



# Efficacy of Type 2 porcine reproductive and respiratory syndrome virus (PRRSV) vaccine against the 2010 isolate of Vietnamese highly pathogenic PRRSV challenge in pigs

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**ABSTRACT.** The efficacy of a commercial attenuated live type 2 porcine reproductive and respiratory syndrome (PRRS) vaccine was tested under experimental infection with a highly virulent Vietnamese virus isolated from a diseased pig affected with highly pathogenic PRRS (HP-PRRS) using specific pathogen-free (SPF) pigs. Twenty-five 4-week-old SPF pigs were divided into three groups as follows: pigs vaccinated with a single dose of the vaccine (Group 1, n=10), unvaccinated pigs (Group 2, n=10) and unvaccinated and non-infectious control pigs (Group 3, n=5). Four weeks later, Groups 1 and 2 were challenged with a 1 ml inoculum containing  $1 \times 10^{5.5}$  50% tissue culture infectious dose (TCID<sub>50</sub>)/ml of a Vietnamese HP-PRRS virus isolated in 2010 via the intranasal route. Animals were monitored during the subsequent two-week period post-challenge and necropsied for virological and pathological assays. Results showed a significant reduction in viral replication and shedding in vaccinated pigs compared to unvaccinated pigs. The non-vaccinated pigs showed severe pyrogenic and respiratory illness with marked systematic lesions including interstitial pneumonia and thymic atrophy. In contrast, vaccinated pigs recovered quickly from fever with only mild pathological manifestations. Therefore, although viral shedding was still noted, immunization with the live PRRS vaccine did indeed reduce viral replication and disease severity, suggesting its utility in minimizing outbreaks of HP-PRRS.

**KEY WORDS:** highly pathogenic porcine reproductive and respiratory syndrome, pig, vaccine

*J. Vet. Med. Sci.*

79(4): 765–773, 2017

doi: 10.1292/jvms.16-0536

Received: 16 October 2016

Accepted: 1 February 2017

Published online in J-STAGE:

25 February 2017

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure in sows and respiratory symptoms in piglets and growing pigs. Following its emergence in the late 1980s, PRRS has become endemic in most pig-producing countries [9, 28]. The etiologic agent of PRRS is the PRRS virus (PRRSV), an enveloped, single stranded, positive sense RNA virus which is a member of the *Arteriviridae* family in the order *Nidovirales* [3]. Two distinct genotypes of PRRSV which share approximately 60% identity at the nucleotide level have been described: type 1 (European genotype) and type 2 (North American genotype) [19]. Within these genotypes, however, substantial genetic and antigenic diversity has been demonstrated [33]. Further, the pathogenesis of isolated viruses ranges widely from subclinical to severe manifestations [33]. In 2006, a highly pathogenic form of PRRS causing high fever and mortality in animals of all ages emerged in China and was named highly pathogenic PRRS (HP-PRRS) [26]. The etiologic, highly virulent type 2 PRRSVs were detected, and a common deletion site in nonstructural protein 2 (nsp2) was identified [26, 30]. HP-PRRS, caused by viruses with a common genetic character [25], has now rapidly spread throughout Southeast Asian countries, where it has caused severe economic losses [4, 20, 21]. Countries presently free from these highly virulent PRRSVs are at high risk of their infection and spread.

An attenuated vaccine (Ingelvac PRRS® MLV) from the VR-2332 strain, a prototype of type 2 PRRSV, has been shown to

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be effective against heterologous viral isolates [2] and is in wide use in many countries. The vaccine has been shown to reduce mortality rate and viremia under experimental conditions with virulent Chinese strains [27]. However, viral strains of HP-PRRS are reported to show diverse virulence [32], which may influence the evaluation of vaccine efficacy. Recently, Lager *et al.* reported that Vietnamese HP-PRRSV isolated in 2007 and Chinese HP-PRRSV have different pathogenicity potential in pigs immunized with Ingelvac PRRS® MLV [12]. The first Vietnamese HP-PRRS outbreak was confirmed in 2007, and PRRSV has since continued to spread to other regions of the country [4]. Thuy *et al.* compared genetic mutations in ORF5 between 2007 and 2010 isolates and reported some differences [25]. In addition, Giang *et al.* described severe clinical and pathological manifestations in pigs affected with HP-PRRS in 2010 in Vietnam [7]. These findings indicate the need for further evaluation of the efficacy of the currently available live vaccine.

Here, we evaluated the *in vivo* pathogenicity and virulence of the 2010 Vietnamese isolate and the efficacy of Ingelvac PRRS® MLV by assessing clinical features, viral load in sera, oral fluid and organs, and gross and microscopic lesions in a specific pathogen-free (SPF) piglet model.

## MATERIALS AND METHODS

### Animals

Crossbreed SPF pigs aged 4 weeks were purchased from a closed SPF herd (ZEN-NOH LIVESTOCK CO., LTD., Tokyo, Japan) and were negative for pathogens for PRRS, pseudorabies, porcine epidemic diarrhea, transmissible gastroenteritis, atrophic rhinitis, *Mycoplasma pneumoniae*, swine dysentery, salmonellosis, toxoplasma and actinobacillosis. Pigs were also confirmed to be negative for antibody to PRRSV before the experiment using a commercially available enzyme-linked immunosorbent assay (ELISA) (HerdChek PRRS ELISA; IDEXX Laboratories Westbrook, ME, U.S.A.). The pigs were kept in a closed animal facility and received a commercial diet.

### Virus

The virus (10186-614 strain) was isolated in 2010 from an affected pig with HP-PRRS in Vietnam using MARC-145 cell culture by three times passaged. The nsp2 and open reading frame (ORF) 5 regions of this isolate shared >99% nucleotide identity with equivalent regions of the prototypical HP-PRRSV JXA1 strain (GenBank accession number: EF112445), and the nucleotide identity of the ORF5 region was 85.6% of the attenuated live vaccine. The isolate was propagated three times by culture in porcine alveolar macrophages (PAMs). PAMs were obtained from pigs aged approximately 4 weeks old, as described previously [15], and then were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (Cansera International INC., Ontario, Canada) and antibiotics (25 U/ml penicillin and 25 µg/ml streptomycin (NAKARAI TESQUE INC., Kyoto, Japan), 40 µg/ml gentamicin (Thermo Scientific, Hudson, NH, U.S.A.), 25 µg/ml neomycin (Thermo Scientific) and 300 U/ml polymyxin (Thermo Scientific)). The isolate was stored at -80°C until use, before amplification by one passage in PAMs before inoculation.

### Experimental design and postmortem examination

Twenty-five pigs were randomly allocated into three groups: Group 1 (n=10) was administered an intra-muscular injection of 2 ml attenuated live genotype 2 PRRSV vaccine (Ingelvac PRRS® MLV; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO, U.S.A.) and inoculated 4 weeks later with 1 ml of nasal spray containing  $1 \times 10^{5.5}$  50% tissue culture infectious dose/ml (TCID<sub>50</sub>/ml) of the 10186-614 strain of PRRSV with a nasal spray device (TOP Corporation; Tokyo, Japan); Group 2 (n=10) was injected with the virus alone; and Group 3 (n=5) was left as an uninfected control group. The pigs were monitored for body temperature, clinical signs and body weight during the experiment. Lethargy (1, mild; 2, severe), dyspnea (1, mild; 2, severe) and anorexia (1) were monitored and scored on a scale from 0 to 5. All pigs were necropsied at 14 days post-inoculation (dpi) with the virus, and tissues were collected at necropsy for pathological, virological and bacteriological assays. This study was conducted in compliance with the animal experimentation code of the National Institute of Animal Health (NIAH) (Approval number: 12-059, 8/17/ 2012).

### Serology and quantities of PRRSV RNA

Serum was collected from pigs on -28, -21, -14, -7, 0, 1, 2, 3, 5, 8, 11 and 14 dpi to analyze antibodies against PRRSV and quantities of PRRSV RNA. Antibodies against PRRSV were determined using the HerdChek PRRS ELISA (IDEXX Laboratories). Oral fluid was collected with a cotton pad from individual pigs at 0, 1, 2, 3, 5, 8, 11 and 14 dpi to quantify PRRSV RNA. At necropsy, lungs, tonsils, tracheobronchial lymph nodes, liver, kidneys and spleen were also collected to examine the quantity of PRRSV RNA in these tissues. Viral RNA extraction kits for sera and oral fluid (QIAamp Viral RNA Mini kit; QIAGEN, Venlo, Netherlands) and for tissues (QIAGEN RNeasy Mini kit; QIAGEN) were used as templates for one-step real-time reverse transcriptase polymerase chain reaction (qRT-PCR) with a kit (TaKaRa One Step SYBR PrimeScript RT-PCR Kit II, Takara Bio Inc., Otsu, Japan). We used a slightly modified version of the published method. Briefly, a primer pair (forward: 5'-TCCAGATGCCGTTTGTGCTT-3', reverse: 5'-GACGCCGACGACAAATG-3') designed for TaqMan qRT-PCR [11] was used to detect a portion of the ORF7 gene (124 nucleotides) without use of a labeled probe. To obtain the standard curve for the quantitative RT-PCR assay, the equivalent of  $1 \times 10^5$  TCID<sub>50</sub>/ml of viral RNA was extracted from the culture supernatant, and serial 10-fold dilutions were analyzed. Subsequently, to analyze the gene copy number included in these dilutions, positive control DNA was generated using the synthetic gene nsp2 (partial, 317 bp) synthesized by GeneArt® Strings DNA Fragments (Life

Technologies Inc., Carlsbad, CA, U.S.A.). A linear standard curve was generated for each quantitative RT-PCR run using serial dilutions. The  $C_t$  value is valid only between the minimum and maximum values obtained using the standard RNA. Fluorescence data were analyzed using PE 7500 Sequence Detection System Software (Version 1.4; Thermo Scientific).

#### *Sequence analysis of open reading frame 5 region*

Extracted viral RNAs from sera were randomly selected from three RNA samples at -7, 0, 3 and 8 dpi of HP-PRRSV challenge. To detect the ORF5 gene, extracted RNA was used for one-step RT-PCR using a QIAGEN One-step RT-PCR Kit (QIAGEN) with the published PCR primer pair (P420; 5'-CCATTCTGTTGGCAATTTGA-3' and P620; 5'-GGCATATATCATCACTGGCG-3') [1]. The primers amplified 716 nucleotides (nt) of the ORF4-6 regions. For DNA sequencing, PCR products were purified from agarose gel or PCR solution using a MinElute PCR Purification Kit (QIAGEN). The PCR products were directly sequenced using the same primers with BigDye Terminator v3.1 cycle sequencing kits (Thermo Scientific). Sequence reaction products were analyzed using an ABI 3,130 × 1 genetic analyzer (Thermo Scientific). Multiple alignment analysis was conducted using Genetics Software v8 (Genetyx, Tokyo, Japan).

#### *Pathological examination*

The scoring system used for visible pneumonia and histopathological pneumonic lesions followed a previous paper [6] with modification to match the characteristics of lesions from an experimental infection of this isolate [8]. At necropsy, visual examination of all organs was performed for each pig, and gross lung lesions were scored from 0 to 100 points to estimate the percentage of lung affected by pneumonia, which occurred as a well-differentiated reddish edematous consolidation or tan-mottled discoloration. The seven lung lobes (cranial, middle and the dorsal caudal lung lobes on the right and left sides, and accessory lobe) were each assigned a number to reflect the approximate lung volume with visible pneumonia and scored to reflect the extent of grossly visible pneumonia, and the summed number for all lobes combined was evaluated. Single sections of tissue were collected from the seven lung lobes mentioned above as well as the liver, kidneys, spleen, heart, brain and lymphoid organs for microscopic examination. Samples were suspended in 10% neutral buffered formalin, then dehydrated, embedded in paraffin wax, sectioned at 4  $\mu$ m and stained with hematoxylin and eosin. Microscopic lung lesions were scored to reflect the severity of interstitial pneumonia as follows: 0=no microscopic lesions, 1=mild focally interstitial pneumonia, 2=mild diffuse interstitial pneumonia, 3=moderate focally interstitial pneumonia, 4=moderate diffuse interstitial pneumonia, 5=severe focally bronchointerstitial pneumonia with hemorrhage and 6=severe diffuse bronchointerstitial pneumonia with severe hemorrhage. The overall mean was then calculated.

#### *Bacterial isolation*

Nasal swabs were collected at the start of the experiment, and tissues (lungs, liver, kidneys, spleen, heart and brain) were collected at necropsy for bacterial assay from individual pigs. Columbia agar with 5% sheep blood (Becton, Dickinson and Co., Franklin Lakes, NJ, U.S.A.), MacConkey agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) and 5% mucin-supplemented PPLO broth (Becton, Dickinson and Co.; San Jose, CA, U.S.A.) was used for swine pathogenic bacteria and mycoplasmas using a general isolation method.

#### *Statistical analysis*

Statistical analyses were performed for multiple comparisons and for respective pairwise comparisons using the 'EZR' software package (Saitama Medical Center, Jichi Medical University; Saitama, Japan) on the R statistical environment.  $P < 0.05$  was considered statistically significant.

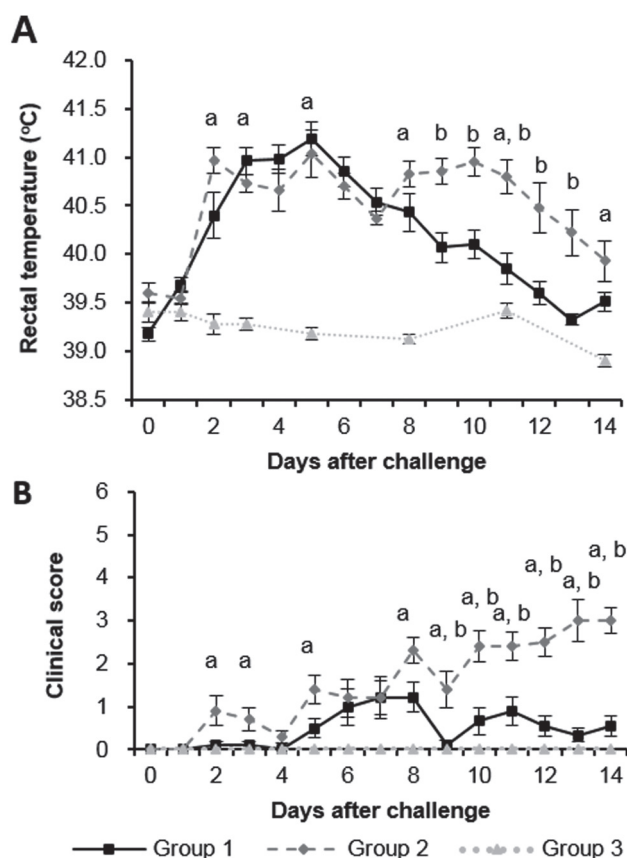
## RESULTS

#### *Body temperature*

Prior to viral challenge, the three groups did not significantly differ with regard to mean rectal temperature. Following viral challenge, in contrast, mean rectal temperature in Groups 1 and 2 increased at 2 dpi, and fever increased to over 40.5°C in both groups and persisted until 7 dpi, with a peak of over 41°C at 5 dpi. Fever in Group 1 gradually decreased to less than 40°C at 11 dpi, while fever in Group 2 remained over 40.5°C from 8 to 12 dpi. Body temperature of the control group ranged from 39.0 to 39.5°C throughout the study. Overall, mean rectal temperatures were significantly higher in Groups 1 and 2 than in the control group from 2 to 14 dpi, while temperature was significantly lower in Group 1 than in Group 2 from 9 to 13 dpi (Fig. 1A).

#### *Clinical signs*

After viral challenge, pigs in both inoculated groups exhibited periocular edema and redness of the skin on the trunk. Both groups showed loss of appetite and tachypnea from 2 or 3 dpi to up to 8 dpi, with most pigs in Group 1 recovering. In contrast, after 9 dpi, pigs in Group 2 still had a poor appetite and respiratory signs, and became seriously ill with coughing and abdominal breathing. These clinical signs persisted until the end of the experiment. A proportion of animals in Group 2 lay collapsed in the recumbent position and exhibited intermittent thrill. Clinical score in Group 1 was significantly lower than that in Group 2 from 9 to 14 dpi. No clinical signs were observed in uninfected animals (Fig. 1B).



**Fig. 1.** Changes in rectal temperature (A) and clinical score (B) of pigs following challenge with virus. a) Statistically significant difference among the three groups ( $P < 0.05$ ). b) Statistically significant difference between Groups 1 and 2 ( $P < 0.05$ ). Bars indicate SE of the mean. Temperature in Group 3 was not measured on 4, 6, 7, 9, 10, 12 and 13 days post-inoculation.

#### Daily weight gain

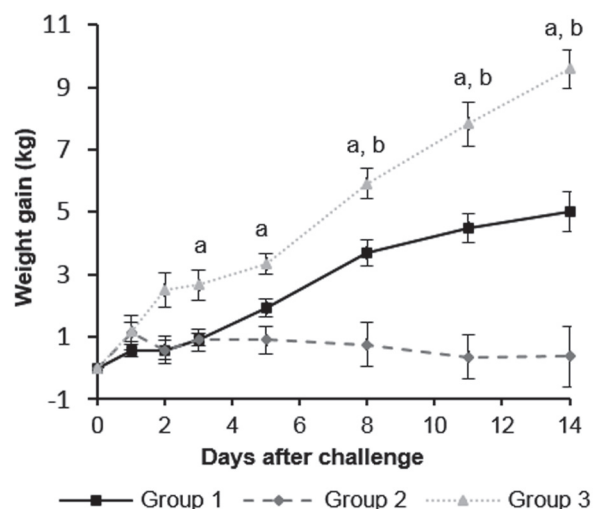
Average daily weight gain significantly differed among the three groups from 3 dpi onwards. No increase in daily weight gain was observed in Group 2 following viral challenge. Significant differences were noted between Groups 1 and 2 from 8 dpi onwards. Body weight gain by the day of necropsy was 5.02 kg in Group 1, 0.37 kg in Group 2 and 9.58 kg in Group 3 (Fig. 2).

#### Viral load in sera and oral fluid

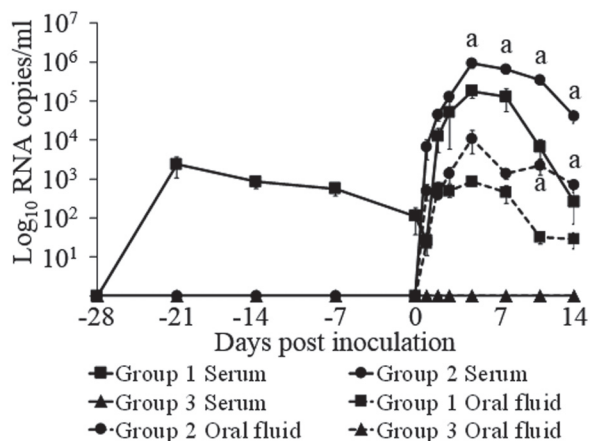
Viral RNA of approximately  $10^3$  copies/ml was observed in the serum of pigs in Group 1 on 7, 14, 21 and 28 days after immunization. Serum viral load in the two inoculated groups sharply rose from 1 to 5 dpi and peaked at 5 dpi with  $1.8 \times 10^5$  copies/ml in Group 1 and  $9.2 \times 10^5$  copies/ml in Group 2. Serum viral load in Group 2 remained high, at  $6.6 \times 10^5$  copies/ml at 8 dpi,  $3.5 \times 10^5$  copies/ml at 11 dpi and  $4.2 \times 10^4$  copies/ml at 14 dpi. In contrast, serum viral load in Group 1 rapidly declined to  $1.3 \times 10^5$  copies/ml at 8 dpi,  $6.5 \times 10^3$  copies/ml at 11 dpi and  $2.6 \times 10^2$  copies/ml at 14 dpi. Viral load in Groups 1 and 2 significantly differed from 5 to 14 dpi by 10 to 1,000 times. Virus was detected in oral fluid from both viral challenge groups from 1 to 14 dpi, with a peak at 5 dpi following viral challenge. Viral shedding significantly differed between the two groups at 11 and 14 dpi. No viral RNA was found in the sera or oral fluid of pigs in Group 3 (Fig. 3).

#### Sequence analysis of ORF5 region

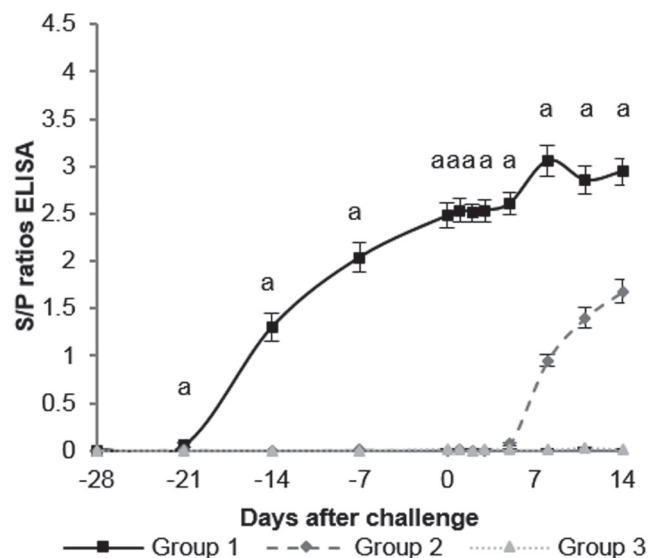
To confirm that the RT-PCR products were amplified from the major viral RNA after 1 dpi in Groups 1 and 2, the sequences of each RT-PCR product were compared with those of the vaccine strain and HP-PRRSV. For PCR products amplified from -7 and 0 dpi, ORF5 identity for nucleotides ranged from 99.5–99.7% and 88.6–89.1% against vaccine strain and HP-PRRSV, respectively. The ORF5 gene was sequenced from the infected sera at 3 and 8 dpi in Group 1 and compared with those of the inoculated virus. The sequences were 100% identical, and no additional new mutations were found.



**Fig. 2.** Mean cumulative daily weight gain following challenge with virus. a) Statistically significant difference among three groups ( $P < 0.05$ ). b) Statistically significant difference between Groups 1 and 2 ( $P < 0.05$ ). Bars indicate SE of the mean.



**Fig. 3.** Quantity of viral RNA in serum and oral fluid before and after challenge with virus. Quantity of porcine reproductive and respiratory syndrome virus (PRRSV) RNA (mean  $\pm$  SE) was measured by one-step real-time reverse transcriptase PCR. a) Statistically significant difference between Groups 1 and 2 ( $P < 0.05$ ).



**Fig. 4.** Mean titer of porcine reproductive and respiratory syndrome virus (PRRSV) antibody measured by ELISA (S/P ratios) before and after challenge with virus. Bars indicate SE of the mean. a) Statistically significant difference between Groups 1 and 2 ( $P < 0.05$ ).

**Table 1.** Porcine reproductive and respiratory syndrome virus (PRRSV) RNA load (mean  $\pm$  SE copies/ml) measured by one-step real-time reverse transcriptase PCR in 10% tissue homogenates in Group 1 and Group 2

Tissues	Group 1	Group 2
Liver	$1.6 \times 10^2 \pm 1.6 \times 10^{2a)}$	$3.2 \times 10^3 \pm 1.3 \times 10^3$
Kidney	$1.5 \times 10 \pm 1.5 \times 10^a)$	$1.7 \times 10^3 \pm 1.5 \times 10$
Lung	$1.2 \times 10^4 \pm 5.1 \times 10^{3a)}$	$2.6 \times 10^5 \pm 8.8 \times 10^4$
Spleen	$8.9 \times 10^2 \pm 2.8 \times 10^2$	$1.2 \times 10^4 \pm 6.6 \times 10^3$
Tonsil	$2.8 \times 10^4 \pm 6.2 \times 10^{3a)}$	$1.6 \times 10^5 \pm 4.3 \times 10^4$
Tracheobronchial lymph node	$1.9 \times 10^4 \pm 1.3 \times 10^4$	$2.5 \times 10^4 \pm 8.1 \times 10^3$

a) Statistically significant difference between Groups 1 and 2 ( $P < 0.05$ ).

### Antibody to PRRSV

Antibodies against PRRSV were observed in Group 1 from 7 days after vaccination in some pigs and were over the cutoff line (S/P=0.4) in those of all animals at 14 dpi except one pig (S/P=0.38). The S/P ratio then gradually rose, and a transient increase was observed at 8 dpi. In Group 2, positive conversion of antibody to PRRSV was observed at 8 dpi, and the S/P ratio of viral antibody gradually rose. In contrast, no antibodies against PRRSV were observed in Group 3 (Fig.4).

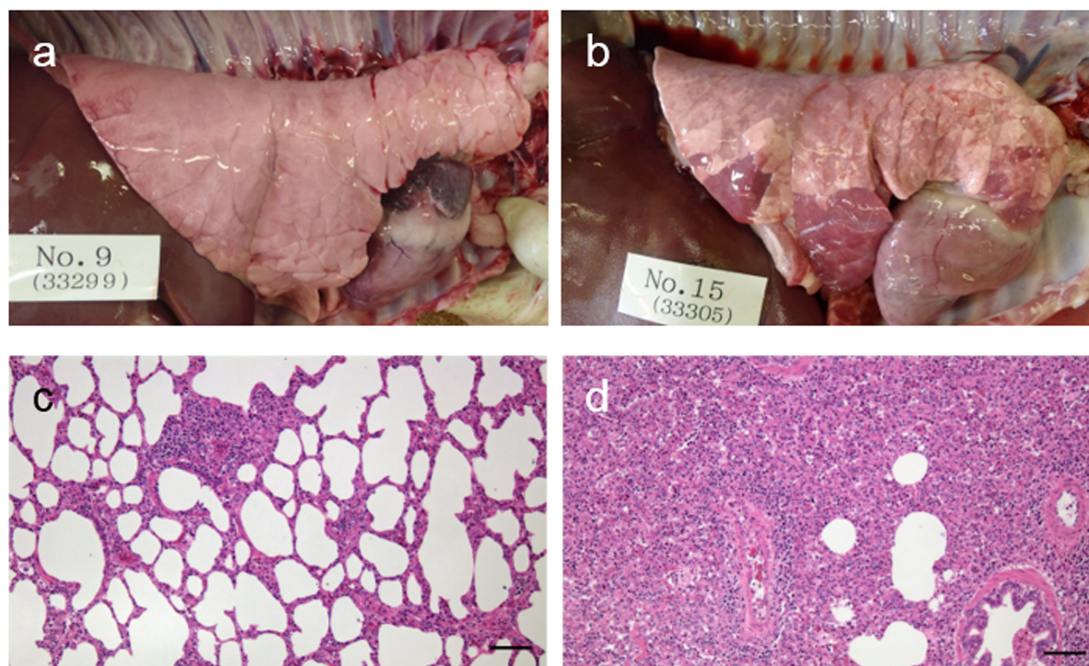
### Viral RNA in tissues

PRRSV RNA was detected from all tested organs, namely the liver, kidneys, lung, spleen, tonsils and tracheobronchial lymph nodes in both Groups 1 and 2. The quantity of PRRSV RNA in the lungs, liver, kidneys and tonsils was lower in Group 1 than in Group 2. However, values for the spleen and tracheobronchial lymph nodes did not significantly differ between Groups 1 and 2 (Table 1). No PRRSV RNA was observed in Group 3.

### Gross and histological lesions

Prominent gross lesions in pigs in Group 2 were pneumonia and thymic atrophy. The lung lesions in these pigs were characterized by tan-mottled swollen consolidation in whole lobes or well-demarcated dark-reddish pneumonia in the cranial, middle and accessory lobes and frontal portion of the caudal lobe (Fig. 5b). In contrast, lungs in the majority of Group 1 pigs showed slightly discolored swollen consolidation (Fig. 5a), and well-demarcated dark-reddish pneumonia was observed in only one pig. Mean ( $\pm$  SD) gross lung lesion scores were  $57.9 \pm 18.3$  in Group 2 and  $12.5 \pm 6.9$  in Group 1, with this difference being significant (Table 2). In addition to these lesions, edema in the cardiac sac was observed in a proportion of pigs in Group 2. Lymphadenopathy was observed in all pigs in Groups 1 and 2, but thymus atrophy was not observed in Group 1.

Microscopically, pneumonic lesions in Group 2 were characterized by severe necrotizing interstitial pneumonia with marked



**Fig. 5.** Gross and microscopic lung lesions. (a) Right dorsal surface of lung in Group 1; (b) right dorsal surface of lung in Group 2; (c) mild interstitial pneumonia in Group 1 (hematoxylin and eosin staining, bar=200  $\mu$ m); (d) severe interstitial pneumonia in Group 2 (hematoxylin and eosin staining, bar=200  $\mu$ m).

**Table 2.** Lung lesion scores in Groups 1 and 2

Score	Group 1	Group 2
Gross pneumonic score <sup>a)</sup>	12.5 $\pm$ 6.9 <sup>c)</sup>	57.9 $\pm$ 18.3
Microscopic pneumonic score <sup>b)</sup>	1.2 $\pm$ 0.5 <sup>c)</sup>	2.7 $\pm$ 0.5

a) Gross pneumonic score (mean  $\pm$  SD) given as estimate of percentage of the lung with grossly visible pneumonia. b) Microscopic pneumonic scores (mean  $\pm$  SD): 0=no microscopic lesions, 1=mild interstitial pneumonia, 2=moderate multifocal interstitial pneumonia, 3=moderate diffuse interstitial pneumonia and 4=severe interstitial pneumonia. c) Statistically significant difference between Groups 1 and 2 ( $P < 0.05$ ).

accumulation of proteinaceous and karyopyknotic cell debris in multiple alveolar spaces, marked type II pneumocyte hypertrophy and hyperplasia, septal infiltration with macrophages, and histiolympocytic infiltration around the vessels in the multifocal alveolar wall (Fig. 5d). In contrast, pneumonic lesions in Group 1 were characterized by slight to moderate histiolympocytic infiltration around the vessels in the multifocal alveolar wall (Fig. 5c). The microscopic pneumonic score (mean  $\pm$  SD) in Group 1 (1.2  $\pm$  0.5) was significantly lower than that in Group 2 (2.7  $\pm$  0.5) (Table 2). Concerning other tissues, lymphocytic reduction of the thymic cortex and extramedullary hematopoiesis of the spleen were noted only in Group 2. Histiolympocytic infiltration in the kidney was found in 6 pigs in Group 2 and 3 pigs in Group 1. Perivascular cuffing in the brain was found in 3 pigs in Group 2 and 1 pig in Group 1. Follicular hyperplasia in lymphoid tissues was found in all pigs in both Groups 1 and 2. Notably, no gross or microscopic lesions were observed in Group 3.

#### Bacterial examination

*Streptococcus* spp. were isolated from nasal swab of 3 pigs in each group at the start of the experiment, but necropsy findings of these pigs were not significant among other animals. No significant pathogens were isolated from the tissues collected from pigs in any of the groups at necropsy.

## DISCUSSION

We experimentally reproduced the pathological conditions of HP-PRRS in SPF pigs by nasal inoculation with a Vietnamese 2010 isolate of highly virulent PRRSV and evaluated the efficacy of commercially available type 2 PRRSV vaccine. Viral replication, viral emission in oral fluid, clinical signs and lesion severity were significantly reduced, and average daily weight gain

was improved in pigs immunized with modified live PRRSV vaccine compared with non-immunized animals.

Unvaccinated animals exhibited prolonged high fever and severe interstitial pneumonia in this study. These findings correspond to the common pathological features in both field and experimental cases of highly virulent PRRSV-infected animals [17, 26, 31]. Furthermore, thymic atrophy developed in the unvaccinated pigs, which Guo *et al.* pointed out as a unique pathological feature in experimental infection with HP-PRRSV [5]. The above results may likely confirm this experimental model for outcomes evaluation following infection with highly virulent PRRSVs. In contrast, our study did not reproduce the high mortality rate of HP-PRRS. Conflicting results have been published concerning mortality among experimental challenge studies of HP-PRRS [5, 17, 31]. Guo *et al.* recently reported that Vietnamese HP-PRRSV isolated in 2007 induced reduced pathogenic outcomes compared to those with the Chinese strain [5]. The determinant of the different pathogenicity between the two strains has not been identified. Meanwhile, given the frequency of concurrent infection with opportunistic pathogens with experimental HP-PRRS [5, 17] and the age-dependent difference in mortality in field cases [26], the difference in lethality in experimental HP-PRRS is likely due to host factors, such as the age and bacterial flora of animals. It is interesting that the high mortality rates were not reproducible using clean SPF animals in the present study. Host factors and bacterial flora might lead to higher mortality in HP-PRRSV even under experimental conditions.

Immunization using the vaccine did not provide perfect protection against HP-PRRSV, but obviously contributed to reducing the symptoms of the disease. Although the vaccinated and unvaccinated groups had elevated rectal temperature after viral challenge, the immunization significantly diminished the duration of high fever. Consistent with the induction of defervescence, respiratory signs were also significantly diminished in the vaccinated group. Gross and histological examinations showed that the extent and severity of pneumonic lesions were lower in the vaccinated group than the unvaccinated group. Improvement in these clinical signs and pathological findings might have resulted in the improvement in weight gain. The antipyretic potency against prolonged high fever and symptoms of relief in this study resembled that of virus-challenged animals immunized with a type 1 PRRSV vaccine [23]. Longitudinal data from qRT-PCR revealed an increase in viremia in the vaccinated group, which then rapidly decreased compared to the unvaccinated group. Interestingly, these two courses of viral load are quite similar to those previously reported for Chinese HP-PRRS (rJXwn06) in pigs vaccinated and unvaccinated with Ingelvac PRRS® MLV [12]. Lager *et al.* also compared the viral load of Vietnamese HP-PRRSV isolate in 2007 (rSRV07) between vaccinated and unvaccinated animals, but patterns differed to those of JXwn06 [12]. Therefore, while the SRV07, JXwn06 and Vietnamese 2010 (10186-614) isolates are genetically quite close (>98.3% in ORF5 nt level; data not shown), the 10186-614 strain we used here had similar pathogenicity to the JXwn06 strain. Considered together with the fever which occurred in the early phase in the vaccinated group, this kinetic alteration of viremia might indicate that immunization with a vaccine contributes to viral clearance in infected tissues rather than inhibition against viral proliferation in the early phase. Moreover, the amount of viral RNA in oral fluid collected from vaccinated animals after challenge infection was significantly reduced compared with that in the unvaccinated group at 11 and 14 dpi. These results indicate that the inhibition of viral replication by vaccination leads to a reduction in viral load in the circulating blood and subsequently suppresses the amount of virus excreted from the body.

Several limitations of the study warrant mention. First, although we used a vaccine with efficacy against the 10186-614 strain, namely Ingelvac PRRS® MLV, it is difficult to predict whether this would be valid for other HP-PRRSV variants. Although the mechanism of host immunity against PRRSV has not been fully determined, a degree of cross-protection has been identified among different viral isolates [16, 22]. Reports have described the difficulty in predicting the strength of protective immunity against PRRSV infections among heterogeneous viral strains by analyzing contributing factors, such as the production of neutralizing antibodies [10] and genetic characteristics [16]. The evaluation of PRRSV vaccine candidates might therefore depend upon challenge studies of whether or not a vaccine can alleviate pathological conditions. Second, we did not clarify how humoral and cellular immunity contributed to inhibit viral replication in pigs. Both immunity may prove essential to PRRSV clearance [18, 29]. In the present study, all pigs in the vaccinated group showed increased production of specific antibodies to PRRSV before viral challenge. As neutralizing antibodies against PRRSV appear at 4 weeks post-infection [14], the detected antibodies may have neutralizing activities against viral infection. However, the strength of the neutralizing antibody response [10, 24] and interferon response [13] in PRRSV infection does not appear to be closely linked with the host response to infection or virulence. Further studies are required to clarify the key elements of host immunity that reduce the disease severity of PRRS.

In summary, we found that immunization with a modified live PRRSV vaccine significantly reduced both viral replication and lesion severity and contributed to the improvement of clinical manifestations in pigs experimentally infected with the 2010-Vietnamese isolate of HP-PRRS. These findings suggest the potentially wide utility of this vaccine in controlling HP-PRRS. Further, secondary infection contributes to the economic cost of PRRS [33]. In this study, however, no thymic atrophy developed in the vaccinated pigs. The reduced influence of the virus on organs of the immune system following immunization with a vaccine may inhibit secondary infection and help reduce economic costs.

**CONFLICT OF INTEREST STATEMENT.** This study was conducted under a contract for collaborative research between NIAH and Boehringer Ingelheim Vetmedica. This study was designed by researchers of NIAH in consultation with Boehringer Ingelheim Vetmedica. All procedures, data collection, registries, manipulation and analysis of samples were conducted by NIAH researchers. None of the authors had any financial or personal relationship with Boehringer Ingelheim Vetmedica that could inappropriately influence or bias the content of the paper.

**DISCLOSURE.** We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

**ACKNOWLEDGMENTS.** We thank Dr. Hideki Kobayashi (Bacterial and Parasitic Diseases Research Division, National Institute of Animal Health, NARO) for bacterial isolation. The authors would like to thank all colleagues of the NIAH for animal handling and technical assistance. Preliminary results were presented as an Abstract at the 6th Asian Pig Veterinary Society Congress, Ho Chi Minh City, 23rd–25th September 2013.

## REFERENCES

- Andreyev, V. G., Wesley, R. D., Mengeling, W. L., Vorwald, A. C. and Lager, K. M. 1997. Genetic variation and phylogenetic relationships of 22 porcine reproductive and respiratory syndrome virus (PRRSV) field strains based on sequence analysis of open reading frame 5. *Arch. Virol.* **142**: 993–1001. [[Medline](#)] [[CrossRef](#)]
- Cano, J. P., Dee, S. A., Murtaugh, M. P. and Pijoan, C. 2007. Impact of a modified-live porcine reproductive and respiratory syndrome virus vaccine intervention on a population of pigs infected with a heterologous isolate. *Vaccine* **25**: 4382–4391. [[Medline](#)] [[CrossRef](#)]
- Cavanagh, D. 1997. Nidovirales: a new order comprising Coronaviridae and Arteriviridae. *Arch. Virol.* **142**: 629–633. [[Medline](#)]
- Feng, Y., Zhao, T., Nguyen, T., Inui, K., Ma, Y., Nguyen, T. H., Nguyen, V. C., Liu, D., Bui, Q. A., To, L. T., Wang, C., Tian, K. and Gao, G. F. 2008. Porcine reproductive and respiratory syndrome virus variants, Vietnam and China, 2007. *Emerg. Infect. Dis.* **14**: 1774–1776. [[Medline](#)] [[CrossRef](#)]
- Guo, B., Lager, K. M., Henningson, J. N., Miller, L. C., Schlink, S. N., Kappes, M. A., Kehrli, M. E. Jr., Brockmeier, S. L., Nicholson, T. L., Yang, H. C. and Faaberg, K. S. 2013. Experimental infection of United States swine with a Chinese highly pathogenic strain of porcine reproductive and respiratory syndrome virus. *Virology* **435**: 372–384. [[Medline](#)] [[CrossRef](#)]
- Halbur, P. G., Paul, P. S., Frey, M. L., Landgraf, J., Eernisse, K., Meng, X. J., Lum, M. A., Andrews, J. J. and Rathje, J. A. 1995. Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. *Vet. Pathol.* **32**: 648–660. [[Medline](#)] [[CrossRef](#)]
- Huong Giang, N. T., Lan, N. T., Nam, N. H., Hirai, T. and Yamaguchi, R. 2016. Pathological characterization of an outbreak of porcine reproductive and respiratory syndrome in Northern Vietnam. *J. Comp. Pathol.* **154**: 135–149. [[Medline](#)] [[CrossRef](#)]
- Kawashima, K., Takagi, M., Iseki, H., Shibahara, T., Tung, N., Inui, K. and Tsunemitsu, H. 2011. Lesions and viral antigen distribution in the pigs inoculated with emerging porcine reproductive and respiratory syndrome virus., ed., *Proceedings of the 5th Asian Pig Veterinary Society Congress*.
- Keffabur, K. K. 1989. Reproductive failure of unknown etiology. (ed), *Am. Assoc. Swine Prac. News*. **1**: 1–9.
- Kim, W. I., Lee, D. S., Johnson, W., Roof, M., Cha, S. H. and Yoon, K. J. 2007. Effect of genotypic and biotypic differences among PRRS viruses on the serologic assessment of pigs for virus infection. *Vet. Microbiol.* **123**: 1–14. [[Medline](#)] [[CrossRef](#)]
- Kleiboeker, S. B., Schommer, S. K., Lee, S. M., Watkins, S., Chittick, W. and Polson, D. 2005. Simultaneous detection of North American and European porcine reproductive and respiratory syndrome virus using real-time quantitative reverse transcriptase-PCR. *J. Vet. Diagn. Invest.* **17**: 165–170. [[Medline](#)] [[CrossRef](#)]
- Lager, K. M., Schlink, S. N., Brockmeier, S. L., Miller, L. C., Henningson, J. N., Kappes, M. A., Kehrli, M. E., Loving, C. L., Guo, B., Swenson, S. L., Yang, H. C. and Faaberg, K. S. 2014. Efficacy of Type 2 PRRSV vaccine against Chinese and Vietnamese HP-PRRSV challenge in pigs. *Vaccine* **32**: 6457–6462. [[Medline](#)] [[CrossRef](#)]
- Lee, S. M., Schommer, S. K. and Kleiboeker, S. B. 2004. Porcine reproductive and respiratory syndrome virus field isolates differ in in vitro interferon phenotypes. *Vet. Immunol. Immunopathol.* **102**: 217–231. [[Medline](#)] [[CrossRef](#)]
- Lopez, O. J. and Osorio, F. A. 2004. Role of neutralizing antibodies in PRRSV protective immunity. *Vet. Immunol. Immunopathol.* **102**: 155–163. [[Medline](#)] [[CrossRef](#)]
- Mengeling, W. L., Lager, K. M. and Vorwald, A. C. 1995. Diagnosis of porcine reproductive and respiratory syndrome. *J. Vet. Diagn. Invest.* **7**: 3–16. [[Medline](#)] [[CrossRef](#)]
- Mengeling, W. L., Lager, K. M., Vorwald, A. C. and Koehler, K. J. 2003. Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. *Vet. Microbiol.* **93**: 13–24. [[Medline](#)] [[CrossRef](#)]
- Metwally, S., Mohamed, F., Faaberg, K., Burrage, T., Prarat, M., Moran, K., Bracht, A., Mayr, G., Berninger, M., Koster, L., To, T. L., Nguyen, V. L., Reising, M., Landgraf, J., Cox, L., Lubroth, J. and Carrillo, C. 2010. Pathogenicity and molecular characterization of emerging porcine reproductive and respiratory syndrome virus in Vietnam in 2007. *Transbound. Emerg. Dis.* **57**: 315–329. [[Medline](#)] [[CrossRef](#)]
- Mulupuri, P., Zimmermann, J. J., Hermann, J., Johnson, C. R., Cano, J. P., Yu, W., Dee, S. A. and Murtaugh, M. P. 2008. Antigen-specific B-cell responses to porcine reproductive and respiratory syndrome virus infection. *J. Virol.* **82**: 358–370. [[Medline](#)] [[CrossRef](#)]
- Murtaugh, M. P., Elam, M. R. and Kakach, L. T. 1995. Comparison of the structural protein coding sequences of the VR-2332 and Lelystad virus strains of the PRRS virus. *Arch. Virol.* **140**: 1451–1460. [[Medline](#)] [[CrossRef](#)]
- Ni, J., Yang, S., Bounlom, D., Yu, X., Zhou, Z., Song, J., Khamphouth, V., Vatthana, T. and Tian, K. 2012. Emergence and pathogenicity of highly pathogenic Porcine reproductive and respiratory syndrome virus in Vientiane, Lao People's Democratic Republic. *J. Vet. Diagn. Invest.* **24**: 349–354. [[Medline](#)] [[CrossRef](#)]
- Nilubol, D., Tripipat, T., Hoonsuwan, T. and Kortheerakul, K. 2012. Porcine reproductive and respiratory syndrome virus, Thailand, 2010–2011. *Emerg. Infect. Dis.* **18**: 2039–2043. [[Medline](#)] [[CrossRef](#)]
- Opriessnig, T., Pallares, F. J., Nilubol, D., Vincent, A. L., Thacker, E. L., Vaughn, E. M., Roof, M. and Halbur, P. G. 2005. Genomic homology of ORF 5 gene sequence between modified live vaccine virus and porcine reproductive and respiratory syndrome virus challenge isolates is not predictive of vaccine efficacy. *J. Swine Health and Production* **13**: 246–253.
- Roca, M., Gimeno, M., Bruguera, S., Segalés, J., Diaz, I., Galindo-Cardiel, I. J., Martínez, E., Darwich, L., Fang, Y., Maldonado, J., March, R. and Mateu, E. 2012. Effects of challenge with a virulent genotype II strain of porcine reproductive and respiratory syndrome virus on piglets vaccinated with an attenuated genotype I strain vaccine. *Vet. J.* **193**: 92–96. [[Medline](#)] [[CrossRef](#)]
- Shibata, I., Mori, M. and Uruno, K. 1998. Experimental infection of maternally immune pigs with porcine reproductive and respiratory syndrome (PRRS) virus. *J. Vet. Med. Sci.* **60**: 1285–1291. [[Medline](#)] [[CrossRef](#)]
- Thuy, N. T., Thu, N. T., Son, N. G., Ha, T. T., Hung, V. K., Nguyen, N. T. and Khoa, V. A. 2013. Genetic analysis of ORF5 porcine reproductive and respiratory syndrome virus isolated in Vietnam. *Microbiol. Immunol.* **57**: 518–526. [[Medline](#)]
- Tian, K., Yu, X., Zhao, T., Feng, Y., Cao, Z., Wang, C., Hu, Y., Chen, X., Hu, D., Tian, X., Liu, D., Zhang, S., Deng, X., Ding, Y., Yang, L., Zhang, Y., Xiao, H., Qiao, M., Wang, B., Hou, L., Wang, X., Yang, X., Kang, L., Sun, M., Jin, P., Wang, S., Kitamura, Y., Yan, J. and Gao, G. F. 2007. Emergence of fatal PRRSV variants: unparalleled outbreaks of atypical PRRS in China and molecular dissection of the unique hallmark. *PLoS ONE* **2**: e526. [[Medline](#)] [[CrossRef](#)]



27. Wei, Z., Zhang, J., Zhuang, J., Sun, Z., Gao, F. and Yuan, S. 2013. Immunization of pigs with a type 2 modified live PRRSV vaccine prevents the development of a deadly long lasting hyperpyrexia in a challenge study with highly pathogenic PRRSV JX143. *Vaccine* **31**: 2062–2066. [[Medline](#)] [[CrossRef](#)]
28. Wensvoort, G., Terpstra, C., Pol, J. M. A., ter Laak, E. A., Bloemraad, M., de Kluiver, E. P., Kragten, C., van Buiten, L., den Besten, A., Wagenaar, F., Broekhuijsen, J. M., Moonen, P., Zetstra, T., Deboer, E. A., Tibben, H. J., Dejong, M. F., Vantveld, P., Groenland, G. J. R., Vangennep, J. A., Voets, M. T., Verheijden, J. H. M. and Braamskamp, J. 1991. Mystery swine disease in The Netherlands: the isolation of Lelystad virus. *Vet. Q.* **13**: 121–130. [[Medline](#)] [[CrossRef](#)]
29. Wesley, R. D., Lager, K. M. and Kehrl, M. E. Jr. 2006. Infection with Porcine reproductive and respiratory syndrome virus stimulates an early gamma interferon response in the serum of pigs. *Can. J. Vet. Res.* **70**: 176–182. [[Medline](#)]
30. Zhou, L. and Yang, H. 2010. Porcine reproductive and respiratory syndrome in China. *Virus Res.* **154**: 31–37. [[Medline](#)] [[CrossRef](#)]
31. Zhou, Y. J., Hao, X. F., Tian, Z. J., Tong, G. Z., Yoo, D., An, T. Q., Zhou, T., Li, G. X., Qiu, H. J., Wei, T. C. and Yuan, X. F. 2008. Highly virulent porcine reproductive and respiratory syndrome virus emerged in China. *Transbound. Emerg. Dis.* **55**: 152–164. [[Medline](#)] [[CrossRef](#)]
32. Zhou, Z., Ni, J., Cao, Z., Han, X., Xia, Y., Zi, Z., Ning, K., Liu, Q., Cai, L., Qiu, P., Deng, X., Hu, D., Zhang, Q., Fan, Y., Wu, J., Wang, L., Zhang, M., Yu, X., Zhai, X. and Tian, K. 2011. The epidemic status and genetic diversity of 14 highly pathogenic porcine reproductive and respiratory syndrome virus (HP-PRRSV) isolates from China in 2009. *Vet. Microbiol.* **150**: 257–269. [[Medline](#)] [[CrossRef](#)]
33. Zimmerman, J. J., Benfield, D. A., Dee, S. A., Murtaugh, M. P., Stadejek, T., Stevenson, G. W. and Torremorell, M. 2012. Porcine reproductive and respiratory syndrome virus (porcine arterivirus), pp. 461–486, *In: Disease of Swine*, 10th ed. (Zimmerman, J. J., Karriker, L. A., Ramirez, A., Schwartz, K. J. and Stevenson, G. W. eds.), Wiley-Blackwell, Ames.