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Sequence and phylogenetic analyses of the M and N genes of porcine epidemic diarrhea virus (PEDV) strains in Anhui Province, China

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ABSTRACT. To assess the homology and phylogenetic relationship between porcine epidemic diarrhea virus (PEDV) Anhui strains and other PEDV strains, molecular homology and phylogenetic analyses of Anhui PEDV field strains were compared with those of reference strains. The results revealed that the M and N genes of PEDV were 681 and 1326 bp long, respectively. The nucleotide sequences of the N genes of Anhui PEDV strains were 95.9-99.9% homologous with each other, and the deduced amino acid sequences were 92.5-99.8% homologous. Compared with the PEDV reference strains, the Anhui PEDV field strains had 94.1-99.5% nucleotide sequence homology in the N gene and 91.2-97.5% amino acid mutation homology in the N protein. The nucleotide sequences of the M genes of Anhui PEDV were 98.3-100% homologous,

and the deduced amino acid sequences were 96.5-99.6% homologous. Compared with the PEDV reference strains, the Anhui PEDV field strains had 96.9-100% nucleotide sequence homology in the M gene and 96.5-99.6% amino acid homology in the M protein. The Anhui strains were genetically similar to USA strains (USA/Iowa/16465/2013 and USA/Indiana/17846/2013) but different from European (CV777;Br1/7), Korean (Chinju99), and Japanese (83p-5) strains.

Key words: Porcine epidemic diarrhea virus; M and N genes; Genetic analysis

INTRODUCTION

The porcine epidemic diarrhea virus (PEDV), which belongs to the Coronaviridae family, is an enveloped, single- and positive-stranded RNA virus. PEDV, which is genetically similar to the transmissible gastroenteritis virus, feline coronavirus, and human coronavirus 229E (Kim and Lee, 2014), is approximately 2.8 kb in length and has both 5' and 3' untranslated regions. PEDV consists of four structural proteins: a spike protein, an envelope protein, a membrane protein (M), and a nucleocapsid protein (N) (Brian and Baric, 2005; Zhao et al., 2014). The N gene, located in the 3' end of the genome, has the highest expression levels during the early phase of infection (Li et al., 2013). It has been reported that the N protein binds to viral RNA (Saif, 1993; Wurm et al., 2001), providing a structural basis for the helical nucleocapsid, which is a basic phosphoprotein associated with viral replication and transcription (Hiscox et al., 2001; Almazán et al., 2004; Fan et al., 2005; Li et al., 2005; Tan et al., 2006). The eukaryotic expression of the N gene in intestinal epithelial cells prolongs the S-phase cell cycle, induces endoplasmic reticulum stress, and upregulates interleukin-8 expression (Xu et al., 2013). The M gene codes for a transmembrane 216-amino acid protein (Egberink et al., 1988), which participates in virion assembly and budding, and stimulates immune protection (de Haan et al., 1998). It has been suggested that the M protein plays a role in interferon induction and mediated virus expression, which makes the protein a good candidate for genetically engineered vaccines (Jinghui and Yijing, 2005; Siu et al., 2009).

Porcine epidemic diarrhea (PED), which was first reported in the European Union in 1978 (Pensaert and de Bouck, 1978), is a devastating enteric disease characterized by acute diarrhea and severe dehydration (Nagy et al., 1996). Since 1978, PED outbreaks have been reported in several swine-producing Asian countries, including China, Japan, and Korea (Zhao et al., 2013; Choi et al., 2014; Hao et al., 2014). The outbreaks are characterized by high piglet mortality rates and economic losses (Park et al., 2013; Temeeyasen et al., 2014). Phylogenetic analyses revealed that Vietnamese PEDV strains are closely related to Chinese strains (Puranaveja et al., 2009). PEDV was first reported in the United States in March 2013; the US strain and the Anhui (Chinese) strain have 99.6% genetic homology (Huang et al., 2013). Anhui Province is a coastal area, which contributes to a PED epidemic.

In this study, DNA clones of M and N genes of field samples from Anhui Province were generated. Sequenced M and N genes were submitted to GenBank and compared with those of other PEDV strains. The data obtained from this study, which will be useful for the production of genetically engineered vaccines and diagnostic reagents, provide the basis for

further studies focused on PEDV strains.

MATERIAL AND METHODS

Sample collection

In this study, 138 porcine samples (fecal and intestinal contents) were collected from piglets with diarrhea and dehydration from 23 different farms in Anhui Province. Six samples were collected from each farm; a farm in Bengbu was sampled twice. Samples were diluted to 10% with phosphate-buffered saline (pH 7.2) and centrifuged at 8000 *g* for 5 min.

Reagents

The reagents used in this study comprised a total RNA extraction kit (Omega, Los Angeles, CA, USA), an agarose gel recovery kit (Omega), LA Taq (TaKaRa, Tokyo, Japan), M-Mlv reverse transcriptase (Omega), deoxynucleotide triphosphate (dNTP) (TaKaRa), RNase inhibitor (Omega), and a pMD18-T vector (TaKaRa).

Primers

Three sets of primers were synthesized (Sangon Biotech, Shanghai, China) based on the PEDV CV777 genome (GenBank No. AF353511) for the amplification of the M and N genes (Table 1).

Table 1. Primers designed for the amplification of the M and N genes in PEDV.

Primer	Sequence (5'-3')	Position	Length of product (bp)
N-f	GTCAAAACACGGCGACTATT	26,281-26,300	1,465
N-r	TGGCACTACCCTGGAACATA	27,728-27,745	
N1-f	TGCGGTTCTCACAGATAGTG	26,320-26,339	1,401
N1-r	GATAAGCCGGTCTAACATTGT	27,700-27,720	
M-f	CCCCAGTACTGTTATTGACGTATAAAC	25,650-25,676	715
M-r	GTTTAGACTAAATGAAGCACTTTC	26,341-26,364	

PEDV detection

Viral RNA was extracted from the samples using the total RNA extraction Kit (Omega) and stored at -70°C. The reverse transcription reaction mixture (20 µL) comprised 3 µL 2.5 mM dNTP, 1 µL 10 µM downstream primer, 5 µL 5X first strand buffer, 6 µL RNA, and 5 µL RNase-free double-distilled water. Reverse transcription was performed at 42°C for 50 min followed by 5 min at 95°C.

The polymerase chain reaction (PCR) mixture comprised 0.5 µL LA Taq, 1 µL 10 µM of each upstream and downstream primers, 2 µL cDNA template, 4 µL dNTP, 5 µL 10X LA Taq Buffer, and 37 µL sterile double-distilled water. M gene amplification was performed at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and

a final extension step at 72°C for 10 min. N gene amplification was performed at 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min, and a final extension at 72°C for 10 min.

Cloning of the target gene

The PCR products were subjected to gel electrophoresis on 2% agarose gel, excised from the agarose gel, and purified using the agarose gel recovery kit. The PCR products were cloned into the pMD18-T vector and sequenced in both directions.

RESULTS

Identification of PEDV by reverse transcription-PCR

In this study, 19 farms were PEDV-positive based on the reverse transcription-PCR results. Both the M and N genes from each sample were sequenced. A farm in Bengbu was sampled twice; therefore, there were a total of 20 positive samples from 19 different farms. The confirmed positive samples were labeled according to year and location (Table 2).

Table 2. Twenty PEDV strains obtained from Anhui province during 2012-2013.

Name	Date	Origin	Accession Nos.
M/NAHHN	2012/02/02	Huainan	KJ001727/KF994795
M/NAHSZ	2012/02/03	Suzhou	KJ001731/KF994799
M/NAHSX	2012/02/05	Huaibei	KJ001730/KF994798
M/NAHFR	2012/02/22	Fuyang	KJ001722/KF994791
M/NAHFD	2012/03/17	Hefei	KJ001721/KF994790
M/NAHLA	2012/03/20	Luan	KJ001728/KF994796
M/NAHFY	2012/03/22	Fuyang	KJ001725/KF994793
M/NAHLX	2012/03/24	Bozhou	KJ001729/KF994797
M/NAHBB	2012/03/25	Bengbu	KJ001723/KF994789
M/NAHGD	2012/03/26	Xuancheng	KJ001726/KF994794
M/NAHFX	2012/05/16	Feixi	KJ001724/KF994792
M/NAHYS	2012/06/01	Fuyuan	KJ001732/KF994800
2013M/NAHLA	2013/01/02	Luan	KJ001738/KF994806
2013M/NAHCH	2013/01/21	Chaohu	KJ001734/KF994802
2013M/NAHLX	2013/01/27	Bozhou	KJ001739/KF994807
2013M/NAHHB	2013/02/27	Huaibei	KJ001737/KF994805
2013M/NAHDY	2013/04/10	Chuzhou	KJ001735KF994803
2013M/NAHMAS	2013/04/14	Maanshan	KJ001740/KF994808
2013M/NAHFD	2013/04/17	Feidong	KJ001736/KF994804
2013M/NAHBB	2013/04/23	Bengbu	KJ001733/KF994801

Amplification of the M and N genes

The M and N genes from the positive samples were amplified and sequenced. The results revealed 715- and 1400-bp fragments (Figures 1 and 2). All the sequences were submitted to GenBank (Table 3).

Sequence analysis of the M and N genes

The M gene of all 20 Anhui PEDV strains was 681 bp long and encoded a protein

of 226 amino acids. Compared with the reference strain CV777, the following nucleotide changes were detected, C→T at positions 125, 198, 213, 222, and 234; G→C at position 37; G→A at position 180; T→A at position 348; and T→C at position 618. The corresponding amino acid changes were E→Q at position 13 (except for MAHFD, MAHFX, and MAHYS), V→A at position 42 (except for MAHFX and MAHYS), and A→S at position 214. The M gene homology between PEDV and reference strains is shown in Table 4.

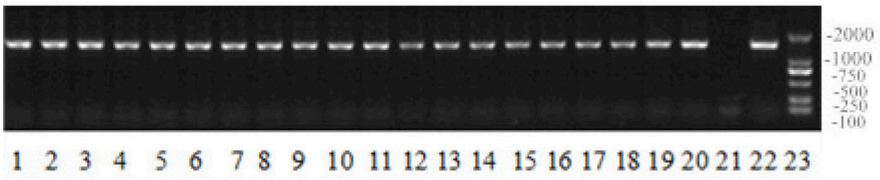

Figure 1. Electrophoresis pattern of RT-PCR products of the N gene.

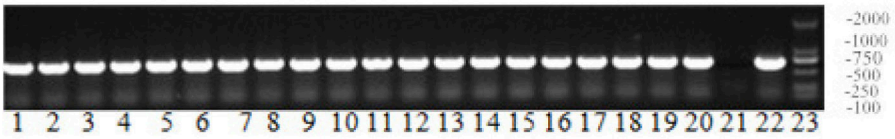

Figure 2. Electrophoresis pattern of RT-PCR products of the M gene.

Table 3. PEDV references used for sequence analysis.

Name	Origin	Accession Nos.
FJPT2011	China	JQ678036
GDYD	China	JN089731
BJ2010	China	JD690778
SD	China	JX560761
PFF1051	Korea	FJ687472
CPF531	Korea	FJ687471
MNAH10054108	Tailand	EU542416
CHGXNN2011	China	JN601062
CHSDRZ22011	China	JN601061
CHGDQY12011	China	JN601056
CHFJND2011	China	JX406145
YT12-4	China	JX406145
WS12-2	China	JX406143
DZ	China	EU031893
USAIowa164652013	USA	KF452322
USAIndiana178462013	USA	KF452323
DR13	Korea	JQ023161
CV777	Britain	AF353511
83p-5	Japan	AB618619/AB618615
Chinju99	Korea	AF237764/DQ845249
JS200402	China	AY653206/AY653205
Br1/7	Belgium	Z14976/Z24733

The N gene of all 20 Anhui PEDV strains was 1326 bp and encoded a protein of 441 amino acids. Compared to the reference strain CV777, the following nucleotide changes were detected, G→A at positions 84, 129, 153, 424, 441, 621, 722, 801, 813, and 897; C→T at positions 126, 459, and 1223; G→C at position 251; T→C at positions 327, 390, 471, 469, and

1142; T→G at position 615; A→C at position 709; A →G at positions 755 and 64; T→A at position 1092; and C→A at position 1192. The corresponding amino acid changes were R→N at position 123; A→T at position 142; R→K at position 241; K→R at position 252; N→S at position 255; L→F at position 381; L→Q at position 395; and H→N at position 398. Anhui and USA PEDV strains shared several identical amino acids (Table 5). The homology of the N genes between Anhui and reference strains is shown in Table 4.

Table 4. Comparison of the nucleotide and deduced amino acid sequence of Anhui strains and reference strains (%).

Strain	N gene		M gene	
	Nucleotide sequence	Amino acid	Nucleotide sequence	Amino acid
Anhui	95.9-99.9	92.5-99.8	98.3-100	96.5-99.6
China	94.4-99.8	92.5-99.3	97.7-99.8	96.9-99.1
USA	97.3-99.7	95.0-99.8	99.1-100	97.4-99.6
Britain	92.3-97.5	94.1-97.1	96.9-98.2	97.4-98.4
Belgium	94.1-97.1	94.1-97.5	97.2-98.2	96.5-97.8
Korea	92.8-97.4	91.2-97.5	97.2-98.2	96.5-98.7
Japan	94.7-97.0	92.8-97.5	97.4-98.4	97.4-98.2

The N gene PCR products of Anhui strains were 1: NAHHN; 2: NAHSZ; 3: NAHSX; 4: NAHFR; 5: NAHFD; 6: NAHLA; 7: NAHFY; 8: NAHLX; 9: NAHBB; 10: NAHGD; 11: NAHFX; 12: NAHYS; 13: 2013NAHLA; 14: 2013NAHCH; 15: 2013NAHLX; 16: 2013NAHHB; 17: 2013NAHDY; 18: 2013NAHMAS; 19: 2013NAHFD; and 20: 2013NAHBB. The M gene PCR products of Anhui strains were 1: MAHHN; 2: MAHSZ; 3: MAHSX; 4: MAHFR; 5: MAHFD; 6: MAHLA; 7: MAHFY; 8: MAHLX; 9: MAHBB; 10: MAHGD; 11: MAHFX; 12: MAHYS; 13: 2013MAHLA; 14: 2013MAHCH; 15: 2013MAHLX; 16: 2013MAHHB; 17: 2013MAHDY; 18: 2013MAHMAS; 19: 2013MAHFD; and 20: 2013MAHBB.

Table 5. Amino acid point mutations of Anhui strains compared to USA strains.

Virus strain	Positions of amino acid point mutations													
	84	123	142	176	205	241	242	252	255	381	395	397	398	408
NAHBB	G-A	K-N	A-T	Q-H	N-K	R-K	H-L	K-R	N-S	L-P	L-Q	Q-L	H-N	A-L
NAHFD	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
NAHFR	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
NAHFX	•	•	•	•	•	•	•	•	•	•	•	•	•	•
NAHFY	•	•	•	•	•	•	•	•	•	•	•	•	•	•
NAHGD	•	•	•	Q	•	•	•	•	•	•	•	•	H-K	A-V
NAHHN	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
NAHLA	•	•	•	•	•	•	•	•	•	•	•	•	•	A-V
NAHLX	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
NAHSX	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
NAHSZ	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
NAHYS	•	•	•	•	•	•	•	•	•	•	•	•	•	•
2014NAHBB	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
2014NAHCH	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
2014NAHDY	G	•	•	Q	N	•	H	•	•	•	•	Q	•	A-V
2014NAHFD	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
2014NAHHB	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
2014NAHLA	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
2014NAHLX	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
2014NAHMAS	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
USAIndiana1784	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
USAIowa1646520	•	•	•	Q	•	•	•	•	•	•	•	•	•	A-V
CV777	G	K	A	Q	N	R	H	K-R	N	L	L	Q	H	A

Phylogenetic analysis of the M gene

Phylogenetic analyses based on the complete M gene fragments of the Anhui PEDV field strains and reference strains (Table 3) confirmed that all PEDV strains fell into three groups (Figure 3). Group I comprised all Anhui strains, one strain from Thailand [MNAH10054108 (2008)], two USA strains [USAIow164652013 (2013) and USAIndiana178462013 (2013)], and two Chinese strains [BJ2010 (2010) and JS200402 (2004)]. Group II comprised three Korean strains [PFF1051 (2008), CPF531 (2008), and DR13 (2009)]. Group III comprised a Korean strain [Chinju99 (2000)], a Belgian strain [Br1/7 (1987)], a British strain [CV777 (1977)], a Japanese strain [83p-5 (2011)], and two Chinese strains [FJPT2011 (2011) and SD (2012)].

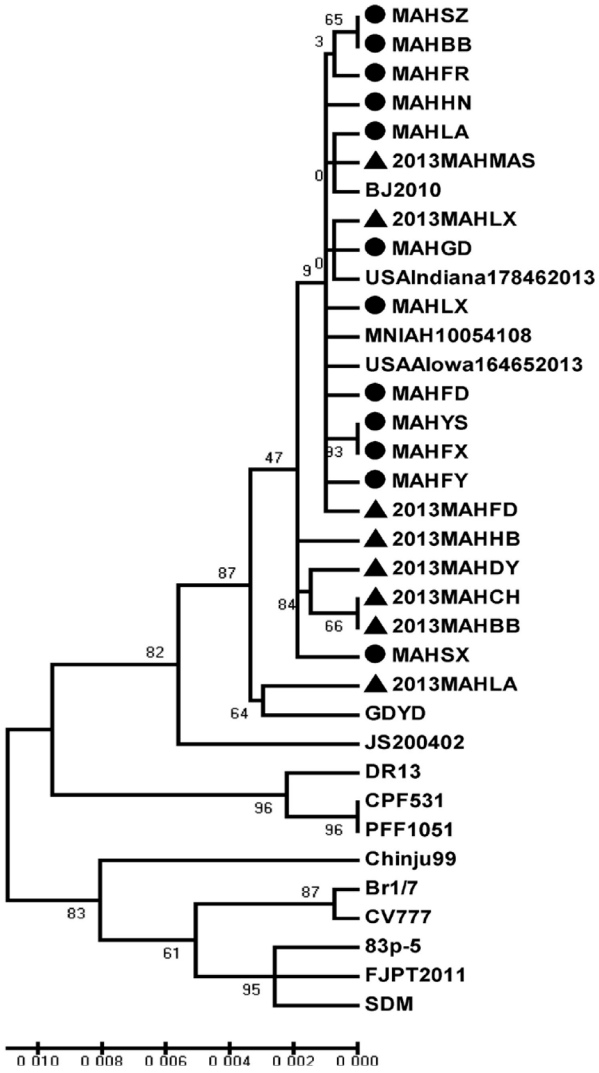

Figure 3. Phylogenetic analyses based on the nucleotide sequences corresponding to the PEDV M gene.

Phylogenetic analysis of the N gene

Phylogenetic analysis of the N gene fragments revealed that all the PEDV Anhui strains belonged to one of two groups (Figure 4). Group I comprised two American strains [USAIow164652013 (2013) and USAIndiana178462013 (2013)], five Chinese strains [YT12-4 (2012), WS12-2 (2012), CHGXNN2011 (2011), DZ (2007), and JS200402 (2004)], and 20 Anhui strains. Group II comprised three Chinese strains [CHBJYQ12011 (2011), CHGDQY12011 (2011), and CHSDRZ22011 (2011)], two Korean strains [DR13 (2009), Chinju99 (2000)], a Japanese strain [83p-5 (2011)], a Belgian strain [Br1/7 (1987)], and a British strain [CV777 (1977)].

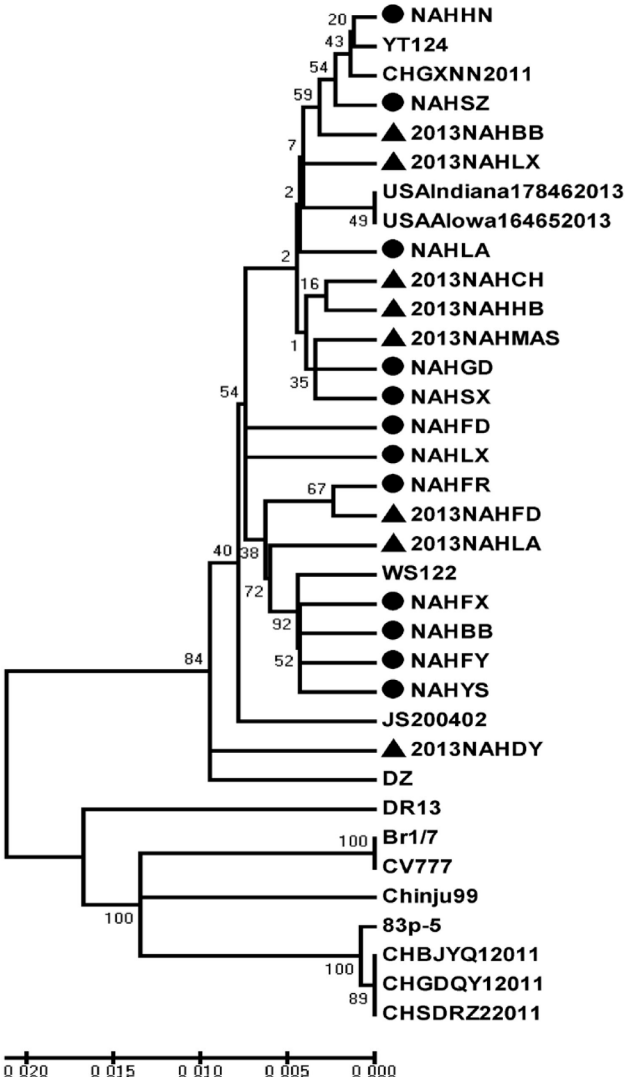

Figure 4. Phylogenetic analyses based on the nucleotide sequences corresponding to the PEDV N genes.

DISCUSSION

In October 2010, PED re-emerged in China (Sun et al., 2012). Variations in viral strains have contributed to the epidemic (Wang et al., 2013). Several Chinese strains have been reported since the variant strain CH/FJND/-03/2011 was detected (Chen et al., 2011, 2012). In PEDV, M genes are highly conserved. On the other hand, N gene homology is low among different PEDV strains. The phylogenetic analyses of the M and N genes confirmed that Anhui strains belong to the same group; however, strains originating in different years have no significant clusters in common. Sequence alignment of strains from the same location revealed that the homology of the M and N genes during the two years was high. Based on the phylogenetic analyses of M and N genes, Anhui strains are linked to Jiangsu strains but not to Shandong strains. The amino acid mutations of the N gene at positions 142, 252, and 255, and of the M gene at positions 13, 62, and 214 were observed in other Chinese strains (Yang et al., 2013).

Phylogenetic analysis, which is based on multiple sequence alignments between the Anhui and reference strains, was performed using the MEG5.0 software. European (CV777, Br1/7) and Anhui strains were distantly related. Most of the farms from which the samples were obtained had immunized animals, which might have attenuated the viral action of PEVD. Nevertheless, the mortality rate of piglets in those farms was high, probably as a result of variation and evolution of the virus. The sequence alignment of the M and N genes revealed that the USA and Anhui strains had the highest homology. Moreover, six Anhui strains (AHFY, AHLA, AHLX, 2013AHFD, 2013AHMAS, and 2013AHLX) had 100% nucleotide sequence identity in the M gene with USA strains (data not shown). The N genes of the Anhui strains had 97.3-99.5% nucleotide identity with USA strains and shared several amino acid mutations. There is currently no swine trade between Anhui and the USA.

PEDV-positive air samples have been obtained in mechanically and naturally ventilated USA farms (Goede et al., 2013). Furthermore, analysis of samples obtained from 669 animal transport trailers before and after unloading revealed that 17.3% of trailers were PEDV-positive on arrival. Additionally, 11.4% of the trailers that were PEDV-negative on arrival were positive after animal loading (Morrison and Goede, 2013). These results suggest that PEDV has both fecal-oral and air transmission routes.

Conflicts of interest

The authors declare no conflict of interest.

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