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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

A novel double antibody sandwich quantitative ELISA for detecting porcine epidemic diarrhea virus infection

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Abstract

Porcine epidemic diarrhea (PED), a contagious intestinal disease caused by the porcine epidemic diarrhea virus (PEDV), has caused significant economic losses to the global pig farming industry due to its rapid course and spread and its high mortality among piglets. In this study, we prepared rabbit polyclonal antibody and monoclonal antibody 6C12 against the PEDV nucleocapsid (N) protein using the conserved and antigenic PEDV N protein as an immunogen. A double-antibody sandwich quantitative enzyme-linked immunosorbent assay (DAS-qELISA) was established to detect PEDV using rabbit polyclonal antibodies as capture antibodies and horseradish peroxidase (HRP)-labeled 6C12 as the detection antibody. Using DAS-qELISA, recombinant PEDV N protein, and virus titer detection limits were approximately 0.05 ng/mL and $10^{3.02}$ 50% tissue culture infective dose per mL ($TCID_{50}$/mL), respectively. There was no cross-reactivity with porcine reproductive and respiratory syndrome virus (PRRSV), porcine rotavirus (PoRV), porcine pseudorabies virus (PRV), porcine deltacoronavirus (PDCoV), or porcine circovirus (PCV). The reproducibility of DAS-qELISA was verified, and the coefficient of variation (CV) for intra- and inter-batch replicates was less than 10%, indicating good reproducibility. When testing anal swab samples from PEDV-infected piglets using DAS-qELISA, the coincidence rate was 92.55% with a kappa value of 0.85 when using reverse transcription-polymerase chain reaction (RT-PCR) and 94.29% with a kappa value of 0.88 when using PEDV antigen detection test strips, demonstrating the reliability of the method. These findings provide fundamental material support for both fundamental and practical studies on PEDV and offer a crucial diagnostic tool for clinical applications.

Key points

- *A new anti-PEDV N protein monoclonal antibody strain was prepared*
- *Establishment of a more sensitive double antibody sandwich quantitative ELISA*
- *DAS-qELISA was found to be useful for controlling the PEDV spread*

Keywords PEDV · Nucleocapsid protein · Monoclonal antibodies · DAS-qELISA · Antigen detection

Introduction

Porcine epidemic diarrhea virus (PEDV), a member of *Alphacoronavirus* in the family *Coronaviridae* order *Nidoviridae*, replicates in the epithelial cells of the small intestinal villi of pigs, causing intestinal disease, which is clinically characterized by diarrhea, vomiting, dehydration, and high lethality in lactating piglets (Stevenson et al. 2013). PEDV was first identified in Belgium in the 1970s and has since spread throughout Europe and Asia (Pensaert and Bouck 1978). In China, PEDV first broke out in southern China in 2010, affecting most pig farms (Li et al. 2023). Since then, 34 countries in the USA and Canada have reported cases of PEDV, with porcine

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epidemic diarrhea (PED) becoming a critical disease in the pig industry (Su et al. 2018).

PEDV has a capsule membrane, is polymorphic, and is mostly spherical, with a 95–190 nm diameter. The PEDV genome is a single-stranded RNA without segments, with a total length of approximately 30 kb (Li et al. 2020). The PEDV genome consists of 5′ and 3′ end untranslated regions, seven open reading frames (ORF). ORF1a and ORF1b encoding 16 non-structural proteins (nsp1-nsp16) are required for transcription and translation. ORF2 and ORF4-6 encoding four structural proteins, namely spinal (S) protein, membrane (M) protein, small membrane (E) protein and nucleocapsid (N) protein, and ORF3 encoding an accessory protein related to PEDV virulence (Guo et al. 2024). PEDV is classified into G1 (G1a and G1b subtypes) and G2 (G2a, G2b, and G2c subtypes) based on the homology of the PEDV S gene or its N-terminal hypervariable region S1 sequence. Before 2010, the predominant PEDV strain in China was subtype G1a. The main circulating strains after 2010 were the G2 genotype and the S-INDEL strain. Currently, multiple genotypes of PEDV exist simultaneously in China (Li et al. 2023).

The N protein, the main component of the PEDV nucleocapsid, is composed of 441 amino acids with a molecular weight of 55–58 kDa (Ding et al. 2014). It contains multiple potential phosphorylation sites, is rich in serine, and has a high isoelectric point (Chen et al. 2005). As a multifunctional alkaline phosphoprotein, it is one of the most highly expressed and conserved viral proteins in infected cells (Ma et al. 2019). The N protein is directly connected to the viral RNA and plays a vital role in viral genome synthesis. During cell infection, the N protein has other unique mechanisms that affect host cells (Su et al. 2021). An in-depth understanding of the function of the PEDV N protein is necessary to clarify further the pathogenic mechanism of PEDV, from which efficient and accurate detection techniques can be developed and antiviral strategies can be proposed (Zhao et al. 2023).

Many virological, histological, immunological, and molecular biology assay techniques have been developed to detect PEDV antigens, viral proteins, and nucleic acids from clinical samples, including stool, intestinal contents, and intestinal tissue. These techniques include enzyme-linked immunosorbent assays (ELISA) (Ma et al. 2021), direct immunofluorescence (IF), immunohistochemistry techniques (IHC), immunochromatographic assays (ICA), reverse transcription-polymerase chain reaction (RT-PCR), real-time quantitative polymerase chain reaction (RT-qPCR) (Zhou et al. 2017), and nucleic acid isothermal amplification (NAIA). ELISA has been widely used in rapid detection at home and abroad because of its strong specificity, good sensitivity, and ease of operation (Ma et al. 2021).

In this study, we used a prokaryotic expression system to express the N protein from the PEDV CH/SX/2016 strain (GenBank accession no. MT787025.1). Rabbits and mice were immunized with purified recombinant N (rN) protein as an immunogen to obtain rabbit polyclonal antibodies (PAb) and monoclonal antibodies (MAb). A double-antibody sandwich quantitative enzyme-linked immunosorbent assay (DAS-qELISA) was performed using rabbit PAb as the capture antibody and horseradish peroxidase (HRP)-labeled MAb. Because of its high sensitivity, specificity, reproducibility, and reliability, this ELISA can be used to detect PEDV antigens in large quantities of clinical samples, providing a crucial diagnostic tool for clinical applications and suppressing the prevalence of PEDV.

Materials and methods

Cells, viruses, and experimental animals

African green monkey kidney (Vero) cells (Procell, Wuhan, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Basal Media, Shanghai, China) containing 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA), and the SP2/0 cells (Keycell, Wuhan, China) were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640; Basal Media, Shanghai, China) containing 20% FBS.

PEDV CH/SX/2016 strain (GenBank accession no. MT787025.1) was isolated and maintained in State Key Laboratory for Animal Disease Control and Prevention in Lanzhou, China. The cell culture supernatants of porcine reproductive and respiratory syndrome virus (PRRSV), porcine pseudorabies virus (PRV), porcine rotavirus (PoRV), and porcine circovirus (PCV) were provided by State Key Laboratory for Animal Disease Control and Prevention in Lanzhou, China.

BALB/c mice and female rabbits were obtained from Lanzhou Veterinary Research Institute, China Agricultural Science Academy. Weaned piglets were purchased from the Kangzhiyuan Planting Farmers' Specialized Cooperative in Linxia.

Preparation of PEDV N protein

Using RT-PCR (with primers F:5′- AAATGGGTCG*GGATCC*GATGGCTTCTGTCAGCTTTCAGGA-3′ (contains a *Bam*HI digestible site) and R: 5′- GGTGGTGGTG*CTCGAG*ATTTCCTGTATCGAAGATCTCGTTGATAATCT-3′ (attached to a *Xho*I site)) the N protein from the PEDV CH/SX/2016 strain was amplified and subcloned into the pET-21b (+) (Merck KGaA, Darmstadt, Germany). The pET-21b-PEDV-N positive plasmid was

transformed into *Escherichia coli* BL21 (DE3) (TransGen Biotech, Beijing, China) competent cells. Then, after induction with 1 mM isopropyl β-D-thiogalactoside (IPTG) (Biotopped, Beijing, China) at 37 °C for 6 h, purification was performed using Ni–NTA resin (TransGen Biotech, Beijing, China). Lastly, identification was performed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis.

Preparation and characterization of PAb and MAb against PEDV N

Immunized 6–8-week-old BALB/c female mice (50 μg/mouse) and 8–12-week-old female rabbits (300 μg/rabbit) were used as the study animals. In the first immunization, the purified rN protein was emulsified with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and injected subcutaneously into the back of the mice and rabbits. Subsequent immunizations were emulsified with equal volumes of Freund's incomplete (Sigma-Aldrich, St. Louis, MO, USA) adjuvant. Immunizations were performed every 2 weeks, and blood samples were collected before each immunization. The titer was tested using an ELISA kit coated with N protein. For mice, after three immunizations, when the titer exceeded 1×10^4, a booster immunization was administered via intraperitoneal injection. Three to five days after immunization, peritoneal washes from blank mice were collected as a source of feeder cells to facilitate hybridoma cell growth. Induced fusion of SP2/0 cells and spleen cells from immunized mice into hybridoma cells was obtained using PEG1450 (Sigma-Aldrich, St. Louis, MO, USA) and cultured using HAT/HT selection medium (Sigma-Aldrich, St. Louis, MO, USA). Indirect ELISA was used to detect the supernatant of the cell cultures, followed by screening for positive hybridoma cells and three rounds of subclone culturing on positive hybridoma cells to obtain hybridoma cells that stably secreted MAb against the PEDV N protein. Female BALB/c mice aged 8–10 weeks were sensitized via intraperitoneal injection of an incomplete adjuvant. After 1 week, 1×10^7 hybridoma cells were injected into the peritoneal cavity of each mouse to prepare the ascitic fluid. The MAb subtype was identified using a mouse MAb IgG subclass identification kit (Biodragon, Suzhou, China). Serum was collected from rabbits that had demonstrated high levels of potency. Subsequently, the rabbit serum and mouse ascites were purified using protein G resin (Biodragon, Suzhou, China) and the antibody titers were determined by indirect ELISA. The antibodies were further validated by indirect immunofluorescent assay (IFA) and western blotting, and MAb were labeled using HRP labeling reagent kits (Biodragon, Suzhou, China).

Enzyme-linked immunosorbent assay

The purified rN protein was diluted to 2 ng/μL in phosphate buffer saline (PBS) and coated onto ELISA plates overnight at 4 °C. The plates were washed four times with phosphate buffer saline containing 0.05% Tween-20 (PBST) and blocked with blocking buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 2 h at 37 °C. For positive selection, 50 μL of hybridoma cell culture supernatant was added to each well and incubated for 60 min at 37 °C. After four washes, 50 μL of 1:10,000 dilution of HRP-labeled goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) was added and incubated for 60 min at 37 °C. After the same four washes, 50 μL of 3,3',5,5'-tetramethylbenzidine (TMB) (Biodragon, Suzhou, China) was added, and the plate was incubated for 15 min in the dark. Next, 50 μL of 2 M sulfuric acid was added to terminate the reaction, followed by reading of the absorbance at 450 nm ($OD_{450\ nm}$). Mouse serum was used as the positive control, while the negative control was SP2/0 cell culture supernatant. A sample was considered positive if the $OD_{450\ nm}$ value divided by that of the negative control was greater than 2.

To test the titers of PAb and MAb, antibodies were diluted from 1:1000 to 1:12,800 for incubation. The remaining steps were the same as those described above.

Indirect immunofluorescence assay

Vero cells grown in 96-well plates were infected with PEDV and cultured for 24 h at 37 °C in a CO_2 incubator. Cells were washed two times with PBS, fixed with 4% paraformaldehyde for 15 min at 37 °C, washed, and permeabilized with 0.1% triton X-100 (Solarbio, Beijing, China) for 15 min. After washing, the cells were blocked cells with 5% bovine serum albumin (BSA) for 30 min at 37 °C, followed by washing three times. Then, the cells were incubated with a 1:1000 dilution of MAb or PAb for 1 h at 37 °C. After three PBS washes, a 1:500 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (or anti-rabbit) IgG (Jackson Immuno Research, West Grove, PA, USA) was added, followed by incubation at 37 °C away from light for 1 h. Following three more washes, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Jackson Immuno Research, West Grove, PA, USA) at a 1:1000 dilution for 10 min protected in the dark. Finally, the cells were observed under a fluorescence microscope.

Western blotting

The treated protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. After being

closed with 5% skim milk diluted in PBST for 2 h at 37 °C, the membranes were incubated in antibody overnight at 4 °C. After washing four times with PBST, the cells were incubated with an HRP-labeled goat anti-mouse (or anti-rabbit) IgG secondary antibody at 37 °C for 1 h. After another four washes, the membrane was exposed using electrochemiluminescence (ECL).

To validate the purified rN protein, anti-His mouse monoclonal antibody (TransGen Biotech, Beijing, China) was used as the detection antibody to confirm protein expression. The protein samples were detected as either PEDV-infected Vero cell lysates or normal Vero cell lysates, and the antibodies were detected as MAb or PAb to confirm the application of the antibodies in western blotting analysis.

Establishment of DAS-qELISA

In DAS-qELISA, rabbit PAb was used as the capture antibody and HRP-labeled MAb (6C12). The optimal concentration for capturing and detecting antibodies was determined using checkerboard titration. The purified PAb was diluted (0.5, 1.0, 2.0, and 4.0 μg/mL) using carbonate-bicarbonate buffer (Sigma-Aldrich, St. Louis, MO, USA), adding 50 μL laterally into ELISA plates and incubated overnight at 4 °C. The plates were then washed four times with PBST, followed by incubation with 5% skim milk diluted in PBST for 2 h at 37 °C. After washing, 100 μL was added to each of the PEDV infection and normal Vero culture supernatant and incubated for 1 h at 37 ℃. After washing four times, HRP-labeled MAb was diluted at 1:500, 1:1000, 1:2000, 1:4000, 1:6000, and 1:8000 with PBST and vertically added to the wells (100 μL/well) before incubating for 1 h at 37 °C. Following another round of washing, 50 μL of TMB was added to each well. The reaction was terminated using 50 μL of 2 M sulfuric acid after color development for 10 min at 37 °C, followed by measurement at $OD_{450\ nm}$. The optimal capture and detection antibody concentrations were determined using the highest positive/negative (P/N) ratio.

Based on the determined antibodies concentration, the optimal coating conditions were determined by coating at 37 ℃ for 1 h, 37 ℃ for 1 h + 4 ℃ for 12 h, 37 ℃ for 2 h, 37 ℃ for 2 h + 4 ℃ 12 h, and 4 ℃ for 12 h, respectively. This was followed by incubation with 1.5%, 3.0%, and 5.0% skim milk and BSA at 37 ℃ for 60 min, 90 min, and 120 min, respectively, to determine the optimal blocking conditions. Similarly, the samples were then incubated with HRP-labeled antibody at 37 ℃ for 30 min, 60 min, 90 min, and 120 min to determine the optimal incubation time. Finally, the samples were incubated with TMB at 37 ℃ for 5 min, 10 min, 15 min, and 20 min, respectively, to determine the optimal conditions.

Cutoff value of DAS-qELISA

PEDV-negative anal swabs were collected from 30 healthy piglets, diluted with 1 mL of PBS, and vortexed for 30 s. The supernatant was collected after centrifugation at 13,000 × *g* for 5 min. Each sample was analyzed three times, detected using the established DAS-qELISA, and measured at $OD_{450\ nm}$. The average value and standard deviation (SD) of the $OD_{450\ nm}$ values were calculated, and the cutoff value was determined as equal to the sum of the average value and three times the SD. When the $OD_{450\ nm}$ value of the test sample was greater than the cutoff value and the sample-to-negative (S/N) ratio was greater than 2, it was deemed to be positive; otherwise, it was considered negative.

Sensitivity, specificity, and reproducibility

Purified rN protein was diluted with PBS from 200 to 0.012 ng/mL, with PBS used as the negative control. The 50% tissue culture infective dose ($TCID_{50}$) of the PEDV cell culture was measured as $10^{5.13}$ $TCID_{50}$/mL, and the PEDV cell culture was diluted with DMEM from 1:16 to 1:4096. The normal Vero supernatant was used as the negative control. The absorbance was measured at $OD_{450\ nm}$ and the S/N value was analyzed to determine the sensitivity of DAS-qELISA.

Next, the specificity of DAS-qELISA was evaluated using PRRSV, PoRV, PRV, porcine deltacoronavirus (PDCoV), and PCV. PEDV-infected cell culture supernatants were used as positive controls and PBS was used as a negative control.

To test the intra-assay reproducibility, four PEDV cell culture supernatants and normal cell culture supernatants were assayed four times. Four batches of DAS-qELISA were used to confirm inter-assay reproducibility based on the average value, SD, and coefficient of variation (CV) of the absorbance measured at $OD_{450\ nm}$.

Compliance rate test

To determine the detection effect of DAS-qELISA on clinical samples, an animal test was conducted on twelve one-day-old piglets by oral gavage with the PEDV (CH/SX/2016) strain. DMEM was used as a negative control for six piglets taken orally, with anal swabs collected daily. The collected anal swabs were then diluted with 1 mL of PBS and vortexed for 30 s. The resulting supernatant was collected by centrifugation at 13,000 × *g* for 5 min. Then, 300 μL of extracted RNA was taken for RT-PCR detection and 100 μL was used for DAS-qELISA detection, and the consistency of the two detection methods was compared based on the results. In addition, 35 samples were randomly selected for the PED Ag

Rapid Test Kit (JNT, Beijing, China), and the results were compared with those of the DAS-qELISA.

Results

Preparation and characterization of PAb and MAb against PEDV N

Rabbits were immunized with the purified rN protein, and the anti-PEDV N protein PAb was obtained. Immunization of mice, followed by hybridoma cell fusion, yielded a strain of MAb, namely 6C12, specific to the anti-PEDV N protein. Indirect ELISA was performed to detect the reactivity of the MAb 6C12 strain and rabbit PAb with PEDV N protein. As shown in Fig. 1A, MAb 6C12 and rabbit PAb were found to react with PEDV N protein. The reactivity of 6C12 and rabbit PAb with PEDV was characterized by western blotting and IFA, which specifically reacted with PEDV (Fig. 1B and C). Subsequently, their titers were determined using indirect ELISA in a 1:12,800 dilution (Fig. 1D). Both 6C12 and PAb were found to maintain an $OD_{450\ nm}$ value greater than 2. These results demonstrate the high reactivity and efficacy of the prepared PAb and MAb.

Development of DAS-qELISA

Checkerboard titration was used to determine the most appropriate concentrations of the capture and detection antibodies. As shown in Fig. 2A, when the rabbit PAb was 4.0

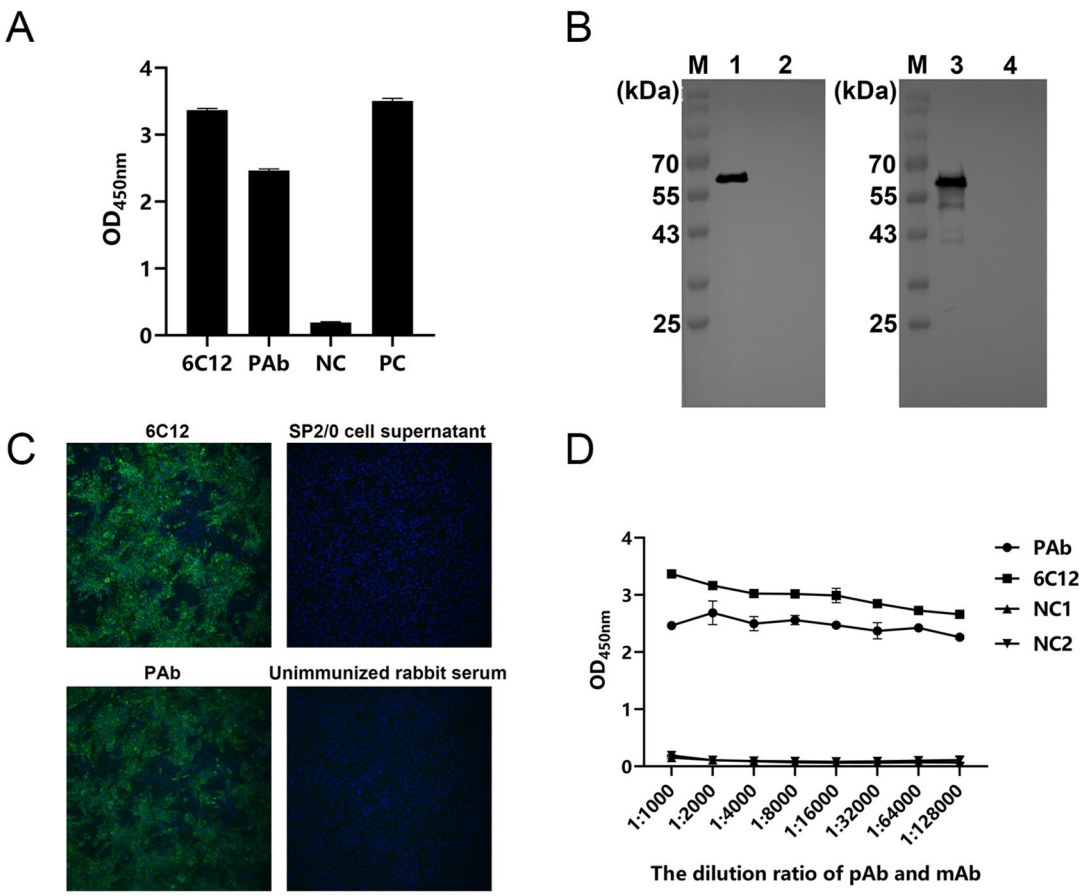

Fig. 1 Preparation and identification of anti-PEDV N MAb and PAb. **A** ELISA analysis of the reactivity of MAb and rabbit PAb with the N protein. Anti-PEDV N protein mouse positive serum was used as the positive control and normal mouse serum was the negative control, with both antibodies incubated at a dilution of 1:1000. **B** Western blotting to determine the reactivity of MAb and rabbit PAb with PEDV. The protein samples of Lane 1 and Lane 3 were PEDV-infected Vero cell lysate, and the protein samples of Lane 2 and Lane 4 were negative control normal Vero cell lysate. MAb was used for Lane 1 and Lane 2, and rabbit PAb was used for Lane 3 and Lane 4. **C** IFA demonstrating the reactivity of MAb and rabbit PAb with PEDV. Cells were infected with PEDV for 24 h. Cells were fixed and strained with the MAb 6C12 and rabbit PAb, wherein the SP2/0 cell supernatant and unimmunized rabbit serum were used as the negative controls, respectively. **D** Determination of the titers of MAb and rabbit PAb through indirect ELISA. The rabbit PAb, purity MAb 6C12, unimmunized rabbit serum (NC1), and unrelated Mab (NC2) were diluted (1:1000, 1:2000, 1:4000, 1:8000, 1:16,000, 1:32,000, 1:64,000, and 1:128,000) in PBST, followed by detection by indirect ELISA

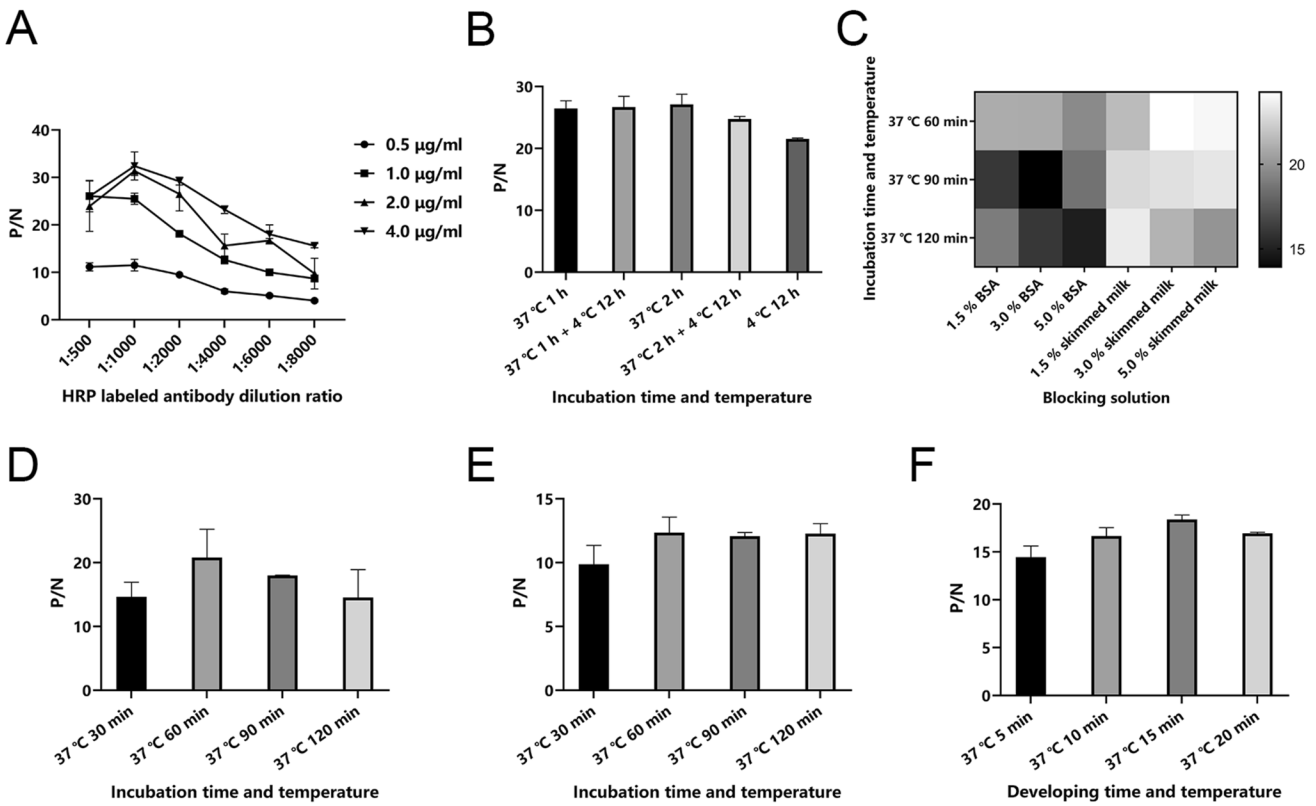

Fig. 2 Establishment and optimization of DAS-qELISA. **A** Optimum concentration of the capture and detecting antibodies. **B** Optimum coating time and temperature. **C** Optimum blocking conditions. **D** Optimum reaction time of antigen. **E** Optimum reaction time of HRP labeled antibody. **F** Optimum developing time. PEDV-infected or mock-infected culture supernatants were used as the positive and negative antigens, respectively. The $OD_{450\ nm}$ value of positive-to-negative (P/N) ratio was calculated

μg/mL and the HRP-labeled 6C12 was diluted at 1:1000, the highest P/N value obtained was 32.43, which is the optimal antibody concentration.

Next, different temperatures and durations were tested for rabbit PAb coating, as depicted in Fig. 2B. The highest P/N value (27.10) was achieved when the coating was performed at 37 °C for 2 h, indicating the optimal coating conditions.

Third, 1.5%, 3.0%, and 5.0% skim milk and BSA were used for blocking at 37 °C for 60 min, 90 min, and 120 min, respectively. As shown in Fig. 2C, when 3% skim milk at 37 °C for 60 min was used, the highest P/N was 24.25, which is the optimal blocking condition.

To determine the optimal incubation time for the PEDV antigen and antibodies, samples and HRP-labeled antibodies were incubated separately for 30, 60, 90, and 120 min at 37 °C, as illustrated in Fig. 2D. The highest P/N value (20.82) was achieved when the samples were incubated for 60 min, highlighting this as the optimal sample incubation conditions. Figure 2E shows that when HRP-labeled antibodies were incubated for 60 min at 37 °C, the P/N value was 12.36, indicative of the optimal antibody incubation condition, with no statistically significant differences compared with 90 or 120 min.

Finally, TMB was applied at 37 °C for different durations (5, 10, 15, and 20 min) for development, as shown in Fig. 2F. The highest P/N value (18.39) was obtained when TMB was incubated for 15 min, which was considered the optimal conditions for color development.

Cutoff value of DAS-qELISA

Thirty negative anal swabs were tested, the results of which are shown in Fig. 3. The average of $OD_{450\ nm}$ value was 0.122 with an SD of 0.015. The cutoff value was calculated as the sum of the average and three times the SD, resulting in a value of 0.167. Therefore, the sample was considered positive when the $OD_{450\ nm}$ value was > 0.167 and the S/N was > 2; otherwise, it was considered negative.

Sensitivity and specificity of DAS-qELISA

Purified rN protein and PEDV cell culture supernatants were assayed in twofold dilutions to determine the sensitivity of DAS-qELISA. As shown in Fig. 4A, within the range of N protein concentrations from 0.05 to 1.56 ng/mL, the $OD_{450\ nm}$ value exhibited a linear relationship with the N

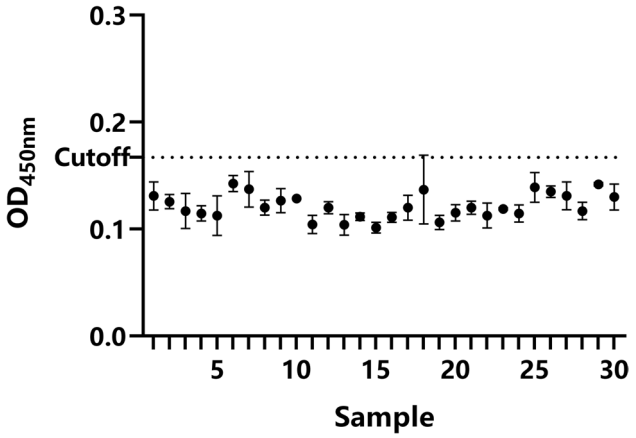

Fig. 3 Determination of the cutoff value. The optimized DAS-qELISA was used to evaluate 30 PEDV-negative anal swabs. The average value and standard deviation (SD) of the absorbances measured at $OD_{450\ nm}$ value were calculated, from which the cutoff value was determined as equal to the sum of the average value and three times SD

protein concentration, characterized by the following linear equation: $Y = 1.1781x - 0.3785$, $R^2 = 0.9726$, and a minimum detection concentration of 0.05 ng/mL. When the virus was diluted 1:128, the average $OD_{450\ nm}$ value was 0.338, exceeding the cut-off value, and P/N > 2 (Fig. 4B). The minimum viral detection rate was $10^{3.02}$ $TCID_{50}$/mL.

In terms of the specificity of DAS-qELISA, the $OD_{450\ nm}$ value of PEDV was 1.92, whereas all other viruses showed values below the cutoff or P/N < 2 (Fig. 5). This indicates that DAS-qELISA does not cross-react with PRRSV, PoRV, PDCoV, PRV, or PCV and exhibits good specificity.

Reproducibility of DAS-qELISA

Next, the reproducibility of the same batch of DAS-qELISA (intra-batch reproducibility) for different samples and the reproducibility of different batches of DAS-qELISA (inter-batch reproducibility) for different samples were verified (Table 1). As a result, the intra-batch CV was found to range from 1.29 to 6.48%, whereas the inter-batch CV ranged from 0.82 to 5.29%. All CV values were less than 10%, indicating that DAS-qELISA established for PEDV detection exhibited excellent stability.

Sample analysis

A total of 94 anal swabs were tested using both RT-PCR and DAS-qELISA, as described above. As shown in Table 2, RT-PCR detected 59 PEDV-positive samples and 35 PEDV-negative samples, whereas DAS-qELISA detected 54 positive and 40 negative samples. The concordance rate between DAS-qELISA and RT-PCR was 92.55% with a kappa value of 0.85. To evaluate these techniques further, an additional 35 samples were randomly selected and tested using the PED Ag Rapid Test Kit (Table 3). Although this test also detected 22 positive samples, one was negative in the DAS-qELISA results. The resulting concordance rate between DAS-qELISA and the test strips was 94.29% with a kappa value of 0.88. Thus, DAS-qELISA exhibited high concordance with RT-PCR and the PEDV antigen test strips, with a kappa value of ≥ 0.75, indicating good consistency.

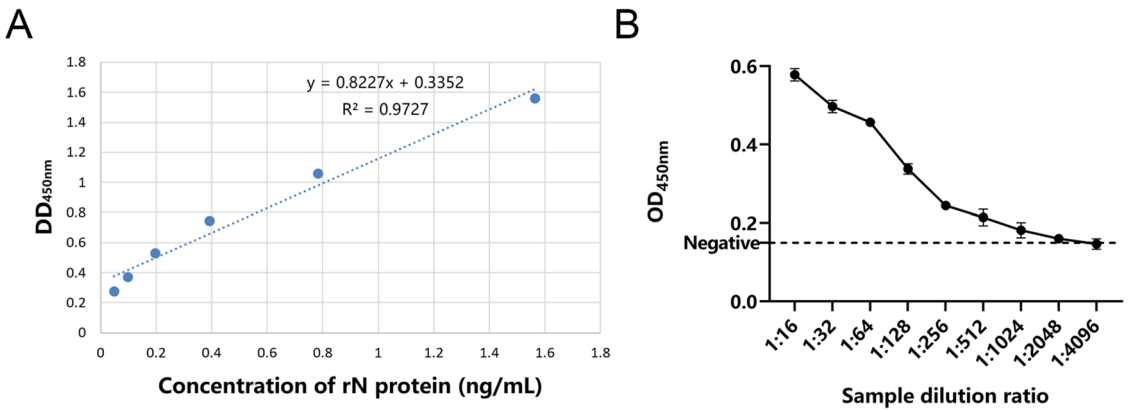

Fig. 4 Sensitivity of DAS-qELISA. **A** The standard curve of the DAS-qELISA. Twofold serially diluted rN protein was assayed using DAS-qELISA with two replicates per concentration. The standard curve was calculated using a linear relationship between the $OD_{450\ nm}$ values and concentrations. **B** Sensitivity of DAS-qELISA for PEDV. PEDV-infected culture supernatants were diluted twofold in DMEM and detected by DAS-qELISA, Vero cell culture was used as a negative control

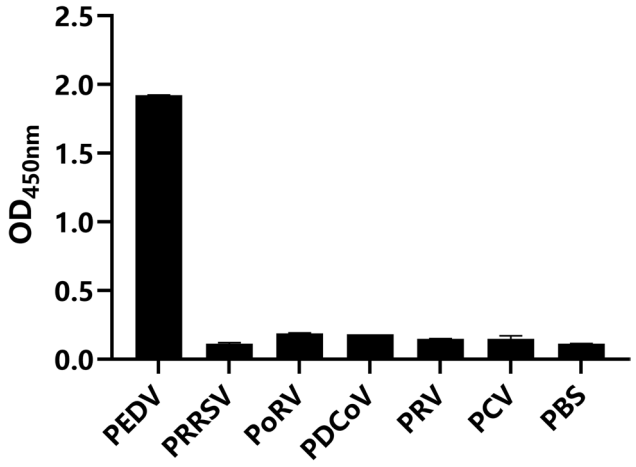

Fig. 5 Specificity of DAS-qELISA for PEDV. The purified recombinant PDCoV N protein and the cell culture supernatants of PEDV, PRRSV, PoRV, PRV, and PCV were detected by DAS-qELISA. PBS was used as a negative control

Table 2 The concordance rate between DAS-qELISA and RT-PCR

	RT-PCR	DAS-qELISA		Coincidence rate	Kappa
		Positive	Negative		
Positive	59	53	6	92.55%	0.85
Negative	35	1	34		
Total	94	54	40		

Discussion

PEDV causes highly contagious intestinal diseases without exhibiting any obvious patterns of seasonal or geographical selectivity. As a result, pigs of all ages have been found to be infected, albeit showing different degrees of symptoms. However, the most serious clinical symptoms tend to occur in piglets, including infection with acute enteritis, vomiting, watery diarrhea, and dehydration (Li et al. 2022). At present, annual global economic losses resulting from piglet deaths caused by PEDV are extremely high because the virus can evade current vaccination programs and biosecurity systems (Liu et al. 2024).

PEDV often presents with other diarrheal viruses (Zhao et al. 2016), and its clinical symptoms bear a high resemblance to those of other porcine diarrheal viruses (Lopez-Figueroa et al. 2023). In the context of developing of specific tools for detecting PEDV infections, N protein is the preferred target antigen for confirming this diagnosis (Ma et al. 2019). MAb produced by a single B cell clone specifically recognize a particular antigenic epitope, which sets them apart from PAb (Duan et al. 2020). MAb offer advantages such as high specificity, good homogeneity, and high potency, making them ideal tools for serological testing, immunological research, and targeted therapies. In this study, the analysis of rN protein expressed in a prokaryotic system, prepared an MAb, validated that this antibody could effectively detect PEDV and N proteins using ELISA, western blotting, and IFA.

At present, conventional diagnostic methods for PEDV are based on laboratory tests, including virus isolation, immunoelectron microscopy (IEM), conventional RT-PCR (Ishikawa et al. 1997), real-time RT-PCR (Liu et al. 2019), IFA assay (Lee 2016), and ELISA (Lin et al. 2018). However, in practice, the S protein of this pathogen is easily shed during the treatment of the disease material, making the virus atypical. As a result, analysis using immunoelectron microscopy requires more sophisticated experimental equipment, thereby restricting the applicability of this method for in situ detection (Gong et al. 2016). Currently, RT-PCR is the primary technique used to diagnose microbial infections. However, although its detection sensitivity is high and the presence of virus can be detected at very low viral levels, it often succumbs to the effects of contaminated environments and produces false positives. Thus, this method requires specific test conditions and is not suitable for in situ diagnosis (Fan et al. 2020). Although virological testing is often the preferred diagnostic method after a PEDV outbreak, serological testing can provide essential information regarding previous viral exposure and the

Table 1 Reproducibility of DAS-qELISA

	Repetitions	Intra-batch			Inter-batch		
		$\bar{x}$	SD	CV (%)	$\bar{x}$	SD	CV (%)
Positive	4	2.185	0.050	2.30	2.156	0.028	1.29
		1.469	0.034	2.28	1.495	0.030	2.02
		2.063	0.062	3.01	2.079	0.033	1.60
		2.561	0.033	1.29	2.585	0.021	0.82
Negative	4	0.142	0.009	6.48	0.148	0.008	5.15
		0.147	0.008	5.15	0.156	0.004	2.24
		0.144	0.004	3.02	0.155	0.005	3.38
		0.150	0.008	5.25	0.155	0.008	5.29

Table 3 The concordance rate between DAS-qELISA and PED Ag Rapid Test Kit

	DAS-qELISA	PED Ag Rapid Test Kit		Coincidence rate	Kappa
		Positive	Negative		
Positive	22	21	1	94.29%	0.88
Negative	13	1	12		
Total	35	22	13		

immunological status of specific animals or herds. ELISA is a simple and rapid method that can be used for large-scale testing in both the field and clinical settings (Ma et al. 2019). Compared to indirect ELISA with higher cross-reactivity, DAS-qELISA offers high specificity by using capture and detection antibodies, both of which are specific (Cao et al. 2023). This method is suitable for complex samples and does not require antigen purification prior to testing. DAS-qELISA also provides flexibility and improves sensitivity. Despite this, to date, there has been only one report on the use of DAS-qELISA for the detection of PEDV, and further exploration is required (Fan et al. 2020). To address this, herein we established a DAS-qELISA using polyclonal and monoclonal antibodies.

Given that PAb can recognize multiple epitopes, we chose a PAb against the PEDV N protein as the capture antibody to enhance the detection sensitivity. Horseradish peroxidase (HRP)-labeled MAb 6C12 was used as a detection antibody to ensure both specificity and sensitivity. The results showed that DAS-qELISA had a detection limit of 0.05 ng/mL for the rN protein, which is more sensitive than the 1 ng/mL limit previously reported by Fan et al. (2020). Additionally, DAS-qELISA exhibited a lower CV and better reproducibility. When optimizing the reaction conditions, although no statistically significant difference was observed in the P/N values of the different HRP-labeled antibody incubation times used, considering the time cost, a shorter time was chosen instead of a higher P/N for the same non-statistically different coating conditions. To better validate the performance of DAS-qELISA on clinical samples, we conducted an animal test using suckling piglets and collected anal swabs for concordance testing. In comparison to Fan et al. (2020), we not only compared these results to those obtained using RT-PCR but also included a comparison with commercial test strips, which adds to the credibility of DAS-qELISA. The results indicated that while the sensitivity of this DAS-qELISA was slightly lower than that of RT-PCR, it was consistent with that of the commercial test strips.

In summary, we expressed the PEDV N protein, prepared polyclonal and monoclonal antibodies against the N protein, and developed a sensitive, specific, reproducible, and reliable DAS-qELISA method for detecting PEDV antigens. These findings provide the necessary material support for practical research on PEDV, as well as an important diagnostic tool for clinical use that will help to control the spread of PEDV.

Author contribution SX and HZ designed the experiments. WH, ZM, and ZL wrote the manuscript. Clinical samples were collected by CC, CZ, ZS, and RF. YY and YL analyzed the samples by IFA, ELISA, and western blotting. WH, ZM, HT, and ZL analyzed the data. All authors read and approved the manuscript.

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Data availability Data are available; contact the author on request.

Declarations

Ethical approval All applicable international, national, and institutional guidelines for the care and use of animals were followed. All animal experimental procedures were reviewed and approved by the Animal Care and Use Committee of the Lanzhou Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (approval no. SYXK(Gan) 2015–0003). This article does not contain any studies with human participants performed by any of the authors.

Competing interests The authors declare no competing interests.

References

Cao L, Kong X, Zhang Y, Suo X, Li X, Duan Y, Yuan C, Zheng H, Wang Q (2023) Development of a novel double-antibody sandwich quantitative ELISA for detecting SADS-CoV infection. Appl Microbiol Biotechnol 107(7–8):2413–2422. https://doi.org/10.1007/s00253-023-12432-4

Chen H, Gill A, Dove BK, Emmett SR, Kemp CF, Ritchie MA, Dee M, Hiscox JA (2005) Mass spectroscopic characterization of the coronavirus infectious bronchitis virus nucleoprotein and elucidation of the role of phosphorylation in RNA binding by using surface plasmon resonance. J Virol 79(2):1164–1179. https://doi.org/10.1128/JVI.79.2.1164-1179.2005

Ding Z, Fang L, Jing H, Zeng S, Wang D, Liu L, Zhang H, Luo R, Chen H, Xiao S (2014) Porcine epidemic diarrhea virus nucleocapsid protein antagonizes beta interferon production by sequestering the interaction between IRF3 and TBK1. J Virol 88(16):8936–8945. https://doi.org/10.1128/JVI.00700-14

Duan H, Ma Z, Xu L, Zhang A, Li Z, Xiao S (2020) A novel intracellularly expressed NS5B-specific nanobody suppresses bovine viral diarrhea virus replication. Vet Microbiol 240:108449. https://doi.org/10.1016/j.vetmic.2019.108449

Fan B, Sun J, Zhu L, Zhou J, Zhao Y, Yu Z, Sun B, Guo R, He K, Li B (2020) Development of a novel double antibody sandwich quantitative enzyme-linked immunosorbent assay for detection of porcine epidemic diarrhea virus antigen. Front Vet Sci 7:540248. https://doi.org/10.3389/fvets.2020.540248

Gong X, Zhu M, Li G, Lu X, Wan Y (2016) Specific determination of influenza H7N2 virus based on biotinylated single-domain antibody from a phage-displayed library. Anal Biochem 500:66–72. https://doi.org/10.1016/j.ab.2015.09.020

Guo Y, Sui L, Kong D, Liu D, Gao Y, Jiang Y, Cui W, Li J, Li Y, Wang L (2024) Porcine epidemic diarrhea virus strain CH/HLJ/18 isolated in China: characterization and phylogenetic analysis. Virol J 21(1):28. https://doi.org/10.1186/s12985-023-02233-6

Ishikawa K, Sekiguchi H, Ogino T, Suzuki S (1997) Direct and rapid detection of porcine epidemic diarrhea virus by RT-PCR. J Virol Methods 69(1–2):191–195. https://doi.org/10.1016/s0166-0934(97)00157-2

Lee C (2016) Erratum to: Porcine epidemic diarrhea virus: an emerging and re-emerging epizootic swine virus. Virol J 13:19. https://doi.org/10.1186/s12985-016-0465-y

Li Z, Ma Z, Li Y, Gao S, Xiao S (2020) Porcine epidemic diarrhea virus: molecular mechanisms of attenuation and vaccines. Microb Pathog 149:104553. https://doi.org/10.1016/j.micpath.2020.104553

Li Z, Ma Z, Dong L, Yang T, Li Y, Jiao D, Han W, Zheng H, Xiao S (2022) Molecular mechanism of porcine epidemic diarrhea virus cell tropism. mBio 13(2):e0373921. https://doi.org/10.1128/mbio.03739-21

Li M, Pan Y, Xi Y, Wang M, Zeng Q (2023) Insights and progress on epidemic characteristics, genotyping, and preventive measures of PEDV in China: a review. Microb Pathog 181:106185. https://doi.org/10.1016/j.micpath.2023.106185

Lin H, Zhou H, Gao L, Li B, He K, Fan H (2018) Development and application of an indirect ELISA for the detection of antibodies to porcine epidemic diarrhea virus based on a recombinant spike protein. BMC Vet Res 14(1):243. https://doi.org/10.1186/s12917-018-1570-5

Liu G, Jiang Y, Opriessnig T, Gu K, Zhang H, Yang Z (2019) Detection and differentiation of five diarrhea related pig viruses utilizing a multiplex PCR assay. J Virol Methods 263:32–37. https://doi.org/10.1016/j.jviromet.2018.10.009

Liu G, Tiang MF, Ma S, Wei Z, Liang X, Sajab MS, Abdul PM, Zhou X, Ma Z, Ding G (2024) An alternative peptone preparation using *Hermetia illucens* (Black soldier fly) hydrolysis: process optimization and performance evaluation. PeerJ 12:e16995. https://doi.org/10.7717/peerj.16995

Lopez-Figueroa C, Cano E, Navarro N, Perez-Maillo M, Pujols J, Nunez J I, Vergara-Alert J, Segales J (2023) Clinical, pathological and virological outcomes of tissue-homogenate-derived and cell-adapted strains of porcine epidemic diarrhea virus (PEDV) in a neonatal pig model. Viruses 16(1). https://doi.org/10.3390/v16010044

Ma Z, Wang T, Li Z, Guo X, Tian Y, Li Y, Xiao S (2019) A novel biotinylated nanobody-based blocking ELISA for the rapid and sensitive clinical detection of porcine epidemic diarrhea virus. J Nanobiotechnology 17(1):96. https://doi.org/10.1186/s12951-019-0531-x

Ma Z, Bai G, Wang T, Li Z, Li Y, Xiao S, Li S (2021) Development of a blocking ELISA based on a single-domain antibody target the S1 protein of porcine epidemic diarrhea virus. Sheng Wu Gong Cheng Xue Bao 37(9):3221–3230. https://doi.org/10.13345/j.cjb.200789

Pensaert MB, de Bouck P (1978) A new coronavirus-like particle associated with diarrhea in swine. Arch Virol 58(3):243–247. https://doi.org/10.1007/BF01317606

Stevenson GW, Hoang H, Schwartz KJ, Burrough ER, Sun D, Madson D, Cooper VL, Pillatzki A, Gauger P, Schmitt BJ, Koster LG, Killian ML, Yoon KJ (2013) Emergence of porcine epidemic diarrhea virus in the United States: clinical signs, lesions, and viral genomic sequences. J Vet Diagn Invest 25(5):649–654. https://doi.org/10.1177/1040638713501675

Su Y, Hou Y, Prarat M, Zhang Y, Wang Q (2018) New variants of porcine epidemic diarrhea virus with large deletions in the spike protein, identified in the United States, 2016–2017. Arch Virol 163(9):2485–2489. https://doi.org/10.1007/s00705-018-3874-y

Su M, Shi D, Xing X, Qi S, Yang D, Zhang J, Han Y, Zhu Q, Sun H, Wang X, Wu H, Wang M, Wei S, Li C, Guo D, Feng L, Sun D (2021) Coronavirus porcine epidemic diarrhea virus nucleocapsid protein interacts with p53 to induce cell cycle arrest in S-phase and promotes viral replication. J Virol 95(16):e0018721. https://doi.org/10.1128/JVI.00187-21

Zhao ZP, Yang Z, Lin WD, Wang WY, Yang J, Jin WJ, Qin AJ (2016) The rate of co-infection for piglet diarrhea viruses in China and the genetic characterization of porcine epidemic diarrhea virus and porcine kobuvirus. Acta Virol 60(1):55–61. https://doi.org/10.4149/av-2016-01-55

Zhao Y, Fan B, Xue J, Guo R, Li J, Zhou J, Song X, Zhang X, Tao S, Li B (2023) Characterization and epitope mapping of monoclonal antibodies against PEDV N protein. Virology 579:29–37. https://doi.org/10.1016/j.virol.2022.12.011

Zhou X, Zhang T, Song D, Huang T, Peng Q, Chen Y, Li A, Zhang F, Wu Q, Ye Y, Tang Y (2017) Comparison and evaluation of conventional RT-PCR, SYBR green I and TaqMan real-time RT-PCR assays for the detection of porcine epidemic diarrhea virus. Mol Cell Probes 33:36–41. https://doi.org/10.1016/j.mcp.2017.02.002