Effects of North American Porcine Reproductive and Respiratory Syndrome Virus (PRRSV)-Based Modified Live Vaccines on Preimmunized Sows Artificially Inseminated with European PRRSV-Spiked Semen

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The objective of the present study was to determine if the European porcine reproductive and respiratory syndrome virus (PRRSV) can be transmitted via spiked semen to preimmunized sows and induce reproductive failure. Sows were immunized with the North American PRRSV-based modified live vaccine (Ingelvac PRRS MLV; Boehringer Ingelheim Animal Health, St. Joseph, MO) and were artificially inseminated. The sows were randomly divided into three groups. The vaccinated (group 2) and nonvaccinated (group 3) sows developed a PRRSV viremia at 7 to 28 days postinsemination with the European PRRSV-spiked semen. The number of genomic copies of the European PRRSV in serum samples was not significantly different between vaccinated and nonvaccinated sows. All negative-control sows in group 1 farrowed at the expected date. The sows in groups 2 and 3 farrowed between 103 and 110 days after the first insemination. European PRRSV RNA was detected in the lungs of 8 out of 11 live-born piglets and 46 out of 54 stillborn fetuses. In addition, PRRSV RNA was detected using in situ hybridization in other tissues from vaccinated sows that had been inseminated with European PRRSV-spiked semen (group 2). The present study has demonstrated that vaccinating sows with the North American PRRSV-based modified live vaccine does not prevent reproductive failure after insemination with European PRRSV-spiked semen.

Porcine reproductive and respiratory syndrome virus (PRRSV) is a member of the Arterivirus genus (family Arteriviridae, order Nidovirales). Genetic analysis has established two predominant PRRSV genotypes, the European genotype (type 1) and the North American genotype (type 2), which share approximately 60% nucleotide sequence identity (1, 3, 16). The European PRRSV emerged in Asian countries. It was first isolated in 2000 in South Korea (6), in 2004 in Thailand (24), and in 2011 in China (4). In South Korea, infection with European PRRSV is prevalent in swine herds (29.4%; 20/68 swine herds) (13).

Recently, immunizing boars with the North American PRRSV-based modified live vaccine (Ingelvac PRRS MLV; Boehringer Ingelheim Animal Health, St. Joseph, MO) has been shown to decrease subsequent shedding of the North American PRRSV after a challenge (8). However, the vaccine was unable to decrease shedding of the European PRRSV in semen after a challenge (8). These results are clinically meaningful because the North American PRRSV-based vaccine could not protect swine from the European PRRSV. In South Korea, it is common to vaccine sows as a whole-herd vaccination program, where all sows are vaccinated every 3 to 4 months with the North American PRRSV-based modified live vaccine as recommended by the manufacturer. This vaccination schedule is often utilized because the commercial European PRRSV-based vaccine is not available yet. However, to date, it is unclear whether preimmunized sows artificially inseminated with semen from European PRRSV-infected boars are susceptible to reproductive failure.

Artificial insemination (AI) has been widely and routinely used in the swine industry worldwide. On South Korean swine farms, >90% of sows are bred by AI and >80% of swine producers purchase semen for AI from commercial AI centers (http://www .pigtech.co.kr). Therefore, the objective of the present study was to determine whether European PRRSV-spiked semen can be transmitted via AI to preimmunized sows which have received the North American PRRSV-based modified live vaccine and induce reproductive failure.

MATERIALS AND METHODS

Commercial vaccine and PRRSV inocula. The commercial modified live PRRSV vaccine (Ingelvac PRRS MLV, Boehringer Ingelheim Animal Health) was used in this study. Sows were vaccinated with a 2.0-ml dose intramuscularly 4 weeks prior to AI, according to the manufacturer’s instructions.

European PRRSV (SNU090485) was used as the inoculum to spike semen. The European PRRSV strain was isolated from lung samples from an aborted fetus in 2009 in Gyeonggi Province, South Korea. The nucleotide sequence homology in ORF5 between the European PRRSV strain (SNU090485; GenBank accession number JN315686) and the vaccine strain (GenBank accession number AF353152) is 68%, as determined by using BioEdit, version 7.0.0, software (Ibis Biosciences, Carlsbad, CA [http://www.mbio.ncsu.edu/BioEdit/bioedit.html]).

Semen collection. PRRSV-free semen was collected from three PRRSV-naïve boars by the gloved-hand technique using an insulated mug containing a disposable plastic liner with tear-away filter gauze (Kukjea Industry Co., Tongjak-gu, Seoul, Republic of Korea). Semen was collected from the boar, and the raw semen was diluted 1:1 in an extender (Minitub;...
Songkang GLC Co., Seongnam, Gyeonggi, Republic of Korea). Each insemination dose was 80 ml, which contained approximately $5 \times 10^6$ sperm. Insemination doses were stored at 17°C and used immediately after extension. The raw semen samples tested negative for the presence of North American and European PRRSV RNA using real-time PCR (8, 26).

To evaluate a potential negative effect of the semen extender on the infectivity of the PRRSV, an equal amount of extender was added to PRRSV in a 1:1 dilution. In addition, the PRRSV was diluted with Dulbecco’s modified Eagle medium (DMEM) in a 1:1 dilution, which also served as a control. The PRRSV titer was estimated at 0, 8, 24, 48, 72, and 96 h postdilution by immunofluorescence assays (IFAs) using a PRRSV-specific antibody (SR30) as described previously (17).

**Experimental design.** Twenty-one conventional crossbred sows with a second parity were obtained from a PRRSV-free herd. All sows were serologically negative for porcine circovirus type 2, porcine parvovirus, PRRSV, and swine influenza virus. All sows were moved to a research facility, housed individually in separate rooms, and randomly allocated into 1 of 3 groups. The estrous cycles for all sows were synchronized as previously described (14).

Group 1 (T01; $n = 5$) sows served as negative controls and were artificially inseminated with 80 ml extended semen-negative semen upon estrus detection. Group 2 (T02; $n = 8$) sows were vaccinated intramuscularly with one 2-ml dose of a commercial modified live PRRSV vaccine 4 weeks prior to AI. Sows in groups 2 and 3 (T03; $n = 8$) were inseminated with 80 ml of extended semen spiked with PRRSV. Insemination doses were stored at 17°C and used immediately post insemination. The PRRSV isolates recovered from semen were previously described (9).

**Serology.** Blood samples were collected from each sow by jugular venipuncture at $-28, -21, -7, 0, 7, 14, 21, 35, 45, 56, 70, 85, 99, 105,$ and 115 days postinsemination (dpi), and the sera were stored at $-20°C$. The serum samples were tested using a commercially available PRRSV enzyme-linked immunosorbent assay (HerdCheck PRRS 2XR; IDEXX Laboratories Inc., Westbrook, ME).

**Virus isolation.** Blood samples were collected at $-28, -25, -23, -21, -7, 0, 7, 14, 21, 35, 45, 56, 70, 85, 99, 105,$ and 115 dpi for virus isolation from all sows used in this study. PRRSV was isolated from semen and semen as previously described (7, 9). Virus titrations were also performed in confluent monolayers of MARC-145 cells in 96-well plates as previously described (9).

**Sequence analysis.** The PRRSV isolates recovered from semen were further analyzed for the ORF5 sequence. RNA was extracted from PRRSV-infected MARC-145 cell lines (6) and amplified from the ORF5 region by reverse transcription-PCR (RT-PCR) (18). Sequencing was performed on the purified RT-PCR products of amplified ORF5.

**Quantitative real-time PCR.** RNA extractions from the semen samples from all sows used in this study were collected at $-28, -25, -23, -21, -7, 0, 7, 14, 21, 35, 45, 56, 70, 85, 99, 105,$ and 115 dpi and performed as previously described (8, 23). Real-time PCR for the European PRRSV and vaccine strains was used to quantify PRRSV genomic cDNA copy numbers using RNA extraction from semen samples, which was performed as previously described (8, 23). A commercially available real-time, single-tube RT-PCR assay (Tetracore Inc., Gaithersburg, MD) for the detection of North American and European PRRSV was used to detect PRRSV RNA. Within the highly conserved ORF7 region and 3′ untranslated region (UTR) of the genome of both virus types, a forward primer (5′-GTTGAAATGCGCAGTTG-3′) and a reverse primer (5′-CGGTCACATGGTCTCCG-3′) for European PRRSV were selected. The forward primer for North American PRRSV is 5′-GTTGGAATGCGCAGTTG-3′, and the reverse primer is 5′-CCCAACAGGCTTCG-3′. Two type-specific Taq probes were European PRRSV-specific probe 5′-TCACTATACAGATCTACGGG-3′, which was labeled with 6-carboxyfluorescein (FAM) attached to the 5′ terminus (reporter) and a nonfluorescent quencher (NFQ) and minor groove binder (MGB) at its 3′ end (quencher), and North American PRRSV-specific probe 5′-TCTCTCATTACACATTAGGCG-3′, which was labeled with FAM (reporter) at its 5′ end and carried 6-carboxytetramethylrhodamine (Tamra) at its 3′ end. The PRRSV RNA was transcribed in a single tube by using a 25-µl reaction volume consisting of Tetracore PRRSV master mixture (18.9 µl of the master mixture, 2 µl of enzyme mixture 1, 0.1 µl of enzyme mixture 2) and 4 µl of extracted RNA. The reaction tubes were loaded into an ABI 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA), and software settings for fluorescence detection were set for automatic calculation of the baseline with the background subtraction turned on. The thermal cycler program for the North American PRRSV (vaccine strain) real-time assay consisted of 52°C for 1,800 s; 95°C for 900 s; and 45 cycles at 94°C for 30 s, 61°C for 60 s, and 72°C for 60 s. For the European PRRSV assay, thermal cycling times consisted of 60°C for 1,200 s, 95°C for 15 s, and 45 cycles at 95°C for 3 s and 61°C for 30 s. The results of the standard curve and interference for the European PRRSV and vaccine strains were reported in previous study (8). A PCR was considered positive if the cycle threshold ($C_T$) level was obtained at $\leq 45$ cycles.

**Necropsy.** Sows were euthanized with an intravenous (auricular vein) overdose of pentobarbital. Live-born piglets were euthanized, and all expelled fetuses (mummified, dead, and live born) from all groups were necropsied and evaluated for gross lesions. Crown-to-rump measurements were used to determine the approximate time of death of mummified and dead fetuses during the gestation (25).

**In situ hybridization.** Fresh and 10% neutral buffered formalin-fixed tissue samples, which included samples of heart, lungs, liver, kidney, brain, spleen, tonsil, thymus, and intestine, were collected for all dead and live-born piglets. A single set of tissues was collected from mummified fetuses if organ recognition was possible during necropsy. For European PRRSV, a 354-base-pair cDNA fragment representing the 5′ region of ORF6 and ORF7 was used as a probe. The forward and reverse primers were 5′-GGCTGTGCACAAACCGCGCGGAC-3′ (nucleotides 14482 to 14501) and 5′-TCAGTTGCAACAGCAGGGG-3′ (nucleotides 14814 to 14835), respectively. For North American PRRSV, a 349-base-pair cDNA fragment representing the 5′ region of ORF6 and ORF7 was used as a probe. The forward and reverse primers were 5′-GGTTTCGGCGTCCCCGCCCTC-3′ (nucleotides 14775 to 14794) and 5′-TGAGCAAGACACACATTGGC-3′ (nucleotides 15122 to 15141), respectively. The PCR was carried out as previously described (11). The purified PCR product was labeled by random priming with digoxigenin-DUTP using a commercial kit (Boehringer Mannheim). In situ hybridization was carried out as previously described (5).

**Statistical analysis.** Summary statistics were calculated for the three groups to assess the overall quality of the data, including normality. For a single comparison of PRRSV RNA quantification, Student’s $t$ test for paired samples (European PRRSV RNA quantification) was used to estimate the difference at each time point.

**RESULTS**

**Effect of extender.** In the extender dilution in vivo assay, the starting titer of the PRRSV diluted with semen extender was determined to be $1 \times 10^{4.4} \text{TCID}_{50}/\text{ml}$. The control virus stock diluted with DMEM had a titer of $1 \times 10^{5.5} \text{TCID}_{50}/\text{ml}$. The PRRSV titers slightly decreased over time, and the final titers of the PRRSV at 96 h postdilution were $1 \times 10^{3.1} \text{TCID}_{50}/\text{ml}$ and $1 \times 10^{3.2} \text{TCID}_{50}/\text{ml}$ for the extender-diluted PRRSV and the DMEM-
PRRSV was isolated from the serum of negative-control sows and the vaccine strain from the serum of sows in the three groups. Attempts were made to isolate and identify the European PRRSV group 1 throughout the experiment (data not shown). As expected, no PRRSV antibodies were detected in serum samples until 0 dpi. In vaccinated sows (T02) only, anti-PRRSV antibodies were detected at 21 dpi. European PRRSV was isolated from several organs from live-born piglets and stillborn fetuses from sows in groups T02 and T03 (Table 1). All European PRRSV isolates recovered were confirmed to be the same propagating virus as in the challenge stock by sequence analysis.

**Log**$_{10}$ TCID$_{50}$/ml quantification and real-time PCR of PRRSV RNA in blood from sows. Genomic copies of the European and vaccine PRRSV isolates were not detected in the serum samples at −28 dpi for all groups. The vaccine strain was detected only until −7 dpi in the serum samples from vaccinated sows in group 2. European PRRSV was detected from 7 to 28 dpi in sows in groups 2 and 3 (Fig. 1). No European or vaccine PRRSV was observed in the serum samples from the negative-control sows in group 1 throughout the experiment. For the intergroup comparison, the number of genomic copies and the log$_{10}$ numbers of TCID$_{50}$/ml of the European PRRSV in the serum samples were not significantly different between vaccinated (T02) and nonvaccinated (T03) sows inseminated with European PRRSV-spiked semen.

**In situ hybridization.** European PRRSV RNA was detected in lung, lymph node, thymus, tonsil, and heart tissues of stillborn fetuses and live-born piglets from sows in groups 2 and 3 (Table 2). European PRRSV RNA was detected exclusively within the cytoplasm of macrophages in the lung. The positive cells generally had large oval nuclei and abundant cytoplasm, a finding which is consistent with macrophages in the lymph nodes. The tonsillar macrophages showed European PRRSV labeling in live-born piglets and stillborn fetuses. In the thymus, the PRRSV-positive cells most often seen in the medulla were round or triangular, resembling macrophages (Fig. 2). A few antigen-positive cells were stellate and had at least one long cytoplasmic process, characteristic of interdigitating cells. Positive cells were similar to macrophages scattered between cardiac myocytes. No North American PRRSV RNA was detected in any tissues of stillborn fetuses or live-born piglets in sows from the 3 groups (T01, T02, and T03).

**TABLE 1** Effect of semen extender on survivability of European PRRSV in cell culture

| Sample          | No. of TCID$_{50}$/ml at the following times (h) |
|-----------------|--------------------------------------------------|
|                 | 0      | 8      | 24     | 48     | 72     | 96     |
| PRRSV + DMEM    | 10$^{3.5}$ | 10$^{3.5}$ | 10$^{3.4}$ | 10$^{3.4}$ | 10$^{3.3}$ | 10$^{3.2}$ |
| PRRSV + extender| 10$^{3.4}$ | 10$^{3.4}$ | 10$^{3.3}$ | 10$^{3.3}$ | 10$^{3.2}$ | 10$^{3.1}$ |

**TABLE 2** Litter characteristics and detection of European PRRSV in live-born piglets and stillborn fetuses at parturition from sows

| Group (n$^a$) | Gestation (days) | Litter characteristics | Length$^c$ (cm) | In situ hybridization/virus isolation$^b$ |
|---------------|------------------|------------------------|-----------------|-----------------------------------------|
|               |                  | Status at birth        | No. | Avg | Range | Lung | LN | Heart | Tonsil | Thymus |
| 1 (5)         | Term             | Live born              | 44  | 31  | 30–32 | −/− | −/− | −/−   | −/−   | −/−   |
|               |                  | Stillborn              | 2   | 30  | 29–31 | −/− | −/− | −/−   | −/−   | −/−   |
|               |                  | Mummified              |     |     |       | NA  | NA | NA    | NA    | NA    |
| 2 (8)         | 103–110          | Live born              | 11  | 27.9| 27.6–28.3 | 8/10 | 8/5 | 9/3   | 11/9  | 11/10 |
|               |                  | Stillborn              | 54  | 27.4| 27.1–27.7 | 46/35| 45/39| 40/31 | 47/38 | 49/41 |
|               |                  | Mummified              | 2   | 15.4| 13.5–17.2 | NA  | NA | NA    | NA    | NA    |
| 3 (8)         | 104–109          | Live born              | 12  | 27.8| 27.5–28.2 | 7/9  | 8/8 | 8/5   | 9/5   | 11/8  |
|               |                  | Stillborn              | 53  | 25.5| 24.8–26.3 | 45/39| 49/42| 46/36 | 49/41 | 51/45 |
|               |                  | Mummified              | 3   | 15.3| 14.1–16.6 | NA  | NA | NA    | NA    | NA    |

$^a$ n, number of sows in group.

$^b$ Data represent number of fetuses or piglets in which European PRRSV was detected by in situ hybridization/number in which European PRRSV was detected by virus isolation.

$^c$ Estimation of the time of fetal death was based on crown-to-rump length.
PRRSV RNA was not detected in any tissues of stillborn fetuses or live-born piglets from sows in group 1 (T01).

**DISCUSSION**

The present study has demonstrated that vaccinating sows with the commercial North American PRRSV-based modified live vaccine does not prevent reproductive failure after insemination with European PRRSV-spiked semen. These results strongly suggest that the North American PRRSV-based modified live vaccine is not effective against the European PRRSV. Because the European PRRSV can be transmitted through AI with semen from infected boars to sows preimmunized with the North American PRRSV-based modified live vaccine, it is critical that boars in AI centers remain free of PRRSV infection. Our results are also clinically meaningful because boars may be infected with either North American or European PRRSV and it would be important to protect sows against both genotypes. Once sows are vaccinated every 3 to 4 months with the North American PRRSV-based modified live vaccine, as recommended by the manufacturer, most swine producers expect that the vaccine will prevent their sows from reproductive failure caused by the European PRRSV infection.

Previous study has shown that the dose of PRRSV in semen plays a major role in the transmissibility of the virus (2). For example, one out of five gilts (20%) inseminated at doses of 40 and 400 TCID$_{50}$/ml of the North American PRRSV seroconverted, whereas a 100% seroconversion rate was found in animals given

| Group (n) | PRRSV type | Method $^b$ | No. of sows at the indicated day postinsemination |
|-----------|------------|-------------|---------------------------------------------------|
| T01 (5)   | Vaccine    | VI          | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
|           | Vaccine    | RT-PCR      | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
|           | European   | VI          | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
|           | European   | RT-PCR      | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
| T02 (8)   | Vaccine    | VI          | 0 3 3 2 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
|           | Vaccine    | RT-PCR      | 0 6 5 3 2 1 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
|           | European   | VI          | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
|           | European   | RT-PCR      | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
| T03 (8)   | Vaccine    | VI          | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
|           | Vaccine    | RT-PCR      | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
|           | European   | VI          | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
|           | European   | RT-PCR      | 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |

$^a$ n, number of sows in group.
$^b$ VI, virus isolation; RT-PCR, real-time RT-PCR.

**FIG 1** Detection of PRRSV in blood samples. Mean group log$_{10}$ European PRRSV RNA load and mean group log$_{10}$ number of TCID$_{50}$/ml European PRRSV from the different treatment groups are shown. T02, sows were immunized with modified live PRRSV vaccine and inseminated with European PRRSV-spiked semen; T03, sows were inseminated with European PRRSV-spiked semen.
extended semen doses of >4,000 TCID₅₀/ml of North American PRRSV (2). In the present study, the dose of PRRSV in semen was determined on the basis of a previous study where the maximal shedding of European PRRSV in semen from an infected boar was approximately 1 × 10⁵.⁵ TCID₅₀/ml (equivalent to 10⁰.¹ to 10⁶.⁵ viral genome copies per ml) at 7 dpi (8). All nonvaccinated (T03) sows inseminated at doses of 6 × 10⁵.⁵ TCID₅₀ of the European PRRSV per 1 ml of extended semen seroconverted at 21 dpi. Therefore, this dosage in semen is enough for sows to become infected with European PRRSV via insemination. Since only spiked semen was used in this study, further study is needed to determine that the transmissibility would be different between free virus and possibly cell-associated virus in naturally infected semen.

In the present study, the European PRRSV induced placental infection of embryos and occasionally caused death in sows preimmunized with the North American-based modified live vaccine and inseminated with semen spiked with European PRRSV. These results agree with previous findings, in which the PRRSV vaccine was more effective against homologous challenges than heterologous challenges (15, 20). In addition, less protection against heterologous challenge than homologous challenge was observed in a trial that included the challenge of pregnant sows with the same vaccine used in this study (12). However, further study is needed to determine the protective effect of the North American PRRSV-based vaccine against various European PRRSV strains since the present study described only a small number of sows and only one European PRRSV strain was used.

All sows in groups 2 and 3 became viremic at 7 dpi, thereby increasing the potential for European PRRSV to cross the placenta. Fetal infection with European PRRSV via vertical transmission was confirmed, as measured by the presence of PRRSV antibodies, viremia, or an in situ hybridization signal in tested serum and tissue samples. However, no North American PRRSV was detected in stillborn fetuses and live-born piglets. Although PRRSV may infect embryos during early gestation, it lacks the ability to kill embryos until late gestation (22). These data agree with those from our study, where most fetuses died in the last trimester of gestation.

To our knowledge, this is the first study using North American PRRSV-based vaccine-immunized sows and European PRRS-vspiked semen to determine the vaccine’s effects on reproductive failure during pregnancy. Boars infected with PRRSV are characterized by no or nonspecific symptoms such as anorexia, lethargy, and loss of libido (8, 10, 19, 21, 23). The absence or limited duration of clinical signs is important from the epidemiological point of view because acutely infected boars can go unnoticed by their caretakers, thus increasing the risk of PRRSV transmission because no control measures will be taken. Since PRRSV surveillance in boars cannot rely on clinical signs, a routine sampling protocol is needed to detect any potential introduction of PRRSV into boar herds.

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