The Thai Journal of Veterinary Medicine

Volume 50
Issue 4 *December, 2020*

Article 8

12-1-2020

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Original Article

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Abstract

Porcine epidemic diarrhea virus (PEDV) is highly pathogenic and infects suckling piglets, thereby imposing a major burden on the swine industry all over the world. The aim of this study is to develop two ELISA systems for the detection of antigens and antibodies against PEDV in pigs. The PEDV N recombinant protein was expressed and was used as the coating antigen to establish an indirect ELISA for detecting PEDV-specific antibodies in pig serum. Moreover, MAb was induced with the recombinant N protein and used for sandwich ELISA to specifically test for PEDV. The specificities of the two ELISAs were validated by comparing the results with other viruses and the reproducibility was evaluated by intra-assay or inter-assay variation. The diagnostic sensitivity and specificity also were evaluated by the receiver operating characteristic (ROC) curve. The two ELISAs both indicated acceptable reproducibility. Neither ELISA cross-reacted with other viruses. ROC curve analysis revealed 100.0% sensitivity and 95.2% specificity of the iELISA, 100.0% sensitivity and 90.0% specificity of the sELISA. Consequently, this work indicates that these two ELISAs can be used clinically to diagnose PEDV.

Keywords: indirect ELISA, N protein, porcine epidemic diarrhea virus, sandwich ELISA

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Received July 21, 2020.
Accepted September 22, 2020.

Introduction

Porcine epidemic diarrhea virus (PEDV) is a member of the genus Alphacoronavirus, causing malabsorption, diarrhea, dehydration, vomiting and wasting disease in suckling piglets, especially in winter, and has a devastating effect on the swine industry because of high mortality rates among suckling piglets (Pensaert and Bouck, 1978). At the beginning of October 2010, an outbreak of diarrhea in piglets re-emerged in China; piglets infected with PEDV exhibited watery diarrhea and dehydration within one week of age, leading to high mortality and large economic loss to the swine industry in China (Chen *et al*., 2010 ;Wang *et al*., 2016;Wang *et al*., 2013). Meanwhile, a large number of suckling piglets in this outbreak had characteristic symptoms and the mortality rate of affected pigs on the farms ranged from 70% to 100% (Li *et al*., 2012;Sun *et al*., 2012). Beyond China, the disease has rapidly spread to several countries around the world. For example, Japan, Canada, Mexico and Colombia have experienced continuous outbreaks (Lara-Romero *et al*., 2017;Lin *et al*., 2016). In particular, the outbreak of Porcine epidemic diarrhea (PED) in the United States in 2013 and in France and Belgium in 2014 threatened the swine industry in North America and Europe (Chen *et al*., 2013;Guo *et al*., 2019).

It is reported that the PED pandemics in recent years are related to the highly virulent PEDV strain, namely PEDV variants, which have circulated and caused considerable economic losses to the global swine industry (Li *et al*., 2012). Moreover, the PEDV and transmissible gastroenteritis virus (TGEV) infection usually cause clinical symptoms, pathological changes and epidemiology, but it is difficult to differentiate these two infections just by clinical symptoms (Chae *et al*., 2000). Therefore, it seems to be important to establish a rapid, safe, accurate and effective assay which can be used to detect circulating PEDV, control the spread of the disease and provide prompt treatment. In addition, the detection of specific antibodies against PEDV seems to be the key measurement for the evaluation of the immunization effect of a vaccine as well as the formulation of a reasonable immune procedure.

The N protein of PEDV is an RNA-binding protein that participates in transcription of the viral genome and provides a structural basis for the helical nucleocapsid (Duarte *et al*., 1994;Li *et al*., 2007). The N protein has been suggested to play an important role in inducing cell-mediated immunity in the host, indicating it is an optimal candidate protein for vaccine development (Saif, 1993). Moreover, the N protein is highly conserved among different strains. Li *et al*., have used phylogenetic analysis to show that the N gene is well conserved among Chinese variants and has a close identity with circulating strains in the Americas, Europe and Asia(Li *et al*., 2013). Thus, the N protein is properly used as a diagnostic target for the development of serologic diagnostic assays.

The purpose of this present study was to develop two ELISAs to detect specific antibodies and antigens for PEDV diagnosis. In this study, a N recombinant protein-based indirect ELISA (iELISA) for the detection of specific antibodies against PEDV and a sandwich ELISA (sELISA) to detect the PEDV N protein using MAb induced by the N protein were investigated. These ELISA systems were validated for the coefficient of variation (CVs), sensitivity, diagnostic sensitivity and specificity. These ELISAs would be helpful for clinical diagnosis and research related to PEDV.

Materials and Methods

Virus isolates and standard specimens: The PEDV, TGEV, porcine rotavirus A (pGARV), classical swine fever virus (CSFV), porcine circovirus-2 (PCV-2), porcine pseudorabies virus (PRV), and porcine reproductive and respiratory syndrome virus (PRRSV) strains were isolated and stored in our laboratory. PEDV negative serum samples were obtained from pigs with no vaccination and no clinical signs of PED. Western blot using PEDV-N protein and RT-PCR was used to determine the presence of antibodies in the serum and PEDV in the fecal samples, respectively.

Cloning and sequencing: Based on the cDNA sequence of CV777 from GenBank (Accession number AF353511.1), one pair of primers was designed and used to amplify the N gene of PEDV. The sense primer was 5′-GGATCCATGGCTTCTGTCAGCTTTC-3′, and the antisense primer was 5′-CTCGAGTTTCAACGGC CGTATCACC-3′. The purified PCR product was cloned into the pEASY®-Blunt E1 Expression vector (TransGen Biotech, Beijing, China) and transformed into *Escherichia coli* BL21 (DE3). A clone of N gene was also sent to Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China) to be sequenced.

Expression of the N protein: The N clone was induced using 1mM IPTG at 16°C for 8 hrs and the protein expression was mixed with SDS-loading buffer, subjected to 12% (v/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The recombinant N protein was purified according to Zhu *et al*., (Zhu *et al*., 2013) and was designated as PEDV-N.

Production of polyclonal antibody and mAbs against PEDV-N: Preparation of polyclonal antibody against PEDV-N protein was performed according to Ren *et al*., described methods (Ren *et al*., 2011). Immunization of mice, selection of positive hybridoma lines, production and purification of monoclonal antibodies (mAbs) were performed according Gui *et al* (Gui *et al*., 2018). The purified antibody was tested by western blot using PEDV-N protein.

Establishment of indirect ELISA to detect PEDV induced antibodies: The optimum concentrations of coated antigen, blocking solution, HRP-conjugated goat anti-pig IgG and serum dilution rate of the indirect ELISA were optimized simultaneously, according to the largest P/N value that appeared. The best reaction conditions for the indirect ELISA were as follows. A dilution of the purified PEDV-N was established with 1.25 µg/ml in 50mM PBS (pH 9.6). Generally, 50 µl of the above diluted antigen was

added to each well and coated overnight at 4°C. The plate was then washed 3 times and blocked with 5% of skimmed milk in PBS for 120 mins at room temperature (RT). Subsequently, in order to screen the optimum reagent, the test serum collected against the PEDV-N was diluted 1:200. A total of 100 ul of the diluted serum was added to each well and incubated at for 90 mins at RT. After washing, the (HRP)-conjugated goat anti-pig IgG was diluted 1:10000 and added to the plates, followed by incubation for 90 mins. The plate was washed 3 times and incubated for 8 mins at RT in the dark, adding 100 ul of 3,3′ ,5,5′-tetramethylbenzidine solution (TMB). After adding stop solution, the optical density was read at 450nm with a microplate reader.

Development of sandwich ELISA to detect PEDV N protein: A sandwich ELISA for the detection of N protein was established. The conditions of the ELISA were optimized, and the results indicated that when the largest P/N value appeared, the optimal conditions were as follows. The purified rabbit polyclonal antibodies prepared against the PEDV-N were coated at dilution concentration (2.0 µg/ml) at 4°C overnight. The plate was washed 3 times and blocked with 5% of skimmed milk in PBS for 120 mins at RT. After 3 washes, the antigen sample was added to the wells. The plate was incubated at RT for 90 mins, and then washed 3 times. After washing, the purified antibody against the PEDV-N was added to the wells at dilution concentration (1.0 µg/ml) for 90 mins at RT. After three washes, the (HRP)-conjugated goat anti-mouse IgG was diluted 1:10000. A total of 50 ul of the diluted antibody was added to each well for ELISA and followed by incubation at RT for 90 mins. The plate was washed 3 times and added TMB at RT in the dark. After 8 mins the plate stop solution was added and the OD_{450} was measured with a microplate reader.

Determination of the cut-off value: For the two ELISAs, the determination of the negative criteria was the average value (X) of negative samples with the standard deviation (SD) of the OD_{450} using the cut-off value prepared for the average value plus three standard deviations.

Check for the coefficient of variation: To assess the iELISA and sELISA developed in this study, amounts of intra-assay variation and inter-assay variation were determined to confirm reproducibility. For investigation of the precision evaluation, 6 samples (3 positive samples and 3 negative samples) were analyzed in duplicate within the same assays, and these repeated over a 3-day period. The average, standard deviations, medians and CV were calculated using the software Statistics Package for Social Science 19.0 (SPSS 19.0).

Assessment of the diagnostic sensitivity and specificity: Forty serum samples including 20 considered to be PEDV antibody positive samples and 20 negative samples were verified by the Western blot for evaluation of the diagnostic sensitivity of the indirect ELISA. The 40 fecal samples (20 positive samples and 20 negative samples) collected from pigs with diarrhea were detected by the sandwich ELISA developed in this study and were verified by RT-PCR to evaluate the diagnostic sensitivity of this sandwich ELISA. The sensitivity and specificity were evaluated by the receiver operating characteristic (ROC) curve using the results of the Western blotting or RT-PCR as the standard for negative and positive determination.

Assessment of the analytic sensitivity: The different anti-sera against TGEV, pGARV, CSFV, PCV-2, PRV and PRRSV were tested to evaluate the specificity of indirect ELISA. The reaction conditions were the same as those used for the indirect ELISA procedures in this study. Samples collected from pigs infected with TGEV, pGARV, CSFV, PCV-2, PRV and PRRSV were evaluated by the sandwich ELISA established in this study to determine the specificity of the ELISA. All data was visualized in GraphPad Prism 8.0 software (GraphPad Software, CA, USA).

Results

Cloning and production of the N protein: The RT-PCR for the N gene of PEDV produced amplicon of 1326 bp, and the specificity of RT-PCR was confirmed by cloning and sequencing (Fig. 1). The N protein was expressed in *E.coli* after IPTG induction at 16°C for 8 hrs. The protein was expressed as soluble in recombinant *E. coli* and its molecular weight was about 55 kDa as expected. The expressed N protein was then purified. SDS-PAGE analysis indicated that expression and purification of the N protein was detectable (Fig. 2 and 3).

Identification for the mouse anti-PEDV-N mAb by Western blot: The mice with the highest antibody titer (1:8000) were selected and splenocytes from these mice were fused with SP2/0 murine myeloma cells. One positive hybridoma clone, named 4F3, was identified and used for the production of ascites monoclonal antibodies. The isotypes of the 4F3 MAb were identified as IgG^1. The purified mAb was identified and characterized by Western blot using the purified N protein. The results showed that the 4F3 MAb could recognize the PEDV-N, which has a relative molecular weight of about 55 kDa, similar to the expected weight (Fig. 4).

Determination of the ELISA criteria: For the iELISA, 20 negative serum samples were tested to determine the cut-off value. The average $OD_{450\ nm}$ value of negative samples were statistically analyzed and calculated for 0.538, and the standard deviation (SD) was 0.136. The cut-off value was 0.946 determined as the mean value of the negative serum + 3×the SD. All OD_S of the serum samples higher than 0.946 were determined as positive, while less or equal to 0.946 the serum was considered to be negative. For the sELISA, 20 negative samples were tested to determine the cut-off value. The cut-off value was 0.451 (mean + 3×SD, 0.303+0.148) and used to determine whether the sample was positive or negative in the sandwich ELISA.

Repeatability of the ELISA: In order to assess the repeatability of iELISA and sELISA, coated ELISA plates with the same protein or antibody batch were

tested with 6 analytes under optimized ELISA conditions, respectively. For the iELISA, the intra-assay CV ranged from 0.23% to 2.33%, while the inter-assay CV was from 0.85% to 4.07%. For the sELISA, the intra-assay CV analysis of 6 samples ranged from 0.26% to 2.81%, and the inter-assay CV was from 0.35% to 3.28%. In general, above CV% values all were < 10%, indicating good reproducibility of iELISA and sELISA.

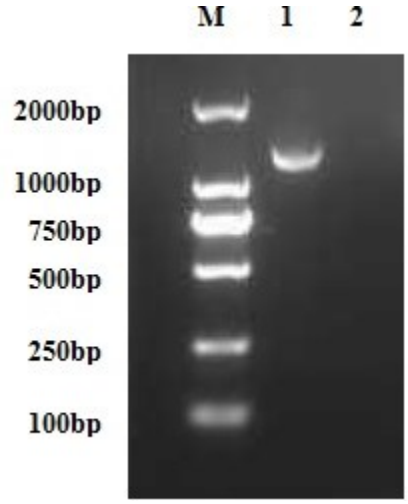


Figure 1 **PCR identification of recombinant plasmids.** Lane M, DL2000 marker; Lane 1, N gene PCR product (1326bp); Lane 2, negative control.

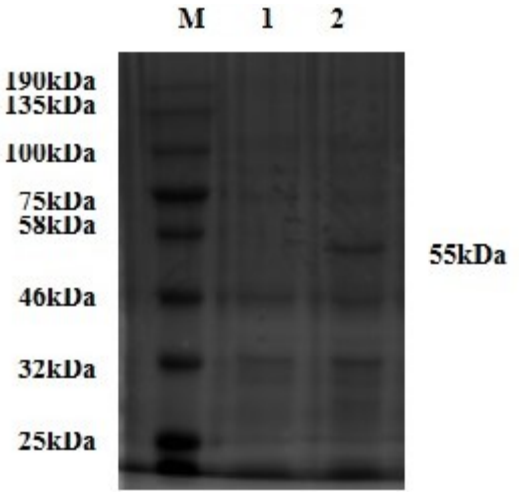


Figure 2 **Prokaryotic expression of the PEDV-N.** Lane M, Blue Plus® Protein Marker (22kDa-190kDa); Lane 1, the IPTG-induced recombinant bacteria of the PEDV-N; Lane 2, the IPTG-induced recombinant bacteria of the pEASY®-Blunt E1 vector.

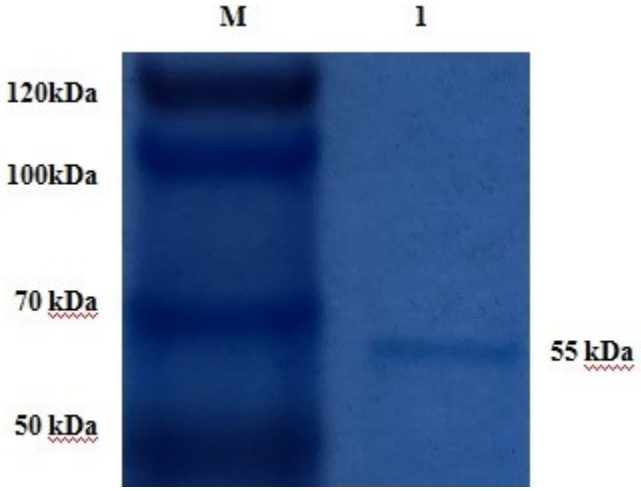


Figure 3 **Purification of the recombinant PEDV-N protein.** Lane M, Blue Plus® Pre-stained Protein Ladder (14kDa-120kDa); Lane 1, purified rPEDV-N protein.

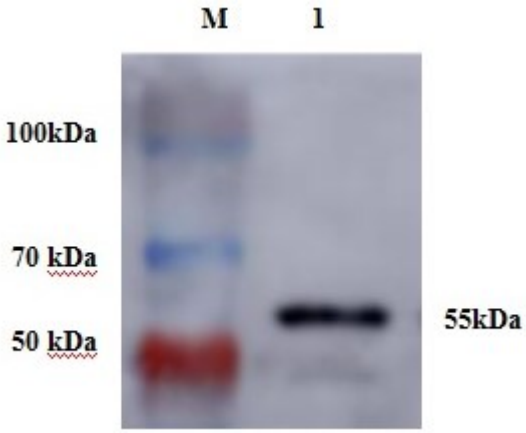


Figure 4 **Identification of the anti-PEDV-N monoclonal-antibodies by Western blot.** The purified antibody was added into the PVDF membranes, and the substrate 3,3-diami-nobenzidine tetra hydrochloride (DAB) was used for color development. Lane M, Blue Plus® Prestained Protein Ladder (14kDa-120kDa); Lane 1, purified rPEDV-N protein.

Diagnostic sensitivity and specificity of ELISA: For the iELISA, in total 19 out of the 20 positive specimens were correctly identified by the test. Of the 20 negative samples, 20 were correctly identified using the test (Table 1). The ROC curve showed the sensitivity was 100.0%, the specificity was 95.2%, and the area under ROC curve (ROC AUC) was 0.97 (Fig. 5a). Of the 40 samples tested by the sandwich ELISA, 18 samples were correctly identified from 20 positive samples. In all of the negative samples, 20 samples were correctly identified from 20 samples (Table 2). The ROC curve indicated the sensitivity was 100.0%, the specificity was 90.0%, and the ROC AUC was 0.945 (Fig. 5b).

Specificity test: The specificity of indirect ELISA was detected by antiserum of TGEV, pGARV, CSFV, PCV-2, PRV and PRRSV. The results showed that there was no cross reaction between the iELISA and the above antiserum (OD value <0.946) (Fig. 6a). In addition, the specificity assessment of sELISA showed the OD_{450nm} values of the known positive samples of TGEV, pGARV, CSFV, PCV-2, PRV and PRRSV were all less than the cutoff value (OD value <0.451), indicated that the anti-N antibody reacted with PEDV exclusively (Fig. 6b).

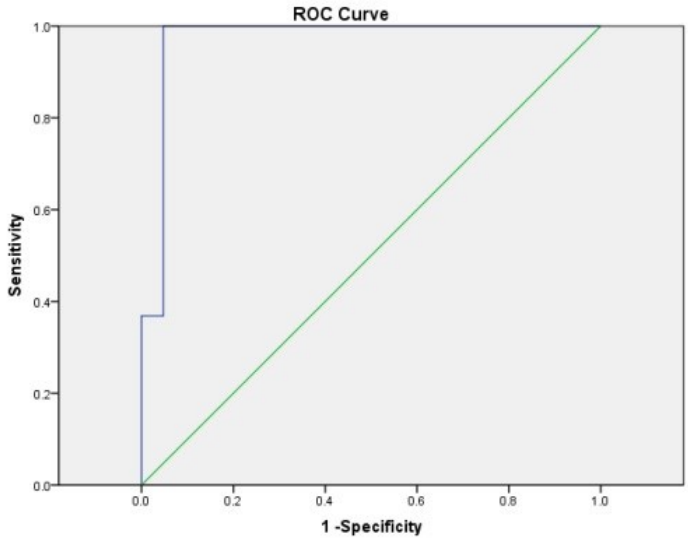
Table 1 Results for 40 serum samples that were tested to compare between western blot and iELISA.

iELISA	**Result of western blot**		**total**
	postive	**negative**	
Positive	19	0	19
negative	1	20	21
total	20	20	40

Table 2 Comparison the result of RT-PCR and sELISA for the detection of PEDV in fecal specimens.

sELISA	**Result of RT-PCR**		**total**
	postive	**negative**	
Positive	18	0	18
negative	2	20	22
total	20	20	40

(a)



(b)

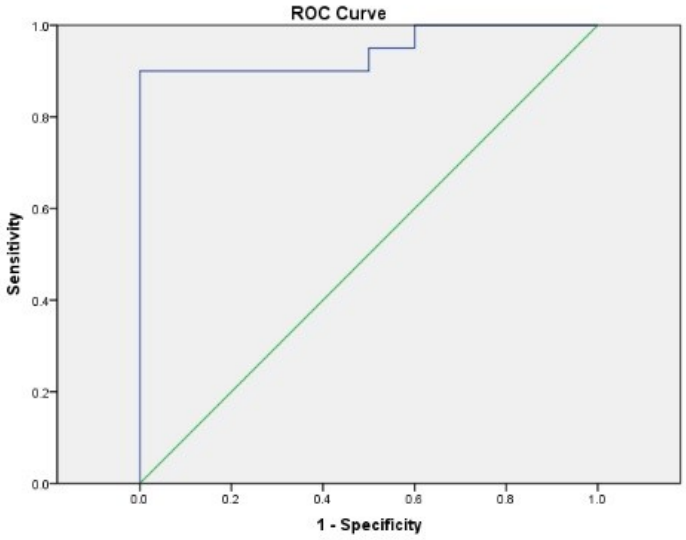
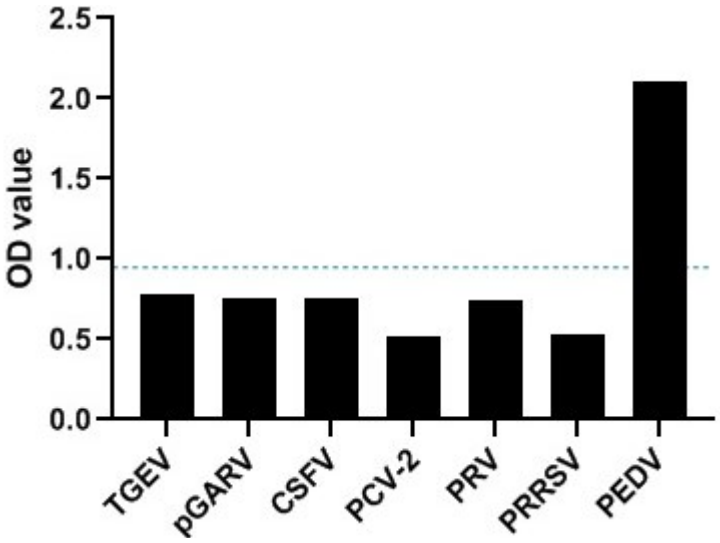


Figure 5 **Diagnostic sensitivity and specificity of ELISA.** (a) The receiver operating characteristic (ROC) curve using Western blotting as a diagnostic standard of iELISA; (b) The receiver operating characteristic (ROC) curve using RT-PCR as diagnostic standard of sELISA.

(a)



(b)

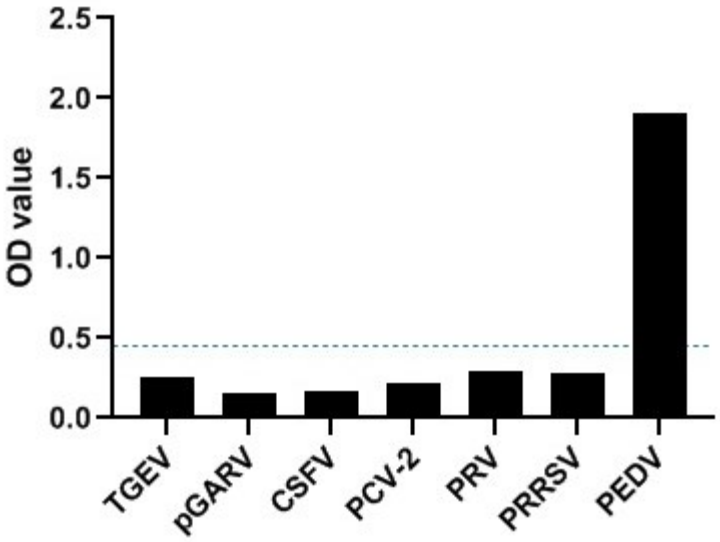


Figure 6 **Assessment of the analytic sensitivity.** (a) The specificity of the iELISA. Several kinds of antiserum, which are TGEV, PRoV, CSFV, PCV-2, PRV and PRRSV were tested by iELISA; (b) The specificity of the sELISA. Several kinds of virus, which are TGEV, PRoV, CSFV, PCV-2, PRV and PRRSV were tested by sELISA.

Discussion

Since the beginning of October 2010, an outbreak of diarrhea in piglets re-emerged in many provinces of China caused by highly virulent PEDV strain variants (Wang *et al*., 2016). So far, the variants are still prevalent in China and other parts of the world from time to time, leading to a potential threat to the pig industry. Therefore, rapid and precise diagnostic assays for detection of PEDV are important to detect rapidly, survey accurately and prevent spread of disease. Previous sequence comparisons with other PEDV strains from GenBank indicate that N genes are highly conserved among each other, especially among PEDV strain variants. Moreover, compared with the S protein of PEDV, which is prone to a greater range of genetic heterogeneity including insertions and deletions, the N protein has less variation among PEDV strains and is abundant after virus infection (Okda *et al*., 2015). Therefore, the N protein from the current PEDV strain circulating in China was selected as the target gene of the iELISA and the sELISA in this study.

Currently, a number of commercial and in-house iELISAs have been developed for detecting PEDV-induced antibodies. Early iELISAs are based on cell cultivated viruses or animal cell expressed viral proteins, leading to low specificity and high background (Hofmann and Wyler, 1990;Knuchel *et al*., 1992;Oh *et al*., 2005). This is because the antiserum inoculated with PEDV antigen vaccine prepared by cell culture may cross-react with the cellular components of ELISA antigen (Okda *et al*., 2015). In the present study, a recombinant PEDV N protein which was expressed from E. coli, was developed to detect antibodies against PEDV. The specificity assay showed that N protein coated antigens did not cross-react with other coronaviruses. Our results are in line with those of an indirect anti-PEDV IgG ELISA based on the recombinant S1 portion of the S protein (Gui *et al*., 2018). To evaluate the iELISA, comparison with Western blotting using expressed N protein as an antigen was carried by analysis of the ROC curve. The result showed that the iELISA exhibited acceptable diagnostic sensitivity and specificity. In addition, the developed iELISA also showed good reproducibility and precision. These results suggest that the iELISA developed in this study can be a useful diagnostic tool to detect anti-PEDV antibodies in serum and monitor passive immunity against the current circulating strains.

At present, RT-PCR is widely used to detect PEDV in the clinical laboratory but it is relatively expensive, time consuming and complex to carry out (Masuda *et al*. 2016). Therefore, the sandwich ELISA is more suitable for PEDV detection, with cost saving and a simpler operation compared with RT-PCR, which can detect a large number of samples. In the current study, a sandwich ELISA was established to detect the PEDV N protein using MAb induced by the N protein. The good reactivity of antibody induced N protein was detected by Western blot, indicating it can specifically recognize PEDV N protein. On the basic of the mAb, the sELISA was established with excellent reproducibility and precision and with no cross-reactivity with TGEV, PRoV, CSFV, PCV-2, PRV and PRRSV. In addition, the sELISA also demonstrated good diagnostic specificity and sensitivity to detect PEDV in fecal samples in comparison with RT-PCR. Taken together, this sELISA can be a useful material to establish a method to detect PEDV. Moreover, it also can directly detect and quantify PEDV N proteins. Considering the N protein is promising to be a vaccine candidates target, the sandwich ELISA can be used for quality control tests, such as measuring antigen content of candidate vaccines.

In conclusion, the iELISA and the sELISA described in this study provided a convenient tool to detect PEDV-induced antibody and PEDV, respectively. These two ELISAs have adequate diagnostic sensitivity and specificity, which provides an alternative tool for investigations of the epidemiology of PEDV.

Conflicts of Interest: The authors declare that there is no conflict of interest.

Acknowledgements

This study was supported by Fujian natural science foundation general project (2019J01804), Major projects of the Key project of Fujian provincial youth natural fund (JZ160481) and the Education and scientific research projects of young and middle-aged teachers in Fujian Province (JAT160483).

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