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# Development of an enzyme-linked immunosorbent assay for the monitoring and surveillance of antibodies to porcine epidemic diarrhea virus based on a recombinant membrane protein

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## ABSTRACT

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The recent dramatic increase in reported cases of porcine epidemic diarrhea (PED) in pig farms is a potential threat to the global swine industry. Therefore, the accurate diagnosis, serological monitoring, and surveillance of specific antibodies in pigs resulting from porcine epidemic diarrhea virus (PEDV) infection or vaccination would be essential in helping to control the spread of PED. We developed and validated an indirect enzyme-linked immunosorbent assay (ELISA) based on the recombinant membrane (M) protein of PEDV. To detect PEDV antibodies in eight herds, 382 serum samples were collected from sows that had been immunized with a PED vaccine, and screened using the developed ELISA in parallel with a serum neutralization (SN) assay. Of the tested samples, 276 were positive for the presence of PEDV antibodies according to both assays, while 98 were negative. An excellent agreement between the ELISA and the SN assay was observed ( $\kappa = 0.947$ ; 95% confidence interval = 0.910–0.984; McNemar's test,  $P = 0.727$ ). No cross-reaction was detected for the developed ELISA with other coronaviruses or other common pig pathogens. The developed ELISA could be used for serological evaluation and indirect diagnosis of PED infection.

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## 1. Introduction

Porcine epidemic diarrhea (PED) is a devastating swine disease and its etiological agent is PED virus (PEDV). Porcine epidemic diarrhea is characterized by watery diarrhea, dehydration, and a high death rate among suckling pigs (Pensaert and DeBouck, 1978). The disease was first documented in the United Kingdom in 1971 as a swine disease that resembled transmissible gastroenteritis (Oldham, 1972). Outbreaks of PED have since been reported in Europe, Asia, and North America (Nagy et al., 1996; Takahashi et al., 1983; Sun et al., 2011; Fan et al., 2012; Stevenson et al., 2013). Porcine epidemic diarrhea virus has spread rapidly across the United States, resulting in the high mortality of piglets, and substantial economic losses (Cima, 2013). Its recent emergence in North America suggests that this virus is a threat to the swine industry worldwide. Accurate diagnosis and serological detection of specific antibodies in pigs as a result of PEDV infection or vaccination are required to control the spread of PED.

Porcine epidemic diarrhea virus is a coronavirus within the *Coronaviridae* family. The genome of PEDV contains genes encoding spike (S), membrane (M), small membrane (sM), open reading frame 3, and nucleocapsid (N) proteins (Park et al., 2011). The M protein is a structural membrane glycoprotein, and the most abundant of all the envelope proteins. It has a short amino-terminal domain that exists outside of the virion, with the long carboxy-terminal domain of the protein present inside the virion (Utiger et al., 1995). Immune reactions to the M protein of coronaviruses play an important role in the induction of protection, and in mediating the course of the disease (Fleming et al., 1989; Vennema et al., 1991). The nucleotide sequence of the PEDV M gene exhibits low homology (around 50%) with the M gene of other coronaviruses; however, its sequence is highly conserved among different PEDV strains (Kim and Chae, 2000; Arndt et al., 2010). Therefore, the M protein could be a suitable candidate for the detection of PEDV-specific antibodies and in diagnosing PEDV infections.

Many enzyme-linked immunosorbent assays (ELISAs) have been developed for the detection of antibodies against PEDV. However, the preparation of an appropriate antigen for most of these methods requires cultivation of PEDV, which is time consuming and expensive. In the current study, we expressed and purified the M protein of PEDV. The purified form of the recombinant M protein

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was used as a coating antigen in an indirect ELISA that we developed. We then screened serum samples from pigs to determine the presence of antibodies specific for the PEDV M protein.

## 2. Materials and methods

### 2.1. Serum samples

We collected 36 blood samples from healthy unvaccinated PEDV-free pigs of various ages. The sera derived from these samples were negative for the presence of antibodies against PEDV according to serum neutralization (SN) assays (SN titers <1:2). The sera from 20 blood samples, taken from PEDV-infected pigs, were used as reference positive sera (SN titers  $\geq$ 1:16). The PEDV infection status of pigs was confirmed by reverse transcription polymerase chain reaction (RT-PCR) assays targeting the PEDV M gene, with viral RNA extracted from fecal samples. Porcine sera containing antibodies against transmissible gastroenteritis virus (TGEV; SN titer 1:413), porcine rotavirus (PRV; 1:305), porcine reproductive and respiratory syndrome virus (PRRSV; 1:376), porcine circovirus 2 (PCV-2; 1:317), and classical swine fever virus (CSFV; 1:289) were obtained from the Hebei Center for Disease Prevention and Control (Shijiazhuang, People's Republic of China). A total of 382 serum samples were collected from eight pig herds, in which the animals had been administered a PEDV vaccine. All samples were tested by the developed indirect ELISA.

### 2.2. Virus

Porcine epidemic diarrhea virus strain HB/BD (GenBank Accession No. JF690777.1) was isolated from the feces of a pig from Hebei Province, China, and adapted to cell culture for 32 passages in Vero cells.

### 2.3. Cloning and sequencing the PEDV M gene

Viral RNA was extracted from PEDV using Trizol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The sequence corresponding to the M gene was amplified from viral RNA by RT-PCR using oligonucleotide primers MP1 (5'-GGA TCC ATG TCT AAC GGT-3') and MP2 (5'-AAG CTT TCT GTT TAG ACT AAA T-3'). The resulting PCR products were separated by agarose gel electrophoresis and purified using a Gel Extraction Mini Kit (Tiangen Biotech Co. Ltd., Beijing, China) according to the manufacturer's recommendations. Purified amplicons were cloned into the pMD18-T vector (Takara Biotech Co. Ltd., Dalian, China) to yield the recombinant plasmid pMD-M, which was then transformed into competent *Escherichia coli* JM109. Positive clones were selected according to their lacZ phenotype, and verified by restriction enzyme digestion, PCR screening, and DNA sequencing.

### 2.4. Expression and purification of the PEDV M protein

The M gene contained within pMD-M was subcloned into the prokaryotic expression vector pGEX-6P-1 (Sunbiotech Inc., Beijing, China) to yield pGEX-6P-M. The pGEX-6P-1 vector also contained a sequence for a GST tag, which was designed so as to be added at the amino terminal of the expressed protein. The pGEX-6P-M vector was verified by DNA sequencing. The recombinant M protein was transformed into *E. coli* BL21 (Tiangen Biotech Co. Ltd., Beijing, China). *E. coli* BL21 cells were cultured and transformed with pGEX-6P-M, in Luria-Bertani broth supplemented with 100  $\mu$ g/mL ampicillin until the optical density at 600 nm ( $OD_{600}$ ) was 0.6–1.0, at which point protein expression was induced via the addition of 1.0 mM isopropyl- $\beta$ -D-thio-galactopyranoside.

At 5 h post-induction, bacterial cells were collected by centrifugation. The recombinant protein was purified from the bacterial lysate using a GST Fusion Protein Purification Kit (TransGen Biotech Co. Ltd., Beijing, China), according to the manufacturer's recommendations. Fractions of purified recombinant M protein were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), with polyacrylamide gels stained with Coomassie Brilliant Blue R-250. Expression of the recombinant M protein was confirmed by immunoblotting, using porcine PEDV antisera. The prokaryotic expression vector pGEX-6P-1 was also transformed into *E. coli* BL21 as a negative control.

### 2.5. Western blotting

Fractions of purified recombinant proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Gibco BRL, Gaithersburg, MD, USA). To prevent non-specific reactions, membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) at 37 °C for 2 h. Membranes were incubated with pig PEDV antisera (diluted 1:200) at 37 °C for 1 h. After three washes with TBST, membranes were incubated with an anti-pig IgG conjugated to horseradish peroxidase (HRP; Sigma–Aldrich, St Louis, MO, USA) at 37 °C for 1 h. We used 3,3'-diaminobenzidine (Sigma–Aldrich) for the development of color reactions to visualize protein bands.

### 2.6. Indirect ELISA

A checkerboard titration involving each combination of antigen (purified recombinant PEDV M protein) and sera was used to determine optimal dilutions for use in the indirect ELISA we developed. The antigen and serum were diluted from 30 to 0.938  $\mu$ g/ml and 1:10 to 1:320 (two-fold dilution), respectively. Ninety six well plates (Nunc MaxiSorp, Denmark) were coated with the recombinant M protein, which was diluted in phosphate-buffered saline (PBS), and allowed the plates to incubate at 4 °C overnight. PBS was used as a blank control. Wells were blocked with 5% fetal bovine serum in PBS (pH 7.4) for 1 h at 37 °C. Wells were then washed three times with PBS containing 0.05% Tween 20 (PBST) and diluted serum samples were added, in duplicate, to the antigen-coated wells. Following incubation at 37 °C for 1 h, plates were washed three times with PBST and 100  $\mu$ L of diluted goat anti-pig IgG conjugated to HRP (KPL Inc., Gaithersburg, MD, USA) was added to each well. After incubation at 37 °C for 45 min, wells were washed with PBST, and 100  $\mu$ L of 3,3',5,5'-tetramethyl-benzidine (TMB) was added to each well and the plates were incubated for 15 min at room temperature. Color development reactions were stopped with 100  $\mu$ L of 2 M H<sub>2</sub>SO<sub>4</sub>. The  $OD_{450}$  was determined for each well using a microplate reader. Dilutions that resulted in the highest  $OD_{450}$  ratio between the positive and negative serum samples (P/N value), along with an  $OD_{450}$  value for positive serum samples close to 1.0, were considered optimal working conditions.

### 2.7. Negative–positive threshold value for the developed ELISA

To establish negative and positive cutoff values for this assay, 36 PEDV-negative serum samples, as determined by SN assays, were tested in triplicate using the ELISA we developed. A cutoff value was defined as the mean absorbance value plus three standard deviations (SD).

### 2.8. Intra- and inter-assay repeatability of the developed ELISA

Repeatability assays were conducted by comparing the ratios of  $OD_{450}$  values for triplicate results from each serum sample tested on the same plate (intra-assay repeatability) or on different plates

(inter-assay repeatability). The coefficient of variation (CV) was calculated according to the following formula:

$$CV = \frac{SD}{\bar{X}} \times 100$$

where  $\bar{X}$  was the average OD<sub>450</sub> for the field serum samples. A CV value less than 10% was considered an acceptable level of variation.

### 2.9. Specificity and sensitivity of the developed ELISA

To determine the specificity of the developed ELISA, cross-reactivity was assessed by determining the reactivity of the purified antigen with serum samples containing antibodies against TGEV, PRV, PRRSV, PCV2, CSFV and PEDV. Reference serum samples were diluted 1:40, with three replicates of each dilution tested using the indirect ELISA. Serum samples from PEDV-infected pigs ( $n = 10$ ) were selected at random to determine the sensitivity of the indirect ELISA. Samples were serially diluted two-fold from 1:2 to 1:4096. Eight replicates of each diluted serum sample were tested to determine the titers of antibodies in samples. The titers were also determined by SN.

### 2.10. Comparison of ELISA and SN assay results

The indirect ELISA that we developed was used to evaluate the presence of PEDV antibodies in 382 serum samples from sows with varying immune status. These serum samples were also subjected to SN assays. The level of agreement between the SN and ELISA assay was determined using a Cohen's kappa, and a McNemar's test was performed to investigate the differences between both assays. A  $P$ -value  $< 0.05$  was considered significant. All analyses were performed in SPSS version 22.0.

## 3. Results

### 3.1. Cloning and sequencing the PEDV M gene

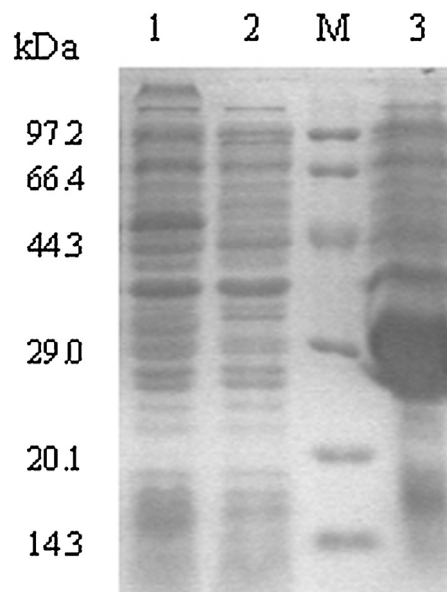
The M gene (approximately 680 bp) of PEDV was successfully amplified and cloned it into a TA vector. Positive clones were identified by sequencing. Sequence analysis indicated that the PEDV M gene was 681 bp, and encoded 227 amino acids. The complete nucleotide sequence of the M gene has been deposited in GenBank (Accession number JF690777).

### 3.2. Expression and purification of the PEDV M protein

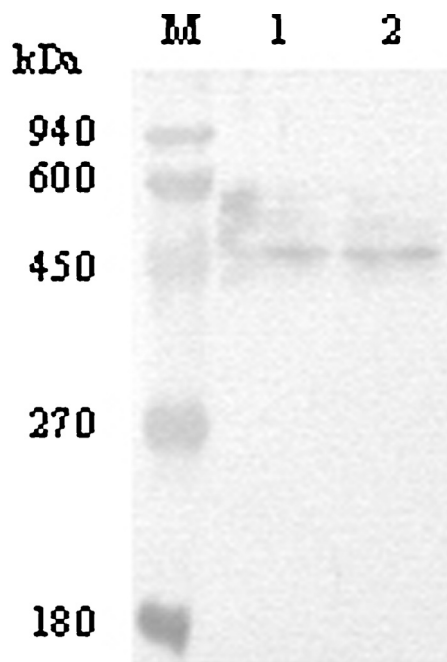
Recombinant PEDV M gene was expressed in *E. coli* BL21. Analysis of the bacterial cell lysate by SDS-PAGE and western blotting revealed a prominent band of around 53 kDa. The recombinant protein we identified was approximately 26 kDa heavier than the predicted molecular weight of 27 kDa, which was due to the GST tag (Fig. 1). The recombinant protein was predominantly in the insoluble fraction of the bacterial cell extract, and purified using a GST Fusion Protein Purification Kit. Western blotting analysis showed that the purified protein was recognized by PEDV antiserum (Fig. 2).

### 3.3. Development of the indirect ELISA

The optimum antigen concentration and serum sample dilution were determined to be 3.75  $\mu\text{g}/\text{mL}$  and 1:40, respectively. The optimum dilution of the anti-pig IgG conjugated to HRP was found to be 1:4000. The incubation conditions for primary and secondary antibodies were 1 h at 37 °C and 45 min at 37 °C, respectively. The optimum blocking buffer was found to be 5% fetal bovine serum in PBS, while PBST was the most appropriate washing buffer.



**Fig. 1.** SDS-PAGE analysis of the expressed protein with Coomassie brilliant blue staining. Lane 1, cell lysate of *E. coli* BL21/pGEX-6P-M induced for 5 h; Lane 2, cell lysate of *E. coli* BL21/pGEX-6P-M before IPTG induced; Lane M, molecular weight marker; Lane 3, cell lysate of *E. coli* BL21/pGEX-6P-1 induced for 5 h.



**Fig. 2.** Identification of the purified recombinant protein by Western blot using anti-PEDV antibody. Lane M: Prestained protein molecular weight marker; Lane 1–2: Purified recombinant protein. A clear band with the expected molecular weight appeared on the nitrocellulose membrane after incubation.

### 3.4. Confirmation of negative–positive threshold

The detection threshold of our indirect ELISA was determined using 36 serum samples that were negative for the presence of PEDV antibodies. The cutoff point was specific for each individual assay and was based on the OD<sub>450</sub> of negative samples included in each assay. The mean OD<sub>450</sub> value for the ELISA was 0.192, with an SD of 0.054. The cutoff value was determined to be 0.354 (mean + 3SD); therefore the negative–positive threshold was set at 0.35. Serum samples returning an ELISA OD<sub>450</sub> value below

**Table 1**

Cross-reaction analysis of the M-based i-ELISA with anti-sera against other pig viruses: OD value (mean  $\pm$  3SD).

Serum	OD
PEDV	1.253 $\pm$ 0.059
TGEV	0.036 $\pm$ 0.016
PRV	0.039 $\pm$ 0.025
PCV-2	0.042 $\pm$ 0.019
CSFV	0.051 $\pm$ 0.028
PRRSV	0.047 $\pm$ 0.031
Non-infected	0.034 $\pm$ 0.012

PEDV, TGEV, PRV, PCV-2, CSFV, PRRSV represent pig sera that were positive for PEDV, TGEV, PRV, PCV-2, CSFV, PRRSV, respectively. 'Noninfected' represents the serum from a pig that was negative for PEDV.

0.354 were regarded as negative; samples that returned an OD<sub>450</sub> value higher than this were considered to be seropositive for PEDV antibodies.

### 3.5. ELISA repeatability

Reproducibility within and between ELISA plates was evaluated by testing ten SN-negative serum samples and ten SN-positive serum samples in triplicate. The inter-assay CV of the indirect ELISA ranged from 4.7% to 7.3% with a median value of 5.8%. The intra-assay CV ranged from 5.4% to 8.9%, with a median value of 6.3%. These results indicate that the indirect ELISA we developed to detect antibodies against the PEDV M protein was repeatable, with low and acceptable levels of variation.

### 3.6. Specificity and sensitivity of the ELISA

The specificity of our ELISA was evaluated by testing the reactivity of the PEDV M protein antigen with antibodies against PEDV, TGEV, PRV, PRRSV, PCV-2, and CSFV. We found that PEDV antisera significantly reacted with the PEDV M antigen, as expected. In contrast, the OD<sub>450</sub> values for all other serum sample containing antibodies against other porcine viruses were significantly lower than the established negative cutoff value (Table 1). Our results suggest that little or no cross-reactivity occurs between the PEDV M protein and antibodies against other porcine viruses, and that the M protein antigen was specific for antibodies against PEDV. We evaluated the sensitivity of our indirect ELISA using ten PEDV-positive serum samples, and found that we could detect PEDV antibodies in a serum sample that had been diluted out to 1:2048, while only 1:532.5 in SN. This result indicated that our indirect ELISA was more sensitive than the SN assay for PEDV antibody detection (Table 2).

### 3.7. Comparison of ELISA and SN assay results

Of the 382 serum samples collected from animals with a known immunization history across eight pig farms, 281 (73.6%) were positive for PEDV antibodies according to our ELISA, while 101 (26.4%) were PEDV antibody-negative. In comparison, according to the SN assay we used in parallel with the indirect ELISA, 73% (279/382) of samples were PEDV antibody-positive and 27.0% (103/382) of samples were antibody-negative (Table 3). According to both assays, 276 and 98 of the screened serum samples were PEDV antibody-positive and -negative, respectively. Three of the serum samples that were positive according to the SN assay, were

**Table 3**

Determination of the agreement of the indirect ELISA test for the detection of antibodies to PEDV with the serum neutralization test.

ELISA test	SN test		Total
	Positive	Negative	
Positive	276	5	281
Negative	3	98	101
Total	279	103	382

SN, serum neutralization. The serum samples were collected from 382 sows immunized with vaccine in eight herds. Kappa=0.947; 95% confidence interval=0.910–0.984; McNemar's test,  $P=0.727$ .

negative according to our indirect ELISA, while five serum samples that were negative by SN were considered positive in the ELISA. An excellent agreement between the ELISA and the SN assay was observed (kappa = 0.947; 95% CI = 0.910–0.984; McNemar's test,  $P=0.727$ ) (Table 3)

## 4. Discussion

Porcine epidemic diarrhea virus is a member of the *Coronaviridae*, and is known to cause fatal diarrhea in newborn piglets. The virus was first identified in 1978 (Pensaert and DeBouck, 1978; Chasey and Cartwright, 1978) and has since been found to be prevalent in many countries. Infection with PEDV has resulted in significant economic losses, mainly in Europe and Asia, and recently the USA (Fan et al., 2012; Chae et al., 2000; Martelli et al., 2008; Stevenson et al., 2013).

Although there are commercial vaccines available to prevent and control PED in China, the damage caused by PEDV is significant and the threat continuous. To effectively control and prevent this disease, detection methods that can assess the current epidemic situation in herds are required. In addition, for subsequent immunoprophylaxis, assays that can monitor serum antibody levels of immunized or infected pigs need to be developed. Before local immunity is actively established, the piglet intestine is protected against PEDV infection by maternal antibodies. Although the presence of antibodies in serum is not directly related to the protection for sows or for piglets, serological examination facilitates the assessment of humoral response to PEDV elicited either through vaccination or natural infection.

Porcine epidemic diarrhea virus was isolated for the first time in 1988 using the Vero cell line and trypsin-supplemented medium (Hofmann and Wyler, 1988). Various diagnostic methods have been described for the detection of PEDV antigen and PEDV antibodies (Carvajal et al., 1995; Knuchel et al., 1992; Kweon et al., 1997; Song et al., 2006). At present, serological diagnostic methods for the diagnosis of PEDV infection are more common. Given that ELISAs are simple, sensitive, and convenient serological detection methods, a number of commercial and in-house ELISAs have been developed to detect PEDV antigens, or antibodies against PEDV (Song and Park, 2012). The majority of in-house assays for the detection of antibodies against PEDV are based on using the whole virus as an antigen (Carvajal et al., 1995; Oh et al., 2005), or preparations of viral antigen (Knuchel et al., 1992). The cultivation of PEDV is laborious and time consuming, especially considering the bio-security measures that must be taken in handling this virus; therefore it is difficult to upscale the production of these serological assays. The use of a

**Table 2**

Determination of the sensitivity of the i-ELISA and SN test. Number 1–10 represents the sample code.

Method	1	2	3	4	5	6	7	8	9	10
SN titer	316.4	87.2	133.7	226.3	56.8	157.9	93.5	385.4	532.5	420.9
ELISA titer	1024	2048	512	1024	128	512	256	1024	2048	1024

recombinant viral protein as an antigen would allow researchers to avoid some of the problems associated with the large-scale preparation of native PEDV antigen. Recently, an ELISA based on a recombinant N protein was validated in China (Hou et al., 2007). However, these researchers did not assess the cross-reactivity of the ELISA with other coronaviruses.

The M protein of PEDV protrudes from the viral envelope, and is considered a superior diagnostic antigen compared with other PEDV proteins (Shenyang et al., 2007). In the current study, we chose the PEDV M protein for use as an ELISA antigen because it is highly conserved among PEDV strains, and because it can elicit the formation of protective antibodies (Zhang et al., 2012). We expressed the M protein in *E. coli* and confirmed its antigenicity using PEDV-specific antibodies. Our results indicated that the expressed recombinant M protein was indeed antigenic, and could feasibly be used as a coating antigen in an indirect ELISA.

The indirect ELISA that we developed exhibited low levels of variability among replicates, according to intra- and inter-assay tests. These minor variations in results indicated that the indirect ELISA was reproducible. Furthermore, this ELISA was able to detect PEDV antibodies in serum samples that had been diluted out to 1:2048, and exhibited no cross-reactivity to antibodies against other common pig pathogens. These findings indicated that the developed indirect ELISA could be used widely in the future.

Vaccination is one of the most effective techniques in controlling PED in China. However, since late 2010, PED has been reemerging in immunized swine herds with devastating impact. To confirm the effects of targeted immunoprophylactic measures, we used our indirect ELISA to screen serum samples from vaccinated pigs. We found that 73.6% and 73% of samples were seropositive according to ELISA and SN assay, respectively. This indicated that vaccination elicited neutralizing antibodies against PEDV in sows to some degree. However, 26.4% and 27% of samples from vaccinated pigs were seronegative by the ELISA and SN assays, respectively. Although these antibodies could not be used to assess protection against PEDV, these vaccinated, but seronegative sows are not able to vertically transmit effective antibodies to their neonates. It indicating that the vaccine used requires some improvement in antigenicity.

A comparison of the ELISA and SN assay results revealed eight differences between positive and negative samples. There were five ELISA-positive samples that were negative according to the SN assay we used, which could be explained by the higher sensitivity of the ELISA. The reason for the ELISA-negative results for the three SN-positive serum samples might be due to errors inherent in the tests that we applied.

In conclusion, this is the first report of an indirect ELISA using the recombinant PEDV M protein as a coating antigen for the detection of antibodies against PEDV in China. The developed assay is quick, convenient, and not labor-intensive, and could facilitate the development of a reliable tool or kit for the large-scale detection of antibodies against PEDV.

#### Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

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