Effects of an Inactivated Porcine Circovirus Type 2 (PCV2) Vaccine on PCV2 Virus Shedding in Semen from Experimentally Infected Boars

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Porcine circovirus type 2 (PCV2) is associated with a number of diseases and syndromes collectively referred to as porcine circovirus-associated disease (PCVAD), which includes postweaning multisystemic wasting disease (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, porcine respiratory disease complex (PRDC), and exudative epidermitis (3).

Several commercial PCV2 vaccines are currently available in the global market. All PCV2 vaccines have been administered to either sows or piglets (2, 5, 16, 18). Commercial PCV2 vaccines are able to reduce the PCV2 load in the serum and reduce PCV2 shedding in nasal and fecal samples in conventional pigs (5, 15, 16, 18). Recently, it has also been reported that vaccination against PCV2 in naturally infected boars can decrease the duration of viral shedding in semen (1). These data strongly imply that vaccination against PCV2 may reduce the subsequent shedding of PCV2 in the semen of boars. Therefore, the objective of the present study was to determine the effect of the PCV2 vaccine on the shedding of the PCV2 virus in the semen of experimentally infected boars.

MATERIALS AND METHODS

Commercial vaccine and PCV2 inoculum. The commercial inactivated tissue homogenate PCV2 vaccine based on PCV2 genotype 2b, CircoPrime (Komipharm International Company Ltd., Shiheung-shi, Kyongki-do, Republic of Korea), was used in this study. It was administered intramuscularly in two 2.0-ml doses separated by 3 weeks.

The PCV2 strain (PCV2 genotype b) isolated from the superficial inguinal lymph node of a field case of PMWS was used as the inoculum. The virus was propagated in PCV-free PK15 cells to a titer of 10^5 TCID_{50} ml^-1. The virus was propagated in PCV-free PK15 cells to a titer of 10^5 TCID_{50} ml^-1

Experimental design. At 8 months of age, 12 purebred, male, Landrace pigs were purchased from a commercial farm. All boars were negative for PCV2 and porcine reproductive and respiratory syndrome virus (PRRSV), according to routine serological testing performed prior to delivery and again on arrival. All boars were housed individually in an environmentally controlled building with pens over completely slatted floors throughout the experiment. To avoid environmental contamination, the building was completely emptied, cleaned three times with hot (>95°C) water, and disinfected with a 2% potassium peroxymonosulfate and sodium chloride-based product (Virkon S; Antec International, Sudbury, Suffolk, United Kingdom) for 3 days. The building was empty for an additional 21 days before the boars were introduced, and each boar was housed separately within the facility.

The boars were randomly divided into three groups. The boars in group 1 (n = 4) were immunized with two 2.0-ml doses of the inactivated PCV2 vaccine at a 3-week interval, and then, at 3 weeks after the second vaccination, the boars were intranasally inoculated with PCV2b (3 ml) with an infectious titer of 10^5 TCID_{50} per ml. The boars in group 2 (n = 4) were intranasally inoculated with PCV2b (3 ml) with an infectious titer of 10^5 TCID_{50} per ml. The concentration of PCV2b (10^5 TCID_{50} per ml) for inoculation was similar to that used in a previous study to allow comparison (13). The boars in group 3 (n = 4) served as negative controls.

Serology. Blood samples from each pig were collected by jugular venipuncture at –42, –21, 0, 14, 21, 28, 35, 42, 49, and 60 days postinoculation (dpi), and the serum samples were tested using a commercial PCV2 enzyme-linked immunosorbent assay IgG kit (Ingezim Circovirus IgG; Ingenasa, Madrid, Spain). Quantification of PCV2 DNA. DNA was extracted from semen (raw) and serum samples collected at –42, –21, –7, 0, 4, 7, 10, 14, 18, 21, 25, 28, 32, 35, 39, 42, 46, 49, 53, 56, and 60 dpi using a QIAamp DNA minikit (Qiagen, Valencia, CA) as described previously (19). DNA extracts were used to quantify the PCV2 genome DNA copy numbers by real-time PCR as described previously (6).

Standard curve. To construct a standard curve, real-time PCRs were performed in quadruplicate in two different assays: (i) 10-fold serial dilutions of the PCV2 plasmid were used as the standard, with concentrations ranging from 10^{10} to 10^0 copies/ml, and (ii) 10-fold serial dilutions of PCV2b cultured in PK-15 cells free of PCV1 were used at concentrations ranging from 10^5 to 10^3 TCID_{50} per ml. The PCV2 plasmid was prepared as described previously (6).
Briefly, the PCV2b ORF2 sequence was cloned into the pCR 2.1 plasmid (Invitrogen, Carlsbad, CA). The recombinant plasmid was purified using a Qiagen plasmid Miniprep kit according to the manufacturer’s instructions, and the concentration of the purified plasmid was determined using a spectrophotometer.

**Virus isolation.** PCV2 was isolated from whole semen as previously described (11). Briefly, whole semen (2 ml) was frozen and then thawed, mixed with 20 ml of Hanks’ balanced salt solution, and centrifuged at 40,000 \( \times g \) for 1 h. The supernatant was discarded, and the pellet was resuspended and vortexed in 1 ml of minimal essential medium plus 4% fetal bovine serum. Confluent monolayers of PCV-free PK-15 cells were inoculated with 200 \( \mu l \) of the suspension and then incubated for 5 days. Duplicate cultures were fixed in 80% acetone and tested for PCV1, PCV2a, and PCV2b by differential in situ hybridization as previously described (8, 9).

**Statistical analysis.** Summary statistics were calculated for all groups to assess the overall quality of the data, including normality. For a single comparison, analysis of variance (ANOVA) with a post hoc Tukey’s test was used to compare the primary variables (PCV2b DNA concentrations in the blood and semen) among groups. Student’s t test for paired samples (PCV2b DNA quantification) was used to estimate the difference at each time point. A \( P \) value of \(<0.05\) was considered significant.

Continuous data for PCV2 serology collected over time were analyzed using a nonparametric Kruskal-Wallis one-way ANOVA at each time point. If the difference was significant (\( P < 0.05 \)), Mann-Whitney U tests were used to assess differences between groups. The Wilcoxon signed-rank test for paired samples was used to estimate the difference at each time point.

Spearman’s rank correlation coefficient was used to assess the relationship among serology, viremia, and shedding in semen. Pearson’s correlation coefficient was used to assess the relationship between viremia and shedding in semen. A \( P \) value of \(<0.05\) was considered significant.

**RESULTS**

**Serology of PCV2.** Anti-PCV2 IgG was not detected in any serum sample at days −42, −21, and 0 dpi in nonvaccinated challenged (T02) boars (Fig. 1). The serum anti-PCV2 IgG level was significantly higher at −21, 0, and 7 dpi but was significantly lower at 35 dpi in vaccinated challenged (T01) boars than in nonvaccinated challenged boars (\( P < 0.05 \); Fig. 1). No anti-PCV2 IgG was detected in serum samples from negative-control (T03) boars.

**Standard curve.** To determine whether there was a correlation between the genome copy number and the TICD\(_{50} / \)ml, standard curves were constructed with the two different templates (plasmid pORF2 DNA and PCV2b DNA). The resulting lines were nearly parallel, with almost the same slope (Fig. 2). Regression analysis revealed a significant correlation (\( R^2 > 0.997 \)).

**Quantification of PCV2 DNA in blood.** PCV2b genomes were not detected in any serum samples at −42, −21, −7, and 0 dpi from vaccinated challenged (T01) and nonvaccinated challenged (T02) boars (Fig. 3). No PCV2b genomes were detected in any of the serum samples from negative-control (T03) boars throughout the experiment. The number of PCV2b genomes in the serum was significantly lower in vaccinated challenged (T01) boars than in nonvaccinated challenged (T02) boars (\( P < 0.05 \); Fig. 3). The number of PCV2b genomes did not correlate with the serum levels of anti-PCV2 IgG in blood from vaccinated challenged (T01; \( R = 0.640 \)) and nonvaccinated challenged (T02; \( R = −0.256 \)) boars.

**Quantification of PCV2 DNA in semen.** PCV2b genomes were not detected in any semen sample at −42, −21, −7, and 0 dpi from vaccinated challenged (T01) and nonvaccinated challenged (T02) boars. No PCV2b genomes were detected in any of the semen samples from the negative-control (T03) boars throughout the experiment.

The number of PCV2b genomes in semen was significantly lower in vaccinated challenged (T01) boars than in nonvacci-
nated challenged (T02) boars at 14, 18, 21, 25, 32, 42, 49, and 53 dpi ($P < 0.05$; Fig. 4). The number of PCV2b genomes in the semen did not correlate with the serum levels of anti-PCV2 IgG in either vaccinated challenged ($R = 0.555$) or nonvaccinated challenged ($R = 0.007$) boars, but it did correlate with the number of PCV2b genomes in the blood in both vaccinated challenged ($R = 0.714$) and nonvaccinated challenged ($R = 0.861$) boars.

FIG. 2. Standard curves of PCV2b and PCV2b recombinant plasmid DNA measured with real-time PCR (A) and correlation between PCV2b genomic copies and numbers of TCID<sub>50</sub>/ml (B). Tenfold serial dilutions of PCV2b and PCV2b recombinant plasmid DNA were used as templates in the reaction. The regression and efficiency analysis revealed a significant $R^2$ value of $>0.997$ in all analyses.
Virus isolation. Attempts were made to isolate and identify PCV1, PCV2a, and PCV2b from whole semen in the three groups. In vaccinated challenged boars (T01), PCV2b was isolated, and the presence of its DNA was confirmed in the cytoplasm of infected cells (Fig. 5) using *in situ* hybridization in two boars (boars 1 and 3) at 14 dpi and in one boar (boar 3) at 39 dpi. In the nonvaccinated and challenged group (T02), PCV2b was isolated, and the presence of its DNA in the cytoplasm of infected cells was confirmed using differential *in situ* hybridization in four boars (boars 1, 2, 3, and 4) at 10...
and 14 dpi, in three boars (boars 1, 3, and 4) at 21 dpi, in two boars (boars 1 and 2) at 32 dpi, and in one boar (boar 1) at 42 and 49 dpi. No PCV1, PCV2a, or PCV2b was isolated from or identified in the semen of negative-control boars.

**DISCUSSION**

Vaccination of boars was unable to prevent the subsequent shedding of wild-type PCV2 but significantly decreased the amount of PCV2 DNA shed in semen in challenged boars. Vaccinated boars shed PCV2b (10^{2.4} to 10^{3.6} PCV2b genome copies per ml, corresponding to 0.002 to 0.02 TCID_{50}/ml, respectively) in semen, whereas nonvaccinated boars shed PCV2b (10^{3.3} to 10^{4.3} PCV2b genome copies per ml, corresponding to 0.04 to 0.4 TCID_{50}/ml, respectively) in semen. Thus, the vaccination of boars reduced shedding of PCV2b by approximately 100% in semen compared to nonvaccinated boars. The reduction in PCV2 DNA shedding is meaningful because the virus titer in the semen plays a major role in the transmissibility of PCV2. For example, PCV2 at 10^{5.6} to 10^{5.8} PCV2 genome copies per ml isolated from the whole semen did not cause reproductive failure, seroconversion, or PCV2 viremia in naive gilts and their offspring when it was used for artificial insemination, although the PCV2 in semen was shown to be infectious in a swine bioassay model (14). The amount of PCV2 present in semen from vaccinated boars may minimize the transmission of PCV2 to sows via artificial insemination. Therefore, it is more efficient and economical to vaccinate boars to reduce PCV2 shedding in semen and to minimize the transmission of PCV2 to sows via artificial insemination.

In the present study, the amount PCV2 present in the semen of nonvaccinated boars was less than that in semen from nonvaccinated boars found in the previous study (14). We do not know why there is a difference between the two studies with respect to the amount of PCV2 in semen from nonvaccinated boars. This difference could be due to differences between breeds of pigs or differences in the virulence and tropism of the PCV2 isolates. The present study used purebred, male Duroc pigs, whereas the previous study used purebred, male Landrace pigs (14). In addition, the PCV2b isolate used in this study was isolated from postweaning 6-week-old pigs with severe PCVAD, whereas the PCV2b isolate used in the previous study was isolated from finishing pigs with severe PCVAD (14).

In agreement with previous findings (12, 19), PCV2 DNA was present in seminal plasma fractions and non-sperm cell fractions, as determined by conventional PCR (data not shown). Because the in vivo tissue and cell tropism of PCV2 in pigs is most likely monocytes/macrophages (10), the potential target cells of PCV2 in the semen include monocytes and macrophages, which could be vehicles for viral shedding in semen. Although the association of PCV2 with semen remains unclear, PCV2 most likely traffics from lymphoid tissue through the peripheral blood to reproductive tissues or directly into the semen.

There was no correlation between the serum levels of anti-PCV2 IgG and the number of PCV2 genomes in a previous study of 3-week-old pigs (17). These data suggest that PCV2 viremia in the blood is not reflected in the levels of PCV2 antibodies in the blood. It has been reported that a reduced PCV2 load in the semen is unrelated to the serum antibody response to the PCV2 vaccine (16, 18). Boars in the vaccinated challenged and nonvaccinated challenged group had detectable serum levels of anti-PCV2 IgG, indicating successful vaccination and/or challenge.

PCV2 DNA may be shed in semen for a considerable period of time, even in vaccinated challenged boars. The presence of PCV2 DNA in the semen of boars indicates that infectious particles exist and that there is a risk for swine (14). Artificial insemination has been widely and routinely used on South Korean swine farms; >90% of sows are bred by artificial insemination, and >80% of swine producers purchase semen for artificial insemination from commercial artificial insemination centers, according to the South Korea Institute of Pig Technology (http://www.pigtech.co.kr). Artificial insemination has also become standard practice in nearly 60% of North American swine farms and in greater than 90% of European countries (7, 20). Although the presence of viral DNA in a clinical sample does not indicate that infectious virus is present, it is strongly recommended that boars be immunized with the PCV2 vaccine regularly to reduce the amount of PCV2 DNA shed in the semen.

The inactivated PCV2 vaccine used in this study is licensed only for the induction of active immunity in piglets. Therefore, the inactivated PCV2 vaccine was used in an extralabel manner to determine its effect against PCV2 shedding in semen from experimentally infected boars. The reduction in the PCV2 load is one of the criteria used to assess the efficacy of PCV2 vaccines (5, 15). PCV2 vaccines decrease the PCV2 load in the serum and reduce PCV2 shedding in nasal and fecal samples in conventional pigs (5). Commercial vaccines were shown to be able to control two of the multiple disease entities, known as PMWS and PRDC (2, 4, 5, 16, 18). To our knowledge, this is the first application of a PCV2 vaccine in boars to determine its effect on PCV2 shedding in the semen. Further studies are needed to determine the efficacy of PCV2 vaccines against PCV2 shedding in semen from vaccinated boars.
needed to evaluate the role of PCV2 vaccination in PCV2-associated reproductive failure and in other clinical entities.

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