



Evaluation of the efficacy of a new modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Fostera PRRS) against heterologous PRRSV challenge



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ABSTRACT

The objective of this study was to evaluate a new modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (Fostera PRRS, Zoetis, Florham, NJ, USA) that was based on a virulent US PRRSV isolate (P129) attenuated using CD163-expressing cell lines. Sixty-four PRRSV-seronegative 3-week-old pigs were randomly divided into the following four groups: vaccinated challenged (group 1), vaccinated unchallenged (group 2), unvaccinated challenged (group 3), and unvaccinated unchallenged (group 4). The pigs in groups 1 and 2 were immunized with a 2.0 mL dose of modified live PRRSV vaccine at 21 days of age, according to the manufacturer's recommendations. At 56 days of age (0 days post-challenge), the pigs in groups 1 and 3 were inoculated intranasally with 3 mL of tissue culture fluid containing 10^5 50% tissue culture infective dose (TCID₅₀)/mL of PRRSV (SNUVR090851 strain, fourth passage in MARC-145 cells). Vaccinated challenged pigs exhibited significantly lower ($P < 0.05$) respiratory scores, viremia, macroscopic and microscopic lung lesion scores, and PRRSV-antigen with interstitial pneumonia than unvaccinated challenged pigs. The induction of PRRSV-specific IFN- γ -SCs by the new modified live PRRSV vaccine produced a protective immune response, leading to the reduction of PRRSV viremia. Although the new modified live PRRSV vaccine is not effective against heterologous PRRSV challenge, the new modified live PRRSV vaccine was able to reduce the levels of viremia and nasal shedding, and severity of PRRSV-induced lesions after challenging virus under experimental conditions.

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1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) virus (PRRSV) is a member of the *Arterivirus* genus (family *Arteriviridae*, order *Nidovirales*) and has 2 major clinical forms: reproductive failure in breeding animals and respiratory diseases in nursery and growing-finishing pigs

(Cavanagh, 1997; Zimmerman et al., 2006). PRRS is the most devastating disease in the swine industry. It causes economic losses of approximately US\$ 560 million annually in the US and 88% of total economic loss is attributed to respiratory diseases in nursery and growing-finishing pigs (Neumann et al., 2005). Hence, the control of respiratory diseases caused by PRRSV is significant for improved growth performance and economic benefit. A modified live PRRSV vaccine is an efficient tool to decrease mortality rate and increase growth performance in nursery and growing-finishing pigs (Corzo et al., 2010; Gillespie and Carroll, 2003).

The commercial modified live PRRSV vaccine (Ingelvac[®] PRRS MLV, Boehringer Ingelheim Vetmedica Inc.,

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St. Joseph, MO, USA) based on type 2 PRRSV, was first licensed for worldwide use in 3- to 18-week-old pigs in 1994 and in pregnant female breeding-stock pigs in 1996. In 2012, another new commercial modified live PRRSV vaccine (Fostera PRRS, Zoetis, Florham, NJ, USA), based on a virulent US PRRS isolate (P129) and attenuated using CD163-expressing cell lines, was introduced into the international market to control respiratory disease in growing pigs.

Because modified live PRRSV vaccines effectively control PRRSV (Corzo et al., 2010; Gillespie and Carroll, 2003), the objective of this study was to evaluate the efficacy of a new modified live PRRSV vaccine to control respiratory disease in growing pigs against PRRSV infection based on clinical, immunological, virological, and pathological analyses under experimental conditions.

2. Materials and methods

2.1. PRRSV inoculum

Type 2 PRRSV (SNUVR090851 strain) was isolated in lung samples from different newly weaned pigs in a 1000-sow herd in Chungcheung Province in 2010 (Han et al., 2013). This PRRSV is normal North American strain and induced severe interstitial pneumonia in 7 days post-inoculation without high fever (Han et al., 2013). This herd showed severe respiratory problems in newly weaned pigs aged 4 to 8 weeks old. The SNUVR090851 strain was identified as a type 2 PRRSV based on the basis of the nucleotide sequences of open reading frame (ORF) 5 (GenBank JN315685) and ORF7 (GenBank JN399072). The SNUVR090851 and vaccine strains (P129) share 87.2% and 92.7% nucleotide identity for ORF5 and ORF7, respectively. The SNUVR090851 is lineage 1 and vaccine strain P129 is lineage 8 based on classification system (Shi et al., 2010).

2.2. Experimental design

A total of 64 colostrum-fed, cross-bred, conventional piglets will be purchased at 14 days of age from a commercial PRRSV free farm. All piglets will be negative for PRRSV according to routine serological testing. All piglets will be negative for type 1 and type 2 PRRSV viremia by real-time polymerase chain reaction (PCR).

Piglets were randomly divided into 4 groups (16 pigs per group). The pigs in groups 1 and 2 were immunized with Fostera PRRS (Zoetis) administered as a 2.0 mL dose at 21 days of age based on the manufacturer's recommendations. At 56 days of age (0 days postchallenge, dpc), the pigs in groups 1 and 3 were inoculated intranasally with 3 mL of tissue culture fluid containing 10^5 50% tissue culture infective dose (TCID₅₀)/mL of PRRSV (SNUVR090851 strain, fourth passage in MARC-145 cells) by setting them on their buttocks perpendicular to the floor and expending the neck fully back. The inoculum was slowly dripped into both nostrils of the pigs taking approximately 3–5 min/pig as previously described (Halbur et al., 1995). The pigs in group 4 remained unvaccinated unchallenged and served as the negative control group. The pigs in each group were housed separately within the facility. Blood samples and

nasal swabs were collected at –35, –32, –30, –28, –25, –21, –14, 0, 7, 10, 14, and 21 dpc. Four pigs from each group were sedated by an intravenous injection of sodium pentobarbital and then euthanized by electrocution at 7, 10, 14, and 21 dpc as previously described (Beaver et al., 2001). Tissues were collected from each pig at necropsy. All of the methods were previously approved by the Seoul National University Institutional Animal Care and Use Committee.

2.3. Clinical observation

Following vaccination and PRRSV challenge, the pigs were monitored weekly for physical conditions and scored daily for clinical respiratory disease severity using scores ranging from 0 (normal) to 6 (severe dyspnea and abdominal breathing) (Halbur et al., 1995). Observers were blinded to vaccination status. Stress was induced daily by pig handler by holding the pig under his arm and taking the rectal temperature (Halbur et al., 1996b). Rectal thermometer (Digital Fever Thermometer, Becton-Dickinson, Franklin Lakes, NJ, USA) was lubricated and inserted approximately 6–7 cm into the rectum and readings were taken when the thermometer beeped (Thoresen et al., 2001). Rectal temperatures were recorded daily at the same time by same personnel.

2.4. Serology

The serum samples were tested using the commercially available PRRSV enzyme-linked immunosorbent assay (ELISA; HerdCheck PRRS 3XR™, IDEXX Laboratories Inc., Westbrook, Maine, USA). Serum samples were considered positive for PRRSV antibody if the S/P ratio was greater than 0.4, according to the manufacturer's instructions.

Serum virus neutralization (SVN) tests were also performed with the vaccine virus and the challenging strain type 2 PRRSV (SNUVR090851), as previously described (Yoon et al., 1994). The presence of virus-specific cytopathic effect (CPE) in each well was recorded after incubating for 7 days. The presence of virus in wells without CPE was further determined by immunofluorescence microscopy using an SDOW17-FITC conjugate. The neutralizing antibodies (NAs) titer of each serum was determined as the reciprocal of the highest dilution in which no evidence of virus growth was detected. Serum samples were considered to be positive for NAs if the titer was greater than 2.0 (log₂) (Zuckermann et al., 2007).

2.5. Quantification of PRRSV RNA

RNA was extracted from serum samples and nasal swabs to quantify PRRSV genomic cDNA copy numbers, as previously described (Wasilk et al., 2004). Sterile polyester swabs (Fisher Scientific Inc., Pittsburgh, PA, USA) were obtained by swabbing the nasal mucosa of both nostrils, reaching deeply into the turbinates. Swabs were stored in 5 mL plastic tubes (Fisher Scientific Inc) containing 1 mL of sterile saline solution. Real-time PCR for the vaccine strain and challenge strain was designed to detect ORF5 and

ORF7 because the vaccine strain (P129) and the challenging strain type 2 PRRSV (SNUVR090851) shared 87.2% (ORF5) and 92.7% (ORF7) homology, respectively, at this locus, as determined by BioEdit software version 7.0.0 (T. Hall, North Carolina State University, Raleigh, NC, USA). For vaccine virus, the forward and reverse primers were 5'-CTTGACACAGTTGGTCTGGTTACT-3' and 5'-GTCTTCGCAAGCCTAATAACG-3', respectively. For the challenge strain type 2 PRRSV, the forward and reverse primers were 5'-GAAGAGAAACCCGGAGAAGC-3' and 5'-CGTAGGCAAACTAAATTCACAG-3', respectively.

A standard curve for each of primers was obtained. The PCR products for the vaccine virus (129 base pair) and the challenge virus (187 base pair) were cloned with the TOP cloner Blunt kit (Enzymomics, Daejeon, Korea) and propagated in DH5 α competent cells (Enzymomics, Daejeon, Korea) according to the manufacturer's instructions. Plasmid DNA was purified with a plasmid purification kit (iNtRON Biotechnology, Sungnam, Kyeonggi-do, Korea) and quantified using a spectrophotometer.

Amplification was carried out in a 20 μ L reaction containing 10 μ L Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Billerica, MA), 7 μ L DEPC DW, and 2 μ L cDNA. The thermal profile for SYBR Green PCR was 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Each standard curve was generated using serially diluted plasmid standards of 3.5×10^1 – 3.5×10^8 copies/ μ L (vaccine virus plasmid) and 8.5×10^2 – 8.5×10^9 copies/ μ L (challenge virus plasmid). In each run, positive and negative reference samples were tested along with the unknown samples. The melting curves of each amplification product were analyzed to verify the specificity of the PCR. The coefficients of variation for the CT value obtained for each dilution were calculated. A sample was considered positive if the cycle threshold level was obtained at ≤ 40 cycles (Chung et al., 2005).

2.6. Enzyme-linked immunospot (ELISPOT) assay

The numbers of PRRSV-specific interferon- γ -secreting cells (IFN- γ -SCs) were determined in peripheral blood mononuclear cells (PBMCs) as previously described (Meier et al., 2003; Diaz and Mateu, 2005) with some modifications. Briefly, 100 μ L containing 5×10^5 PBMCs in RPMI 1640 medium supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., SelectScience, Bath, UK), 1 mM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 1 mM sodium pyruvate, 5 mM 2-mercaptoethanol, 50,000 IU/l penicillin, and 50 mg/l streptomycin was seeded into plates that were pre-coated overnight at 4 °C with anti-porcine IFN- γ monoclonal antibody (10 μ g/mL, MABTECH, Mariemont, OH, USA). The cells were stimulated with either the vaccine virus (P129 strain) or the challenge strain type 2 PRRSV in RPMI 1640 medium for 20 h at 37 °C in 5% humidified CO $_2$; the linear response was tested between 0.01 and 0.1 MOI (multiplicity of infection). Phytohemagglutinin (10 μ g/mL, Roche Diagnostics GmbH, Mannheim, Germany) and culture medium were used as positive and negative controls, respectively. The wells were washed five times with phosphate buffered

saline (PBS, 0.01 M, pH 7.4, 200 μ L per well). Thereafter, the procedure was conducted according to the manufacturer's instructions using the commercial ELISPOT Assay Kit (MABTECH). The spots on the membranes were read by an automated ELISPOT Reader (AID ELISPOT Reader, AID GmbH, Strassberg, Germany). The results were expressed as the numbers of IFN- γ -SCs per million PBMCs.

2.7. Flow cytometry analysis

The phenotypes of the CD4 $^+$ CD8 $^+$ IFN- γ $^+$ PBMCs were analyzed using flow cytometry with the following three monoclonal antibodies: anti-swine CD4a conjugated to R-PE (SouthernBiotech, Birmingham, AL, USA), CD8a conjugated to FITC (SouthernBiotech), and IFN- γ conjugated to Alexa Fluor 647 (BD Pharmingen, San Diego, CA, USA), as previously described (Ferrari et al., 2013).

Briefly, 1×10^6 purified PBMC (200 μ L) were seeded in RPMI into 48-well plates and incubated for 96 h at 37 °C and 5% CO $_2$ with 0.01 MOI of each PRRSV (vaccine or challenge strain) alone or without antigen (negative controls). The protein transport inhibitor brefeldin A (BFA, 3 μ g/mL, eBioscience, San Diego, CA, USA) was added to all samples 12 h prior to cell harvesting to block IFN- γ secretion. After incubation, cells were transferred into FCM tubes, washed with PBS containing 1% FBS and surface-stained with mouse anti-porcine CD4a-R-PE and CD8a-FITC antibodies (Southern Biotech, Birmingham, AL, USA) for 30 min in the dark. Cells were fixed in fixation buffer (BD Pharmingen, San Diego, CA, USA) for 20 min, washed and subsequently incubated for 20 min with mouse anti-porcine IFN- γ antibody (BD Pharmingen) in Perm/Wash $^{\text{TM}}$ solution (BD Pharmingen) in the dark for cytokine intracellular staining. After washes, cells were suspended in PBS containing 1% FBS and analyzed by FACSCaliber flow cytometer (Becton, Dickinson and Company, San Jose, CA, USA). The results were based on lymphocyte gating on a forward scatter versus side scatter graph after acquisition of at least 30,000 cell events.

2.8. Quantification of interleukin-10 secretion

The protein levels of interleukin-10 (IL-10) were quantified in the supernatants of PBMC (2×10^6 cells per well, 250 μ L) cultures in vitro for 20 h with the vaccine virus or challenging type 2 PRRSV (0.01 of MOI) or PHA (10 μ g/mL) by using commercial ELISA kits (Pig Interleukin-10 ELISA kit, Cusabio Biotech, Wuhan, China) according to the manufacturer's instructions. Detection limits for IL-10 were 1.5 pg/mL.

2.9. Immunohistochemistry

Immunohistochemistry (IHC) for PRRSV was performed using SR30 monoclonal antibody (Rural Technologies Inc., Brookings, SD, USA) as previously described (Han et al., 2012). SR30 monoclonal antibody (Rural Technologies Inc.), capable of specifically recognizing nucleocapsid protein of PRRSV, was diluted 1:1000 in PBS (0.01 M, pH 7.4) containing 0.1% Tween 20.

2.10. Morphometric analysis

For morphometric analysis of the macroscopic pulmonary lesion score, each lung lobe was assigned a number to reflect the approximate percentage of the volume of the entire lung and the percentage volume from each lobe added to the entire lung score (ranging from 0 to 100% of the affected lung) as previously described (Halbur et al., 1995).

For morphometric analysis of the microscopic pulmonary lesion score, the pathologist scoring the lung sections ranging from 0 (normal) to 4 (severe diffuse) was blind to the treatment of the animal from which the pulmonary tissue samples being examined were derived from, as previously described (Halbur et al., 1995).

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

2.11. Virus isolation

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

2.12. Statistical analysis

Prior to statistical analysis, all real-time PCR and NAs data were transformed to $\log_{10}$ and $\log_2$ values, respectively. The normality of the distribution of the examined

variables was evaluated by the Shapiro–Wilk test. Continuous data (rectal temperature, PRRSV cDNA, PRRSV serology, PRRSV-specific IFN- γ -SCs, IL-10, PRRSV antigen score, and lymphocyte subsets) were analyzed with a repeated analysis of variance (ANOVA). If the repeated measures ANOVA showed a significant effect, a one-way ANOVA with pairwise testing using Tukey's adjustment was performed at each time point. Discrete data (macroscopic and microscopic pulmonary lesion score, respiratory score, and proportion of viremic and nasal shedding pigs) were analyzed by Chi-square and Fisher's exact test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Clinical observation

The pigs, vaccinated with a new modified live PRRSV vaccine (groups 1 and 2) remained normal throughout the study, as measured by their respiratory scores and rectal temperatures. After the pigs were challenged with type 2 PRRSV, unvaccinated challenged pigs (group 3) developed fevers, with their temperatures ranging from 40 to 40.5 °C, and increased respiratory scores. The mean rectal temperature was significantly higher ($P < 0.05$) in unvaccinated challenged pigs than in vaccinated challenged pigs at 2, 3, 4, 5, 6, and 7 dpc (Fig. 1). The mean respiratory scores were significantly higher ($P < 0.05$) in unvaccinated challenged pigs than in pigs in other 3 groups from 4 to 15 dpc. The mean respiratory scores were significantly higher ($P < 0.05$) in vaccinated challenged pigs than in vaccinated unchallenged and unvaccinated unchallenged pigs from 7 to 10 dpc (Fig. 2). Vaccinated unchallenged and unvaccinated unchallenged pigs maintained normal temperatures without respiratory signs throughout the experiment. No bacterial pathogens were isolated at necropsy.

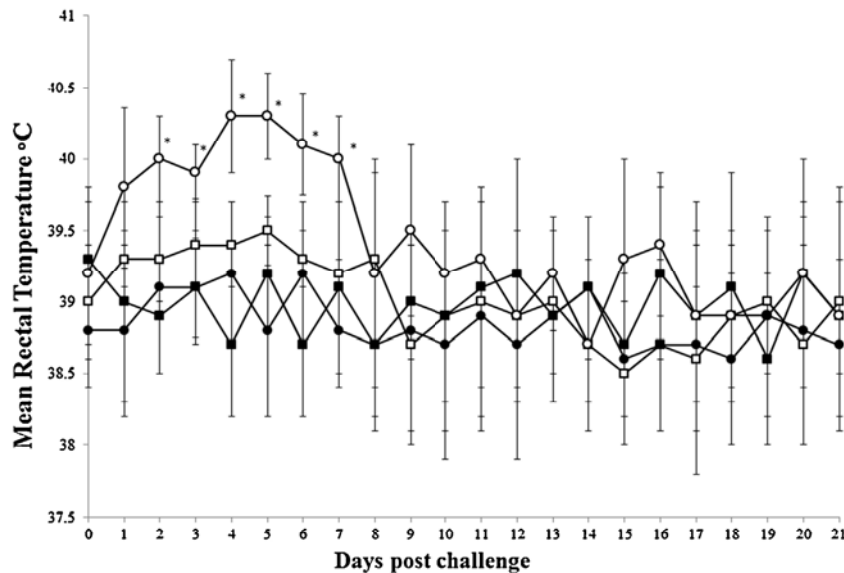


Fig. 1. Mean rectal temperature in vaccinated challenged (□), vaccinated unchallenged (■), unvaccinated challenged (○), and negative control pigs (●). Significant difference is indicated at P value $< 0.05^*$.

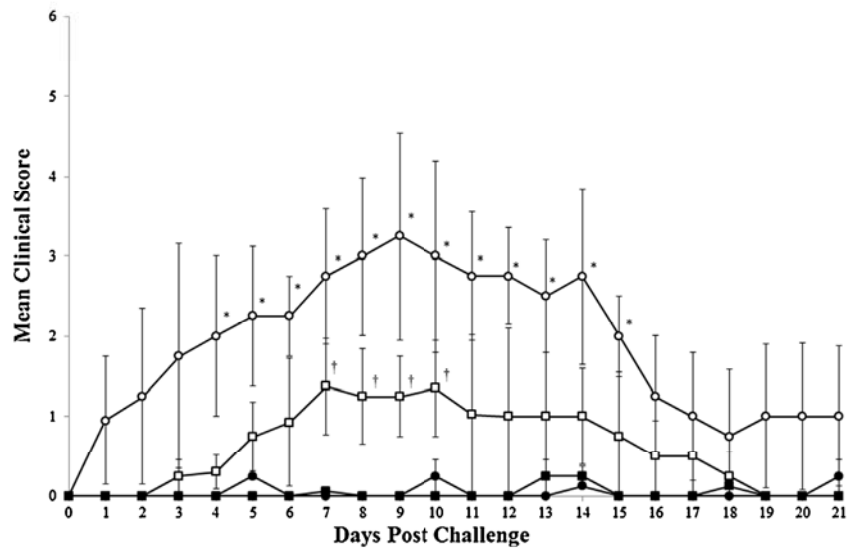


Fig. 2. Mean respiratory score in vaccinated challenged (□), vaccinated unchallenged (■), unvaccinated challenged (○), and negative control pigs (●). Significant difference (□, ■, and ● vs. ○) is indicated at P value $<0.05^*$. Significant difference (□ vs. ■ and ●) is indicated at P value $<0.05^\dagger$.

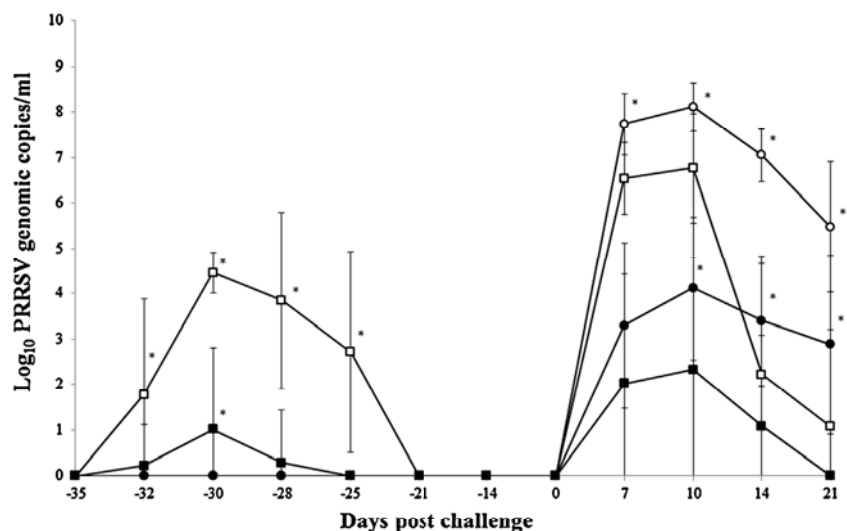


Fig. 3. Mean values of the genomic copy number of PRRSV RNA in serum and nasal swabs in vaccinated challenged (□ for serum and ■ for nasal swab) and unvaccinated challenged (○ for serum and ● for nasal swab) pigs. Variation is expressed as the standard deviation. Significant difference is indicated at P value $<0.05^*$.

3.2. Quantification of PRRSV RNA in sera and nasal swabs

Genomic copies of the vaccine virus were detected in the serum from vaccinated challenged and vaccinated unchallenged pigs (groups 1 and 2) at -32 (3 days postvaccination) but were not detected at -21 dpc (14 days postvaccination). Thereafter, no vaccine strain was detected in the serum from pigs in these two groups throughout the experiment. Genomic copies of the challenge strain were detected in the serum samples and nasal swabs of the vaccinated challenged and unvaccinated challenged pigs (groups 1 and 3) after inoculation with this virus.

Vaccinated challenged pigs had a significantly lower ($P < 0.05$) number of genomic copies of PRRSV in the blood than unvaccinated challenged pigs at 7, 10, 14, and 21 dpc

(Fig. 3). The percentage of viremic pigs was not significantly different in vaccinated challenged pigs (16/16 at 7 dpc and 12/12 at 10 dpc) and unvaccinated challenged pigs (16/16 at 7 dpc and 12/12 at 10 dpc). However, the percentage of viremic pigs was significantly lower ($P = 0.03$) in vaccinated challenged pigs (3/8 at 14 dpc) compared with unvaccinated challenged pigs (8/8 at 14 dpc).

Vaccinated challenged pigs had a significantly lower ($P < 0.05$) number of genomic copies of PRRSV in the nasal swab than unvaccinated challenged pigs at 10, 14, and 21 dpc (Fig. 3). The percentage of nasal shedders was significantly lower ($P < 0.05$) in vaccinated challenged pigs (7/16 at 7 dpc, 6/12 at 10 dpc, and 2/8 at 14 dpc) than in unvaccinated challenged pigs (13/16 at 7 dpc, 11/12 at 10 dpc, and 7/8 at 14 dpc). No challenge strain RNA was

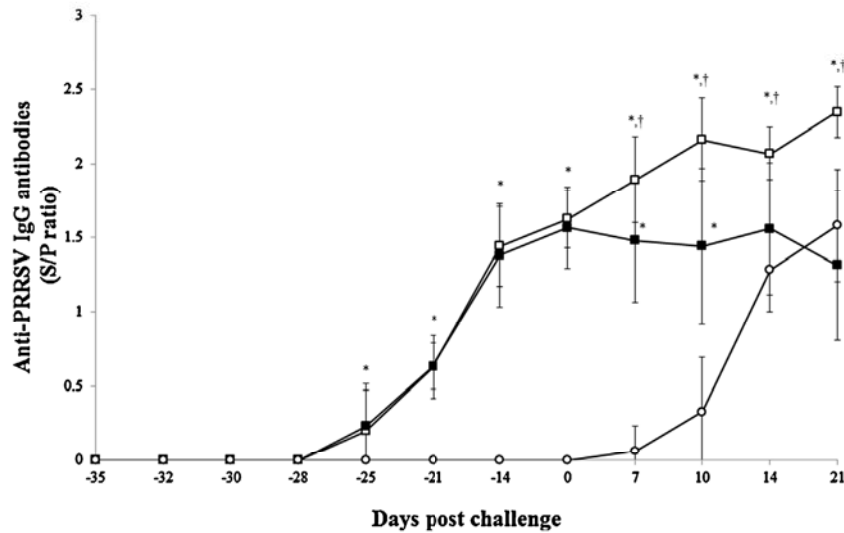


Fig. 4. Mean values of the anti-PRRSV IgG antibodies in the different groups. Vaccinated challenged ($\square$), vaccinated unchallenged ($\blacksquare$), and unvaccinated challenged ($\circ$) pigs. Variation is expressed as the standard deviation. Significant difference ($\square$ and $\blacksquare$ vs. $\circ$) is indicated at P value $<0.05^*$. Significant difference ($\square$ vs. $\blacksquare$ and $\circ$) is indicated at P value $<0.05^{\dagger}$.

detected in the blood or nasal swabs from vaccinated unchallenged and unvaccinated unchallenged pigs throughout the experiment.

3.3. Anti-PRRSV IgG antibodies

At the time of PRRSV vaccination (3 weeks of age; -35 dpc), pigs in all 4 groups were seronegative. Antibodies specific for PRRSV were detected by ELISA in vaccinated pigs (groups 1 and 2) from -25 dpc (10 days postvaccination) and unvaccinated challenged pigs (group 3) from 10 dpc. Anti-PRRSV IgG antibodies titers were significantly higher ($P < 0.05$) in vaccinated challenged pigs than in vaccinated unchallenged and unvaccinated challenged pigs at 7, 10, 14, and 21 dpc (Fig. 4). Anti-PRRSV IgG antibodies titers were not detected in unvaccinated unchallenged pigs at any time.

3.4. PRRSV-specific neutralizing antibodies

NAs were not detected in any pigs in any group (NAs titer $< 2 \log_2$) throughout the experiment.

3.5. Frequency of PRRSV-specific interferon- γ -secreting cells

After stimulation with vaccine virus, mean frequencies of vaccine virus-specific IFN- γ -SCs remained at basal levels (< 20 cells/ 10^6 PBMCs) all pigs until 0 dpc. Upon challenge with type 2 PRRSV, the frequency of vaccine virus-specific IFN- γ -SCs increased to an average of 112 ± 16.3 cells/ 10^6 PBMCs at 21 dpc in vaccinated challenged pigs. The frequency of vaccine virus-specific IFN- γ -SCs reached an average of 18.4 ± 8.5 cells/ 10^6 PBMCs at 7 dpc and decreased gradually until 21 dpc in vaccinated unchallenged pigs. The frequency of vaccine virus-specific IFN- γ -SCs remained at basal levels and reached an average of 23.5 ± 7.7 cells/ 10^6 PBMCs in unvaccinated challenged pigs at 21 dpc (Fig. 5A). The mean frequencies of vaccine virus-specific IFN- γ -SCs remained at

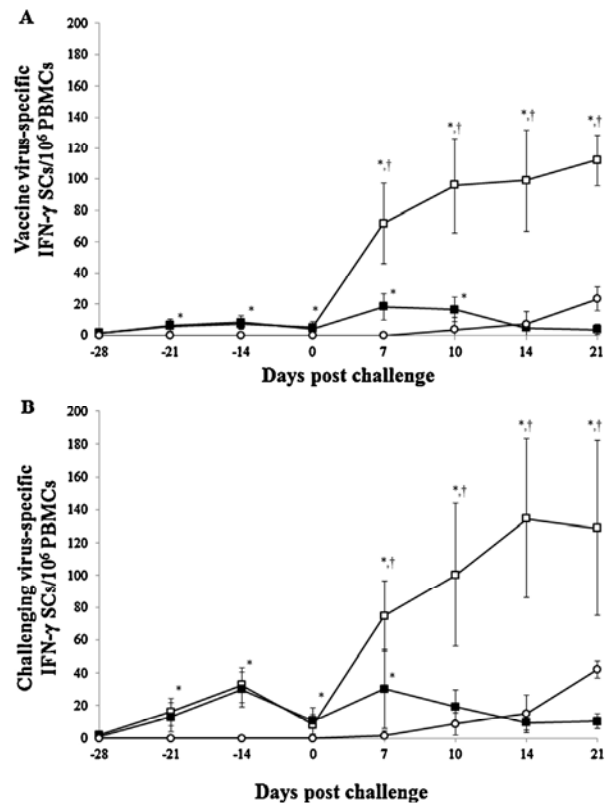


Fig. 5. Frequency of vaccine virus (A) and challenging Type 2 (B) PRRSV-specific IFN- γ -SCs/ 10^6 PBMCs. Vaccinated challenged ($\square$), vaccinated unchallenged ($\blacksquare$), and unvaccinated challenged ($\circ$) pigs. Variation is expressed as the standard deviation. Significant difference ($\square$ and $\blacksquare$ vs. $\circ$) is indicated at P value $<0.05^*$. Significant difference ($\square$ vs. $\blacksquare$ and $\circ$) is indicated at P value $<0.05^{\dagger}$.

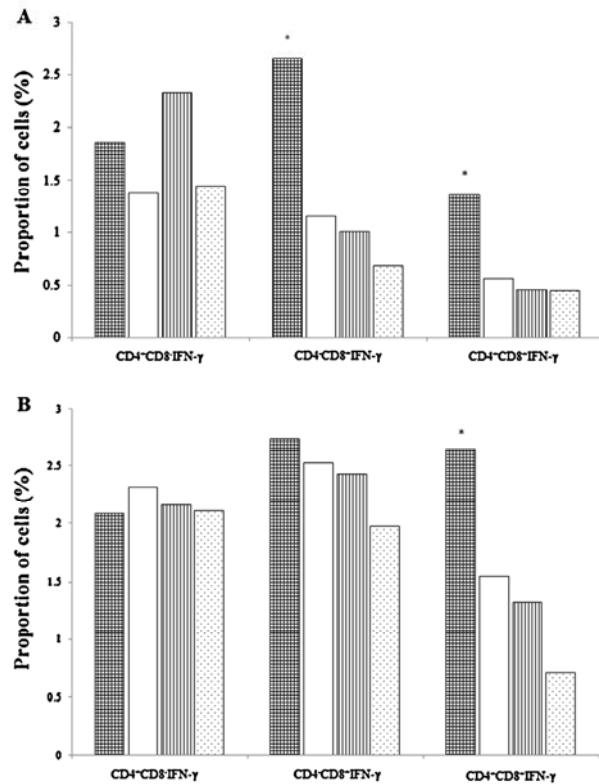


Fig. 6. Proportion of vaccine virus (A) and challenging type 2 (B) PRRSV-specific CD4⁺CD8⁺IFN-γ⁺ cells at 21 dpc. Vaccinated challenged (■), vaccinated unchallenged (□), unvaccinated challenged (▨), and unvaccinated unchallenged (○) pigs. Significant difference (■ vs. ○) is indicated at P value <0.05*.

basal levels (<20 cells/10⁶ PBMCs) in unvaccinated unchallenged pigs throughout the experiment.

After stimulation with the challenge strain type 2 PRRSV, the mean frequencies of type 2 PRRSV-specific IFN-γ-SCs remained at basal levels (<20 cells/10⁶ PBMCs) in pigs in all 4 groups until -21 dpc. Afterwards, the frequency of type 2 PRRSV-specific IFN-γ-SCs/10⁶ PBMCs reached an average of 32.5 ± 10.6 cells/10⁶ PBMCs at -14 dpc and decreased to basal levels at 0 dpc in vaccinated pigs (groups 1 and 2). Upon challenge with type 2 PRRSV, the frequency of type 2 PRRSV-specific IFN-γ-SCs increased to an average of 134.5 ± 48.5 cells/10⁶ PBMCs at 14 dpc and then decreased to an average of 128.7 ± 53.4 cells/10⁶ PBMCs in vaccinated challenged pigs. The frequency of type 2 PRRSV-specific IFN-γ-SCs remained at basal levels and reached an average of 42.2 ± 5.1 cells/10⁶ PBMCs in unvaccinated challenged pigs at 21 dpc (Fig. 5B). After type 2 PRRSV challenge, the mean frequencies of type 2 PRRSV-specific IFN-γ-SCs remained at basal levels (<20 cells/10⁶ PBMCs) in vaccinated unchallenged and unvaccinated unchallenged pigs throughout the experiment.

3.6. Identification of lymphocyte subsets

Upon in vitro stimulation with either the vaccine virus or the challenge strain, there were no differences in the

responses of the 4 groups before or after PRRSV challenge, except at 21 dpc. At 21 dpc, vaccinated challenged pigs had significantly ($P < 0.05$) more CD4⁺CD8⁺IFN-γ⁺ cells than unvaccinated unchallenged pigs (Fig. 6).

3.7. Interleukin-10

After stimulation with the vaccine virus, IL-10 was detected in vaccinated challenged and vaccinated unchallenged pigs only at -14 dpc (21 days postvaccination), and it was maintained at steady levels from 143 ± 32.5 to 165 ± 21.7 pg/ml until 0 dpc (35 days postvaccination). Upon challenge with type 2 PRRSV, IL-10 levels were dramatically decreased and maintained at minimal levels in vaccinated challenged and vaccinated unchallenged pigs throughout the experiment (Fig. 7A). IL-10 was not detected in unvaccinated challenged and unvaccinated unchallenged pigs until the end of the experiment.

After stimulation with type 2 PRRSV, IL-10 was gradually increased at 0 dpc, after which it decreased until the end of the experiment in vaccinated challenged and vaccinated unchallenged pigs. IL-10 was not detected in unvaccinated challenged and unvaccinated unchallenged pigs until 0 dpc. Upon challenge with type 2 PRRSV, IL-10 levels were slightly increased but maintained

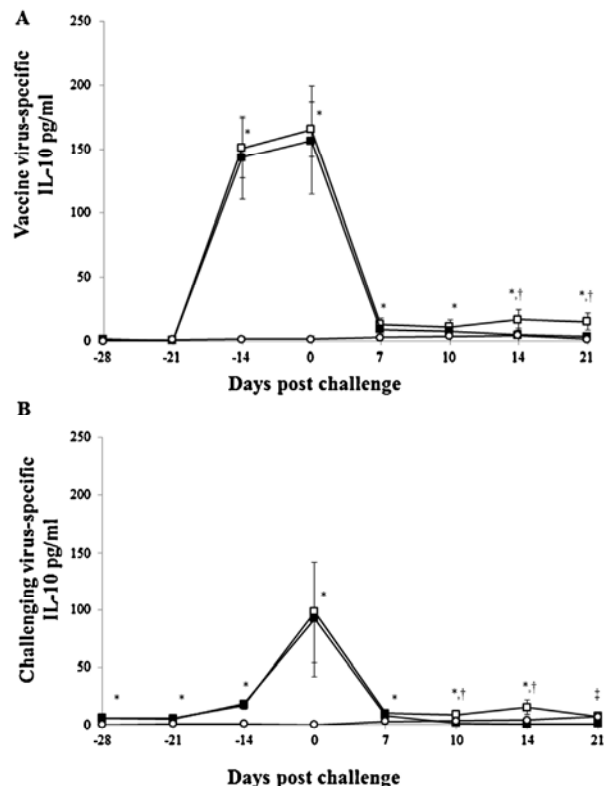


Fig. 7. Level of vaccine virus (A) and challenging type 2 (B) PRRSV-specific IL-10. Vaccinated challenged (■), vaccinated unchallenged (□), and unvaccinated challenged (○) pigs. Variation is expressed as the standard deviation. Significant difference (□ and ■ vs. ○) is indicated at P value <0.05*. Significant difference (□ vs. ■ and ○) is indicated at P value <0.05†. Significant difference (□ and ○ vs. ■) is indicated at P value <0.05‡.

minimal levels in vaccinated challenged and unvaccinated challenged pigs until the end of the experiment (Fig. 7B). IL-10 was not detected in unvaccinated unchallenged pigs until the end of the experiment.

3.8. Macroscopic lung lesions

Macroscopic lung lesions were characterized by multifocal, tan-mottled areas, with irregular and indistinct borders. There was no pleuritis and no visible exudate in the airways. The mean macroscopic lung lesion scores were significantly lower ($P < 0.05$) in vaccinated challenged pigs than in unvaccinated challenged pigs at 7, 10, 14 and 21 dpc (Table 1). No macroscopic lung lesions were observed in vaccinated unchallenged and unvaccinated unchallenged pigs throughout the experiment.

3.9. Microscopic lung lesions

Microscopic lung lesions were observed in vaccinated challenged and unvaccinated challenged pigs, but the lungs of vaccinated unchallenged and unvaccinated unchallenged pigs were normal. In vaccinated challenged and unvaccinated challenged pigs, microscopic lung lesions were typical of those associated with PRRSV infection; the lesions were characterized by thickened alveolar septa with increased numbers of interstitial macrophages and lymphocytes and by type II pneumocyte hyperplasia. Microscopic lung lesion scores were significantly ($P < 0.05$) lower in the vaccinated challenged group than in the unvaccinated challenged group at 7, 10, 14, and 21 dpc (Table 1). No microscopic lesions were observed in lung sections from vaccinated unchallenged and unvaccinated unchallenged pigs.

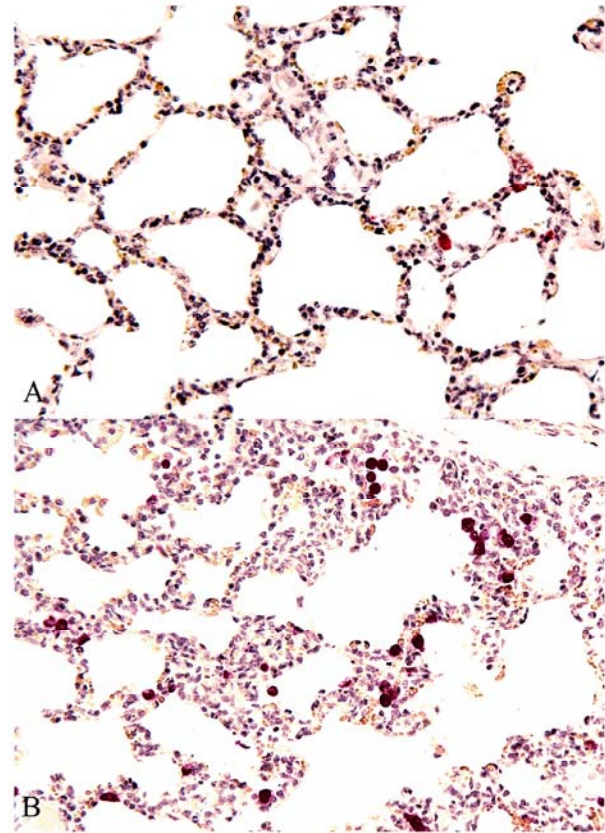


Fig. 8. Immunohistochemistry for the detection of PRRSV antigen in lungs. PRRSV antigen was detected in lungs from vaccinated challenged (A) and unvaccinated challenged (B) pigs at 10 days post challenge.

Table 1

Proportion of viremic pig and nasal shedder, pulmonary gross and microscopic lesion score, immunohistochemical porcine reproductive and respiratory syndrome virus (PRRSV) antigen score among 4 groups at different days post challenge (dpc).

	dpc	Group			
		Vaccinated challenged	Vaccinated unchallenged	Unvaccinated challenged	Unvaccinated unchallenged
Viremic pigs	7	16/16 ^a	0/16 ^b	16/16 ^a	0/16 ^b
	10	12/12 ^a	0/12 ^b	12/12 ^a	0/12 ^b
	14	3/8 ^b	0/8 ^b	8/8 ^a	0/8 ^b
	21	1/4 ^{a,b}	0/4 ^b	4/4 ^a	0/4 ^b
Nasal shedders	7	7/16 ^b	0/16 ^c	13/16 ^a	0/16 ^c
	10	6/12 ^b	0/12 ^c	11/12 ^a	0/12 ^c
	14	2/8 ^b	0/8 ^b	7/8 ^a	0/8 ^b
	21	0/4	0/4	3/4	0/4
Macroscopic lung lesion score	7	32.3 ± 11.3 ^b	0 ^c	52.1 ± 11.3 ^a	0 ^c
	10	27.9 ± 10.8 ^b	0 ^c	53.3 ± 13.2 ^a	0 ^c
	14	20.6 ± 2.5 ^b	0 ^c	37.6 ± 8.5 ^a	0 ^c
	21	13.2 ± 3.9 ^b	0 ^c	25.3 ± 2.5 ^a	0 ^c
Microscopic lung lesion score	7	2 ± 1.1 ^b	0 ^c	3.5 ± 0.8 ^a	0 ^c
	10	1.7 ± 0.9 ^b	0 ^c	3.5 ± 0.6 ^a	0 ^c
	14	1.5 ± 1 ^b	0 ^c	3.2 ± 0.5 ^a	0 ^c
	21	0.5 ± 0.9 ^b	0.2 ± 0.5 ^b	2.5 ± 1.2 ^a	0 ^b
PRRSV-antigen score	7	29.2 ± 8 ^b	0 ^c	44 ± 4.6 ^a	0 ^c
	10	30.7 ± 4.5 ^b	0 ^c	41.5 ± 6.9 ^a	0 ^c
	14	22 ± 5.3 ^b	0 ^c	34 ± 3.1 ^a	0 ^c
	21	11 ± 3.7 ^b	0 ^c	20.7 ± 4.4 ^a	0 ^c

Different letters (a–c) indicate significant ($P < 0.05$) difference among groups.

3.10. Immunohistochemistry

PRRSV antigen was detected by immunohistochemistry in the lungs in vaccinated challenged and unvaccinated challenged pigs throughout the experiment. PRRSV antigens were detected exclusively within the cytoplasm of macrophages and pneumocytes. Positive cells typically exhibited a red reaction product by immunohistochemistry. The score for the mean number of PRRSV-positive cells per unit area of lung was significantly ($P < 0.05$) lower in vaccinated challenged pigs (Fig. 8A) than in unvaccinated challenged pigs at 7, 10, 14, and 21 dpc (Fig. 8B and Table 1). No immunohistochemical signals for PRRSV antigen were observed in lung sections from vaccinated unchallenged and unvaccinated unchallenged pigs.

3.11. Virus isolation

PRRSV was isolated from the lungs at different dpc. No vaccine or challenge strain virus was isolated from vaccinated unchallenged or unvaccinated unchallenged pigs. The PRRSV isolated from vaccinated challenged (4/4 at 7 dpc, 3/4 at 10 dpc, 3/4 at 14 dpc, and 2/4 at 21 dpc) and unvaccinated challenged pigs (4/4 at 7, 10, 14, and 21 dpc) was confirmed by sequencing as the same propagating type 2 PRRSV in the challenge stock.

4. Discussion

A new modified live PRRSV vaccine was able to reduce the levels of viremia and nasal shedding, and severity of PRRSV-induced lesions after heterologous challenge. As other commercial PRRSV vaccines (Murtaugh and Genzow, 2011), new modified live PRRSV vaccine is also not effective against heterologous strain as there were still viremia, nasal shedding and virus replication in the lung after challenge. It has been already reported that the commercial modified live PRRSV vaccine is more effective in controlling homologous rather than heterologous PRRSV challenge (Kimman et al., 2009; Labarque et al., 2000, 2003; Murtaugh and Genzow, 2011). However, a modified live PRRSV vaccine protects against heterologous virus challenge in a respiratory disease model (Prieto et al., 2008; Labarque et al., 2004; Mengeling et al., 1999).

The efficacy of PRRSV vaccines is usually assessed by the reduction in viremia after challenge with a virulent virus (Labarque et al., 2000; van Woensel et al., 1998; Roca et al., 2012). PRRSV viremia is central to its pathogenesis. High PRRSV viremia is associated with the development of severe interstitial pneumonia (Han et al., 2013). Hence, the vaccine mediated reduction of PRRSV viremia is critical for controlling the infection in pigs. In the present study, a new modified live PRRSV vaccine reduced PRRSV viremia significantly in a vaccinated challenged group compared with an unvaccinated challenged group. These results agree with previous findings that commercial modified live PRRSV vaccines reduce viremia after challenge with heterologous type 2 PRRSV in pre-weaning pigs (van Woensel et al., 1998; Charerntantanakul et al., 2006; Park et al., 2013).

Although reduction of PRRSV viremia is a critical parameter for the evaluation of PRRSV vaccines, the mechanisms of the viral clearance are still poorly understood. PRRSV viremia is often resolved even before NAs are detected in infected pigs (Nelson et al., 1994; Mateu and Diaz, 2008) and vaccinated pigs (Mengeling et al., 2003). In this study, the induction of NAs in vaccinated challenged and vaccinated unchallenged pigs was also limited (titers $< 1:2$). Hence, NAs are neither essential for PRRSV immunity nor clearance (Kimman et al., 2009; Mateu and Diaz, 2008). Despite the limited NAs response to vaccination, pigs do efficiently clear subsequent homologous and heterologous virus infections (van Woensel et al., 1998), as confirmed in the present study. Prevention of PRRSV viremia by vaccination depends on the induction of cell-mediated immunity, especially the host IFN- γ response. IFN- γ inhibits PRRSV replication (Bautista and Molitor, 1999; Rowland et al., 2001). In the present study, PRRSV vaccination alone did not elicit a strong cellular immune reactivity, but upon experimental challenge, the immune response was more robust in vaccinated challenged pigs compared with unvaccinated challenged pigs. Our results are further supported by the increased numbers of CD4⁺CD8⁺IFN- γ ⁺ cells. PRRSV-specific IFN- γ -SCs are predominantly CD4⁺CD8⁺ double-positive cells (Mateu and Diaz, 2008) that play key roles in the protective cell-mediated immunity against PRRSV infection (Zuckermann et al., 2007; Meier et al., 2003). These results strongly suggest that PRRSV-specific IFN- γ -SCs are the main factors in viral clearance.

The pattern of IFN- γ -SCs and IL-10 evolution in vaccinated unchallenged pigs is interesting. During the first 5 weeks postvaccination, the modified live PRRSV vaccine induced minimal IFN- γ -SCs but stimulated high levels of IL-10 in vaccinated pigs. After 5 weeks postvaccination, the rapid decrease in IL-10 was correlated with an increased IFN- γ -SCs in vaccinated unchallenged pigs. These results suggest that the activation of IFN- γ -SCs may be modulated by the induction of IL-10, as previous noted (Diaz et al., 2005; Suradhat et al., 2003). After this IL-10 modulatory effect disappeared, the frequencies of IFN- γ -SCs rose, and simultaneously, the level of PRRSV viremia decreased in vaccinated challenged pigs. These observations suggest that IFN- γ -SCs induction by a modified live PRRSV vaccine protects pigs against PRRSV infection. A similar pattern of IFN- γ -SCs and IL-10 evolution has been observed in type 1 PRRSV-based modified live vaccine study (Diaz et al., 2006).

Microscopic pulmonary lesions were typical of those associated with PRRSV infection. Therefore, pathological evaluation that includes detection of the PRRSV antigen within these lesions is also critical for determining the efficacy of PRRSV vaccines. The challenge strain of PRRSV is virulent, resulting in the most extensive and severe lesions at 7 days postinfection and resolved lesions at 21 days postinfection (Han et al., 2013). Even so, statistical power is decreased in the experimental design due to small sample size at each necropsy ($n = 4$). Some believes it may be better to conduct necropsy of all pigs at the same time points to increase sample size since pathological lesions induced by challenging virus typically peaked at 14 days postinfection

(Han et al., 2013). Nevertheless, temporal pathological analysis is useful in this experiment because attenuated virus from a new modified live PRRSV vaccine may affect the pathological lesions induced by challenging virus. Necropsy of 4 pigs at 4 different time periods allows for variation in both virus and genetic responses of the pig. The vaccination of pigs against PRRSV significantly reduces pulmonary lesions and PRRSV antigen in lungs from vaccinated challenged pigs compared with unvaccinated challenged pigs.

To our knowledge, this is the first experimental challenge study for a new modified live PRRSV vaccine. It is reasonable to determine the parameters for PRRSV vaccine efficacy based on the induction of protective immunity and the reduction of viremia, macroscopic and microscopic lung lesions, and PRRSV antigen in lesions. A new modified live PRRSV vaccine is another tool for swine practitioners and producers to protect pigs from PRRSV infection based on clinical, immunological, virological, and pathological evaluations.

5. Conclusion

A new modified live PRRSV vaccine was able to reduce the levels of viremia and nasal shedding, and severity of PRRSV-induced lesions after heterologous challenge. Vaccinated challenged pigs exhibited significantly lower ($P < 0.05$) respiratory scores, viremia, macroscopic and microscopic lung lesion scores, and PRRSV-antigen with interstitial pneumonia than unvaccinated challenged pigs.

Competing interests

None.

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