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Multiple Factors Contribute to Persistent Porcine Epidemic Diarrhea Infection in the Field: An Investigation on Porcine Epidemic Diarrhea Repeated Outbreaks in the Same Herd

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Abstract: In the recent years, severe reoccurrence of Porcine Epidemic Diarrhea (PED) has been recorded in China. In the present study, specific PED related symptoms were observed repeatedly up to six times in the same herds. To investigate the possible factors involved in the PEDV persistent infections and repeat outbreaks. Feces, semen and air samples were collected and examined by RT-PCR and quantified by real-time RT-PCR. The results showed that all of the spike gene sequences of samples at different time were highly homologous. PEDV was shed in fecal excretions at high concentration (up to $1\times10^{6.85}$ copies mL^{-1}) and the shedding time lasted for at least 56 days under field condition. Semen collected from boars without PED clinical phenomena and air samples were positive for PEDV RNA as well.

Key words: PED persistent infection, PEDV transmission, PEDV shedding time, semen, aerosol

INTRODUCTION

Porcine Epidemic Diarrhea (PED) is characterized by vomiting and watery diarrhea. By inducing further infection, the disease can lead to a high mortality rate up to 90% in piglets (Callebaut *et al*., 1982; Yeo *et al*., 2003). The disease was first recognized in 1978 (Pensaert and de Bouck, 1978) and since then has continuously occurred in Europe and Asia causing tremendous economical losses to the swine industry (Chen *et al*., 2008; Puranaveja *et al*., 2009). In China, PED was first reported in 1976 (Cheng and Niu, 1992). PED is caused by a group I Coronavirus (CoV) virus (PEDV). As first described by Pensaert and de Bouck (1978), PEDV was an enveloped virus possessing a single-stranded positive-sense RNA genome which is approximately 28.3 kb in length (Kocherhans *et al*., 2001).

Recently, PEDV has been spread from the Southeast of China to nationwide and made millions of piglet dead. In several farms, the PED reoccurrences were observed for up to six times in the same herd in piglets. These repeat outbreaks were caused by the same PEDV variant strain as demonstrated by the *S* genes sequence data.

PEDV has been detected by RT-PCR, immunohistochemistry and in situ hybridization in mesenteric lymph nodes from piglets experimentally infected with PEDV. And it has been found in nasal fecal samples in experimentally and naturally infected pigs (Song *et al*., 2006) and in colostrum and milk from naturally infected sows as well (Sun *et al*., 2012). However, the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) and Porcine Circovirus type 2 (PCV2) have also been found in semen from experimentally infected pigs (Madson *et al*., 2009; Revilla-Fernandez *et al*., 2005) and in air samples from swine confinement buildings (Otake *et al*., 2010; Verreault *et al*., 2010).

PEDV infection can be transmitted through several routes. One of them is fecal-oral route which is deemed as the classic transmission (Jung *et al*., 2006a; Kim and Chae, 2003; Shibata *et al*., 2000). Previously, the study reported that sow-milk-piglet was another way of PEDV infection (Sun *et al*., 2012). However, if virus-laden particles become concentrated aerosol under appropriate environmental conditions, infections and potentially PED infection can occur by another route the aerosol route. Additionally, several viruses have been found in experimentally infected pig semen samples (Madson *et al*., 2009; Revilla-Fernandez *et al*., 2005).

In summary, the purpose of this study was to investigate the possible factors involved in the PEDV persistent infections and repeat outbreaks. Feces, semen and air samples were collected and examined by RT-PCR and quantified by real-time RT-PCR.

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MATERIALS AND METHODS

Backgrounds of the farms: Three farms were selected for the investigation and the detailed information was listed in Table 1.

Samples collection and treatment

Swabs samples: Fecal swab samples were collected weekly from piglets recovered from PED. All samples were soaked in PBS buffer supplemented with penicillin G (10, 000 IU mL^{-1}) and streptomycin (2 mg mL^{-1}) and centrifuged at 1,500× g for 15 min to collect the supernatant fluids. The supernatant fluids were stored at -80°C until used.

Semen samples: Raw semen samples from boars without diarrhea were slipped in 10 mL vials immediately after collection and shipped on ice from the boar studs to the Veterinary Medicine Institute, Guangdong Academy of Agricultural Sciences. Samples were stored at -80°C once received until RNA extraction.

Air samples: The air samples were collected in triplicate using sample air lite (AES, France) at five different sites of barn 1 m above ground level (Fig. 1) under the conditions as listed Table 2. In brief, the agar plate was placed in machine platform and turn on the machine followed the flow rate with 100 L min^{-1} for 5 min. When finished, the plate was removed and washed with 500 μL PBS, the wash fluids were collected into a 1.5 mL sterile tube and stored in -80°C freezer prior to RNA extraction.

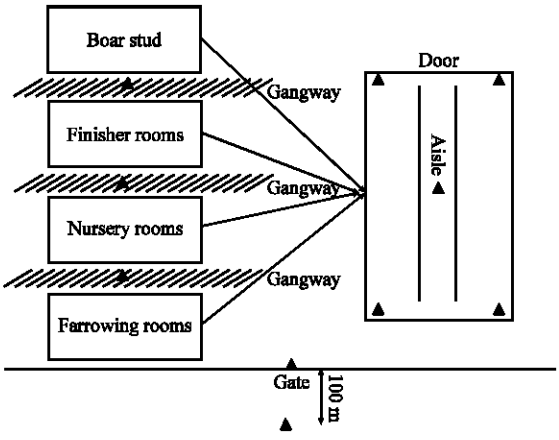

Fig. 1: Sketch map of air sample sites. Sites of air samples are indicated as dark triangle representing postal code areas (Sun *et al*., 2012)

RNA extraction: RNA was extracted from the samples using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA) following the manufacturer's instructions. Briefly, the liquid (200 μL) treated above was mixed with 1 mL TRIzol Reagent. Then 200 μL of chloroform was added to the mixture and the suspension was centrifuged for 15 min at 12,000× g. The RNA-containing aqueous phase was precipitated with isopropanol of the same volume, maintained at -20°C for 1 h and centrifuged for 10 min at 12,000× g. The RNA pellet was washed with 1 mL of 75% ethanol, centrifuged for 10 min at 12,000× g and dried then dissolved in 30 μL Diethyl-Pyrocarbonate (DEPC)-treated deionized water.

Primers: To detect and quantify the PEDV in the air samples, pairs of sense and antisense primers based on the nucleotide sequence of the *S* genes described previously (Park *et al*., 2007) were used in the real-time RT-PCR. The forward primer, 5'-TAATGATTACCTGTCT TTTAGC-3' and reverse primer, 5'-GTGATACCTTCAA GTGGTTTAG-3' were synthesized by Invitrogen Co., Ltd. The size of the amplified product was 651 bp.

RT-PCR: RT-PCR was performed using PrimeScript® One Step RT-PCR Kit Ver.2 (TaKaRa Biotechnology Co., Ltd. Dalian, China). Briefly, 3 μL RNA was mixed with PCR reaction mix containing 25 μL 2×1 Step Buffer, 2 μL each specific primer (10 pmol), 2 μL PrimeScript 1 Step Enzyme Mix and 16 μL RNase Free water. The amplification were performed with reverse transcription at 50°C for 30 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min and a final extension step of 72°C for 10 min. RT-PCR products were visualized by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Bands of the correct size were excised and purified using TaKaRa MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0 (TaKaRa Biotechnology Co., Ltd. Dalian, China) according to the manufacturer's instructions.

Table 1: Characteristics of the swine farms in this study

Farm	Circumstance	Production models[a]	Sows no.	Boars no.	Vaccination[b]	Match way	Times of PED outbreak
A	Bushes near by village	No	100	3	No	AI[c]	4
B	Mountain	Yes	3500	40	Yes	AI	6
C	Open space near the national road	Yes	8600	150	Yes	AI	3

[a]Indicated that the farm carried out the all-in and all-out system; [b]Indicated that sows inoculated with bivalent inactivated Transmissible Gastroenteritis (TGEV H) and porcine epidemic diarrhea (CV777) vaccine; [c]Artificial insemination

To sequence the partial *S* gene, a 651 bp fragment amplified from PEDV was purified using the TaKaRa MiniBEST DNA Fragment Purification Kit Ver.3.0 (TaKaRa Biotechnology Co., Ltd. Dalian, China). The purified products were sequenced by BGI Co., Ltd. (Shenzhen, China).

Quantitative RT-PCR analysis: Quantitative PCR analysis of PEDV was conducted using One Step SYBR® PrimeScript® RT-PCR Kit II (Perfect Real Time) (TaKaRa Biotechnology Co., Ltd. Dalian, China) following the protocol provided by the manufacturer. Briefly, each reaction contained 10 μL 2×One Step SYBR® RT-PCR Buffer, 1 μL each prime (10 pmol), 2 μL RNA sample, 1 μL PrimeScript® 1 Step Enzyme Mix and 5 μL RNase Free dH_2O. The RT-PCR was performed in LightCycler® 480 real-time PCR System (Roche, USA) using 96 well plates. Samples used as positive control template (Standards) were synthesized by Invitrogen DNA (from 10^9-10^4 copies). Water was used as template for the negative control). All test samples were amplified in triplicates. The reaction started with a reverse-transcription step of 42°C for 10 min followed by pre-incubation of 95°C for 10 sec, continued with 60 cycles of 95°C for 5 sec (denature step), 60°C for 20 sec (amplification step). Fluorescence data were captured at the amplification step. A melting curve analysis was performed after the PCR. The analysis was done at 95°C for 0 sec, 60°C for 15 sec then 95°C continuous.

Sequence analysis: The obtained partial S sequence of PEDV was blast using tool provided by NCBI (http://blast.ncbi.nlm.nih.gov) and aligned using Bioedit Software Clustal X v1.83 program. The aligned data was analyzed and converted for use in the phylogenetic analysis using Bioedit program. A Neighbor-Joining (NJ) tree was constructed by MEGA 4.0 program and the percent frequencies of the groupings were determined after 1000 bootstrap evaluations.

Table 2: Characteristics of the swine confinement buildings for air samples collection

Farm	Date	Waste management	Farm type	Animal density (m^2/pig)	Temp. (°C)	RH (%)
A	2011-10-30	Concrete	Boar	6.00	17-27	45-85
		Concrete	Sow	6.00	17-27	50-85
		Saltted	Farrowing	3.96	24-28	40-75
		Saltted	Nursery	1.00	23-28	40-80
B	2011-04-01	Concrete	Boar	9.00	13-20	65-95
		Saltted	Sow	0.70	13-20	65-93
		Saltted	Farrowing	4.05	23-30	60-80
		Saltted	Nursery	0.75	20-26	65-85
C	2012-2-5	Concrete	Boar	1.61	10-16	50-90
		Saltted	Sow	2.00	10-16	50-85
		Saltted	Farrowing	4.05	22-28	60-75
		Saltted	Nursery	0.60	20-28	50-80

RESULTS

Shedding time of PEDV in feces: In this study to monitor the PEDV shedding time, PEDV was detected in the rectal swabs collected from recovery piglets by RT-PCR using specific primers based on the *S* gene. Weekly RT-PCR testing of feces demonstrated that all swabs remained positive for PEDV RNA throughout the study. The swab samples of 3 day old piglets remained positive for 59 days post infection. Intermittent shedding was not recorded. The detailed PEDV quantity detected from field specimens is summarized in Table 3.

Detection of PEDV in air samples: RNA was extracted from air samples and the RNA extract was screened for PEDV presence. All samples from Farm A were PEDV negative, the overall PEDV positive rate of Farm B air samples was 6.7% for all sites and 20% for the delivery room, the PEDV positive rate of air samples from Farm C was 12.2% for all sites and 40% for the delivery room.

Detection of PEDV in semen samples: The prevalence of PEDV in semen samples was summarized (Table 4). For Farm A, only one out of three semen samples was positive showing a PEDV RNA concentration of $1\times10^{2.93}$ copies/mL. The quantity of detectable PEDV RNA in semen from Farm B was ranging from $1\times10^{3.55}$ copies/mL to $1\times10^{1.46}$ copies/mL. In Farm C, 7 out of 10 boars shed PEDV RNA in raw semen with lowest and highest quantity of $1\times10^{1.82}$ copies/mL and $1\times10^{3.65}$ copies/mL, respectively. All boars selected for this study did not show any symptoms of PED. Although, the amount of PEDV RNA presented in semen varied among individual pigs there was no significant difference in mean PEDV genomic copies/mL among the samples.

Table 3: Detection of PEDV RNA amount in fecal swabs collected from recovery piglets in Farm B

DPI[a]	Samples no.	Positive ratio (%)	Quantity (10×copies no.) Minimum	Quantity (10×copies no.) Maximum
7	10	100.0	3.27	6.85
14	9	100.0	3.41	6.58
21	10	90.0	2.65	5.78
28	8	87.5	2.41	4.87
35	8	75.0	1.81	4.87
42	10	60.0	1.90	4.53
49	6	50.0	1.69	3.27
56	10	30.0	1.45	2.13

[a]Days postinfection

Table 4: Quantification of PEDV RNA in semen collected from the field

Farm	Boars no.	Samples no.	Ratio (positive/total)	Quantity[a] (10×copies no.)
A	3	3	1/3	2.93
B	40	8	8/8	3.55-1.46
C	150	10	7/10	3.65-1.82

[a]Amount of PEDV RNA from the maximum to minimum

PEDV partial *S* gene sequences analysis: The PEDV partial *S* genes from samples selected from different farms during different outbreaks were amplified, sequenced and analyzed. The results showed that these sequences had 98.9-100% homology in nucleotide and 99.5-100% in amino acid.

DISCUSSION

PEDV infection is widespread in swine herds (Carvajal *et al*., 1995; Jung *et al*., 2006b). However, the transmission of PEDV has not been yet well characterized. Detection of PEDV RNA has been reported in feces, nasal, colostrum and milk of infected pigs (Cruz and Shin, 2007; Ren and Li, 2011; Sun *et al*., 2012). The rapid spread of PED across Southeast Asia in recent years indicates the possibility of transmission of PEDV by other routes. The main objective of this study was to discover possible new PEDV transmission routes by studying semen and air samples collected from infected swine herds and the PEDV shedding time in feces from recovery piglets.

In this study, shedding of infectious PEDV was observed frequently in feces from naturally infected pigs and from those recovery piglets without diarrhea. The positive PEDV results in feces from recovery piglets were as expected and agree with previous reports. However, it was not anticipated that the PEDV could still be detected in feces from recovery piglets after 56 days of post-infection at the concentration of $1\times10^{2.13}$ copies/mL. Diversity between farms may be related to differences in PEDV infection challenge and virulence, levels of PEDV-antibodies in sows and different test sensitivities. Although, the PEDV shedding in nasal, fecal excretions and oral fluids from infected pigs has been investigated previously (Kim and Chae, 2003) this is the first time that the PEDV shedding time (at least 56 days) in feces from recovery piglets is reported.

Semen was one of the most popular mediators for virus transmission such as PCV2, PRRSV in swine diseases. Semen transmission of PCV2 and PRRSV viruses has been well documented and considered economically important for the swine industry due to the wide usage of artificial insemination (Revilla-Fernandez *et al*., 2005; Schmoll *et al*., 2008). The results demonstrated for the first time that the semen samples from affected farms were positive for PEDV RNA. The dose of viruses in semen proved to play a major role in transmissibility (Madson *et al*., 2009). The amount of PEDV presented in semen and its corresponding transmissibility were not evaluated in this study but will be further investigated. Although, the study results did not confirm whether PEDV could be transmitted by semen, it was possible that the PEDV infection could occur during fertilization. The justification for this was that spermatozoa might be associated with PEDV RNA.

Airborne viral transmission was previously investigated on respiratory virus such as PCV2 and PRRSV. This transmission route was deemed with low risk for enterovirus PEDV and therefore was not investigated extensively in this study. In this study, only samples from delivery barns showed low level of PEDV RNA, indicating the possibility that pigs could be exposed to aerosol mixed with PEDV particles. The air samples were collected from the farms during the second PED outbreak. Due to repeated PED outbreaks, shedding of the virus from these sick and recovery animals could be increased and consequently, the airborne virus concentrations during the PED outbreaks could be much higher than what were detected in this study. As sufficient data were unavailable to shed a light on the viral quantity that required for an effective infection, it was impossible to make any predictions on the impact of these viruses on animal health. Dee *et al*. (2010) reported that PRRSV was transmissible by aerosol. Verreault *et al*. (2010) successfully detected and quantified airborne PCV2 at very high concentrations in the swine confinement buildings. However, PEDV aerosol in the swine barn was not considered important because PEDV was clarified as enterovirus and no data were available regarding the transmission of PEDV by aerosol. It was well-known that PEDV and PRRSV belonged to Coronaviridae (Hofmann and Wyler, 1988; Thiel *et al*., 1993). Given the fact that PEDV was resistant to environmental stress like PRRSV, it could possibly transmit at least as much ease as PRRSV. Additionally, correlations between animal health and airborne concentrations of PEDV would need to be established in order to better understand the significance of the data and to determine whether a threshold concentration could be observed before the onset of the disease. In the present study, the milk samples from sows were PEDV positive with high percentage which was consistent with previous study (Sun *et al*., 2012).

The results demonstrated that feces were positive for PEDV RNA at high concentration and the shedding time lasted for at least 56 days under field condition. Also, the presence of PEDV in semen in boars with no clinical symptoms suggested that PEDV shed intermittently in semen and it could well be a potential semen-sow-piglet as well as fecal-oral transmission route. Additionally, the fact that only air samples from farrowing barns were contaminated with PEDV RNA implied that aerosol might be a potential risk of PEDV infection. The results described in this study demonstrated PEDV viruses could be persistent for long periods of time in fecal excretions at

high concentration and the study also explored the interactions between the airborne contaminated with PEDV RNA and the pigs infected PEDV that may attribute to the persistent PEDV outbreaks.

CONCLUSION

The continuous shedding of PEDV from convalescent piglets (fecal-oral way), the contaminated semen from boars (boar-semen-sow) and the milk from sows (sow-milk-piglet) could be possible reasons for the PEDV repeated outbreaks in the same herd and the aerosol could be a potential supplementary factor.

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