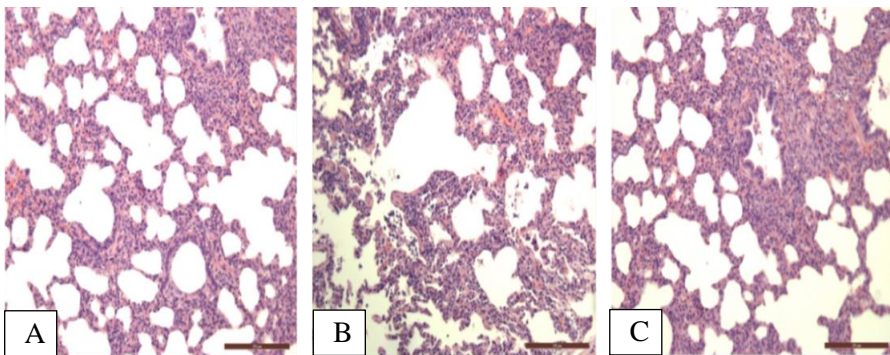
### 3.8 Gross Pathology and histological evaluations of lungs

All pigs were euthanized and anatomized at 21 dpi. No macroscopic (gross) lesions were observed in the lungs collected from the control animals at necropsy (panel A in Figure 9). Animals from the FZ06A-infected group exhibited severe multifocal lesions and consolidation in the lungs (panel B in Figure 9), whereas the FZ16A-infected group only exhibited mild multifocal lesions in the lungs (panel C in Figure 9).



**Figure 9.** Macroscopic observation of the lungs of the control (A) and HP-PRRSV infected (B and C) pigs. No macroscopic (gross) lesions were recorded in the lungs collected from the control animals. (B) Severe multifocal lung lesions and consolidation of lung tissue in the HP-PRRSV FZ06A-infected group. (C) Mild multifocal lung lesions and consolidation of lung tissue in the HP-PRRSV FZ16A-infected group.

Under histological examination, none of the lungs from control pigs exhibited any features indicative of acute PRRSV infection (panel A in Figure 10). However, the lung tissues collected from FZ06A-infected and FZ16A-infected animals displayed interstitial pneumonia with alveolar septa thickening and alveoli disappearance (panels B and C in Figure 10).



**Figure 10.** Histopathological lesions in the lung of control (A) and HP-PRRSV infected (B and C) pigs. Histological lesions were examined in the HP-PRRSV FZ06A- and FZ16A-infected groups and the challenge control by using H&E staining. (A) All lungs from control pigs failed to exhibit features of acute PRRSV infection. (B) Lung tissues collected from the HP-PRRSV FZ06A-infected pigs developed severe interstitial pneumonia with alveolar septal thickening and alveoli disappearance. (C) The lung tissues collected from HP-PRRSV FZ16A-infected pigs developed mild interstitial pneumonia and alveoli disappearance. Scale bars -200  $\mu$ m (A-C).

## 4 Discussion

PRRS is one of the highly-ranked swine diseases and affects industries related to pigs around the world. In China, PRRS was first reported in 1996. In June 2006, there was an unparalleled outbreak of HP-PRRS in China. Since then PRRSV has become widespread and today various types of PRRS has been isolated, and the number of strains is growing rapidly (Tian et al., 2007). Traditional control strategies and conventional vaccines have been unable to provide sustainable disease control; therefore, commercial vaccines specifically against the PRRS have been developed. Because of the considerable genetic and antigenic diversities in HP-PRRSV isolates, surveillance and identification of the recently emerged new virus strains have become crucial (Shi et al., 2010).

In the current study, two PRRSV strains were isolated from pig farm in Fujian, China, one isolated in 2006 and the other in 2016. The complete nucleotide sequence of the FZ06A and FZ16A strains were determined and then compared with other representative PRRSV strains that had been isolated in China and other countries. Based on genome analysis, the FZ06A and FZ16A strains had a discontinuous deletion of 30 AA (1AA and 29AA) in Nsp2, which is suggested to be a marker of HP-PRRSV (Tian et al., 2007). Based on phylogenetic analysis, a high degree of genetic homology was observed among most of the Chinese isolates.

Clinical signs of PRRSV infection developed in pigs challenged with the FZ06A and FZ16A strains. In the FZ16A-infected group, no mortality was observed, but FZ06A-infected group exhibited high mortality. The absence of mortality detected during the challenge study indicated that the virulence of FZ16A was relatively weak. The low virulence might be related to the evolution of Chinese HP-PRRSV under selective pressure.

The Nsp2 gene exhibited the highest genetic diversity in the genome of PRRSV (Fang et al., 2012; Nelsen et al., 1999). Nsp2 is crucial for viral replication and modulation of host immunity due to its protease activity (Chen et al., 2010; Wang et al., 2013). The HP-PRRSV strains that emerged in 2006 had a unique 30 AA (1 AA+29 AA) deletion in the Nsp2 hyper-variable region. In recent years, a variety of atypical PRRSV strains with new AA deletions or insertions in Nsp2 have been reported (Du et al., 2012). The virulence of these strains differs from their typical PRRSV counterparts. A recent study reported that the 30 AA deletions in the Nsp2-coding region could be considered as a putative marker for high virulence in a low virulence strain (Li et al., 2010). In this study, we detected a discontinuous 30 AA deletion of 1 AA + 29 AA) in the Nsp2 region of both FZ06A and FZ16A strains at positions 481, and 533 to 561, which is similar to that of Chinese HP-PRRSV isolates such as JXA1, HUN4, and BJ-4. We also observed AA mutations in other positions including 307 (A→V), 361 (G→E), 373 (E→G), 482 (A→V), 486(L→P), 514 (V→M), 677 (D→N), 712 (K→E) and 771(I→T) in Nsp2 of FZ16A when compared to HP-PRRSV in China; however, there were no obvious mutations in Nsp2 of FZ06A when compared to HP-PRRSVs in China. The phylogenetic tree analysis showed that the FZ16A strain had similarity to the

Chinese JX143 strain, whereas there were no similar AA mutations in Nsp2 in the FZ16A and JX143 strains. Likewise, these two (JX143 and FZ16A) strains have different levels of virulence. However, further study is essential to determine whether these mutations caused the virulence difference. In a previous study, it was reported that the 30 AA deletions was not associated with the virulence of emerging HP-PRRSVs (Zhou et al., 2009), whereas in our research, both of these two isolate strains had the 30 AA deletion. There were various kinds of mutations in Nsp2 of FZ16A compared to those in the Nsp2 of FZ06A, and there were obvious difference in experimental inoculation results from the two strains. Thus we suggest that these mutations may have an influence on the virulence of the current FZ16A isolate.

GP5 is a glycosylated viral transmembrane protein, which is responsible for virus attachment to the host cell and contains important immunological domains associated with virus neutralization (Li et al., 2009). It has been reported that GP5 is the least conserved ORF of PRRSV, and AA sequence variations in GP5 are mainly observed in the extra virion domain of the protein, which has been shown to be responsible for cell attachment. N-glycosylation of GP5 might be critical for proper functioning of the protein. Potential involvement of NGSs in GP5 has been observed in viral immune evasion and virus-neutralizing antibody responses (Ansari et al., 2006; Zhou et al., 2012). Glycosylate H38 (L/F) 39 residues in this domain are considered to be critical to the immunogenicity of this epitope (Ostrowski et al., 2002). Both major and minor viral envelope glycoproteins can help PRRSV to escape, block, or minimize the viral neutralizing antibody response (Murtaugh et al., 1998).

Glycoprotein 5 is essential for virus infectivity due to its significant genetically variable effect on structural proteins of PRRSV. Residues 13 and 151 in the GP5 proteins of PRRSVs have been shown to be associated with virulence (Allende et al., 2000). Compared to the first Chinese strain CH-1a, the other PRRSV strains exhibited variation in their potential glycosylation level or sites. Based on our results, residues 13 and 151 in the GP5 proteins of PRRSVs are associated with virulence. With the changing and increasing number of NGSs, a low value of antigenicity was obtained. In our study, potential NGSs were also observed in the GP5 of FZ06A and FZ16A strains at AA positions 30, 34, 35, 44, and 51. Interestingly, the FZ06A shared six common NGSs with other HP-PRRSV in China (Table 4); regardless, there was one possible NGS in the FZ16A strain at position 33 (N33→K33) that, thus far, has not been observed in any other PRRSV strains. Whether this mutation is associated with virulence remains to be determined.

To confirm the pathogenicity of the FZ06A and FZ16A strains, SPF pigs were inoculated with  $10^5$  TCID<sub>50</sub> of FZ06A or FZ16A. At 3 to 11 dpi, all inoculated animals presented clinical signs of PRRSV infection. In the FZ16A-infected group, there was no mortality, while in FZ06A-infected group four of five inoculated pigs died from 7 and 21 dpi. Development of specific antibodies in inoculated pigs was detected at 7 dpi by ELISA, and PCR analysis showed PRRSV-positive tissue samples including the lung, spleen, kidney, liver and lymph nodes. Pigs in the control group remained seronegative throughout the experiment. Histological

examination revealed that the lungs from the FZ16A-infected animals developed interstitial pneumonia with slight alveolar septa thickening, alveoli disappearance and atrophy than the FZ06A-infected animals. It has been reported that the age of piglets might be associated with their resistance against PRRSV infection (Ladinig et al., 2015). The FZ06A and FZ16A strains produced different mortality; thus, we conclude that after ten years evolution of Chinese HP-PRRSV under selective pressure, the FZ16A strain has adapted with the environment to a greater extent than FZ06A.

In summary, the two PRRSV isolate strains (FZ06A and FZ16A strains) isolated in 2006 and 2016, respectively, showed similar genomic characteristics with HP-PRRSV in China but different mortality effects; the virulence difference indicating that HP-PRRSV strains prevailing in China may have gradually adapted during repeated passages in animals. Furthermore, the types of mutation in Nsp2 and the unique mutation in GP5 are crucial to the virulence of HP-PRRSVs. The high degree of genetic and antigenic diversity among field isolates underlined the complexity to be overcome when attempting to control and eradicate PRRS in China. This study provides useful information on the evolutionary characteristics of Chinese PRRSVs; such information could be utilized as reference material in the development of future live attenuated vaccines against this economically devastating disease.



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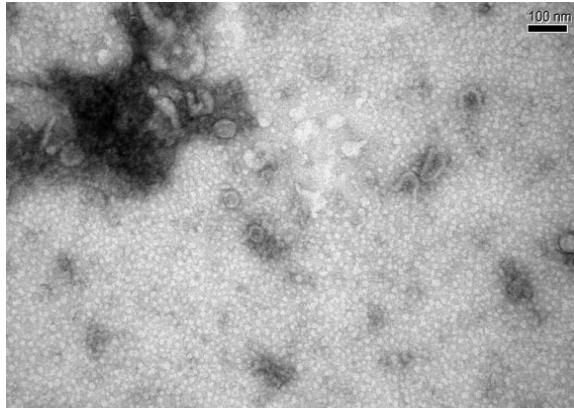
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## 6 Supplementary data



**Figure S1.** Transmission electron microscopy image of isolated porcine reproductive and respiratory syndrome virus (100,000 $\times$ ).



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## **Genomic sequence and virulence of a novel NADC30-like porcine reproductive and respiratory syndrome virus isolate from the Hebei province of China**

From Sui X., Guo X., Willems L., Zhu H., Xin T., Hou S\*. 2018. Genomic sequence and virulence of a novel NADC30-like porcine reproductive and respiratory syndrome virus isolate from the Hebei province of China. *Microbial Pathogenesis*. 125:349-360.





**Abstract:** Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of porcine reproductive and respiratory syndrome (PRRS), which results in immense economic losses in the swine industry. Outbreaks of disease caused by NADC30-like PRRSV are of great concern in China. Here, a novel variant, NADC30-like PRRSV strain HB17A, was analyzed and its pathogenicity in pigs was examined. The full-length genome sequence of HB17A shared 83.6-95.1 % nucleotide similarities with NADC30-like and NADC30 PRRSV without any gene insertions, but with a unique 2-amino acid deletion in Nsp2. A phylogenetic analysis showed that HB17A clustered with NADC30 strains. Different degrees of variation in the signal peptide, transmembrane region (TM), primary neutralizing epitope (PNE), non-neutral epitopes, and N-glycosylation sites were observed in GP5. Challenge experiments showed that HB17A infection resulted in persistent fever, moderate respiratory clinical signs, low levels of viremia and viral loads in serum, and mild gross and microscopic lung lesions. Moreover, IFN- $\gamma$ , IL-6, and IL-10 cytokine levels were significantly elevated in serum, but the levels of IFN- $\alpha$  and IL-2 were similar to those of the negative controls. HB17A was less pathogenic but was secreted longer in nasal discharge than HP-PRRSV FZ06A. Our findings indicate that HB17A is a novel NADC30-like strain with certain deletions and mutations but with no evidence of genomic recombination. This strain exhibits intermediate virulence in pigs. This research will be help to define the evolutionary characteristics of Chinese NADC30-like PRRSV.

**Keywords:** highly pathogenic porcine reproductive and respiratory syndrome virus, NADC30-like, variation analysis, pathogenicity

## 1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is recognized globally and is one of the costliest diseases affecting the piglet industry (Neumann et al., 2005). It was first reported in the United States in 1987 and in Europe in 1990 (Benfield et al., 1992; Wensvoort et al., 1991), and then it spread to other parts of the world (Cavanagh et al., 1997; Done et al., 1996). In 2006, a highly pathogenic form of PRRS that resulted in high mortality in both juvenile and adult animals (including sows) was reported in China and has since spread to the rest of China (Tian et al., 2007). PRRS is mainly associated with reproductive failure in gilts and sows and severe respiratory dysfunction in young pigs (Terpstra et al., 1991). In recent years, it has become one of the leading causes of economic losses in the livestock industry worldwide.

PRRSV belong to the *Arteriviridae* family and the genus *Nidovirales* (Benfield et al., 1992; Cavanagh et al., 1997). It is a single-stranded, positive-sense RNA virus. PRRSVs are classified into two genotypes, namely genotype I (European type) and genotype II (North American type). The Lelystad virus (LV) strain represents the European type, whereas strain VR-2332 serves as the representative of the North American type. These two genotypes share only about 55-70 % nucleotide and 50-80 % amino acid sequence identity (Forsberg et al., 2002; Kapur et al., 1996; Li et al., 2014).

The genome of PRRSV is approximately 15 kb in length and contains a 5'-capped structure and a 3'-polyadenylated tail. It also has at least nine open reading frames (ORF1a, ORF1b, ORF2, ORF3, ORF4, ORF5a, ORF5, ORF6, and ORF7) (Firth et al., 2011; Johnson et al., 2011; Meulenbergh, 2000). ORF1a and ORF1b encode the non-structural proteins (NSPs), including Nsp1a, Nsp1b, and Nsp2 to Nsp12 (Conzelmann et al., 1993). ORFs 2 to 7 encode the PRRSV structural proteins, including the small envelope (E), GP2, GP3, GP4, GP5a, GP5, matrix (M), and nucleocapsid (N) proteins (Huang et al., 2015). Moreover, the Nsp2 and ORF5 regions of the PRRSV genome display substantial genetic variation. These regions have been selected to monitor the evolution of PRRSV and for molecular epidemiological research on PRRSV (Fan et al., 2014).

In recent years, several field isolates of PRRSV have shown high nucleotide similarity with NADC30 type 2 PRRSV prototype strains that were isolated in United States of America in 2008 (Zhao et al., 2015; Zhou et al., 2015). These new isolates from China were designated NADC30-like PRRSV. The first two NADC30-like PRRSVs, HENAN-XINX (access number KF611905) and HENAN-HEB (access number KJ143621), were isolated in Henan province (Li et al., 2016). Now types of NADC30-like PRRSV isolates were identified. Genomic analysis showed that all of the NADC30-like PRRSV isolates share a unique discontinuous deletion of 131 amino acids in the Nsp2-coding region, which can be used as a genetic marker for novel PRRSVs in China. However, only some NADC30-like strains result in significant mortality (Zhao et al., 2015; Zhou et al., 2015; Li et al., 2016). Since 2013, PRRS has been reported in several provinces in China (Zhou et al., 2015), and

it has become one of the leading causes of economic losses in global swine operations.

Because of the high genetic and antigenic diversity of PRRSV isolates, it is necessary to monitor emerging epidemic strains (Fan et al. 2014). In this study, the complete genome of PRRSV strain HB17A, isolated from a pig farm with a clinical outbreak of PRRS in Hebei province, northern China, in 2017 was sequenced and analyzed with other strains of the North American and European genotypes. Clinical signs, pathological changes, and cytokines produced in response to select PRRSV strains were then analyzed for an increased understanding of the variation and evolution of PRRSV. The study revealed that the recent isolation is a NADC30-like and HP-PRRSV strain. The pathogenicity of this virus strain is less than that of the FZ06A strain, which is highly pathogenic and causes severe disease. The genotype and genomic characteristics of strain HB17A were determined. This information may be used in the development of vaccines against this economically devastating disease.

## **2 Materials and methods**

### ***2.1 Clinical samples***

Blood and organs, including samples from lung, liver, spleen, kidney, and lymph nodes, were collected from pigs with severe respiratory signs from Hebei in China. Samples were immersed in sterile phosphate-buffered saline (PBS) with antibiotic (100 U/mL penicillin and 100 mg/mL streptomycin), and the suspension was centrifuged at 10000  $\times$ g for 10 min. The clarified samples were filtered and frozen at  $-80$  °C.

### ***2.2 Virus isolation***

Porcine alveolar macrophages (PAM) cells were used for virus isolation. Blood and tissue culture supernatants were filtered through 0.22- $\mu$ m filters and inoculated at a rate of 200  $\mu$ L per 25-cm<sup>2</sup> flask into cell monolayers. After adsorption for 1 h at 37 °C and 5 % CO<sub>2</sub>, the inocula were removed and the cells were grown in RM1640 with 2 % fetal bovine serum (Gibco, USA) for 1 h at 37 °C and 5 % CO<sub>2</sub>. Cell cultures were observed daily for the appearance of cytopathic effects (CPE). When 75 % CPE was observed, the culture medium was freeze-thawed, and cell lysates harvested and stored at  $-80$  °C.

### ***2.3 Primer design and synthesis***

PRRSV primers for DNA amplification were designed based on the complete genomic sequences of PRRSV in the GenBank database. Fourteen overlapping fragments covering the whole viral genome were amplified by reverse transcription PCR (RT-PCR). The primers Nsp2-up and Nsp2-down (Nsp2-up: 5'-GCTGGAAA GAGAGCAAGGAAAG-3'; Nsp2-down: 5'-GCCCAATAACCTGCCAAGAATG -3'), amplified the entire Nsp2 gene. The primers ORF5-up and ORF5-down

(ORF5-up: 5'-CGCGGATCCATGTTGGGGAAGTGCTTGACC-3'; ORF5-down: 5'-CCGCTCGAGCTAGAGACGACCCCATTTGTTTC-3') amplified the entire ORF5 gene sequence. All primers were designed using Primer 5.0 software. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd., and the purity level was verified using polyacrylamide gel electrophoresis.

## ***2.4 RNA extractions and RT-PCR***

Viral RNA was extracted from the tissue homogenates and cell culture supernatants using an AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Biosciences, USA) according to the manufacturer's recommendation. DNA and RNA were subjected to PCR or RT-PCR for the detection of pathogens such as PRV, PCV, and CSFV. The genomic material was amplified as described previously (Gu et al., 2015; Gao et al., 2013; Ma et al., 2012). The PCR conditions comprised an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec, 52-55 °C for 30 sec, and 72 °C for 2 min, and then a final extension at 72 °C for 10 min. The amplified products were analyzed by electrophoresis on a 1 % agarose gel. Fragments of strain HB17A were amplified and cloned into the pMD18-T vector. Amplicons were sequenced by a commercial corporation (Huada, Beijing).

## ***2.5 Genome and phylogenetic analysis***

The complete nucleotide sequences of HB17A and every ORF, as well as deduced amino acid sequences, were compared with representative PRRSV strains in GenBank using Clustal W and DNASTar. A phylogenetic tree was constructed using the neighbor-joining method in MEGA software (version 5.0). The full-length genome, ORF5, and Nsp2 nucleotide sequence trees were evaluated by bootstrapping with 1000 replicates.

## ***2.6 Amino acid analysis***

Using the BioEdit sequence alignment editor, nucleotide sequences were translated into predicted amino acid sequences after genomic sequencing. For the comparative analysis, representative sequences of PRRSV were selected from GenBank (GenBank accession numbers: AY150564, AY032626, KT143621, EF112445, JN654459, DQ176019, AF331831, AF066183, EF635006, EU144079, EU860248, EU825724, AY150312, EU807840, and KT945018).

## ***2.7 Recombination analyses***

To screen for recombinants, HB17A sequences determined in this study were compared with sequences of up to 80 isolates. RDP V4.24 was used to evaluate potential recombinants (Martin & Rybicki 2000). Briefly, seven methods (RDP, GeneConv, BootScan, MaxChi, Chimera, SiScan, and 3Seq) were applied, with highest acceptable p-value of 0.01 and Bonferroni correction used as general settings. Recombination was further analyzed in SimPlot software v.3.5.1 (Lole et al., 1999). A window size of 200 bp and step size of 20 bp was used in the analysis, and the complete genome of HB17A was chosen as the query sequence.

## ***2.8 Animal challenge experimental design***

Fifteen six-week-old pigs that were free of PRRSV, PCV type 2, and PRV infections were obtained from the Beijing Center for SPF Swine Breeding & Management for use in the experiment. The animals were randomly divided into three groups. Animals in group 1 (n=5) were inoculated intramuscularly and intranasally with 3 mL inocula containing  $10^5$  50 % tissue culture infective doses (TCID<sub>50</sub>) of HB17A (P3). Animals in group 2 were inoculated with FZ06A (P3), which was used as an HP-PRRSV positive control, at the same doses and methods as above. Animals in group 3 were inoculated with a Marc-145 cell culture as above for use as a negative control.

## ***2.9 Clinical observations***

After challenge, general clinical signs, including depression, loss of appetite, coughing, labored abdominal breathing, and increased respiratory rate, in the animals were monitored daily. The rectal body temperature of each pig was recorded daily until 28 dpi. Body weight was measured at 0, 7, 14, 21, and 28 dpi. Clinical signs after inoculation were scored as previously described (Li et al., 2014).

## ***2.10 Quantification of PRRSV***

Viral RNA was extracted from serum using an AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Biosciences, USA) and from tissues using TaKaRa MiniBEST Universal RNA Extraction Kit (Takara Bio, Japan). Viral RNA extracts were then used as templates for one-step qRT-PCR with One Step SYBR PrimeScript RT-PCR Kit II (Perfect Real Time; Takara Bio, Japan). RNA was extracted and the real-time RT-PCR assay was performed according to the manufacturers' instructions. Real-time quantitative reverse transcription PCR (RT-qPCR) was used to quantify PRRSV genomic cDNA copies (Guo et al., 2013). A recombinant plasmid containing PRRSV ORF7 was used to construct a standard curve. The plasmid was 10-fold serial diluted, with concentrations ranging from  $10^2$  to  $10^{10}$  copies/mL.

## ***2.11 Humoral immune response***

Serum samples were collected at 1, 3, 5, 7, 10, 14, 18, 21, 24, and 28 dpi, and PRRSV-specific antibodies were measured using a commercially available ELISA kit (IDEXX, U.S.A.). Based on the manufacturer's guidelines, sample-to-positive control (S/P) ratios greater than 0.4 were considered positive.

## ***2.12 Cytokine detection in serum***

Levels of IL-2, IL-6, IL-10, IFN- $\alpha$ , IFN- $\gamma$ , and tumor necrosis factor (TNF)- $\alpha$  were measured in serum samples using commercial pig IL-2, IL-6, IL-10, IFN- $\alpha$ , IFN- $\gamma$  and TNF- $\alpha$  ELISA kits (Uscn Life Science Inc., China) according to the manufacturer's instructions. The minimum detectable concentrations for IL-2, IL-6, IL-10, IFN- $\alpha$ , IFN- $\gamma$ , and TNF- $\alpha$  were 5.9, 6.2, 6.5, 6.3, 2.8, and 6.1 pg/mL,

respectively.

### ***2.13 Detection of virus in nasal secretions and tissues***

Nasal secretions samples were collected at 1, 3, 5, 7, 10, 14, 18, 21, 24, and 28 dpi and used for RT-PCR detection of PRRSV. At the end of the experiment, all infected and control animals were euthanized. Lungs, spleens, kidneys, livers, and lymph nodes were harvested, and virus was detected in these tissues by means of RT-PCR assays. All tissue samples were also analyzed by PCR for the presence of PPV, PCV, and CSFV.

### ***2.14 Gross pathology and histological examination***

Pathological examinations were performed at 28 dpi, after which time all piglets were euthanized. Lung sections were collected from each pig and then fixed in 4 % paraformaldehyde fix solution for macroscopic (gross) pathological analysis. In addition, some lungs tissues were collected and processed for histopathologic examination followed by hematoxylin and eosin staining, as described previously (Halbur et al., 1995). The lung lesions were graded as described previously (Li et al., 2014). IHC was also performed with a mouse monoclonal antibody to PRRSV N protein to detect the distribution of PRRSV antigen in lung samples as described previously (Weesendorp et al., 2014).

### ***2.15 Statistical analysis***

GraphPad Prism 5 software (GraphPad Software, Inc., USA) was used for the data analysis. The data are expressed as the mean  $\pm$  standard error of the mean. Differences in humoral immune responses, as measured by cytokine production and viremia, between the different groups were determined by one-way ANOVA. Differences were considered statistically significant at  $p < 0.05$ .

## **3 Results**

### ***3.1 Characterizations of the full genome of PRRSV isolate HB17A***

The genome sequence of strain HB17A was deposited in GenBank under accession no. MG844181. The complete sequence of the HB17A genome is 15,011 nucleotides in length, with a 191-nt 5' untranslated region (UTR) and a 148-nt 3' UTR, excluding the poly (A) tail. The complete nucleotide sequence of the isolate was compared with those of six other PRRSV isolates with sequences deposited in GenBank (Table 5). We found that the HB17A strain shared 85 % sequence identity with VR-2332 (North America type), but only 60.4 % identity with Lelystad (LV, the European type), indicating that HB17A belonged to PRRSV type 2. The 5'UTR and 3'UTR of HB17A shared 91 % and 89.1 % nucleotide identities, respectively, with those of HP-PRRSV JXA1; 96.3-96.6 % and 94.6-96.6 % nucleotide identities, respectively, with NADC30 and NADC30-like PRRSV (NADC30 and HNyc15);

and 60.4-92.1 % and 78.2-91.2 % nucleotide identities with the vaccine strains CH-1a, VR-2332, and LV. ORF1a and ORF1b of HB17A shared 80-86.6 % nucleotide identity with those of HP-PRRSV JXA1, 91.6-95.7 % nucleotide identity with those of NADC30 and NADC30-like PRRSV (NADC30 and HNyc15), 80.9-87.2 % nucleotide identity with those of the vaccine strain CH-1a, and 82.3-87.4 % nucleotide identity with those of VR-2332, but only 55.3-63.5 % nucleotide identity with those of LV. ORF2-7 were comparatively conserved, with nucleotide identities of 83.9-90.9 % with HP-PRRSV JXA1 and 81.4-98.3 % with NADC30 and NADC30-like PRRSVs (NADC30 and HNyc15). In contrast, HB17A only showed 61.9-71.6 % identity with LV (Table 5). Strain HB17A shared high nucleotide identity with strains of NADC30 and NADC30-like PRRSV.

**Table 5.** Detailed comparison of the full-length genomes of HB17A and six reference strains of PRRSV.

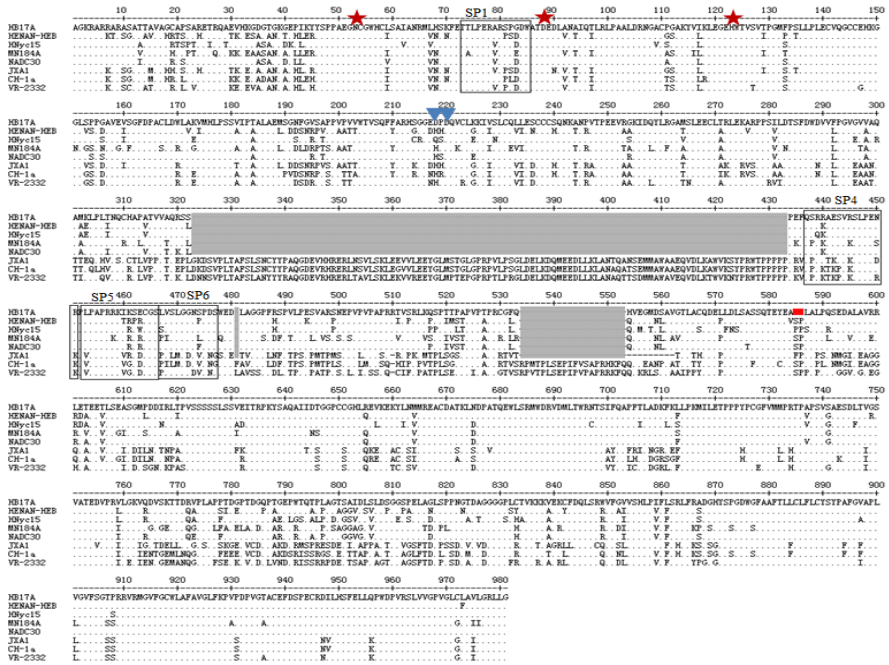
HB17A %	Identity to HB17A					
	LV	VR- 2332	CH-1a	JXA1	NADC 30	HNyc 15
	Nucleotide (%)					
5' UTR	63.2	91.5	92.1	91	96.8	96.3
ORF1a	55.3	82.3	80.9	80	94.2	91.6
ORF1b	63.5	87.4	87.2	86.6	95.7	93
ORF2	66	86.8	86.9	86.1	95.1	88.1
ORF3	65.9	84.7	83.7	83.9	94.6	85.2
ORF4	68.9	88.1	86.8	85.8	96.8	85.5
ORF5	61.9	84.6	86.2	85.6	95.5	81.4
ORF6	71.6	89.5	88.2	89.1	98.3	96.2
ORF7	66.4	91.9	90.3	90.9	96.5	94.9
3' UTR	78.2	91.2	87.8	89.1	96.6	94.6
Comple te	60.4	85	84.1	83.4	95.1	83.6
	Amino acid (%)					
NSP2	32.3	69.1	66.3	65.7	90.1	85.6

GP2	62.8	87.2	87.5	85.2	95.7	89.9
GP3	58.3	84.3	83.1	83.1	92.9	85.5
GP4	70.9	87.7	87.7	87.7	97.8	88.8
GP5	55.3	82.1	84.1	83.6	92	80.6
M	81	93.1	92	93.1	98.9	96.6
N	62	93.5	91.1	92.7	96.8	96.8

### 3.2 Comparative analysis of the *Nsp2* region

*Nsp2* is the least conserved protein in PRRSVs, with the presence of various deletions. To study the deletion regions, the HB17A *Nsp2* gene was compared with those of representative strains of type 2 PRRSV from China and other countries. In addition to the unique discontinuous 30-amino acid deletion in Chinese HP-PRRSV strains (Figure 11), deletions (131-amino acid) identical to those in NADC30 (Brockmeier et al., 2012) and MN184A isolated in the United States (Han et al., 2006) and NADC30-like HNyc15 emerging in China (Zhou et al., 2015) were observed, namely a 111-amino acid deletion at positions 323-433, a 1-amino acid deletion at position 481, and a 19-amino acid deletion at position 534-553, relative to the sequence of prototype type 2 strain VR-2332. Meanwhile, an additional 2-amino acid deletion at positions 585-586 was also found in the *Nsp2* of HB17A (Figure 11). Moreover, the protease domain 2 (PLP2) region was highly conserved in strain HB17A, with 41, 19, 39, 14, 43, 38, and 30 substitution sites, as compared to HENAN-HEB, HNyc15, MN184A, NADC30, JXA1, CH-1a, and VR-2332, respectively (Figure 11). There was a mutation in the ovarian tumor (OTU) catalytic triad (C54, H218, and D220) in H218D (Figure 11). In the present study, isolate HB17A was found to have four putative B cell epitopes (with deletions in the SP2, SP7, and SP8 epitopes). Overall, the epitope sites SP1, SP4, SP5, and SP6 of strain HB17A were most similar to those of NADC30 and NADC30-like PRRSVs, and least like those of JXA1-like PRRSVs.

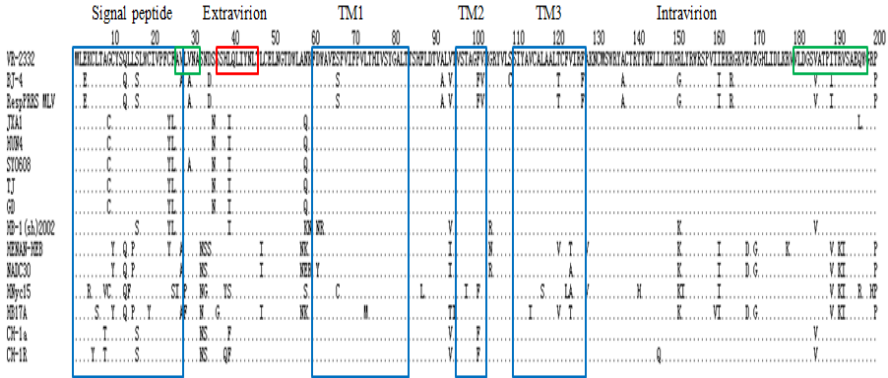




**Figure 11.** Alignment of the putative amino acid sequence of Nsp2. Putative B cell epitopes are highlighted in black boxes. The amino acid residues forming the putative catalytic triad C54, D88, and H123 are marked with red stars. The residues recognized as critical for OTU activity include C54, H218, and D220; H218 and D220 are marked with blue arrows. Deletions sites are indicated in grey rectangles, the new deletion is indicated with a red rectangle.

### 3.3 Sequence alignment and analysis of GP5

Because of the high sequence variation and its presumed immunological significance in PRRSV infection, GP5 has often been used for the analysis of PRRSV genetic diversity. In this study, the amino acid sequence of GP5 in strain HB17A was similar to those in virulent strains NADC30, HENAN-HEB, and HNyc15 at Q13 and K151, and different from that of HP-PRRSV in China. Compared to the reference strains (VR-2332, CH-1a, CH-1R, JXA1, HUN4, BJ-4, TJ, SY0608, GD, HB-1(sh)/2002, RespPRRS MLV, NADC30, HENAN-HEB, and HNyc15), the HB17A strain showed significant differences in the signal peptide, transmembrane (TM), primary neutralizing epitope (PNE) (amino acids 37-45), and non-neutral epitope (amino acids 27-30 and 180-197) regions. In other regions, there were varying degrees of difference between the strains (Figure 12).



**Figure 12.** Alignment of ORF5 amino acid sequences. Functional domains such as the signal peptide and extra-virion, transmembrane (TM), and intra-virion domains are labeled with dashed boxes. The primary neutralizing epitope (PNE) and non-neutral epitopes are indicated in green and red boxes, respectively.

Compared with the VR-2332 strain, the HB17A isolate had no amino acid substitutions in the PNE region, but there were two V27A and L28F mutations in the GP5 decoy epitope. To gain further insight into the genetic evolution of HB17A, we analyzed the variation in potential N-glycosylation sites (NGSs) using the NetOGlyc 4.0 Server. The potential NGSs were observed at six different positions: N30, N33, N34, N35, N44, and N51. GP5 of the HB17A strain shared six common N-glycosylation sites at amino acid positions 30, 33, 34, 35, 44, and 51 (Table 6) with those of the HP-PRRSV JXA1 strain, but at positions 33 and 35, strain HB17A showed differences from other NADC30 or NADC30-like strains.

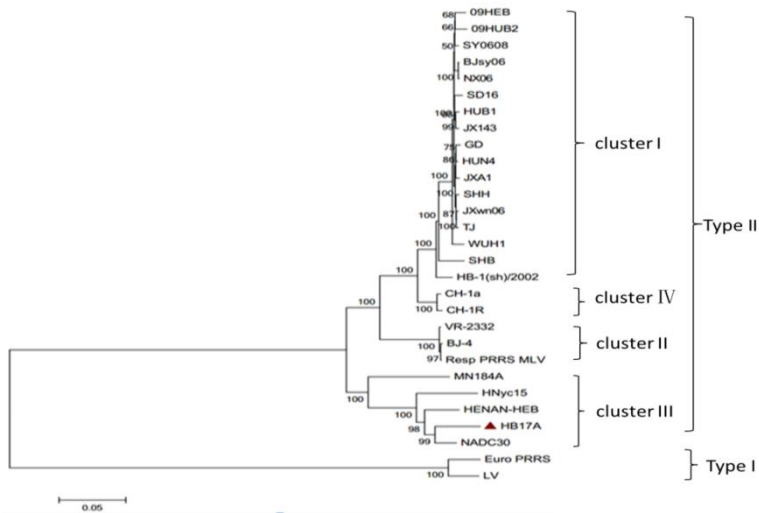
**Table 6.** Potential N-glycosylation sites in PRRSV strains.

Strain name	N-glycosylation sites					
	30	33	34	35	44	51
VR-2332	N	N	D	S	N	N
JXA1	N	N	N	N	N	N
CH-1a	N	S	N	S	N	N
HENAN-HEB	N	S	S	S	N	N
NADC30	N	S	N	S	N	N
HNyc15	N	G	N	S	N	N

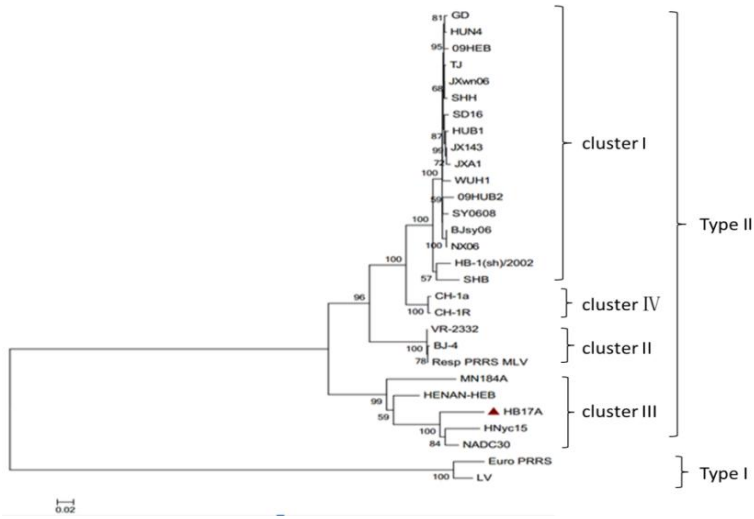
MA184A	N	S	N	S	N	N
HB17A	N	N	N	N	N	N

### 3.4 Phylogenetic analysis

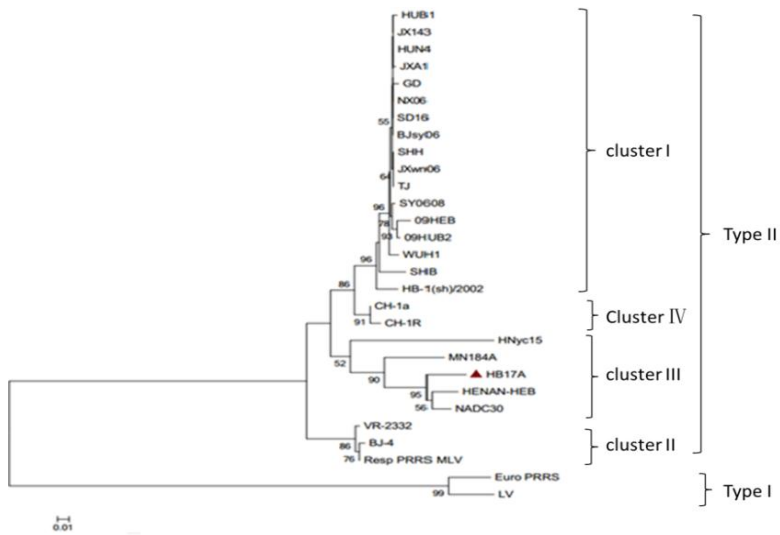
To understand the genetic relationships of HB17A and other representative strains, a phylogenetic analysis of HB17A was performed based on the nucleotide sequences of the complete genome, Nsp2, and ORF5 using a distance-based neighbor-joining method with 1000 bootstrap replicates in MEGA5 software, as shown in Figure 13. A phylogenetic analysis showed that the PRRSV isolates separated into two types. PRRSV type II, with HNyc15, MN184A and HENAN-HEB, HB17A, were clustered with NADC30 (cluster III). The HP-PRRSV field isolates formed another cluster with JXA1 (cluster I), and all classical genotype 2 PRRSV vaccine strains and field isolates, represented by Ingelvac MLV and Ch-1a, clustered in a separate branch (cluster II and cluster IV).



(a)



(b)



(c)

**Figure 13.** Phylogenetic trees of the nucleotide sequences of complete genomic sequences (a), Nsp2 (b), and ORF5 (c) of 28 PRRSV strains constructed using the distance-based neighbor-joining method in MEGA software (Version 5.1). Bootstrap values were calculated from 1000 replicates.

### 3.5 Recombination analysis

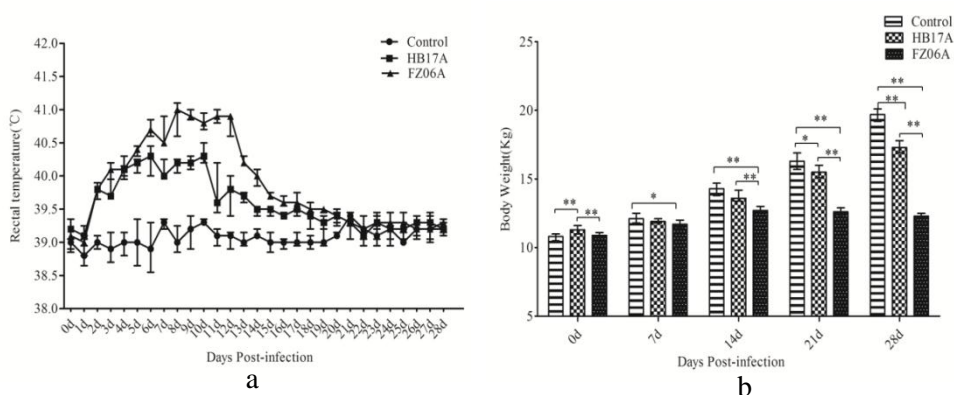
To determine whether recombination events played a role in the generation of HB17A, possible recombination events were examined in the Recombination Detection Program and confirmed in SimPlot. The complete genome of HB17A was used as the query sequence. The results showed that there were no obvious recombination signals, as there were in HP-PRRSV strain JXA1 and the prototype strain CH-1a (data not shown).

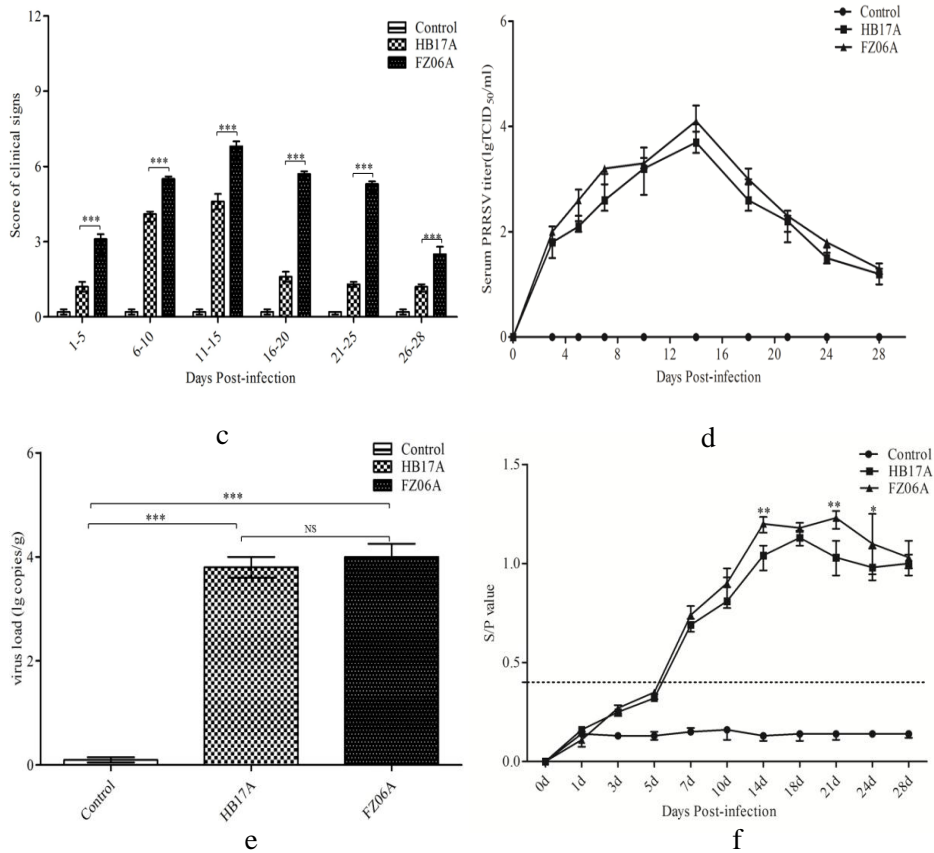
### 3.6 Observation of clinical signs during infection

The mean rectal temperature and clinical signs of infection were recorded daily throughout the experiment, and no clinical signs typical of PRRS were observed in negative control animals. The mean rectal temperature of animals challenged with the HB17A strain was mildly elevated to 40 °C at 3 or 4 days post infection (dpi) for 4-6 days; however, this temperature did not exceed 40.5 °C during the observation period. In the FZ06A infection group, rectal temperatures were significantly increased. From 3 dpi, rectal temperatures sharply increased to 40 °C, and this temperature persisted for 8-10 days; in some challenged animals, the temperature exceeded 41 °C at 8 dpi. There was a significantly higher rectal temperature in FZ06A group than other groups (Figure 14a).

Weight gain is shown in Figure 14b. Through the experiment, the control group exhibited significant weight gain. Compared to the control group, the HB17A infection group showed only mild weight gain. In contrast, pigs in the FZ06A infection group lost a significant amount of weight. They were obviously emaciated after 21 dpi.

FZ06A-inoculated pigs developed more severe clinical symptoms, including depression, anorexia, lethargy, and respiratory distress (coughing and sneezing), whereas HB17A-infected pigs showed moderate clinical signs, with significantly lower clinical symptom scores than those in the FZ06A-inoculated group ( $p < 0.001$ ) throughout the experiment (Figure 14c). No obvious clinical signs were observed in the control group during the experimental period.





**Figure 14.** Rectal temperatures, body weights, clinical scores, viremias and viral RNA loads in lungs, and virus-specific antibody responses in piglets. (a) Mean rectal temperatures and (b) body weights in pigs infected experimentally with PRRSV FZ06A and HB17A and negative control pigs. (c) Clinical scores are shown. (d) Viral loads in the sera of pigs at different dpi. (e) Viral RNA loads in the lungs of pigs. Viral RNA loads in the lungs of pigs at 28 dpi (log copies/g) were measured by qRT-PCR. (f) Values represent average titers from five animals. The dashed line at an S/P ratio of 0.4 designates the threshold above which titers were considered positive for anti-PRRSV antibodies. Data from groups of n=5 pigs were evaluated by one-way ANOVA (\* $p < 0.05$ ) (\*\* $p < 0.01$ ) (\*\*\*) $p < 0.001$ ).

### 3.7 Viral loads in serum samples

Total RNA was extracted from serum and tissues, and one-step SYBR quantitative real-time (qRT)-PCR was performed to determine the PRRSV RNA copy number as described (Guo et al., 2013). Sera from FZ06A-infected pigs showed increased viral titers since 4 dpi, which peaked at 14 dpi. The HB17A infection group also showed increased viral titers. However, PRRSV RNA copy numbers in pigs in the HB17A infection group were lower than that in pigs in the FZ06A infection group (Figure

14d), but this difference was not significant. The levels of PRRSV RNA in lungs were also evaluated by qRT-PCR. The results showed little or no significant difference between the FZ06A and HB17A challenge groups. PRRSV RNA was not observed in the controls at any point in the experiment (Figure 14e).

### ***3.8 Antibody detection post infection***

Blood samples were collected at 1, 3, 5, 7, 10, 14, 18, 21, 24, and 28 dpi to measure PRRSV-specific antibodies using a commercially available enzyme-linked immunosorbent assay (ELISA) (IDEXX Laboratories, U.S.A.) kit. Beginning at 7 dpi, all PRRSV-inoculated pigs were seropositive for PRRSV-specific antibody (S/P>0.4) by ELISA. In PRRSV-infected pigs, the S/P ratio then gradually increased until the end of the experiment, whereas no PRRSV-specific antibodies were detected in control animals (Figure 14f).

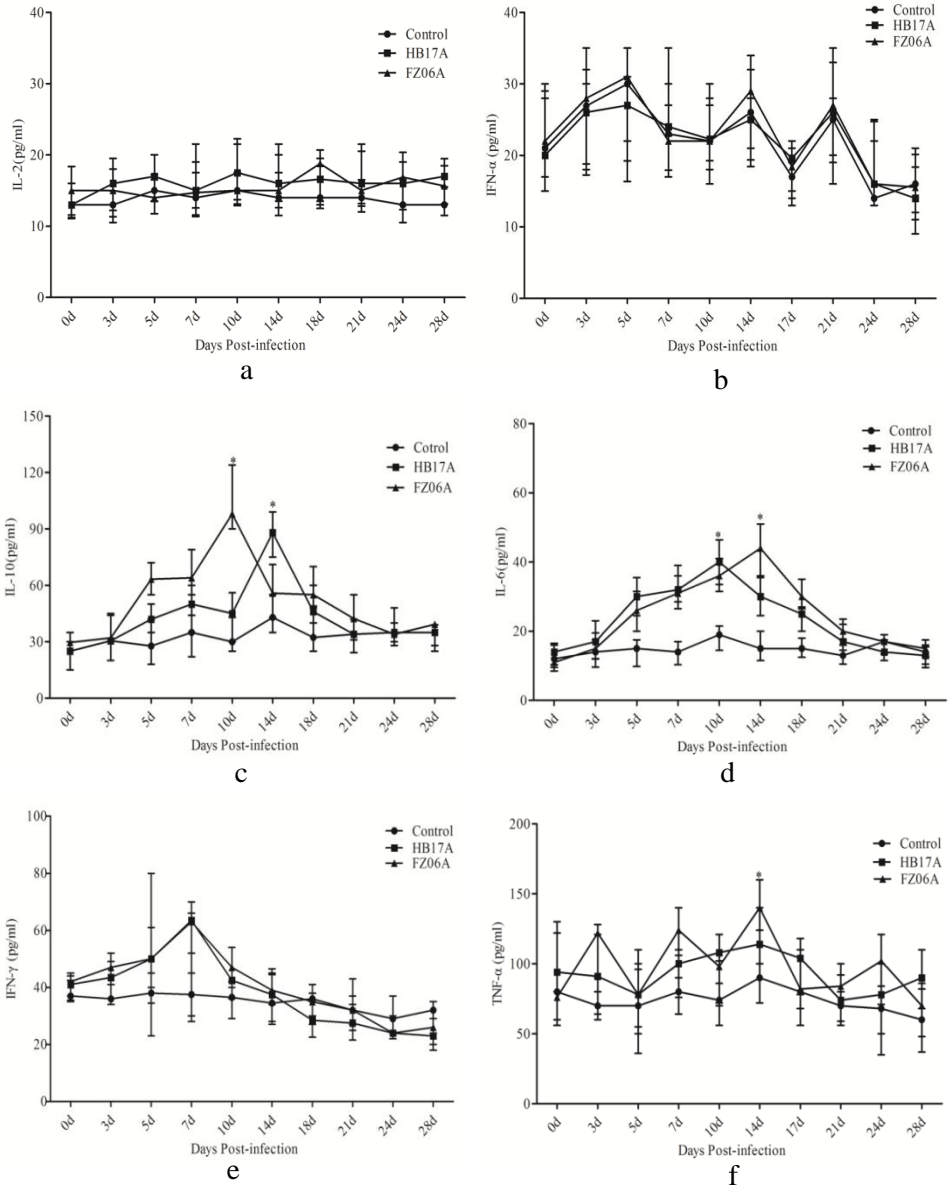
### ***3.9 Virus detection in nasal secretions and tissue samples***

PRRSV could not be detected in nasal swab samples from any of the control pigs, but PRRSV was detected in most pigs in the PRRSV infection groups at 3, 7, and 10 dpi (data not shown). At 10 dpi, no virus was detected in nasal swab samples from pigs in the FZ06A infection group, whereas PRRSV was detected in 2 out of 5 pigs in HB17A infection group. In both infection groups, no virus was detected in pigs from 10 dpi to the end of the experiment.

Virus detected in tissue samples collected from negative controls was negative for PRRSV, whereas virus was detected in all evaluated organs from inoculated pigs. All tissue samples from the control and PRRSV-inoculated animals were negative for pseudorabies virus (PRV), porcine circovirus (PCV), or classical swine fever virus (CSFV) (data not shown).

### ***3.10 Cytokine concentrations in serum***

Cytokine levels in sera were evaluated by multiplex ELISA and results showed that serum concentrations of interleukin (IL)-2 and interferon (IFN)- $\alpha$  were not significantly different in the PRRSV infection groups compared with the control group ( $p>0.05$ ; Figure 15a and b). IL-6 and IL-10 levels were enhanced at 10 and 14 dpi, respectively, in the HB17A infection group compared with the control group ( $p<0.05$ ; Figure 15c and d), but enhanced at 14 dpi and 10 dpi, respectively, in the FZ06A infection group ( $p<0.05$ ; Figure 15c and d). IFN- $\gamma$  levels in both the HB17A-infected and FZ06A infection groups were enhanced at 7 dpi compared with those in the control group ( $p<0.05$ ; Figure 15e). The serum concentrations of TNF- $\alpha$  in the HB17A infection group were not significantly increased, but concentrations in the FZ06A infection group at 14 dpi were elevated compared to those in the control group ( $p<0.05$ ; Figure 15f).



**Figure 15.** Mean pro-inflammatory cytokines levels. Pro-inflammatory cytokines were monitored throughout the experiment. Mean levels of IL-2 (a), IFN- $\alpha$  (b), IL-10 (c), IL-6 (d), IFN- $\gamma$  (e), and TNF- $\alpha$  (f) are presented.

### 3.11 Gross pathology and histological evaluations of lungs

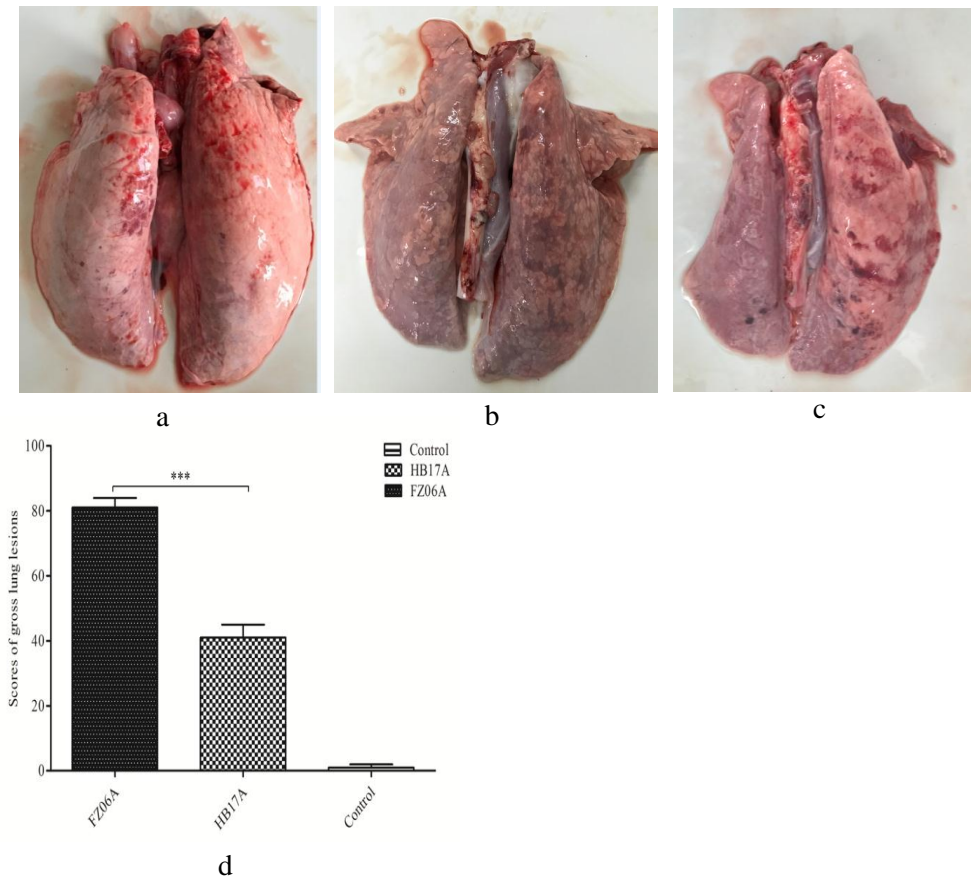
No macroscopic (gross) lesions were observed in lungs from pigs in the control

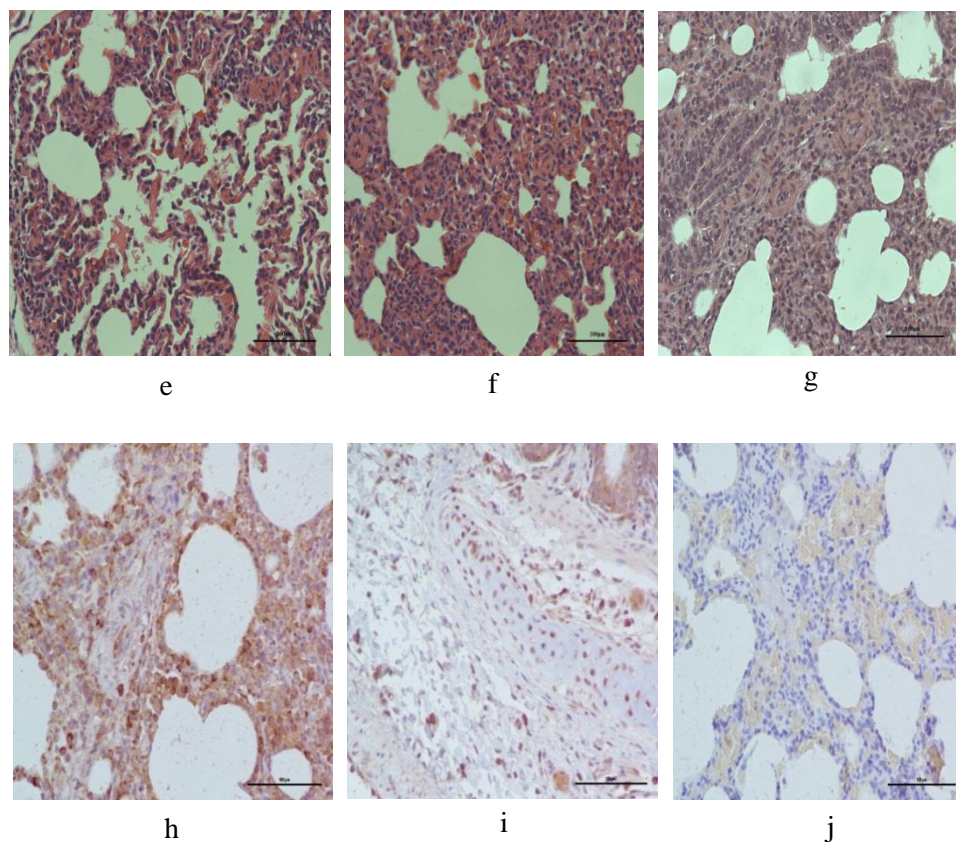


group (Figure 16a). Lung lesions in pigs infected with FZ06A exhibited severe multifocal lesions and lung tissue consolidation (Figure 16b). In contrast, mild pathological lesions were observed in the lungs collected from HB17A-infected pigs (Figure 16c). There was no statistically significant difference in clinical score of pigs in the two PRRSV-challenged groups. The average score of gross lung lesions was significantly higher ( $p<0.01$ ) in the FZ06A infection groups than in the HB17A infection groups (Figure 16d).

Histological examination revealed interstitial pneumonia associated with hemorrhage and infiltration of mononuclear cells in lung tissues collected from FZ06A-infected and HB17A-infected pigs (Figure 16e, 16f), whereas no lung tissues from control pigs exhibited any features of acute PRRSV infection (Figure 16g).

Immunohistochemistry (IHC) results showed positively stained epithelial cells and macrophages in the lungs of pigs challenged with either FZ06A or HB17A (Figure. 16h, 16i). In contrast, no stained cells were observed in tissues from pigs in the control group (Figure. 16j).





**Figure 16.** Gross and microscopic observations of lungs and the detection of PRRSV in lungs of piglets by IHC. (a) No macroscopic (gross) lesions were found in lungs collected from the control animals. (b) Severe multifocal lung lesions and consolidation of lung tissue in an FZ06A-infected pig. (c) Mild multifocal lung lesions and consolidation of lung tissue in a HB17A-infected pig. (d) The mean scores of gross lung lesions in each group are shown. Gross lesions were graded based on the percentage of lung area affected. (e) The lung tissues collected from pigs infected with HPPRRSV strain FZ06A showed severe interstitial pneumonia with thickening of the alveolar septa and disappearance of the alveoli. (f) Lung tissues collected from pigs infected with strain HB17A showed mild interstitial pneumonia and disappearance of the alveoli. (g) Lungs from control pigs showed no features of acute PRRSV infection. Positively stained bronchial epithelial cells and macrophages were found in lungs from piglets challenged with PRRSV strains FZ06A (h) and HB17A (i). No stained cells were observed in tissues from the negative control group (j).

## 4 Discussion

PRRS is a globally important disease of swine caused by a virus that has continued to evolve rapidly worldwide. The disease has led to substantial economic losses in

the global swine farming industry since the virus emerged in the 1990s (Lunney et al., 2010). Although considerable efforts have been made to control this disease, there are still no effective vaccines available. In 2006, a highly pathogenic strain of PRRSV emerged in an outbreak in southern China and become the dominant strain in subsequent years (Zhou et al., 2009). In recent years, a new strain, NADC30-like, with extensive mutations was reported in China (Zhou et al., 2009). The first case of NADC30-like PRRSV was reported in 2013 (Zhou et al., 2015). After that, several outbreaks of NADC30-like PRRSV were reported in vaccinated pigs in at least 8 provinces in China (Li et al., 2016). Since its first outbreak, researchers had paid high attention to NADC30-like PRRSV. Compared with VR-2332, the Nsp2 gene of NADC30-like strain contains a discontinuous 131-amino acid deletion, which resembles those in the NADC30 and MN184A strains. Genetically diverse PRRSV strains, especially NADC30 or NADC30-like strain, have been associated with local outbreaks and new epidemics of PRRSV in China (Key et al., 2001; Wang et al., 2012). However, knowledge on the molecular diversity and evolution of this virus has been insufficient. The aim of this study was to elucidate the molecular and evolutionary characteristics of a new PRRSV strain isolated from a pig on a farm in northern China in 2017 as a reference for strategies to prevent and control PRRSV.

HB17A, an NADC30-like PRRSV, that was isolated from a diseased pig in 2017 in Hebei province, is well characterized. The complete nucleotide sequence of HB17A was previously determined and compared with representative PRRSV strains isolates from China and other countries. Genome-wide analysis revealed that HB17A shared 95.1 % and 83.4 % nucleotide similarity with NADC30 (JN654459) and HP-PRRSV representative strain JXA1 (EF112445), respectively.

The Nsp2 gene exhibits the highest genetic diversity in the PRRSV genome (Nelsen et al. 1999). This gene is crucial for viral replication and modulation of host immunity because of its protease activity. Compared with Nsp2 of VR-2332, the Nsp2 genes of NADC30-like strains contain three discontinuous deletions (131-amino acids), which can be used as molecular markers to distinguish them from other PRRSV strains (Zhao et al., 2015). These deletions were also found in strains NADC30 and MN184A. However, the regions with deleted amino acids were different from those previously reported. In addition to the 131-amino acid deletions, we also found a 2-amino acid deletion at amino acid positions 585-586 that was not found in other NADC30 or NADC30-like strains. PRRSV Nsp2, a multidomain protein located immediately downstream of the first two nonstructural proteins, possesses a papain-like protease domain 2 (PLP2) at amino acids 47-220 that is well conserved not only among PRRSV strains but also among other members of the *Arteriviridae* family. Additionally, PLP2 of Nsp2 belongs to the OTU protease family, which inhibits NF- $\kappa$ B activation (Sun et al., 2010). In this study, the Nsp2 PLP2 domain of PRRSV strain HB17A was characterized. Compared to those in HENAN-HEB, HNyc15, MN184A, NADC30, JXA1, CH-1a, and VR-2332, amino acids in the HB17A stain were highly conserved at sites 41, 19, 39, 14, 43, 38, and 30, respectively (Figure 11). The HB17A amino acid sequence was highly similar to that of strain NADC30. Amino acid residues (C54, D88, C110, H123, W124, C141,

and C145) of PLP2 that are responsible for Nsp2 protease activity were found to be conserved in the HB17A strain, but a mutation was identified in the OTU catalytic triad (amino acids C54, H218, and D220) in strain H218D, relative to that in other strains (Figure 11) (Han et al., 2006; Komander et al., 2010). Previous reports indicated that a deletion in the Nsp2 PL2 domain conferred lethality to PRRSV. Determining whether the mutations identified are related to the virulence of the virus needs will require more research. Previous studies have also shown that Nsp2 contains the highest frequency of B cell epitopes (Yan et al., 2007; Oleksiewicz et al., 2001), indicating that Nsp2 in PRRSV is an immunogenic protein capable of eliciting the production of specific antibodies during infection. The HB17A isolate exhibited four putative B cell epitopes (with deletions in the SP2, SP7, and SP8 epitopes). Overall, the epitope sites SP1, SP4, SP5, and SP6 in the HB17A strain were most similar to those in NADC30 or NADC30-like PRRSVs, but they differed from those in JXA1-like PRRSVs, and these epitopes showed a high proportion of variable sites. Taken together, these data indicate that related monoclonal antibodies might be useful as novel PRRSV vaccines to prevent NADC30-like PRRSVs.

ORF5, which encodes the GP5 protein, was the most frequently mutated region in the PRRSV genome. It has often been used to analyze the genetic variation in PRRSV. The primary neutralizing epitope (PNE) at amino acids 37-45 of GP5 and decoy epitope V27LVN is important to induce immune responsiveness (Li et al., 2009). Extensive amino acid substitutions were also observed in GP5 of strain HB17A, particularly in the putative neutralization epitope, N-glycosylation sites, and other immunologically relevant positions. In our study, compared with the VR-2332 strain, isolate HB17A did not exhibit any amino acid mutations in the PNE region. Additionally, the epitopes at amino acid positions 27 to 30 can act as a decoy epitope, eliciting antibodies against GP5 and delaying the production of neutralizing antibodies, thereby diminishing an immune response. In this study, there were two mutations (V27A and L28F) in the GP5 decoy epitope. Interestingly, strains in cluster 3 almost all had the V27A mutation, which was not present in strains in other clusters, but only HB17A had the L28F mutation. N-glycosylation sites in GP5 have been reported to be involved in viral immune evasion and neutralizing antibody responses (Ostrowski et al., 2002; Ansari et al., 2006). Putative N-glycosylation sites were observed in GP5 of HB17A at amino acid positions 30, 33, 34, 35, 44, and 51, respectively (Table 6). Compared with positions 33 and 35 in other NADC30 or NADC30-like strains, strain HB17A strain showed differences; however, amino acids in these positions were the same as those in HP-PRRSV JXA1.

In addition to mutations, genetic recombination also plays an important role in the evolution of PRRSV. Several recent studies have shown genetic exchanges between NADC30 and previously dominant circulating strains in China (Zhao et al., 2015; Zhou et al., 2015). Moreover, HB17A, analyzed in SimPlot, presented no obvious recombination signals. This also similar to the isolate strain HNjz15, which is also belonging to cluster III but have no recombination. Given that HB17A was isolated from a pig on a farm with clinical outbreaks of PRRS, we investigated the pathogenicity of this strain in pigs. We compared the virulence of HB17A and

Chinese HP-PRRSV strain FZ06A. The results showed that strain HB17A induced a febrile response that was similar to that of FZ06A, but that the pigs' temperatures soon returned to normal. FZ06A caused an extended febrile response. Mild clinical signs such as weight loss were also observed in HB17A-infected pigs. In the HB17A infection group, there was no mortality, whereas, in the FZ06A infection group four (out of five) inoculated pigs died from 7 dpi to 21 dpi.

Overall, increased virulence was associated with higher titers of virus *in vivo*. We initially detected HB17A and FZ06A in serum samples at 3 dpi; both viremia peaked at 14 dpi and then decreased; however, the average quantity of serum HB17A RNA was significantly lower than that of FZ06A. The levels of HB17A and FZ06A RNA in lungs were not significantly different. Interestingly, virus persisted longer (to 10 dpi) in pigs in the HB17A infection group, based on testing of nasal swabs. This was longer than that in the FZ06A infection group. The longer duration but lower pathogenicity may also indicate that the virulence of HB17A is less than that of FZ06A. This may also indicate an invisible threat to pigs. In addition, development of specific antibodies in both HB17A-infected and FZ06A-infected pigs was observed by ELISA as early as 7 dpi. Pigs in the control group remained seronegative throughout the experiment.

Histopathological examinations of tissues were also performed. Pathological changes were found in almost every tissue examined in this study. The severity of lung lesions was used to compare the virulence, because respiratory symptoms are the main clinical manifestation of PRRSV infection. In this study, lungs from HB17A-infected animals showed interstitial pneumonia with slight thickening of the alveolar septa and the disappearance and atrophy of alveoli, relative to that in the FZ06A-infected animals.

Elevated levels of multiple cytokines were associated with severe morbidity and mortality (Guo et al., 2013; Gomez-Laguna et al., 2013). In this study, we tested the serum of pigs for three innate immunity cytokines (IFN- $\alpha$ , TNF- $\alpha$ , and IL-6) and three adaptive immunity cytokines (IL-2, IL-10, and IFN- $\gamma$ ). The results showed that the IFN- $\gamma$ , IL-6, and IL-10 cytokine levels were significantly elevated in the serum, but the levels of IFN- $\alpha$  and IL-2 in infected and control animals were not significantly different. In addition, cytokines in serum were generally low in those animals infected with HB17A, which may be the reason for the diminished clinical signs and lesions in this group. This may be one reason that why HB17A appeared less virulent than FZ06A. Several studies have shown that IFN- $\gamma$  can inhibit PRRSV infection (Van Reeth et al., 2000). In our study, an increase in the expression of IFN- $\gamma$  levels was observed at 7 dpi after infection with HB17A and FZ06A. However, viral replication still occurred following the induction of IFN- $\gamma$ . We concluded that this temporary IFN- $\gamma$  response was not sufficient to eliminate PRRSV. IL-6 was accompanied with the onset of clinical respiratory disease and increased body temperature (Van Reeth et al., 2000). In our study, IL-6 levels in animals infected with HB17A and FZ06A were elevated at 10 and 14 dpi, respectively. At these time points, pneumonia and other clinical signs were apparent. Increased TNF- $\alpha$  expression has been associated with apoptosis in the lungs of

swine experimentally infected with PRRSV (Labarque et al., 2003); in the present study, we found that TNF- $\alpha$  level in serum in response to HB17A were maintained at a similar level throughout the experiment, but levels in the FZ06A infection group were high. These results were consistent with the difference in clinical signs and lung damage observed in pigs in these two groups. IL-10 levels were elevated in response to HB17A and FZ06A, which were consistent with Mateu's (Mateu & Diaz 2008) result.

In summary, we report the complete genome sequence of a novel Chinese PRRSV-2 isolate isolated in the Hebei province of China. Genomic characterization showed that HB17A had a unique 2-amino acid insertion in Nsp2, and a phylogenetic analysis indicated that HB17A was closely to NADC30-like strains and subtype 2 strains. There was no evidence of recombination in HB17A. In addition, mutations in GP5 were also observed in strain HB17A. HB17A showed less pathogenicity but longer persistence, relative to these measures in HP-PRRSV. The pathogenesis of HB17A was similar to that of most NADC30-like strains tested. At present, NADC30-like PRRSVs are dominant circulating strains. They have undergone rapid evolution and recombination with other HP-PRRSVs and attenuated vaccine strains. Further, we identified a new deletion strain with no recombination. This can serve as a reminder to search NADC30-like PRRSV strains for different deletions. Our findings contribute to an understanding of the evolutionary characteristics of Chinese PRRSV and data on NADC30-like isolates in China. Our study may also suggest strategies to protect against and control PRRSV infection in swine in China.

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## **The preliminary evaluation of efficacy of PRRS inactivated vaccine based on the viral purification and inactivation methods**

Sui X, Xin T, Willems L, Zhu H\*. The preliminary evaluation of efficacy of PRRS inactivated vaccine based on the viral purification and inactivation methods. (Manuscript in preparation)



**Abstract:** Vaccine immunization is an important method for preventing and controlling porcine reproductive and respiratory syndrome (PRRS). Purified viruses and optimal method for inactivation of viruses are essential for effective vaccine studies. In this study, we compared different purification and inactivation method for the PRRSV, and the purified PRRSV vaccine inactivated with  $\beta$ -propiolactone (BPL) was used for immunization of healthy piglets by intramuscular injection, and the serum antibody levels and degree of protection were determined. Highly pure viral particles were obtained after ultrafiltration and liquid chromatography. The viral particles were analysed by SDS-PAGE and Western blotting. We found that up to 98.5 % of the medium and cellular proteins were removed by ultrafiltration and liquid chromatography. Female BALB/c mice were immunized with the purified viruses, and immunogenicity of the virus was tested by using an immunoperoxidase monolayer assay (IPMA). The results of IPMA indicated that virus purified by ultrafiltration and liquid chromatography had better immunogenicity and higher specificity. The titer of inactivated virus was detected by microfiltration and verified by blind passage for three generations in Marc-145 cells. RT-PCR and Western blot analysis were used to detect viral antigen integrity. We observed that treatment with BPL at 1:2000 for 24 h at 4 °C resulted in quick and complete inactivation of the virus. Both the vaccinated group (five pigs) and mock-vaccinated group (five pigs) were challenged with PRRSV (strain FZ06A) and were then euthanized at 21 days post-infection (dpi). Following viral challenge, the mock group developed significant clinical signs, and at 10 dpi, one of the mock-vaccinated pigs died. In contrast, no obvious clinical signs were observed in pigs vaccinated with the inactivated vaccine. The mean anti-PRRSV neutralizing antibody titers were higher in the vaccinated group than in the mock group. The mean virus copy number in the vaccinated group was significantly lower than that of the mock group. By pathological examination, we observed less severe macroscopic and microscopic lesions in vaccinated pigs than in mock-vaccinated pigs. In conclusion, use of our novel experimental inactivated PRRSV vaccine could induce a degree of virus neutralizing (VN) antibody response and protect pigs against homologous PRRSV challenge.

**Key words:** porcine reproductive and respiratory syndrome virus (PRRSV), virus purification, inactivation, inactivated vaccine, immune effect

## 1 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is caused by porcine reproductive and respiratory syndrome virus (PRRSV) and is one of the most serious infectious diseases in the swine industry. Since its first emergence in the 1980s, PRRSV has had a significant economic impact on the swine industry worldwide. It is estimated that the total economic losses caused by PRRS amount to approximately \$664 million annually in United States (Neumann et al., 2005). PRRS mainly causes respiratory disease in growing pigs and reproductive failure in pigs of breeding age (Christianson 1992; Bøtner 1997; Meulenberg 2000). PRRSV is an enveloped, positive-sense single-strand RNA virus belonging to the *Arteriviridae* family in the order *Nidovirales* (Lunney et al., 2016; King et al., 2018). According to its genetic diversity, PRRSV can be classified into North American (type 2, VR-2332 strain) and European (type 1 Lelystad virus [LV]) genotypes. These two types share approximately 65 % sequence identity (Allende et al., 1999).

To control PRRS, two types of commercial vaccines, modified live-attenuated vaccines (MLVs) and inactivated vaccines, are currently used in many countries, including China (Huang & Meng 2010; Meng 2000; Murtaugh et al., 2011). Inoculation with modified live-attenuated vaccines can provide some degree of protection against PRRSV infections and prevent the spread of this disease. However, live-attenuated vaccines do not provide complete protection against heterologous PRRSV infection, and there is a risk of reversion of MLV strains to virulence as well as a risk of recombination (Meng, 2000; Mengeling et al., 2003). Compared with the MLV vaccines, the inactivated PRRSV vaccine is much safer, though it induces a poor immune response in naïve pigs and fails to protect pigs against heterologous strains (Nilubol et al., 2004; Charerntantanakul, 2012). Previous research demonstrated that vaccination of sows with an inactivated PRRSV vaccine could improve the reproductive performance and health status of piglets (Papatsiros et al., 2006). Pigs vaccinated with an inactivated PRRSV vaccine had significantly higher virus-neutralizing (VN) antibody titers than control pigs (Misinzo et al., 2006).

To our knowledge, the inactivated vaccines currently used against PRRSV display a poor quality of viral antigens. However, high purity, high recovery rate, and good stability of the virus are basic requirements for production of a vaccine of superior quality. Therefore, it is crucial to understand the chemical, physical, and biological properties of the virus (Ali & Roossinck, 2007; Rodrigues et al., 2007). An effective virus purification strategy needs to account for the biochemical properties of the virus and its production characteristics. In previous research, many methods have been investigated for the purification of various viruses, such as density gradient ultracentrifugation methods (Ali & Roossinck, 2007), size-exclusion chromatography methods (Transfiguracion et al., 2003), ion-exchange chromatography methods (Karger et al., 1998) and affinity chromatography methods (Segura et al., 2006; Anderson et al., 2000). Traditionally, sucrose density gradient ultracentrifugation method has been the preferred method for purification of PRRSV

(Meulenberg et al., 1995; Wu et al., 2005). Unfortunately, it has been reported that the strong forces involved in ultracentrifugation may damage the viral particles (Caul et al., 1978). In recent years, liquid chromatography has been used for the purification of PRRSV (Kramberger et al., 2007).

Complete inactivation of PRRSV is critical to ensure the production of a safe vaccine. There are several kinds of inactivants that are used in the preparation of various viral inactivated vaccines, including formaldehyde (F), binary ethylenimine (BEI), and  $\beta$ -propiolactone (BPL) (Diez-Domingo et al., 2005; Zangwill et al., 2004; Bahnemann et al., 1990; Kamaraj et al., 2008). Formaldehyde is the most commonly used inactivant and is widely used for the preparation of a variety of viral inactivated vaccines. BEI is also an effective viral inactivation product, which can destroy viral nucleic acid but does not affect the structure of its protein components. BPL is also a useful inactivant of bacteria, fungi and viruses. Although most of the current inactivated PRRSV vaccines were produced using BEI and BPL, the results were not optimal (Renukaradhya et al., 2015; Scortti et al., 2007). Vaccine manufacturers also assess the efficacy of inactivation procedures by various in-process control tests, such as tissue culture amplification tests and measurement of inactivation kinetics. Incomplete protection of inactivated vaccines against PRRSV infection may be caused by an over-inactivation of the virus, resulting in destruction of neutralizing viral epitopes (Kim et al., 2011). Thus, it is critical to determine the optimal inactivation parameters. Validation of the inactivation process is an essential component of obtaining a high quality vaccine.

In this study, we evaluated the efficacy of a highly purified BPL-inactivated HP-PRRSV vaccine in pigs.

## **2 Methods**

### ***2.1 Virus propagation***

Marc-145 cells were used for virus propagation. Cells were grown to approximately 90 % confluency in Dulbecco's modified Eagle medium (DMEM) (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA) and  $1 \times$  antibiotics in a 5 % CO<sub>2</sub> atmosphere at 37 °C. The cell monolayers were washed three times with DMEM, and inoculated with  $10^5$  TCID<sub>50</sub> PRRSV FZ06A. The infected cells were maintained in DMEM supplemented with 2 % FBS at 37 °C in 5 % CO<sub>2</sub>. When approximately 90 % of the infected cells developed cytopathic effect (CPE), the viruses were harvested by three consecutive freeze/thaw cycles of the infected cells. The harvested viral stock solution was frozen at -80 °C until use.

### ***2.2 Concentration of the virus***

The virus stock containing cell lysate was clarified by low-speed centrifugation at  $5000 \times g$  for 30 min at 4 °C (Gonin et al., 1998). Clarified samples were transferred into sterile containers and further concentrated using an Amicon® 100K device at 4 °C for approximately 2 h.

## **2.3 Purification**

### **2.3.1 Ultrafiltration and liquid chromatography**

Virus purification experiments were performed at room temperature using an ÄKTA Explorer 150 (GE Healthcare, Sweden) automated chromatography system controlled by Unicorn software (version 3.10). Briefly, 2 mL of concentrated clarified PRRS virus stocks were loaded onto a C26/100 sepharose 4 fast flow column (GE Healthcare, Sweden) equilibrated with 0.02 M Tris-HCl (pH 7.5) buffer. The column was washed with 3 bed volumes of equilibration buffer at a flow rate of 0.2 mL/min. The OD<sub>280</sub> absorbance of eluents was monitored continuously. The fractions from the first elution peak were collected and further purified by ion exchange chromatography (IEC).

HiTrap Q FF column was used for IEC. The column was equilibrated with 20 mM Tris-HCl (pH 7.5) until the absorbance reached the baseline (the absorbance shows the components of the flow-through). Purified virus samples were injected into the loading column. Elution was performed with a linear gradient of 0.1 to 1.0 M NaCl in 20 mM Tris-HCl (pH 7.5). The OD<sub>280</sub> absorbance of the eluents was monitored continuously. The fractions were collected and concentrated by using a Millipore ultrafiltration tube.

### **2.3.2 Sucrose density gradients ultracentrifugation**

Purified PRRS viruses from clarified supernatants were harvested by ultracentrifugation through a sucrose cushion. Polyallomer 50 Ultra-Clear tubes (Beckman, CA) were used for the ultracentrifugation. In brief, 8 mL of ice-cold 30 % (w/v) sucrose prepared in TE7 buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7) was gently under-laid in each Ultra-Clear tube. Approximately 4 mL of the clarified supernatant was gently transferred to the top of the sucrose cushion. The tubes were balanced and loaded into pre-cooled rotor buckets (rotor SW27). Virions were pelleted through the sucrose cushion at 30,000 rpm (Optima L-100K Ultracentrifuge, CA) for 3 h at 4 °C. The final viral pellet was resuspended in 2 mL of TE 7 buffer. Purified virions were stored at 4 °C until use.

## **2.4 Quantitative analysis of purified PRRS virus**

The virus suspension was serially diluted 10-fold in DMEM, and 100 µL of virus solution was inoculated onto Marc-145 cells in 96-well cell culture plates at 37 °C in 5 % CO<sub>2</sub> atmosphere for 1 h. The inoculum was then discarded, and 200 µL maintenance medium was added to each well. The plate was then incubated at 37 °C in a 5 % CO<sub>2</sub> atmosphere for 5 days until the cytopathic effect (CPE) appeared. The viral titer was determined by the Reed-Muench method (Pizzi, 1950).

## **2.5 Protein assays**

Total protein concentrations were calculated using the Bradford Protein Assay kit (BioRad, USA) according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as the standard. The formula used was as follows: (virus stock total



protein content - the total protein content of the purified virus solution) / total protein of the virus stock solution; the viral content was determined by TCID<sub>50</sub> and the recycle rate of virus was calculated according to the formula: (virus content of the purified virus solution × its volume) / (virus content of virus solution × its volume) × 100 %.

## ***2.6 Virus inactivation***

### **2.6.1 Binary ethylenimine (BEI)**

BEI was prepared as a 0.1 M stock solutions by stirring 0.1 M 2-bromoethylamine hydrobromide (Sigma, USA) in 0.175 M NaOH at 37 °C for 1 h, as described previously (Bahnmann, 1990). The BEI stock solution was added to the virus suspension to final concentrations of 0.6 %, 0.8 %, 1 % and 1.2 %. The mixture was swirled at 30 °C for 6, 12, 24, 36 and 48 h. The remaining BEI was subsequently neutralized by the addition of 10 % 1 M sterile sodium thiosulfate (Sigma) solution for 2 h.

### **2.6.2 Formaldehyde (F)**

Formaldehyde solution (35 % v/v) (Merck, Germany) was added to the viral suspension to final concentrations of 0.05 %, 0.1 %, 0.2 % and 0.3 %. Formalin-treated viral suspensions were incubated at 37 °C for 6, 12, 24, 36 and 48 h, respectively.

### **2.6.3 β-propiolactone (BPL)**

Viral suspensions were mixed with BPL to final dilutions of 1:1000, 1:2000, 1:3000, and 1:4000 (v/v) on a magnetic stir plate at 4 °C for 6, 12, 24, 36 and 48 h as described previously (Parker 1975). The viral suspensions were then heated to 37 °C for 2 h.

## ***2.7 Analysis of virus inactivation***

### **2.7.1 Virus inactivation verification test**

Inactivated virus was added to monolayers of Marc-145 cells grown in a T-25 flask. After 3 generations of transmission, virus titration was performed on cultivated Marc-145 cells using the standard procedure. After 5 days, the cytopathic effect (CPE) was observed and the 50 % tissue culture infective dose (TCID<sub>50</sub>) was calculated.

### **2.7.2 RT-PCR**

At 6, 12, 24, 36 and 48 h after inactivation, total RNA was extracted, reverse transcribed into cDNA and amplified by PCR with primers of PRRSV ORF7. The PCR reaction conditions were set as follows: an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec, and final extension at 72 °C for 7 min. The amplified products were analysed by electrophoresis in a 1 % agarose gel.

### **2.7.3 Detection of the integrity of inactivated virus antigens by SDS-PAGE and Westernblot**

Inactivated virus was concentrated and separated by SDS-PAGE. Following SDS-PAGE, gels were transferred at 300 mA for 80 min to a nitrocellulose membrane (BioRad, USA) in 1 ×NuPAGE transfer buffer (Invitrogen, Carlsbad, USA) supplemented with 10 % methanol and 0.1 % antioxidant (Invitrogen, Carlsbad, USA). The membrane was then incubated for 2 h with gentle agitation in 5 % (w/v) non-fat dry milk in TBST buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.1 % (v/v) Tween 20, pH 7.5) at room temperature. The blot was incubated with 1:100 dilution of PRRS antiserum (PRRSV) in TBST overnight at 4 °C. Following consecutive washing steps in TBST buffer, the blot was incubated at room temperature for 1 h in a 1:5000 dilution of rabbit anti-swine HRP conjugate (BioRad, USA) in 5 % (w/v) non-fat dry milk in TBST buffer. After three consecutive wash steps in TBST, the blot was exposed with enhanced HRP-DAB chromogenic substrate kit (Tiangen, Beijing) according to the manufacturer's instructions.

### **2.8 Experimental design of animal studies**

A total of ten 28-day-old healthy piglets were randomly assigned to two groups (five pigs per group). Five piglets (V1) were intramuscularly vaccinated twice at 35, and 56 days with 2 mL (contain  $10^{5.2}$  TCID<sub>50</sub>/mL) of the BPL-inactivated PRRSV vaccine. Five piglets (C1) were vaccinated with 2 mL of the aluminum adjuvant and served as controls. Three weeks after the second vaccination, all pigs were challenged intranasally with 2 mL of the  $10^{5.0}$  TCID<sub>50</sub>/mL PRRSV FZ06A strain.

### **2.9 Clinical signs**

Body temperature, appetite, and behaviour were monitored daily during the experiment. Clinical sign evaluated included evidence of depression, respiratory distress, coughing, and sneezing. Body weight was recorded daily after challenge.

### **2.10 ELISA and virus neutralization assay**

Blood samples were collected from all pigs at the first and second vaccinations, and at 3, 5, 7, 10, 14, and 21 days post-infection (dpi) for serological testing or virus titration; serum samples were stored at -80 °C until use. The effects of antibodies against PRRSV were analysed using IDEXX PRRS X3 ELISA test kit (IDEXX laboratories, USA). ELISA was performed in accordance with the manufacturer's instructions. A sample-to-positive (S/P) ratio value  $\geq 0.4$  was considered as positive.

VN titers were determined by SN test in Marc-145 cells. Sera was collected at 21 dpi, and was heat-inactivated at 56 °C for 30 min. Serial 2-fold dilutions of serum sample were prepared, and an equal volume of virus solution with a titer of 100 TCID<sub>50</sub>/mL was added to each dilution and incubated at 37 °C for 1 h. The serum-virus mixtures were transferred to a 96-well cell culture plate containing a monolayer culture of the Marc-145 cell. CPEs were analysed at 7 dpi. The VN

antibody titer was defined as the reciprocal of the highest serum dilution that inhibited CPE in 50 % of the inoculated cells (Kim et al., 2011).

### ***2.11 Quantification of PRRSV***

Viral RNA was extracted from the serum using an AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Biosciences, USA). Viral RNA extracts were then used as templates for one-step quantitative real-time RT-PCR (qRT-PCR) with One Step SYBR PrimeScript RT-PCR Kit II (Perfect Real Time, Japan) according to the manufacturers' instructions. Viral RNA was quantified by real-time PCR according to the method described by Sui et al. (Sui et al., 2018).

### ***2.12 Pathological examinations***

Pathological examinations were performed at 21 dpi. Lung sections were collected from each pig and fixed in 4 % paraformaldehyde fix solution for macroscopic pathological analysis. In addition, some lung tissues were collected and processed for histopathology examination followed by hematoxylin and eosin staining, as described previously (Halbur et al., 1995).

## **3 Results**

### ***3.1 Comparison of the efficacy of the two purification methods***

#### **3.1.1 Virus recovery and concentration**

After three consecutive freeze/thaw cycles, cell lysate was clarified by low-speed centrifugation to remove the cell debris. Clarified supernatants were concentrated by using an ultrafiltration tube with an Amicon® 100K device. The final protein concentration was 1230 g/mL (Table 7).

The titers of the samples were determined using an end-point dilution assay (Table 7). The clarified and concentrated culture supernatant exhibited a TCID<sub>50</sub> of  $1.0 \times 10^{4.5}$ . Following purification by sucrose density gradients ultracentrifugation, the titer of the virus was  $1.0 \times 10^{5.1}$  TCID<sub>50</sub>/mL, and the recovery rate was 39.7 %. In contrast, the titer of the virus purified by size-exclusion chromatography was  $1.0 \times 10^{5.4}$  TCID<sub>50</sub>/mL, and the recovery rate was 79.2 %. Following further purification by IEC, the titer of the purified virus was  $1.0 \times 10^{5.2}$  TCID<sub>50</sub>/mL and the recovery rate was 63.09 %. The final recovery rate of the purified sample was approximately 50 %. In conclusion, the virus recovery rates for ultrafiltration and liquid chromatography were higher than that of sucrose density gradient ultracentrifugation.

**Table 7.** The recovery and protein removal of virus purified by ultrafiltration and liquid chromatography and sucrose density gradients ultracentrifugation.

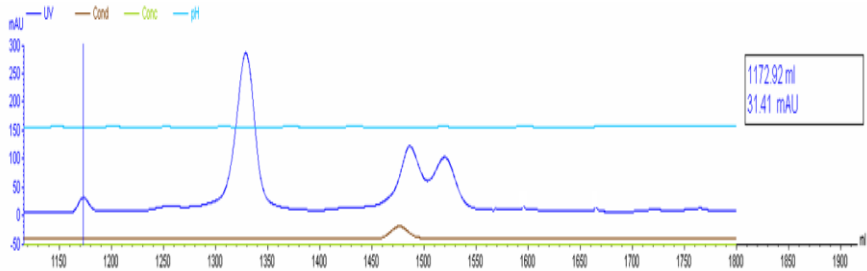
Samples	TCID <sub>50</sub>	Protein conc.	Volume	Protein removal	Virus recovery
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steps		( $\mu\text{g/mL}$ )	(mL)	(100%)	(100%)
Viral stock	$10^{4.5}$	865	100	0	
Tangential flow filtration virus	$10^{6.2}$	1230	2	97.2	100
Size exclusion	$10^{5.4}$	253	10	97.1	79.2
Anion exchange	$10^{5.2}$	135	10	98.5	63.09
					50
Virus purified by ultra	$10^{5.1}$	453	10	94.8	39.7
					39.8

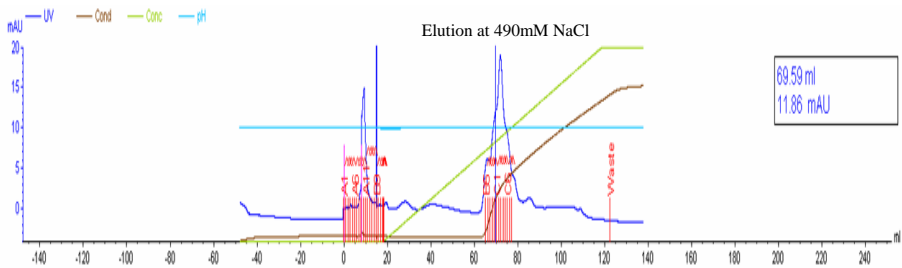
### 3.1.2 SDS-PAGE and Western blot analysis

As shown in Figure 17, we observed four major peaks of absorption at  $\text{OD}_{280}$  using the size-exclusion chromatography. The fractions were analysed by SDS-PAGE. PRRSV was detected in the first peak fraction. The majority of contaminating proteins were removed from the virus preparation by size-exclusion chromatography. The protein banding pattern of the first peak (Figure 19; (a) Lane 4) was similar to that described previously for purified PRRSV (Zhang et al., 2010). The first peak fraction from size-exclusion chromatography was further purified using anion exchange chromatography. Fractions were collected and analysed for the presence of PRRSV particles. The virus was eluted at 490 mM NaCl (Figure 18). The molecular weights of the purified products were analysed by SDS-PAGE (Figure 19a) and were found to be consistent with the sizes of the main structural proteins of PRRSV (Allende et al., 1999). The purity of the PRRSV purified by column chromatography followed by anion exchange was higher. In this sample, there were fewer faint bands corresponding to cellular proteins. Western blotting indicated that the major protein bands were grouped into 15 kDa (N), 18-19 kDa (M), 25 kDa (GP5), 29 kDa (GP2), 31 kDa (GP4), and 42 kDa (GP3) (Figure 19b). PRRSV purified using the sucrose ultracentrifugation purification method was also analysed by SDS-PAGE (Figure 19a) and Western blot (Figure 19b). The most

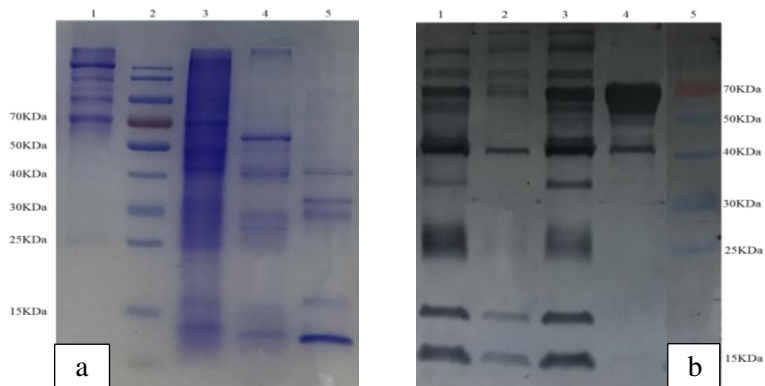
prominent bands appeared at 68 kDa, as determined by Commassie blue staining of the SDS-PAGE gel. A previous study demonstrated that these bands correspond to serum albumin (Vellekamp et al., 2001). Western blot analysis (Figure 19b) also showed that the virus purified by sucrose gradient ultracentrifugation could also react with the predominant viral structural proteins, but displayed a weak reaction with GP5 protein. Western blot analysis (Figure 19b) showed that the purity of the virus purified by ultrafiltration and liquid chromatography was higher than that obtained by sucrose density gradients ultracentrifugation.



**Figure 17.** Purification of 2 mL PRRSV on a C26/100 sepharose 4 fast flow column.



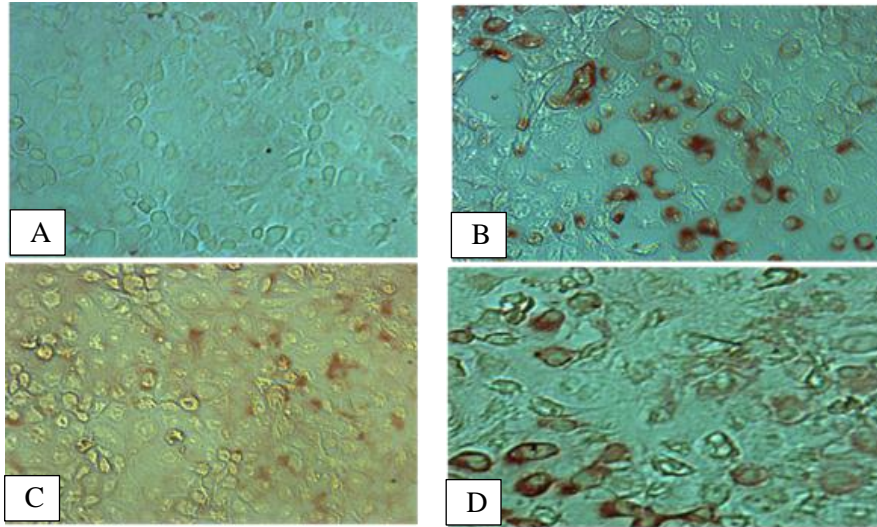
**Figure 18.** Linear gradient elution of PRRSV in Q Sepharose High Performance column. Pure viral particles were eluted in second peak.



**Figure 19.** Coomassie stained SDS-PAGE gel and Western blot analyses. (a) SDS-PAGE analyses of fractions from sucrose ultracentrifugation, ultrafiltration and liquid chromatography. Lane 1, fraction from sucrose ultracentrifugation; Lane 2, protein standard. Lane 3, sample after tangential flow filtration; Lane 4, fraction from first peak of size exclusion chromatography; Lane 5, fraction from second peak of anion exchange column. (b) Western blot of fractions produced in the purification process and revealed with PRRSV antiserum. Lane 1, concentrated sample after size exclusion chromatography; Lane 2, concentrated sample after anion exchange column; Lane 3, concentrated sample after sucrose ultracentrifugation; Lane 4, virus stock; Lane 5, protein standard.

### 3.1.3 Analysis of the immunogenicity of the purified virus

The serum from vaccinated mice was used to test the immunogenicity of the PRRSV by IPMA. Cells infected with the purified PRRSV obtained by ultrafiltration and liquid chromatography exhibited red staining in the cytoplasm (Figure 20B). The negative control samples did not display any staining (Figure 20A). These findings suggest that the virus purified by ultrafiltration and liquid chromatography has superior immunogenicity. Cells infected with virus purified by sucrose density gradient ultracentrifugation displayed light staining in the cytoplasm (Figure 20C). Red-stained particles were also observed in the negative control Marc-145 cells (Figure 20D), indicating that the virus purified by sucrose density gradient ultracentrifugation had a poor immunogenicity and specificity.

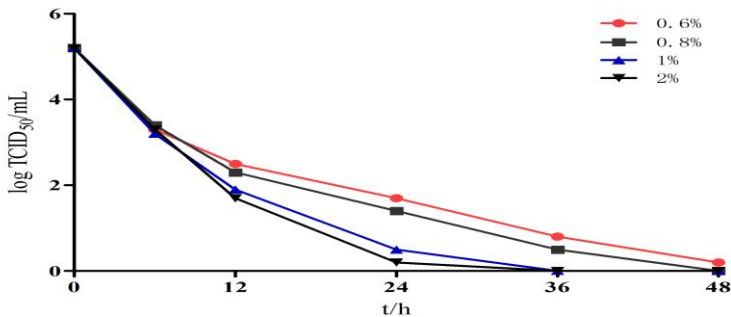


**Figure 20.** Immunogenicity of purified PRRSV was tested by immunoperoxidase monolayer assay. A: Mock-infected Marc-145 cells incubated with sera of mice immunized with PRRSV purified by ultrafiltration and liquid chromatography; B: PRRSV-infected Marc-145 cells incubated with sera of mice immunized with PRRSV purified by ultrafiltration and liquid chromatography; C: Mock-infected Marc-145 cells incubated with sera of mice immunized with PRRSV purified by sucrose density gradients ultracentrifugation; D: PRRSV-infected Marc-145 cells incubated with sera of mice immunized with PRRSV purified by sucrose density gradients ultracentrifugation.

### ***3.2 Comparison of the efficacy of the inactivants***

#### **3.2.1 Inactivation of PRRSV-FZ06A using different concentrations of BEI at 30 °C**

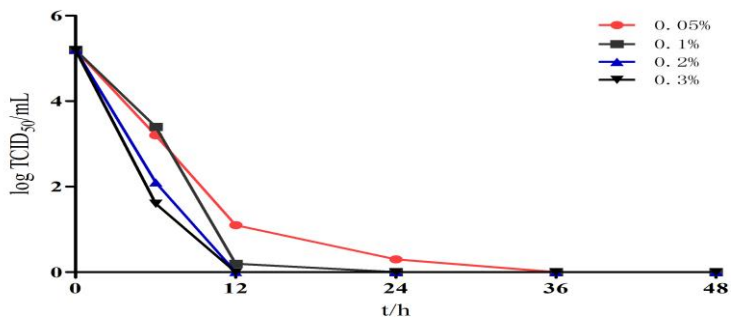
The abilities of different concentrations of BEI to inactivate PRRSV-FZ06A at 30 °C are summarized in Figure 21. After 6 h of BEI treatment, the viral titer decreased from  $10^{5.2}$  TCID<sub>50</sub> to  $10^5$  TCID<sub>50</sub> in a dose-dependent manner. After 24 h, the virus was almost completely inactivated by the 1 % and 2 % BEI treatments. Complete inactivation was achieved with these concentrations at 36 h.



**Figure 21.** The inactivated effect by BEI at 30 °C with different concentrations.

### 3.2.2 Inactivation of PRRSV-FZ06A using different concentrations of formaldehyde at 37 °C

The abilities of different concentrations of formaldehyde to inactivate PRRSV-FZ06A at 37 °C are summarized in Figure 22. After 6 h of formaldehyde treatment, the viral titer decreased from  $10^{5.2}$  TCID<sub>50</sub> to  $10^3$  TCID<sub>50</sub> below. After 12 h, the virus was almost completely inactivated by the 0.1 %, 0.2 % and 0.3 % formaldehyde treatments.



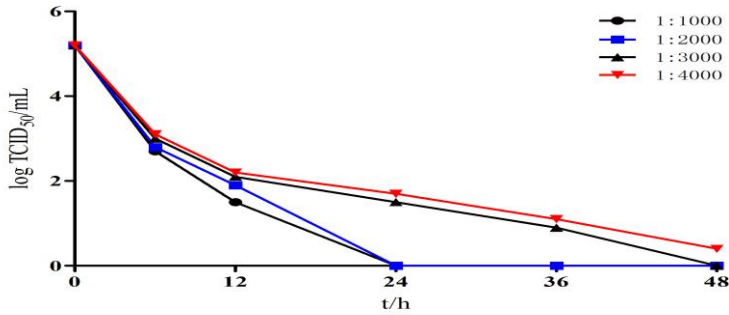
**Figure 22.** The inactivated effect by formaldehyde at 37 °C with different concentrations.

### 3.2.3 Inactivation of PRRSV-FZ06A using different concentrations of BPL at 4 °C

The abilities of different concentrations of BPL to inactivate PRRSV-FZ06A at 4 °C are summarized in Figure 23. After 6 h of BPL treatment, the viral titer decreased from  $10^{5.2}$  TCID<sub>50</sub> to  $10^3$  TCID<sub>50</sub>, similar to that following BEI treatment. After 24 h, the virus was completely inactivated by the 1:1000 and 1:2000 BPL concentrations. However, the virus was not completely inactivated with the 1:4000 concentrations of



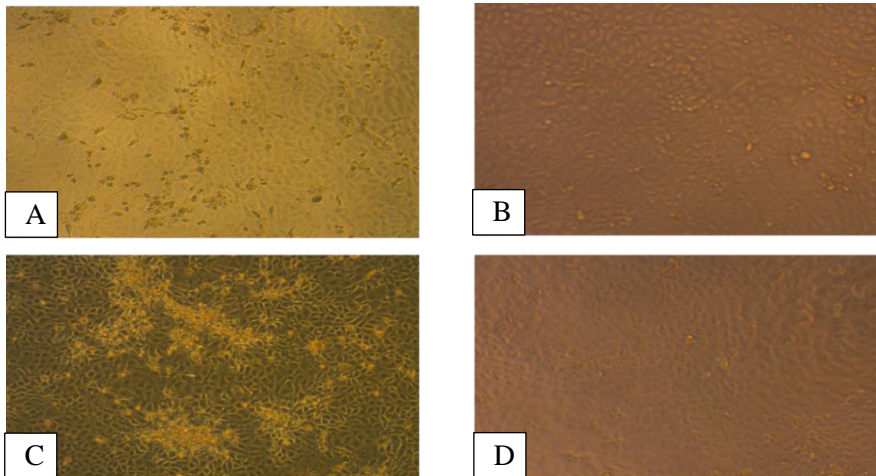
BPL until 48 h.



**Figure 23.** The inactivated effect by BPL at 4 °C with different concentrations.

### 3.2.4 Verification of inactivation

The inactivated virus was inoculated into the Marc-145 cells, and the cells were blindly passaged for 3 generations. After 24 h of inactivation with 1:1000 and 1:2000 BPL and after 36 h inactivation with 1 % and 2 % BEI, no cytopathic effects were observed (Figure 24), demonstrating that the virus was completely inactivated. After 12 h, the virus was almost completely inactivated by each concentration of formaldehyde. However, when we inoculated Marc-145 cells with the inactivated viruses, the cells lost their shape. This change may be due to cytotoxic effects of formaldehyde.



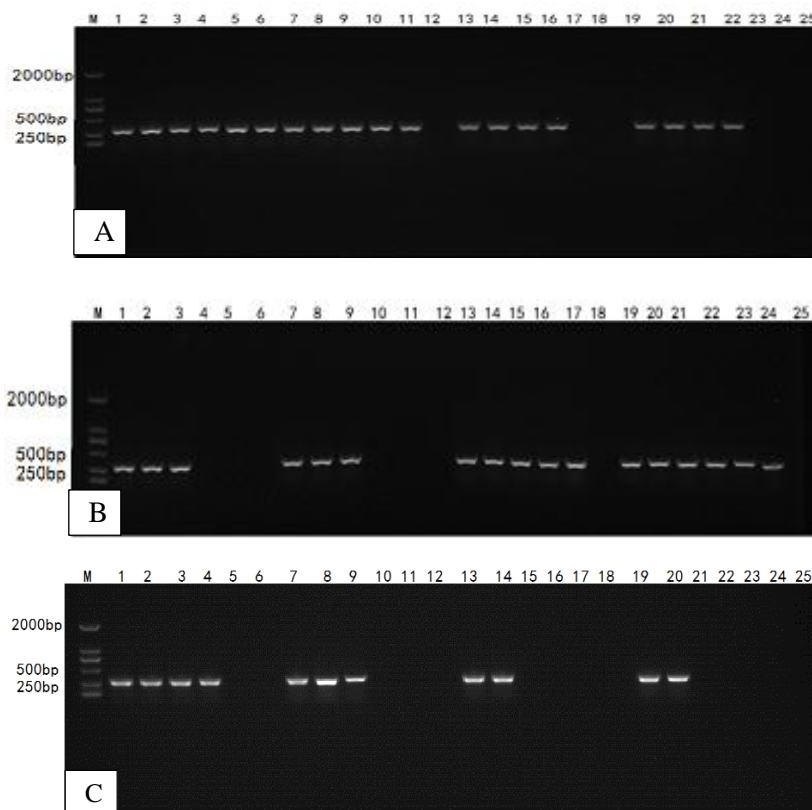
**Figure 24.** Inactivated virus passage on Marc-145 cell. A: Marc-145 cells were infected when inoculated with incompletely inactivated virus; B: Marc-145 cells were inoculated with

completely inactivated virus; C: Marc-145 cells were inoculated with FZ06A virus as positive control; D: Marc-145 cells mock-inoculated with DMEM as negative control.

### 3.2.5 Inactivated virus antigen integrity test

#### 3.2.5.1 RT-PCR

The inactivated virus was analysed by RT-PCR. After 36 h of inactivation with 0.6 % and 0.8 % BEI, we were able to detect the target product (approximately 300 bp) (Figure 25A). After 24 h of inactivation with 1:1000 and 1:2000 BPL, no viral nucleic acid was detected (Figure 25B). Similarly, after 12 h of inactivation with 0.2 % and 0.3 % formaldehyde, no viral nucleic acid was detected (Figure 25C).



**Figure 25.** PCR identification for inactivated PRRSV. (A) PRRSV was inactivated with different concentration of BEI by 0, 6, 12, 24, 36, and 48 h. M: DNA Maker; 1-6: 0.6 % BEI; 7-12: 0.8 % BEI; 13-18: 1 % BEI; 19-24: 2 % BEI; 25: negative control. (B) PRRSV was inactivated with different concentration of BPL by 0, 6, 12, 24, 36, and 48 h. M: DNA Maker; 1-6: 1:1000 BPL; 7-12: 1:2000 BPL; 13-18: 1:3000 BPL; 19-24: 1:4000 BPL; 25: negative control. (C) PRRSV was inactivated with different concentration of formaldehyde by 0, 6, 12,

24, 36, and 48 h. M: DNA Maker; 1-6: 0.05 % formaldehyde; 7-12: 0.1 % formaldehyde; 13-18: 0.2 % formaldehyde; 19-24: 0.3 % formaldehyde; 25: negative control.

### 3.2.5.2 Western blot analysis

The inactivated virus was concentrated and analysed with the positive PRRS antiserum (PRRSV). The major protein bands were as follows: 15 kDa (N), 18-19 kDa (M), 25 kDa (GP5), 29 kDa (GP2), 31 kDa (GP4), and 42 kDa (GP3) (Figure 26). The specific binding of the PRRSV polyclonal antibody indicated that the inactivated PRRSV retained its antigenicity and reactogenicity.



**Figure 26.** Western blot analysis. M: Protein standard; Lane 1: Inactivated virus reactivated with PRRSV positive serum.

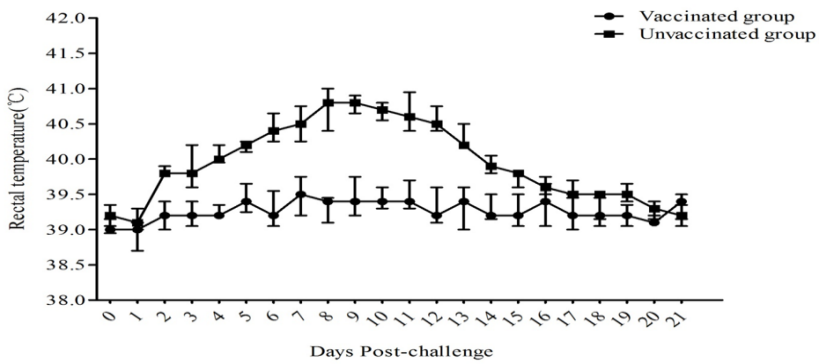
## 3.3 Clinical examination

All animals remained in good health condition. No local or systemic vaccine side effects were noted throughout the trial period. After challenge, some pigs in the vaccinated group exhibited a moderate degree of listlessness; no other clinical signs were recorded in these groups (Figure 27A). Unvaccinated pigs had severe cough, sneezing, shiver, and gathered together (Figure 27B). One pig in the unvaccinated group died at 10 dpi. There was a statistically significant difference in body temperature in unvaccinated group. After 4 dpi, the rectal body temperature elevated to 40 °C and persisted at that level for 10 days. The body temperature of some unvaccinated pigs exceeded 41 °C (Figure 28).

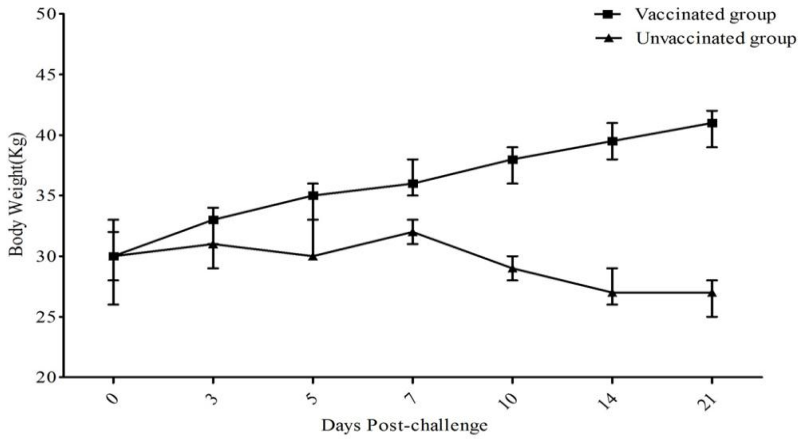
After challenge, the unvaccinated pigs exhibited significant weight loss. They were obviously emaciated after 21 dpi. Compared to unvaccinated pigs, the vaccinated pigs showed moderate weight gain (Figure 29).



**Figure 27.** Clinical signs observation of vaccinated pigs and unvaccinated pigs infected experimentally with FZ06A strain.



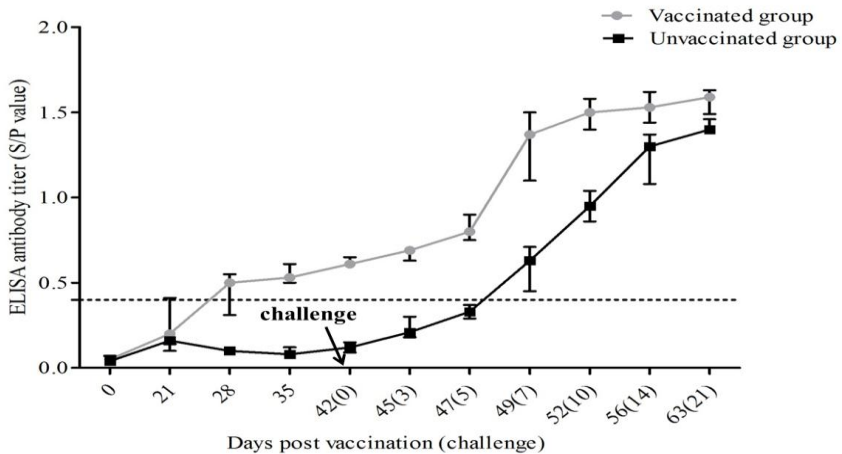
**Figure 28.** Mean rectal temperatures in vaccinated pigs and unvaccinated pigs challenged experimentally with FZ06A strain.



**Figure 29.** Body weight in vaccinated pigs and unvaccinated pigs challenged experimentally with FZ06A strain.

### 3.4 Antibody detection

The PRRSV-specific antibodies were measured using a commercial ELISA kit and was summarized in Figure 30. All unvaccinated pigs were seronegative for PRRSV-specific antibodies by ELISA before challenge and until 7 dpi. In the vaccinated group, a specific antibody response could be detected after two immunizations. The mean sample-to-positive (S/P) values of the vaccinated groups were higher than those of the unvaccinated pigs at each post-infection time point.



**Figure 30.** PRRSV-specific antibody titer in pigs.

PRRSV-specific antibody titers were measured in serum from vaccinated pigs challenged with PRRSV strains FZ06A. Values represent average titers from five animals. The dashed line designates the threshold above which titers were considered positive for anti-PRRSV antibodies (at an S/P ratio of 0.4). S/P means sample to positive ratio.

### 3.5 PRRSV VN titer assay

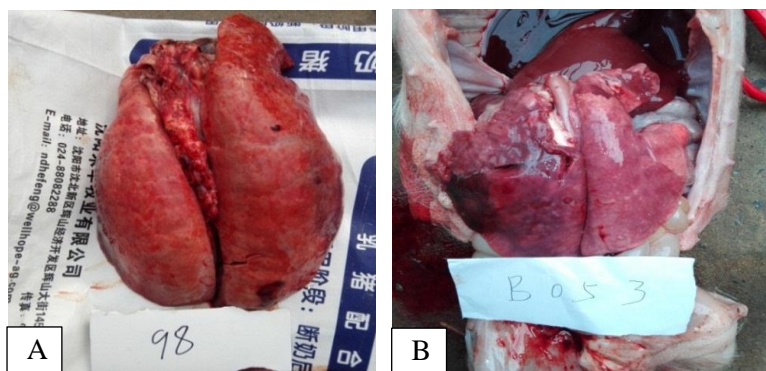
On the last day of the challenge, the serum samples were analysed to determine the difference of the VN antibody titer. Before challenge, all groups were negative for VN antibody titers. After challenge, the mean VN antibody titer of the vaccinated pigs (four of five) was higher than that of unvaccinated pigs (data not show).

### 3.6 Viral loads in serum samples

Total RNA was extracted from serum and tissues. One-step SYBR quantitative real-time qRT-PCR was performed to determine the PRRSV RNA copy number, as previously described (Guo et al., 2013). The vaccinated pigs had a lower level of viremia than that of unvaccinated pigs at 5 dpi. At 21 dpi, virus-positive blood was observed in both groups, but the mean copy number of virus in the vaccinated pigs was significantly lower than that of unvaccinated pigs (data not show).

### 3.7 Gross pathology and histological evaluations of lungs

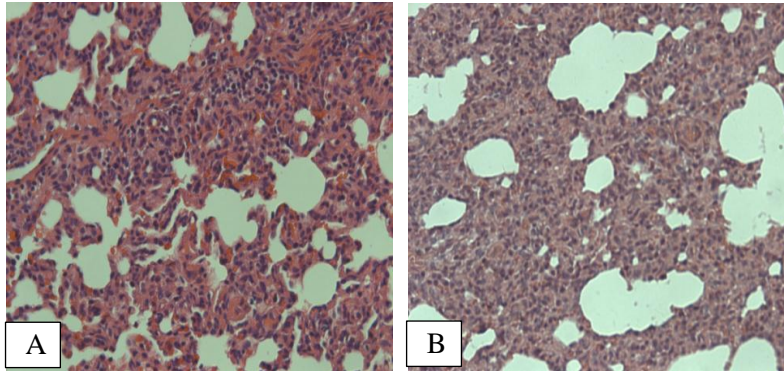
After challenge, no macroscopic (gross) lesions were observed in lungs collected from the vaccinated pigs at necropsy (Figure 31A). Lungs from unvaccinated pigs exhibited severe multifocal lesions and consolidation (Figure 31B).



**Figure 31.** Macroscopic observation of the lungs of the vaccinated pigs and unvaccinated pigs after challenge with FZ06A. (A) No macroscopic lesions were observed in the lungs collected from the vaccinated pigs after challenge with FZ06A. (B) Severe multifocal lung lesions and consolidation of lung tissue in unvaccinated pigs after challenge with FZ06A.

By histological examination, lung tissues collected from unvaccinated pigs displayed interstitial pneumonia associated with slight alveolar septa thickening, alveoli disappearance (Figure 32A). However, lung tissues collected from

vaccinated pigs exhibited very mild peribronchiolar and perivascular lymphoid hyperplasia (Figure 32B).



**Figure 32.** Histopathological lesions in the lung. (A) Severe interstitial pneumonia with thickening of the alveolar septa and disappearance of the alveoli in unvaccinated pigs. (B) Lungs from the vaccinated pigs showed no features of acute PRRSV infection.

## 4 Discussion

Currently, HP-PRRSV is widespread in China (Liang et al., 2018). It causes severe reproductive disorders in sows and boars. Despite several commercial attenuated and inactivated vaccines are currently used to control this disease, however, the prevalence of PRRSV has not dramatically decreased. Although these vaccines also have exhibited variable degrees of success in the field, outbreak of clinical PRRS in vaccinated pigs has led to doubts regarding the efficacy of the currently available vaccines (Thanawongnuwech & Suradhat, 2010). An improved vaccine is clearly needed to eliminate PRRSV. Attenuated vaccines can provide a certain level of protection against the virus but can revert to virulence (Yu et al., 2015; Renukaradhya et al., 2015). Therefore, inactivated vaccine is preferred over an attenuated vaccine. Unfortunately, the existing commercial inactivated vaccines have failed to induce a strong immune response (Nilubol et al., 2004; Scotti et al., 2007; Zuckermann et al., 2007). So, the main objective of the current study was to develop a highly purified inactivated vaccine against homologous challenge.

To date, vaccination is the most effective approach to prevent and control PRRSV. Purified vaccine can induce high-titer antibody production; thus, pigs immunized with lower vaccine dosages can develop stronger resistance to highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) infection. As non-viral proteins have been removed, allergic reactions and stress responses caused by vaccine inoculation can be avoided. Purified vaccine will potentially be a safer and more effective candidate for the control and prevention of PRRS.

Traditionally, ultracentrifugation has been used for the purification of different viruses. Density gradient ultracentrifugation was also a popular method for the

purification of PRRSV. Wu et al obtained high concentrations of PRRSV using the sucrose gradient centrifugation (Wu et al., 2005). However, there are inherent problems and limitations associated with ultracentrifugation, such as long processing time, small processing volume, and potential damage to the viral particles. To solve these issues, a novel downstream process was investigated to allow scale-up of highly purified virus. Over last few years, researchers paid more attention to the chromatography techniques which has been widely employed in the production of viral vaccines and viral vectors for human gene therapy. Compared with density gradient ultracentrifugation, chromatography is substantially faster, more consistent and allows for automation (Huyghe et al., 1995). However, chromatography also displays a few disadvantages, such as low loading volume. Overall, this method offers the advantages of being a gentle purification method resulting in a high yield and high-quality virus. Additionally, the purity of the PRRSV after chromatography was higher than that of virions purified using sucrose density gradient ultracentrifugation, and the virus recovery was greater than 50 %.

In our study, a clearly visualized, hybrid protein band with a molecular mass of about 68 kDa was observed using SDS-PAGE following ultracentrifugation. Western blot analysis showed that this 68 kDa band was found in both the virus stock solution purified by ultracentrifugation and the negative control cell culture solution, which was not infected with PRRSV. This may be caused by bovine serum albumin antibody in the positive pig serum. However, this band was not observed in the PRRS virus purified by ultrafiltration and liquid chromatography, indicating that our ultrafiltration and liquid chromatography method was able to remove more non-viral protein than ultracentrifugation.

The immunogenicity of virus purified using different purification methods was assessed by IPMA. We found that the cytoplasm was clearly dyed red in cells infected with PRRSV purified using ultrafiltration and liquid chromatography, while negative control cells displayed no background signal, suggesting that the virus purified by ultrafiltration and liquid chromatography had superior immunogenicity. When the serum from mice immunized with virus purified by sucrose density gradient ultracentrifugation was tested on infected cells, the cytoplasm of the cells also stained red. In addition, the negative control also displayed dyed particles and faint signal in the background, which significantly interfered with our interpretation. The observed differences in immunogenicity following these purification methods may be caused by differences in purity and contaminating protein content, resulting in serum containing more anti-cell antibodies when vaccinated with virus purified by ultracentrifugation. The PRRSV purification method we describe resulted in an efficient high recovery of pure and stable virus.

The inactivation of PRRSV is an important step in the production of a safe PRRS vaccine. Optimally, after inactivation, the virus should lose its infectivity, toxicity and proliferative capacity, but should retain major structural proteins and antigenicity. An effective inactivation method should maintain viral immunogenicity while removing pathogenicity (Logrippo, 1960).



A commonly used inactivant, formaldehyde can crosslink and denature vial proteins or cause viral particles to aggregate. These effects may prevent the formaldehyde from acting on the viral nucleic acid, causing the pathogen to escape inactivation (Brown, 1995). BEI is an alkylating agent widely used for inactivation of viruses. It is an effective preservative, less hazardous than other inactivants, and is inexpensive (Bahnemann, 1990). The mechanism of BEI inactivation is mainly through the alkylation of guanine or adenine in the viral nucleic acid. This alkylation can modify the nucleic acid structure by causing single-stranded chain breaks or double-stranded chain crosslinking. It also can interfere with RNA synthesis and inhibit cell mitosis. BPL, a heterocyclic compound, exerts a strong inactivating effect on viruses (Kamaraj et al., 2008; Chowdhury et al., 2015). BPL treatment maintains immunogenicity by reacting with the purine bases and destroying the structure of nucleic acids without acting on the protein directly. In contrast to formaldehyde, both BPL and BEI can inactivate the virus while maintaining its antigenicity and immunogenicity (Uittenbogaard et al., 2011; Kamaraj et al., 2008; Aarthi et al., 2004). However, different viruses display differential resistance to the inactivants. Some inactivants can also exhibit significant mutagenic effects on nucleic acids, which may result in production of mutant strains (Brown, 1993). The risk of producing a mutant virus increases if the virus is not fully inactivated. Thus, it is of utmost significance for production of the inactivated vaccine to determine the optimal inactivation conditions.

In this study, the PRRSV strain was inactivated using different concentrations of BPL, BEI, and formaldehyde. We found that BEI was able to inactivate PRRSV within 36 h at concentrations of 1 % and 2 % at 30 °C. However, after 48 h of inactivation with 0.6 % BEI at 30 °C, viral nucleic acid also could be identified by RT-PCR. After 24 h of inactivation with 1:1000, 1:2000 BPL at 4 °C, the virus was totally inactivated. We also found that the inactivant concentration and the inactivation time affected the extent of inactivation. When the virus was inactivated with different concentrations of formaldehyde at 37 °C, viral nucleic acid could not be detected by RT-PCR in any treatment after 24 h. When we inoculated Marc-145 cells with the formaldehyde-inactivated virus, the shape of the cells was destroyed. This phenomenon was not observed in BPL- and BEI-treated cells. The formaldehyde likely reacted with proteins to “close” the outer protein shell of the virus before the viral nucleic acid was damaged (Habib et al., 2006).

Both BPL and BEI treatments effectively inactivated PRRSV. However, after 24 h of inactivation with 1:2000 BPL at 4 °C, we were unable to detect viral nucleic acid by RT-PCR after 36 h of inactivation with 1 % BEI at 30 °C. These observations indicated that BPL can destroy viral nucleic acids quickly and can display high viral inactivation efficiency. Western blot analysis also showed that the BPL-inactivated virus reacts with PRRSV antiserum. Taken together, the optimal inactivation conditions for PRRSV by BPL is a 1:2000 concentration for 24 h at 4 °C.

To detect whether inactivated vaccine could induce a robust level of neutralizing humoral immunity in vaccinated animals, Misinzo et al. immunized pigs with an inactivated vaccine and they observed an enhanced VN antibody response after

challenge (Misinzo et al., 2006). Vanhee et al challenged pigs with a BEI-inactivated PRRSV vaccine and found that the inactivated vaccine could enhance the VN antibody response and produce partial protection against challenge with a homologous strain (Vanhee et al., 2009).

In the present study, we found that purified inactivated PRRSV vaccine is effective in partially protecting pigs against the homologous strain. After challenge, the unvaccinated pigs exhibited serious clinical signs. After 4 days, the rectal temperature elevated to 40 °C and persisted for 10 days, and the body temperature of some unvaccinated pigs exceeded 41 °C. What's more, the unvaccinated pigs exhibited significant weight loss. In contrast, there was no significant change in the vaccinated pigs.

ELISA and VN tests were performed to evaluate the capacity of the vaccines to induce virus-specific (neutralizing) antibody response. The inactivated PRRSV vaccine was inoculated twice at three-week intervals. The inactivated pigs had significantly higher S/P ratios than the unvaccinated pigs ( $p < 0.05$ ). However, no VN titers were detected during the entire experimental period before challenge (data not shown). One week after the second immunization, virus-specific antibody was detected in the group inoculated with purified BPL-inactivated vaccine. VN antibodies are important for the prevention of viremia and also play an important role in clearance of PRRSV from the lungs and blood (Labarque et al., 2000; Osorio et al., 2002). However, the VN titer was measured at 21 dpi. Compared to the unvaccinated group, the vaccinated group (four of five) had significantly higher neutralizing antibody titer. There was a significant difference in VN titer between the unvaccinated group and the vaccinated group (Geldhof et al., 2012). This indicated that the inactivated vaccine could induce a VN antibody response. Compared with the unvaccinated group, vaccinated pigs showed a shorter period of viremia, indicating that the virus concentration in the blood of vaccinated animals was lower than that in the unvaccinated animals. Vaccination with inactivated vaccine could reduce the duration of viremia as reported by other researchers (Misinzo et al., 2006; Lopez et al., 2007). Unexpectedly, one pig in the vaccinated group did not induce any VN antibody response but did have a high level of virus-specific antibody and a shorter period of viremia than were observed in the unvaccinated group. Histological examinations of the two treatment groups revealed that the lungs from the mock groups developed interstitial pneumonia associated with slight alveolar septa thickening, alveoli disappearance, and atrophy, which were not observed in the vaccinated group.

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## **General discussion and perspectives**



Porcine reproductive and respiratory syndrome (PRRS) is an economically important viral disease in the pig industry worldwide. It is estimated that the total economic losses caused by PRRS total approximately \$664 million annually in the United States, an increase from the \$560 million annual cost estimated in 2005 (Neumann et al., 2005). As one of the biggest pork producers and consumers in the world, China also faces a great threat from PRRS. Recently, many countries have taken some effective measures to eliminate PRRSV from affected pig herds, including whole herd depopulation/repopulation, testing and removal, and herd closure. However, there are many differences between the production mode and management practices of pig farms in China and other developed countries, such that PRRSV is undoubtedly a more difficult problem for Chinese pig farms.

For preventing and controlling PRRS in China, vaccination is the primary choice for most of pig producers. There are two kinds of commercial vaccines, modified live-attenuated vaccines (MLVs) and inactivated vaccines, are currently used in China. MLVs can provide partial immune protection in pigs, but their safety and the risk of virus reversion should be considered. Traditional inactivated PRRSV vaccines induce a poor immune response in naïve pigs and sometimes fail to protect pigs against heterologous strains but are much safer than MLVs. Although these two vaccine types both present concerns, control and prevention of PRRS is a top priority for the swine industry. Thus, vaccines that are safe, cannot revert to virulence in vaccinated animals, and can induce a sufficient immune response are required. On the other hand, many factors influence the efficacy of a vaccine. Presently, most research focuses on the efficacy and protection of inactivated PRRSV vaccines. Less is known regarding vaccine candidate strains, the procedures used to fully inactivate the virus, and the resulting quality of the vaccines. In this work, we focused on the development of a high-quality inactivated vaccine capable of adapting to new circulating virus variants and inducing neutralizing antibodies. This chapter provides a discussion of the contributions and conclusions of the study and its limitations and future prospects.

## 1 Surveillance and genetic evolution analysis

Viruses are important component of the biological chain in the earth. There are kinds of viruses in the ecosystem (Fuhrman 1999; Rohwer & Thurber 2009). Virus infection has caused damage to the swine industry. After several years of evolution, most of swine viruses, such as PRRSV, PRV, and PCV, have adapted to their environment and exhibit different characteristics. Surveillance and identification of recently emerged virus strains are crucial for infection control. Tracing the molecular epidemiology of infectious diseases is key to disease prevention, and genetic sequences of pathogen samples are an important source of information in infectious disease epidemiology (Kenah et al., 2016).

Virus isolation and identification are the most accurate diagnostic approach and is currently widely used to distinguish various viruses. Many factors, such as collection time, the transport and preservation conditions of the samples, and the cells used for

virus isolation, will influence the results. Generally, several kinds of cells, such as BHK21, Vero, and Marc-145 cell, are used for virus isolation. The most suitable sampling locations for isolation of PRRSV are the lungs, lymph nodes, and tonsils (Schommer et al., 2001).

Phylogenetic analysis, a key approach in bioinformatics, can quickly determine the evolutionary relationships among organisms using their molecular sequence data. In general, there are five steps in a phylogenetic analysis, including sequence data preparation, sequence alignment, selection of a phylogenetic reconstruction method, identification of the best tree, and evaluating the tree (Kosiol et al., 2006). Phylogenetic analysis has been widely used in numerous research areas, including identification of novel functional structures in genomes (Lander et al., 2001; Waterston et al. 2002), the detection of homologues within and between genomes (Lander et al., 2001; Waterston et al. 2002), protein structure prediction (Goldman et al., 1996), and the elucidation of how biochemical molecules function (Suzuki & Gojobori, 1999).

In the first part of our study, we isolated several PRRSV strains from different farms. We compared three representative PRRSV strains, designated FZ06A, FZ16A, and HB17A. Based on our phylogenetic analysis, the three isolate strains were all found to belong to the North American genotype. The HB17A genome contains three discontinuous deletions (131 AA), which were also found in the NADC30 strain and MN184A strain, a type 2 PRRSV that was isolated in the United States in 2008. Surveillance and genetic evolution analysis will improve our monitoring and understanding of the evolution of prevalent strains.

## **2 Chromatography methods for virus purification**

Use of a high quality vaccine, which contains a sufficient concentration of antigens and low levels of non-viral proteins, is key for inducing an anti-virus reaction. The current commercial inactivated vaccine does not display good immunogenicity, which is due, in part, to the vaccine quality. The vaccine does not contain enough antigens, and thus an increase in the vaccine dosage is required during immunization. However, a high-dosage vaccine is more likely to cause allergic reactions and stress responses. For these reasons, vaccine quality has become a primary focus in vaccine production in recent years. Traditionally, ultracentrifugation has been used for purification of different viruses, and density gradient ultracentrifugation was the most popular method for the purification of PRRSV. However, this method requires a long processing time, small processing volume, and may damage viral proteins. Each viral structural protein plays an important role in viral attachment and replication, and thus, a novel downstream processing step that is easy to scale-up and results in highly purified virus is needed.

As a powerful separation and analysis approach, chromatography has been integral to developments in biomedicine, food safety and human health. It has also been widely employed in the production of viral vaccines and viral vectors for human

gene therapy. In recent years, chromatography science has experienced a rapid development and achieved a great achievement in China. There are several types of chromatography methods, including affinity chromatography, ion-exchange chromatography, and size-exclusion chromatography. These methods offer important opportunities to better characterize complex vaccine antigens, including recombinant proteins, virus-like particles, inactivated viruses, polysaccharides, and protein-polysaccharide conjugates (Hickey et al., 2016). Compared to the traditional purification method, chromatography can provide sufficient purification and maintains the general viral structure.

Besides the size-exclusion chromatography method, we also employed the Q sepharose high performance, because it had a larger external resin surface area per unit volume of adsorbent. It could provide better binding capacity to get enough virion. The purification ability of sucrose density gradients and ultracentrifugation and liquid chromatography methods was compared by detecting virus particles and protein contents. We found that ultracentrifugation and liquid chromatography method could remove more non-viral proteins. It also had a good immunogenicity. Interestingly, western blot analysis of virus purified by Q sepharose high performance using PRRSV positive serum revealed, structural protein GP5 appearing as diffusive bands. There was only a single band when GP5 monoclonal antibody was used as primary antibody.

### 3 Persistent infection

In general, virus infection will cause clinical sign and pathological changes to the host. Most infections are quickly cleared by the host's immune system. There are also viruses, such as PRRS, PRV, persist latently in the host. The virus cannot be detected and the host does not exhibit any clinical sign. During this phase, viral replication occurs only in the lymphoid tissues (Beyer et al., 2000; Rowland et al., 2003a). Since the virus can be persistent in the tissues, it can easily spread from infected pigs to naive pigs and can cause infection (Ke & Yoo, 2017). Additionally, some of the virus, such as PRRS, can delay the cell-mediated immune responses. Thus, we must pay more attention to achieve a rapid and complete sterilizing immunity against the virus. As we know, innate and adaptive immune responses are two mechanisms that protect against virus infection (Murtaugh et al., 2002). Like PRRSV, innate cytokines (i.e. both TNF $\alpha$  and type I IFN) can inhibit PRRSV replication and activate porcine antigen-presenting cells (APC) to produce pro-inflammatory cytokines and stimulate the lymphocyte proliferation (Albina et al., 1998; Lopez-Fuertes et al., 2000; Murtaugh & Foss, 2002). What's more, neutralizing antibodies are crucial for protecting pigs from viremia and clinical diseases following homologous PRRSV challenge (Lopez & Osorio, 2004). Adaptive cytokine, (i.e. IFN- $\gamma$ ) can effectively inhibit PRRSV replication and activate porcine APC to produce pro-inflammatory cytokines (Bautista et al., 1999; Rowland et al., 2001). Viruses can escape host immunity and evolve as new variant strains. This mechanism is a major threat for pig industry.

## 4 Vaccine adjuvant

Vaccine adjuvant can enhance adaptive immune responses to antigens, which is very important in research and development of novel vaccines (Yu et al., 2018). In recent years, more and more novel vaccine adjuvants have been developed. These adjuvants include cytokines, chemical reagents, and bacterial products. For most of inactivated vaccine, previous studies have proved that they cannot activate intracellular antigen presentation pathways to induce a strong cytotoxic T-cell (CTL) response (Chadwick et al., 2010). So finding a suitable vaccine adjuvant is important to enhance the immunity of the vaccine. IL-1 and IL-6, as the cytokines adjuvant, can effectively promote lymphocyte trafficking to the draining lymph nodes, lymphocyte proliferation, hematopoiesis, and natural killer (NK) activity of peripheral blood lymphocytes (PBL) (Binns et al., 1992; Murtaugh, 1994; Murtaugh et al., 1996). The adjuvanticity of IL-1 and IL-6 has been reported in pigs immunized with *Streptococcus suis* vaccine (Blecha et al., 1995) and pseudorabies virus (PRV) MLV vaccine, respectively (Ling-Hua et al., 2006). CpG ODN, chemical reagents, containing palindromic hexamer 'ATCGAT' has been reported for its immunostimulatory effects in pigs (Kamstrup et al., 2001). The adjuvant effects of CpG Oligodeoxynucleotides (CpG ODN) have been reported in numerous vaccine studies in mice and non-human primates, and some in pigs (Yu et al., 2018; Kaur et al., 2013; Van der Stede et al., 2002). There are also other kinds of adjuvants, such as  $\text{INF}\alpha$ , which has the ability to activate APC, increase antibody and cytotoxic T-cell response (Bracci et al., 2008); *Salmonella* flagellin, which is a ligand of TLR5 and has the ability to induce activation of dendritic cells (DC) and T cells (Means et al., 2003; Bates et al., 2008). A suitable adjuvant is thus essential to enhance the immune response to the vaccine.

## 5 Regional elimination

For certain animal diseases, regional elimination of the pathogen has been the only effective way to control the disease (Corzo et al., 2010). This strategy has been successful for foot and mouth disease (FMD) (Perez et al., 2004). After an effective movement restriction and mass vaccination, the disease can be controlled. Several countries have reported a successful control and elimination of PRRSV (Archibald et al., 2008). This is thus a good method to eliminate the virus in combination with a vaccine. Regional elimination of the pathogen also needs to coordinate cooperation between governmental authorities, local industry and swine veterinarians. There are a series of challenges, including participation, funding, communication and location and regional pig flow dynamics. This programme also will take a long time to be fully efficient. Until now, regional elimination of the pathogen programme has been carried out in several industrialized countries. However, this is very hard to apply in most developing countries.

## 6 Limitations and prospects for future research

Vaccination is a useful method to control and prevent disease. However, the use of the vaccine also is a complex equation. Many factors may influence whether or not a vaccine will work. It is highly dependent on the interaction of the pathogen and host (Renukaradhya et al. 2015). Usually, a useful vaccine is produced based on a specific isolate. Although commercial vaccines have been efficient, occurrences of new mutations and deletions have limited their efficacy. Therefore we evaluated three new isolates. These strains shared high amino acid sequence identity with North American genotype isolates. They also contained several AA deletions and mutations in Nsp2 and GP5. Some of them were unique. To confirm the pathogenicity of the isolate strains, Specific-pathogen-free (SPF) pigs were inoculated with the 3 isolates. These strains showed a difference in virulence and mortality rate. Whether the deletions and mutations influence the virulence is currently unknown. To clearly understand the role of viral proteins in the virus life cycle and pathogenicity, reverse genetic system has been widely used (Huang & Meng, 2010). This also provides a way to develop live-attenuated virus vaccines by introducing attenuating mutations on viral genome (Boyer & Haenni, 1994).

Viral inactivation should be carefully controlled. After inactivation, the virus should lose its infectivity, toxicity and proliferative capacity, but the major structural proteins or antigenicity should not be destroyed. Incomplete inactivation may cause viremia and may even allow reversion to virulence. In contrast, over-inactivation may destruct the viral neutralizing epitopes that are important for the induction of protective immunity. Until now several inactivants, such as formaldehyde, temperature, ultraviolet (UV) light, BEI, and BPL, are widely used for virus inactivation. High temperature can inactivate viruses by denaturation of proteins (Lelie & Lucas, 1987). Because PRRSV proteins will be denaturated at a high temperature, this method is not recommended. Inactivate the virus with UV light may also be a good choice. Besides DNA alterations, this method also affects capsid proteins (Miller & Plagemann, 1974). Formaldehyde can cross-link and denature viral proteins or aggregate virus particles. However, these effects may block the viral capsid assembly process and prevent the inactivating agent from acting on the viral nucleic acid, causing the pathogen to escape inactivation (Brown, 1995). In our study, when the virus was inactivated with different concentrations of formaldehyde at 37 °C, the virus could be quickly inactivated. When we inoculated the inactivated virus on Marc-145 cell, the shape of cells was destroyed. This may be caused by the cross-linking or denaturation of viral domains that were involved in attachment and internalization of PRRSV. As a result, the inactivated virus would probably give a poor preservation of viral immunogenicity. Both BPL and BEI can destroy the structure of nucleic acids but do not directly act on the protein. These chemicals have an effect on the genomic level, preserving entry-associated viral domains. Thereby a good immunogenicity was preserved after inactivation. In our study, after 36 h inactivation with 1 % and 2 % concentrations of BEI, the virus was totally inactivated. But after 48 h inactivation with 0.6 % concentration of BEI, the viral

nucleic acid also could be detected. After 24 h inactivation with 1:1000, 1:2000 concentrations of BPL at 4 °C, the virus was totally inactivated. We also found that the concentration of inactivants and the inactivation time affected the result of inactivation. Although both BPL and BEI effectively inactivate the virus, BPL could quickly destroy nucleic acids and had a high efficiency for the virus inactivation. Additionally, BPL also inactivated the virus at a lower temperature.

Virus inactivation can be monitored by different methods. Inactivation of influenza virus can be detected by measuring the hemagglutinating activity (Di Trani et al., 2003). The attachment of neutralizing antibodies to viral epitopes of human immunodeficiency virus (HIV) can be investigated by ELISA (Grovit-Ferbas et al., 2000). There is no similar method for PRRSV. To investigate the effect of inactivation, we used RT-PCR to detect the nucleic acids of the inactivated virus and Western blot to evaluate antibody reactivity.

Recently, a new modified live virus vaccine was obtained by passaging wild-type strains in sensitive cells (Minor, 2015). Although it has been widely used for vaccination, there is a risk of reversion to virulence. What's more, vaccination trials have been carried out in controlled conditions, but their efficacy in the field is still unknown. In our research, we gave a preliminary evaluation of the purified BPL-inactivated PRRSV vaccine. After immunization and challenge, the vaccinated pigs developed VN antibodies and were protected against homologous strain infection. Based on these results, large-scale field trials should be implemented.

Given the degree of genetic diversity of the virus, the effectiveness of a vaccine against heterologous infection will depend on the antigenic and genetic relatedness of the virus strain (Huang & Meng, 2010). Therefore, a universal vaccine, consisting of antigenically distinct strains should be considered. Common epitopes are considered to induce the primary immune response and protection in different strains (Mateu and Diaz, 2008). The polyepitope vaccine, which includes conserved epitopes, is an interesting novel strategy for the development of a future vaccine. This kind of vaccine can allow the immune system to focus on rationally selected conserved B-cell and/or T-cell epitopes (Hu & Zhang, 2014). This is important for the development of the vaccine. Potential B-cell and T-cell epitopes can be detected with bioinformatics tools. The predicted epitopes can then be screened by peptide ELISA (for B-cell epitope) and enzyme-linked immunospot assay (ELISpot) (for T-cell epitopes). Then, B-cell epitopes yielding adequate ELISA signals and T-cell epitopes stimulating a T-cell response will be considered as the building blocks of a polyepitope vaccine (Hu & Zhang, 2014). This approach has been evaluated in HIV (Rosa et al., 2015). Therefore polyepitope vaccines could also be particularly important for development of effective anti-PRRS vaccines.

After several years of struggle, we have made great progress to control and eradicate animal diseases. In recent years, with the changes in farming models and ecological models and the acceleration of economic globalization, the prevalence of animal diseases has changed. The number of new diseases is increasing, and the epidemic transmission scope is expanding. This situation is a great challenge for



control and prevention of animal diseases. There is a need for a global cooperation and specific measures to different areas. There are indeed major differences in production modes and management in pig farms between China and other developed countries. We propose to different measures to decrease PRRSV. Firstly, an adequate protocol of vaccination avoiding interference with maternal antibodies should be implemented. Secondly, we also need to strengthen farm infrastructure construction. Clean and comfortable environments are important for animals to be in a good health. Thirdly, comprehensive disease surveillance system and diagnostic platform are necessary to take quick and effective measures in case of outbreaks.

## 7 Conclusion

PRRSV causes huge economic losses to the swine production worldwide. During the nearly 30 years of extensive research on the PRRSV vaccine, there have been many unexpected obstacles. Development of effective vaccines against PRRSV has become a significant challenge. Given the safety concerns associated with attenuated vaccines, development of an effective inactivated vaccine is preferred. Ideally, an inactivated PRRS vaccine will have cross-protective efficacy at least equivalent to the current attenuated vaccines. In the present study, during the development of the novel experimental inactivated PRRSV vaccine, the following aspects were fulfilled: (1) a prevalent PRRSV strain was isolated and chosen as the candidate vaccine strain to develop a novel inactivated vaccine against PRRSV; (2) quality-controlled viral inactivation procedure was designed and applied assuring complete killing of PRRSV; (3) a process for purifying PRRSV particles with higher purity and yield was obtained; and (4) a degree of VN antibody response and part protection against homologous PRRSV challenge were achieved. Although the purified inactivated PRRSV vaccine exhibited protective efficacy against the homologous strain used in this study, it was not possible to conclude that the inactivated vaccine could provide cross-protection against infection with heterologous virus. The inactivated vaccine used in this study may require further improvement. In addition to knowledge regarding PRRSV epidemiology and mechanisms of pathogenesis, additional research on novel adjuvants and immunomodulators, inactivation methods, and DNA subunit approaches, is also required to achieve this goal.

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## Appendix- publications

### 1. Publications included in the thesis

1) **Sui X\***, Xin T\*, Guo X, Jia H, Li M, Gao X, Wu J, Jiang Y, Willems L, Zhu H, Hou S#. Genomic characterization and pathogenic study of two porcine reproductive and respiratory syndrome viruses with different virulence in Fujian, China. *J Vet Sci*, 2018, 19: 339-349.

2) **Sui X\***, Guo X\*, Jia H, Wang X, Lin W, Li M, Gao X, Wu J, Jiang Y, Willems L, Zhu H, Xin T#, Hou S#. Genomic sequence and virulence of a novel NADC30-like porcine reproductive and respiratory syndrome virus isolate from the Hebei province of China. *Microbial Pathogenesis*, 2018, 125: 349-360.

### 2. Other publications

1) Li M, Xin T, Gao X, Wu J, Wang X, Fang L, **Sui X**, Zhu H, Cui S, Guo X. Foot-and-mouth disease virus non-structural protein 2B negatively regulates the RLR-mediated IFN- $\beta$  induction. *Biochem Biophys Res Commun*. 2018. 504: 238-244.

2) Xin T, Gao X, Yang H, Li P, Liang Q, Hou S, **Sui X**, Guo X, Yuan W, Zhu H, Ding J, Jia H. Limitations of Using IL-17A and IFN- $\gamma$ -Induced Protein 10 to Detect Bovine Tuberculosis. *Front Vet Sci*. 2018. 5: 28.

3) Li Z, **Sui X**, Wu J, Xin T, Li M, Gao X, Jiang Y, Hou S. Pathogenic Analysis of Two Strains of Porcine Reproductive and Respiratory Syndrome Virus. *China Animal Husbandry & Veterinary Medicine*. 2017, 44: 1477-1483. (in Chinese)

4) **Sui X**, Hou S, Lin W, Zhang S, Jia H, Guo X, Yuan W, Jiang Y, Xin T, Zhu H. Molecular and pathogenicity analyses of a new highly pathogenic porcine reproductive and respiratory syndrome virus strain FZ16A. *Chinese Veterinary Science*. 2018, 48: 1511-1521. (in Chinese)

5) **Sui X**, Xin T, Willems L, Zhu H\*. The preliminary evaluation of efficacy of PRRS inactivated vaccine based on the viral purification and inactivation methods. **(Manuscript in preparation)**