Research Article

The role of porcine reproductive and respiratory syndrome virus as a risk factor in the outbreak of porcine epidemic diarrhea in immunized swine herds

Mei-Zhou HUANG*, Hui WANG*, Sheng-Yi WANG, Dong-An CUI, Xin TUO, Yong-Ming LIU**
Engineering and Technology Research Center of Traditional Chinese Veterinary Medicine of Gansu Province, Key Lab of New Animal Drug Project of Gansu Province, Key Lab of Veterinary Pharmaceutical Development of Ministry of Agriculture, Lanzhou Institute of Husbandry and Pharmaceutical Sciences of Chinese Academy of Agricultural Sciences, Lanzhou, Gansu, P.R. China

Abstract: The aim of the study was to investigate the relation between porcine epidemic diarrhea (PED) and porcine reproductive and respiratory syndrome virus (PRRSV). Here, we constructed a dynamic clinical experimental model in which sows were infected with PRRSV. The stability of the experimental model was surveyed by detecting the levels of antibodies against classical swine fever virus (CSFV) or PRRSV, respectively. In both group A (infected group) and group B (healthy group), the CSFV antibody level was not significantly different (P > 0.05). In group B, the PRRSV antibody level was also not significantly different (P > 0.05) at the three times of testing. The incidence of porcine epidemic diarrhea virus (PEDV) in piglets and PEDV-carrying rate of sows was detected by real-time fluorescent RT-PCR. In group A, PEDV infection of piglets and the PEDV-carrying rate of sows were significantly higher than in group B (P < 0.01) and the PEDV strains were within the same group according to the phylogenetic analysis based on the complete *S* gene. The results suggested that PRRSV was a risk factor in the outbreak of PED in immunized swine herds. Besides that, the experimental model was stable and no interference factors affected the results of the study.

Keywords: Porcine, diarrhea, lactation, piglet, virus, immunity

1. Introduction

Porcine epidemic diarrhea (PED) is considered to be a devastating disease for producers, and the spread of infection is very rapid (1). Clinical symptoms include severe enteritis, vomiting, and watery diarrhea, with high infectivity and lethality in piglets, which causes great financial losses (2,3). Therefore, intensive swine commercial farms vaccinate with a combination of killed or attenuated vaccines against transmissible gastroenteritis virus (TGEV) and PEDV infection (4–6). Since the outbreak of PEDV in 2010, it has rapidly spread throughout China (7,8). Although most large-scale pig farms vaccinate according to a proper immunization schedule, PEDV still emerges in immunized swine herds (4).

Porcine epidemic diarrhea virus (PEDV) is an enveloped, single-stranded, positive-sense RNA virus that mutates easily. Molecular epidemiology studies have shown that virus mutation is one of the causes of PEDV outbreaks (9–11). Some intensive swine commercial farms have used autogenous vaccines that complied with

basic quality and safety requirements, but failed to control outbreaks of PEDV (12–14). Porcine reproductive and respiratory syndrome (PRRS) is considered to be one of the most important diseases affecting pigs. This is mainly due to its impact on production, especially as the virus is putatively immunosuppressive and concurrent diseases are common (15). Animals known to have been infected with PRRSV have a noticeable increase in the number of secondary viral and bacterial infections (15–18). Piglets are protected against PEDV by specific IgG antibodies from the colostrum and milk of immune sows until they are 4 to 13 days old. The duration of immunity depends on the maternal antibody titer (3,19). If a sow's immune system was suppressed, the piglet's antibody level of PEDV would be decreased. Moreover, PEDV mainly infects piglets at 3 to 7 days of age. Hence, whether PRRSV-infected sows are a risk factor for PED occurrence in immunized swine herds has not been documented. Here, we provide proof to verify this hypothesis by constructing a model of PRRSV infected-sows and by detecting the PEDV-positive piglets and the rate of PEDV carrier sows in the model.

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*   These authors contributed equally to this work.

** Correspondence: myslym@sina.com

2. Materials and methods

2.1. Study subjects

The trial was conducted between April 2014 and January 2015 in the Gansu Province of Northwest China. There was a persistent outbreak of PEDV in the area during that time period. The study included a total of 2 intensive swine commercial farms with 840 ternary breeding sows. They were managed under an intensive husbandry system with similar health, nutrition, and husbandry practices. Following the protocols approved by the Institutional Animal Care and Use Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences (animal use permit: SCXK20008–0010), the animals utilized in these experiments were treated humanely and with respect for the alleviation of suffering.

2.2. Experimental protocol

The experiment consisted of five parts (Figure 1). The first part involved constructing the experimental model by enrollment and exclusion. The experimental model was divided into two groups. The sows were enrolled into group A (PRRSV-infected group) according to the following enrollment criteria. The sows were infected with PRRSV but did not show any clinical symptoms and had a good mental and physical state and appetite. The sows were confirmed not to carry any other contagions, such as classical swine fever virus (CSFV) or porcine circovirus (PCV), and there was a clinical history of veterinary quarantine and clinical records. The sows were enrolled into group B (healthy group) according to the same enrollment criteria as above except that the sows were negative for PRRSV. If sows were negative for PRRSV in group A, they
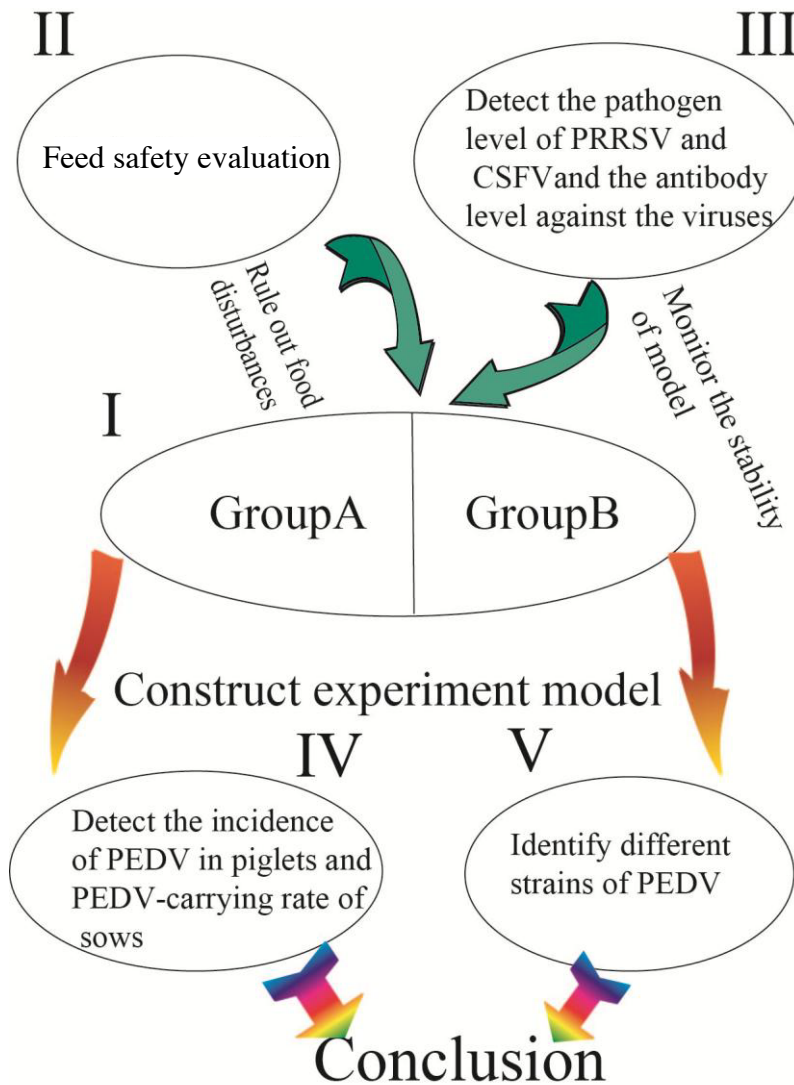

Figure 1. The framework of experiment.

were excluded and their piglets were not included in the final results. In group B, if sows were positive for PRRSV, they were excluded from the study. Additionally, seriously ill sows were excluded from the study. All sows had similar parities (3 to 5 births) in both group A and group B.

The second part was feed safety evaluation, including the detection of PEDV and basic nutritional composition analysis, which was used to rule out food disturbances in the experiment because previous research has documented that contaminated feed is a risk factor for PEDV (20).

The third part was monitoring the stability of the experiment model by detecting pathogen levels of PRRSV and CSFV and the antibody levels against the viruses. The experiment was designed by randomized complete blocks and differentiated stages by block. The different stages of sows and piglets were divided into three blocks: gestation sows, lactation sows, and 1- to 7-day-old suckling piglets.

The fourth part involved detecting the incidence of PEDV in piglets and the PEDV-carrying rate of sows.

The fifth part was identifying different strains of PEDV based on the complete *S* gene, which confirmed the PEDV strains at the two farms during the experiment.

2.3. Sample collection
To evaluate feed safety, the feed of pregnant and lactating sows was collected from the two farms every 3 months during the experiment.

Blood samples were collected from the anterior vena cava at the two farms every 3 months from April 2014 to January 2015. The blood was held at room temperature for 2 h and then centrifuged at $3000 \times g$ for 10 min. The serum was transferred to new centrifuge tubes and stored at –20 °C prior to detecting PRRSV and the antibody levels against CSFV and PRRSV. Fecal scores were used to identify diarrhea in piglets following the following fecal scoring system: 1 = hard, dry pellet; 2 = firm, formed stool; 3 = soft, moist stool that retains shape; 4 = soft, unformed stool that assumes the shape of the container; 5 = watery liquid that can be poured. The piglets were considered to have diarrhea when the fecal scores were at level 4 or 5

(21,22). The feces of diarrheal piglets were collected by squeezing the abdomen, and sows' feces were collected by rectal swab.

2.4. Feed safety evaluation
Feed samples were evaluated using PEDV-TGEV-PRV triple real-time fluorescent RT-PCR kits (Anheal Laboratories, China). Specific steps were carried out in accordance with the manufacturer's protocols. The feeds of the sows during pregnancy and lactation were sent to the Center for Quality Supervising, Inspecting, and Testing of Animal Fiber, Fur, and Leather Products of the Ministry of Agriculture, P.R. China, to analyze the basic nutrients.

2.5. Experimental model surveillance by detecting CSFV and PRRSV antibody levels
During the experiment, blood samples were collected once every 3 months. Ten blood samples were taken at random from each block of the experimental model each time. The blood samples were sent to a laboratory for testing. The antibody levels against CSFV and PRRSV were detected by ELISA kits (IDEXX, USA). Samples were tested in duplicate; the specific steps were carried out in accordance with the manufacturer's protocols. The results were determined using an enzyme standard instrument (MDC Spectramax M2e, USA).

2.6. Detection of CSFV and PRRSV
We used CSFV real-time fluorescent RT-PCR kits and PRRSV real-time fluorescent RT-PCR kits (Anheal Laboratories, China) to detect the virus in the serum samples of piglets and sows, respectively. Real-time fluorescent RT-PCR protocols are shown in Table 1. Results were detected using real-time fluorescent RT-PCR instrument (Bio-Rad CFX96, Germany).

2.7. Detection of the incidence of PEDV in piglets and the PEDV-carrying rate of sows
We used PEDV-TGEV-PRV triple real-time fluorescent RT-PCR kits (Anheal Laboratories, China) to detect the virus in the fecal samples of piglets and sows. Real-time fluorescent RT-PCR protocols are shown in Table 1.

Table 1. Real-time fluorescent quantitative RT-PCR protocols.

Items	Step 1 Extract RNA template	Step 2 Reaction system	Step 3 Reaction conditions	Step 4 Reporter dye	Step 5 Results analysis
Detection of CSFV	Extract virus RNA from samples according to manufacturer's protocols	Add RNase-free dH2O, RT-PCR buffer, enzyme mix TaqMan probe, and RNA template according to manufacturer's protocols	The amplification was performed as follows: one cycle at 42 °C for 5 min and 95 °C for 10 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 35 s, and 72 °C for 5 min	FAM	If positive control shows specific amplification curve and negative control has no Ct value. 0 < Ct value ≤ 30 was recognized as a positive sample.
Detection of PRRSV					
Detection of PEDV					

Results were determined using a real-time fluorescent RT-PCR instrument (Bio-Rad CFX96, Germany).

2.8. Identification of different strains of PEDV

2.8.1. Designing the primer
To obtain the complete *S* gene sequence of PEDV, primers were designed based on the sequence of a reference PEDV strain (Accession Number: AF353511.1). Forward Primer: 5'-ATGAGGTCTTTAATTTACTTCTGGTTG-3'; Reverse Primer: 5'-TCACTG CACGTGGACCTTT- 3'.

2.8.2. PEDV RNA extraction
The fecal pretreatment protocol was based on previous literature reports. Each sample was diluted with phosphate-buffered saline to make a 10% (v/v) suspension. The suspensions were vortexed for 1 min and clarified by centrifugation for 10 min at 5000 × *g*. The supernatants were collected for extraction of PEDV RNA and dissolved in 50 μL of RNase-free dH$_2$O, as described in the TaKaRa miniBEST viral RNA/DNA Extraction Kit (TaKaRa, China). Samples were stored at –80 °C.

2.8.3. RT-PCR
The *S* gene of PEDV was amplified by RT-PCR using the PrimeScript one-step RT-PCR kit. The amplification was performed as follows: one cycle at 50 °C for 30 min, then 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 5 min. Samples were stored at 4 °C.

2.8.4. Sequencing of RT-PCR products
The RT-PCR products were identified by electrophoresis on 0.5% agarose gel. The positive results were sent to the Beijing Genomics Institute for sequencing. All sequencing reactions were performed in duplicate. The size of the RT-PCR products was 4152 bp.

2.8.5. Sequence analysis of the S gene
The nucleotide sequences of the *S* gene from the strains were aligned and analyzed by MEGA 6.0 software. The CV777 strains were used for sequence alignment and analysis with the PEDV strains.

2.8.6. Phylogenetic analysis of PEDV
Phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis (MEGA 6.0) software with the neighbor-joining method based on the sequence of the S gene.

2.9. Statistical analysis
Antibody levels against CSFV and PRRSV were expressed as means and standard deviations (±SD). Statistical analysis was performed with one-way analysis of variance (ANOVA). Table analysis was used to detect the incidence of PEDV in piglets and the PEDV-carrying rate of sows. All statistical analyses used SAS software (version 9.2, USA), and P < 0.05 was considered significant.

3. Results

3.1. Construction of the experimental model
We used PRRSV real-time fluorescent RT-PCR kits (Anheal Laboratories, China) to detect PRRSV in the serum of sows. Each sow received a clinical examination, including identification of pathological conditions, rectal temperature, and mental and physical state. During the experimental period, 183 sows were enrolled in the experimental model and divided into two groups (group A, n = 81; group B, n = 102) according to the enrollment and exclusion criteria. In addition, there were 1723 suckling piglets (group A, n = 726; group B, n = 997) in the experimental model. All suckling piglets were assured colostrum intake from their mothers within 1 to 4 h after birth, and the nipples of the sows were disinfected prior to suckling.

3.2. Feed safety evaluation
The basic nutrition of the sows' feed during pregnancy and lactation agreed with the national standards (Table 2).

The results of the PEDV-TGEV-PRV triple real-time fluorescent RT-PCR showed that all feed samples were negative for PEDV (Ct = 0) (Table 3).

3.3. Detection of CSFV and PRRSV antibody levels
The levels of antibodies against CSFV or PRRSV were detected by ELISA kits (Figure 2). In both group A and group B, the level of antibodies against CSFV was not significantly different at the three times of testing (P > 0.05). In group B, the level of antibodies against PRRSV was also not significantly different (P > 0.05). However, the level of antibodies against PRRSV in group A was

Table 2. The basic nutrition of the sow feed during pregnancy and lactation.

Indexes	DM (%) GB/T 6432-1994	EE (g/kg) GB/T 6433-2006	CF (g/kg) GB/T 6434-2006	Moisture (%) GB/T 6435-2006	Ca (%) GB/T 6435-2006	TP (%) GB/T 6435-2006	Ash (%) GB/T 6435-2006
Pregnant sows' feed	14.28 ± 0.50	19 ± 1	59 ± 2	12.89 ± 0.53	4.25 ± 0.17	0.39 ± 0.09	5.31 ± 0.26
Lactating sows' feed	17.13 ± 0.32	43 ± 1	45 ± 3	12.34 ± 0.69	4.23 ± 0.35	0.43 ± 0.03	5.13 ± 0.18

Values are mean ± standard deviation. DM: Crude protein; EE: crude fat; CF: crude fiber; TP: total phosphorus.

Table 3. The results of PEDV detection in fecal and feed samples by PEDV-TGEV-PRV triple real-time fluorescent RT-PCR.

Indexes	Group A	P (Ct value)	P/T*	Group B	P (Ct value)	P/T*
Positive rate of PEDV in feed	0%	0 (0)	0/12	0%	0 (0)	0/12
Positive rate of PEDV in piglet feces	76.4%**	204 (12.6–28.7)	204/267	53.0%	108 (17.3–27.9)	108/198
Positive rate of PEDV in sow feces	40%**	8 (18.5–29.6)	8/20	15%	3 (14.3–23.6)	3/20

P: Positive samples; P/T*: positive samples/ total samples. $0 < $ Ct value ≤ 30 was recognized as a positive sample. **: $P < 0.01$.
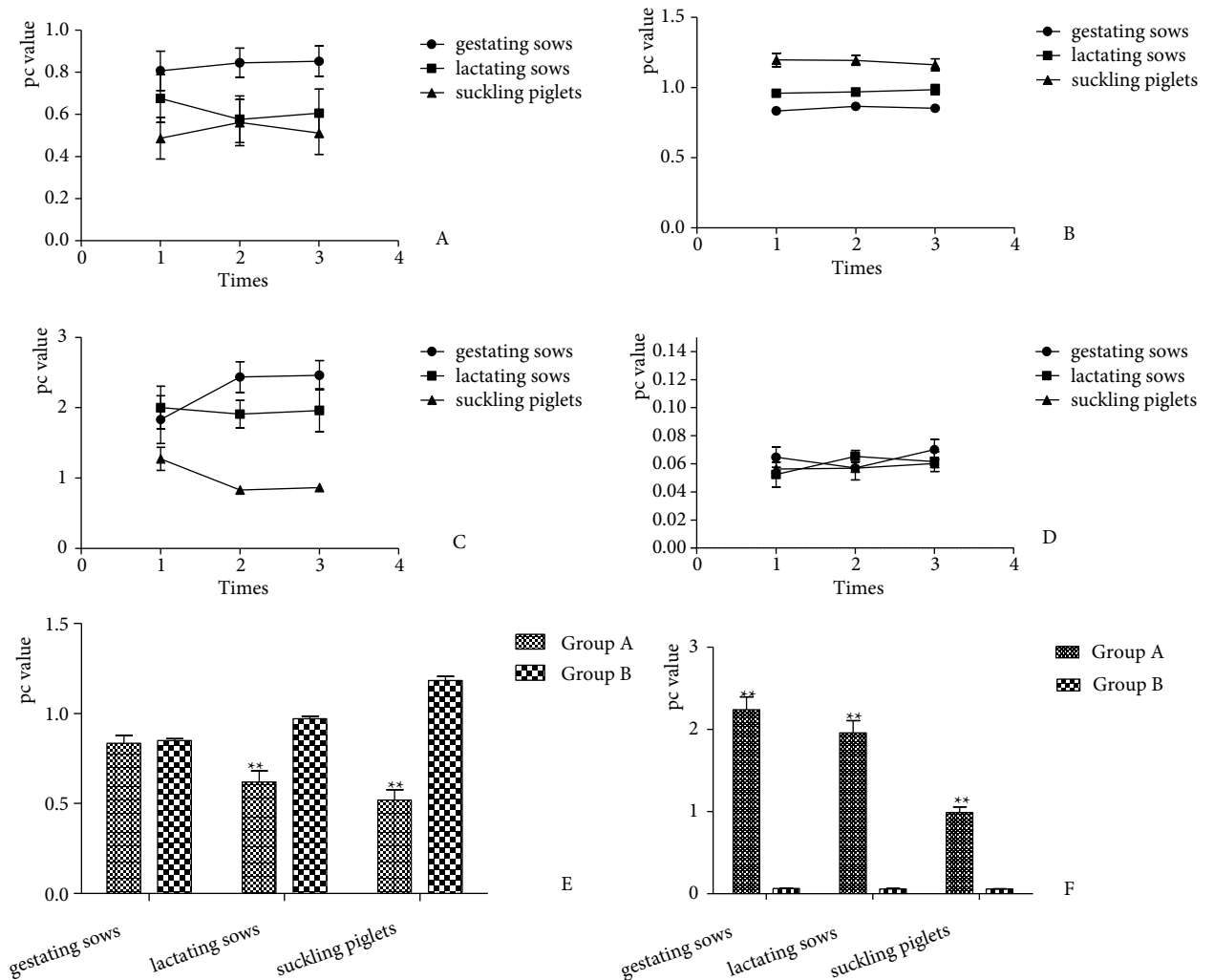

Figure 2. The fluctuation of CSFV and PRRSV antibody levels during different periods from April 2014 to January 2015. A is the antibody level against CSFV in group A, and B is the antibody level against CSFV in group B. C is the antibody level against PRRSV in group A. D is the antibody level against PRRSV in group B. E is the comparative antibody levels against CSFV in swine in farm A and farm B. F shows the comparison of antibody levels against PRRSV in swine in group A and group B. Values are represented as mean ± standard deviation and the treatments in one experiment were repeated twice. **: $P < 0.01$.

significantly different ($P < 0.01$) between the first time point and the latter two time points in the block of suckling piglets. In group A, the level of antibodies against PRRSV was significantly higher than in group B in all blocks ($P <$

0.01). The level of antibodies against CSFV in group A was significantly lower than in group B in the block of lactating sows and suckling piglets ($P < 0.01$).

3.4. Detection of CSFV and PRRSV

The results of the detection of PRRSV and CSFV are shown in Table 4. The sows and piglets in group B were CSFV- and PRRSV-negative. The sows and piglets in group A were CSFV-negative. The sows in group A were PRRSV-positive, while the piglets in group A were PRRSV-negative.

3.5. Detection of the incidence of PEDV in piglets and the PEDV-carrying rate of sows

There were 465 piglets with diarrhea (group A, n = 267; group B, n = 198). In group A, the diarrhea rate of the piglets was significantly higher than in group B (group A was 36.78%, group B was 19.8%, P < 0.01). Analysis of the cycle threshold (Ct) values of the PEDV real-time RT-PCR-positive samples indicated that all of the samples with Ct values below 30 were PEDV-positive. The fecal samples of 312 piglets were PEDV-positive (group A, n = 204; group B, n = 108). In group A, PEDV infection was significantly higher than in group B (P < 0.01). Eleven samples (group A, n = 8; group B, n = 3) out of 40 sow fecal samples (group A, n = 20; group B, n = 20) were PEDV-positive. The rate of PEDV carrier sows in group A was significantly higher than in group B (P < 0.01) (Figure 3; Table 3).

3.6. Identification of different strains of PEDV

During the experiment, we extracted 24 PEDV RNA samples from PEDV-positive fecal samples. The 24 RNA samples were converted to cDNA samples by reverse-transcription. The 10 cDNA samples were successfully sequenced. The nucleotide sequences of the 10 cDNA samples had 99% sequence identity. More precisely, the nucleotide sequences of the 10 cDNA samples had 98% sequence identity with CV777 (Accession No: JN599150.1). We chose 2 cDNA sequences from a different group (Accession No: KR902706 and KR902707) together with the PEDV CV777 strain (Accession No: JN599150.1) to perform a phylogenetic analysis. The results showed that all of the sequences fell into two groups (Figure 4). The two PEDV field isolates were within one group.

4. Discussion

An experimental model, in which sows were infected with PRRSV, was constructed based on enrollment and exclusion criteria. The selection of the appropriate experimental model for the research was complex due to animal welfare considerations and the changeable clinical environment. The selection of a suitable experimental model largely depends on whether it is stable and meets the specific research demands in question (23). Many static experimental models can be built to manually control influencing factors and infection, which allows scientists to evaluate research factors (24,25). The static experimental models have shortcomings in emerging infectious disease research. The animals are euthanized after infection in these models. The outrage of society reminds investigators to adhere to high standards of humane animal usage (25). To monitor the stability of the experimental model, we chose to survey the antibody levels against PRRSV and CSFV. The use of laboratory data for passive disease surveillance is limited by its lack of ability to identify disease outbreaks, reemerging diseases, or novel pathogens (26,27). Monitoring the test results of commonly used first-order tests for a known disease may be a unique form of syndromic data collection for the timely identification of novel disease outbreaks in swine populations (28). In both group A and group B, the level of anti-CSFV antibodies was not significantly different at the three time points (P > 0.05). The results suggested that the experimental model was stable. The piglets had antibodies against CSFV from the colostrum and milk of immune sows until they were first vaccinated. If a sow's immune system was suppressed, the piglet's antibody levels against CSFV would decrease. The antibody level against CSFV in group A was significantly lower than that of group B in the block of lactating sows and suckling piglets (P < 0.01), which showed that PRRSV may suppress the host's immune system. PRRSV has a tropism for immune cells and has been shown to suppress the host's immune system (15–18). PEDV-contaminated feed may be a risk factor for PEDV transmission, which may lead to PEDV occurrence

Table 4. PRRSV and CSFV levels in serum samples using PRRSV real-time fluorescent RT-PCR and CSFV real-time fluorescent RT-PCR, respectively.

Indexes	Group A	P (Ct value)	P/T*	Group B	P (Ct value)	P/T*
Positive rate of PRRSV in piglet serum	0%	0 (0)	0/30	0%	0 (0)	0/30
Positive rate of PRRSV in sow serum	100%	30 (9.67–28.7)	30/30	0%	0 (0)	0/30
Positive rate of CSFV in sow serum	0%	0 (0)	0/30	0%	0 (0)	0/30
Positive rate of CSFV in piglet serum	0%	0 (0)	0/30	0%	0 (0)	0/30

P: Positive samples; P/T*: positive samples/ total samples. 0 < Ct value ≤ 30 was recognized as a positive sample.
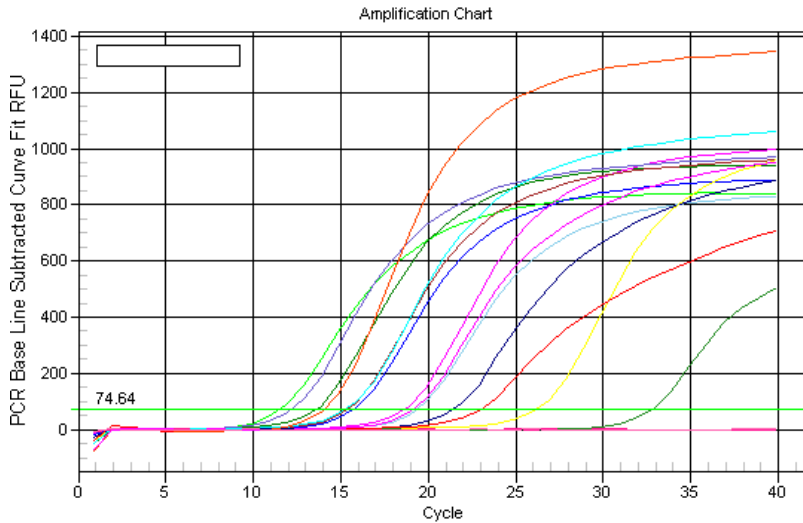
Figure 3. Real-time fluorescent quantitative RT-PCR amplification curve of PEDV.
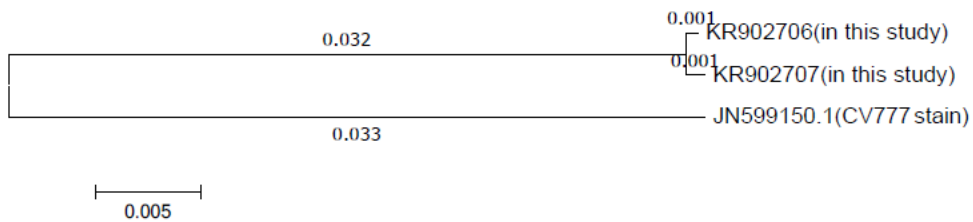

Figure 4. The relationship between the PEDV field isolates of the experimental model and PEDV CV777 stain (Accession No: JN599150.1) based on the full-length *S* gene.

(19). Therefore, we performed a feed safety evaluation. The results of the feed safety evaluation showed that all of the feed samples were negative for PEDV (Ct = 0), and the basic nutrition of the feed met country standards. The results indicated that the feed was not related to the outbreak of PEDV in the experimental model. Moreover, many researchers have documented a remarkable increase in PEDV outbreaks attributable to the emergence of new strains (14,29). The PEDV S glycoprotein plays a pivotal role in regulating interactions with specific host cell receptor glycoproteins to mediate viral entry, which could be a primary target for the development of effective vaccines against PEDV (30). The mutation of the PEDV *S* gene is possible because of the PEDV outbreak. This paper showed that the PEDV strains had high similarity and were within one group, based on the construction of phylogenetic trees using the complete *S* gene sequence from the experimental model. Therefore, we deduced that the outbreak of PEDV was not related to PEDV mutation in the experimental model. The incidence of PEDV in piglets and the PEDV-carrying rate in sows were significantly increased in group A. Therefore, we deduced that the sows were infected

with PRRSV, which led to immunosuppression in swine in group A. If the immunity of the sows and piglets was inhibited, there would be a high risk of infection by secondary viruses and bacteria. The rate of PEDV-positive sows was significantly increased, and although few sows showed any clinical signs, they could excrete the virus in their feces, which could infect piglets by the fecal-oral route (1,19). Therefore, we propose that PRRSV infection might be related to an outbreak of PEDV in immunized swine herds.

In conclusion, many risk factors can cause an outbreak of PEDV, such as virus mutation and feed contamination. The results of this study showed that PRRSV was a risk factor in the outbreak of porcine epidemic diarrhea in immunized swine herds.

Acknowledgments

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