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A competitive ELISA based on nanobodies for the detection of serum neutralizing antibodies against porcine epidemic diarrhea virus

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Abstract

Porcine epidemic diarrhea (PED), caused by porcine epidemic diarrhea virus (PEDV), can induce 80–100% mortality in newborn piglets; therefore, specific and rapid detection methods are important for the prevention of this viral infection. In particular, methods for detecting neutralizing antibodies (nAbs) can be used to evaluate the immunization effect of PEDV vaccines. The spike protein of PEDV (PEDV-S) has been universally used as an antigen to develop immunoassays to detect nAbs. Nanobodies (Nbs) offer advantages such as ease of genetic engineering and low production costs, making them promising for diagnostic applications. In this study, PEDV-S was expressed via the baculovirus system and was used as an antigen to immunize Bactrian camels. A total of 10 Nbs against PEDV-S were first screened and expressed as fusion proteins with horseradish peroxidase (HRP) in HEK293T cells. A Nb-HRP fusion protein named PEDV-S-Nb13-HRP was subsequently selected and used as a probe for developing a competitive enzyme-linked immunosorbent assay (cELISA) to detect anti-PEDV nAbs. Optimization assays identified 80 ng/well of PEDV-S as the optimal coating antigen concentration. The optimal dilution of PEDV-S-Nb13-HRP was 1:200, and the optimal serum dilution was 1:10. The cutoff value of cELISA was determined as 28.1%, demonstrating high specificity, repeatability, stability, and good agreement rates with two commercial ELISA kits (93.6%) and a serum neutralization test (96.34%). Additionally, the results of the detection of IgA antibodies in oral and milk samples from sows were in good agreement with those of the IDEXX PEDV IgA kit. These results demonstrate that the cELISA is a reliable and cost-effective method for detecting anti-PEDV nAbs.

Keywords Porcine epidemic diarrhea virus (PEDV), Neutralizing antibody, Spike protein, Nanobody, Competitive ELISA

Introduction

Porcine epidemic diarrhea (PED) is a highly contagious porcine enteroviral disease caused by porcine epidemic diarrhea virus (PEDV), which can infect pigs of all ages (Zhao et al. 2024). The mortality rate in neonatal piglets under 5 days of age can reach 100%, whereas infected older pigs usually exhibit milder symptoms with low mortality rates (Jung et al. 2020; Li et al. 2023a). Diarrhea, vomiting and dehydration are the typical symptoms

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of PED (Li et al. 2023b). PEDV was first identified in the United Kingdom in 1971 (Wood 1977) and then spread mainly to Asia and Europe (Chen et al. 2008; Debouck et al. 1980). Since 2013, a PEDV variant has quickly swept across most swine-producing countries, especially in Asia and North America, and causing significant economic losses (Chung et al. 2015; Mole 2013; Ojkic et al. 2015; Stevenson et al. 2013; Vlasova et al. 2014; Zhang et al. 2017). Currently, even though vaccines are available in some countries, PED is still a devastating disease that severely threatens the global pig industry (Yang et al. 2020; Yao et al. 2023).

As a member of the *Coronaviridae* family and the *Alphacoronavirus* genus, PEDV is a single-stranded enveloped RNA virus (Liu et al. 2024). Seven open reading frames are located in the viral genome, encoding several nonstructural replicase proteins and four major structural proteins (Lee 2015). As the main viral protein, the spike (S) protein of PEDV (PEDV-S) forms a coronal structure on the viral surface and faciliates viral membrane fusion, interacts with cell receptors and induces neutralizing antibodies (nAbs) in pigs (Chang et al. 2019a; Li et al. 2017, 2015; Sun et al. 2008). Thus, PEDV-S is an ideal target protein for vaccine development and an antigen for nAb detection (Oh et al. 2014; Song et al. 2012; Zang et al. 2020). Currently, diagnosis of PEDV infection in pigs involves the assays used include virus isolation, indirect immunofluorescence, real-time RT–PCR, virus neutralization tests and enzyme-linked immunosorbent assays (ELISAs) (Ma et al. 2019). Several ELISAs based on whole virus, nucleocapsid, and membrane proteins have been developed and commercialized to detect antibodies against PEDV (Fan et al. 2015; Lin et al. 2018). However, these ELISAs have been reported to cross-react with antibodies against other porcine coronaviruses and cannot detect nAbs (Chang et al. 2019b; Gimenez-Lirola et al. 2017). In contrast, cross-reaction with other porcine coronaviruses and could be used to detect nAbs in pig sera with approximately 80% agreement with a serum neutralization test (SNT) (Li 2015; Shan et al. 2022). However, these assays are usually based on polyclonal antibodies and monoclonal antibodies (mAbs), which require a pure antigen and horseradish peroxidase (HRP)-labeled mAbs or secondary antibodies. Thus, their production is expensive, technically demanding and time-consuming. Additionally, traditional antibodies are typically conjugated with alkaline phosphatase and horseradish peroxidase (HRP) in vitro via chemical methods (Shan et al. 2022), and the batch-to-batch variation in conjugation significantly impacts the stability and repeatability of these assays. These limitations hinder the universal application of these assays for detecting nAbs in pig sera.Nbs are derived from the variable region of heavy chain antibodies (VHHs) in camelids and are considered promising reagents for developing of immunoassays in disease diagnosis (Minatel et al. 2023). Compared with traditional antibodies, Nbs have several attractive features, including a small molecular weight, easy gene modification, and low production cost, and have been used to develop diagnostic immunoassays for diseases with promising market applications (Duan et al. 2021; Ji et al. 2020; Lu et al. 2020; Zhao et al. 2022). Moreover, Nbs can be fused with enzymes to maintain both antibody affinity and enzyme activity, which avoids the labeling of antibodies with enzymes in vitro*; thus*, Nbs may be considered novel biological molecules for immunoassays (Sheng et al. 2019; Zhu et al. 2024).

To address these limitations, this study aimed to develop a competitive ELISA (cELISA) using nanobody-HRP fusion proteins for specific detection of PEDV-neutralizing antibodies. Nbs against PEDV-S were first screened and then expressed as a fusion protein with HRP. One Nb-HRP fusion protein (Nb-HRP) was chosen to develop a competitive ELISA (cELISA) for the detection of anti-PEDV nAbs. Notably, the results of the cELISA were highly consistent with those of the SNT, commercial ELISA kits and IDEXX PEDV IgA kits. In addition, compared to available commercial ELISA kits, nanobody-HRP-based cELISAs are easier to perform and have a lower production costs. Therefore, the developed assay may be an ideal method for PEDV infection surveillance and for evaluating of the effectiveness of PEDV vaccines.

Results

Construction of a phage display VHH library from the immunized Bactrian came

SDS–PAGE analysis revealed that PEDV-S was successfully expressed by the baculovirus system, which was purified with the expected size of approximately 180 kDa (Fig. 1A). After six immunizations with an inactivated PEDV vaccine, the titer of antibodies against PEDV-S in the immunized camel reached 1:256,000 (Fig. 1B), indicating that the camel can be induced to strong immune responses to the inactivated PEDV vaccine.

The *VHH* genes, with an expected size of approximately 400 bp, were successfully amplified through two rounds of PCR from peripheral blood mononuclear cells (PBMCs) (Fig. 2A). After constructing the phage display library, colony PCR revealed that the positive rate of this library was 96% (Fig. 2B). Moreover, the phage display VHH library was contained approximately 3.8×10^8 individual clones. Sequence analysis of 48 random clones revealed that each contained a distinct VHH sequence, indicating a high level of library diversity (Fig. 2C).

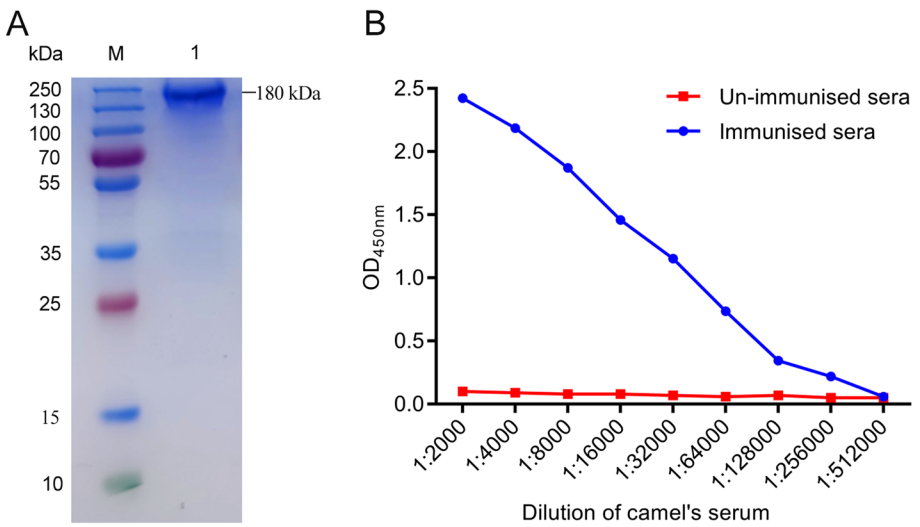

Fig. 1 Determination of purified recombinant PEDV-S and titers of anti-PEDV-S antibodies in the immunized camel. **A** SDS–PAGE analysis of recombinant PEDV-S expressed by the baculovirus system. M, protein molecular weight marker; 1, purified recombinant PEDV-S. **B** Titers of anti-PEDV-S antibodies in the sera from a camel immunized with the inactivated PEDV vaccine

Screening for specific Nbs against PEDV-S

After three rounds of panning, the phages carrying VHH genes against PEDV-S were strongly enriched (Table 1). The results of indirect ELISA with the periplasmic extract as the primary antibody indicated that 36 colonies bound specifically to PEDV-S (Fig. 3A). From the 36 colonies, 10 sequences were obtained and named PEDV-S-Nb6, 13, 25, 41, 45, 49, 58, 61, 79 and 86 (Fig. 3B). The titers of the 10 periplasmic extracts revealed that most of them reached 1:100 (Fig. 3C).

Production of Nb-HRP fusion proteins against PEDV-S

Sequencing results revealed that the recombinant plasmids containing the genes encoding the 10 Nbs fused with HRP were successfully constructed (data not shown). After HEK293T cells were transfected with the plasmids, the immunofluorescence assay (IFA) results revealed that the 10 Nb-HRP fusion proteins against PEDV-S were successfully expressed (Fig. 4A). Additionally, the results of direct ELISA using the supernatants from the transfected HEK293T cells as a primary antibody revealed that the 10 Nb-HRP fusion proteins exhibited secretory expression (Fig. 4B). When the 10 Nb-HRP fusion proteins were used as primary antibodies, the IFA results revealed that all the proteins could bind to PEDV-S in the PEDV-infected Vero cells (Fig. 4C). Additionally, a neutralization test with these Nbs-HRP was also performed. The results revealed that all the PEDV-S-Nbs, except for PEDV-S-Nb86-HRP, had neutralizing activity. The neutralizing titers of PEDV-S-Nb6, PEDV-S-Nb25, PEDV-S-Nb61 and PEDV-S-Nb79-HRP were as high as 1:8, those of PEDV-S-Nb45 and PEDV-S-Nb58-HRP were 1:16, and those of PEDV-S-Nb13, PEDV-S-Nb41, and PEDV-S-Nb49-HRP were the highest at 1:32 (Fig. 4D).

Optimization conditions of the developed cELISA

The results of cELISA revealed that the P/N value was the smallest when the assay used PEDV-S-Nb13-HRP as the competing reagent (Fig. 4E). Therefore, PEDV-S-Nb13-HRP was chosen to develop a cELISA for the detection of anti-PEDV antibodies. As a coating antigen, the optimized amount of PEDV-S was 80 ng/well, and the optimized dilution of PEDV-S-Nb13-HRP was 1:200 (200 μL/well) (Table 2). The best dilution of tested pig sera for the cELISA was 1:10 (Table 3). The incubation time of the mixture containing pig sera and PEDV-S-Nb13-HRP with PEDV-S was 60 min, and color development time was 15 min (Table 4).

Cutoff value of the developed cELISA

The average percentage inhibition (PI) value obtained from 176 negative pig serum samples was 5.6%, with a standard deviation of 7.5%. The cutoff value was subsequently set at 28.1% (5.6% + 3 × 7.5%). This indicates that pig sera with a PI value at or greater than 28.1% would be considered positive for anti-PEDV antibodies; sera below this threshold would be considered negative.

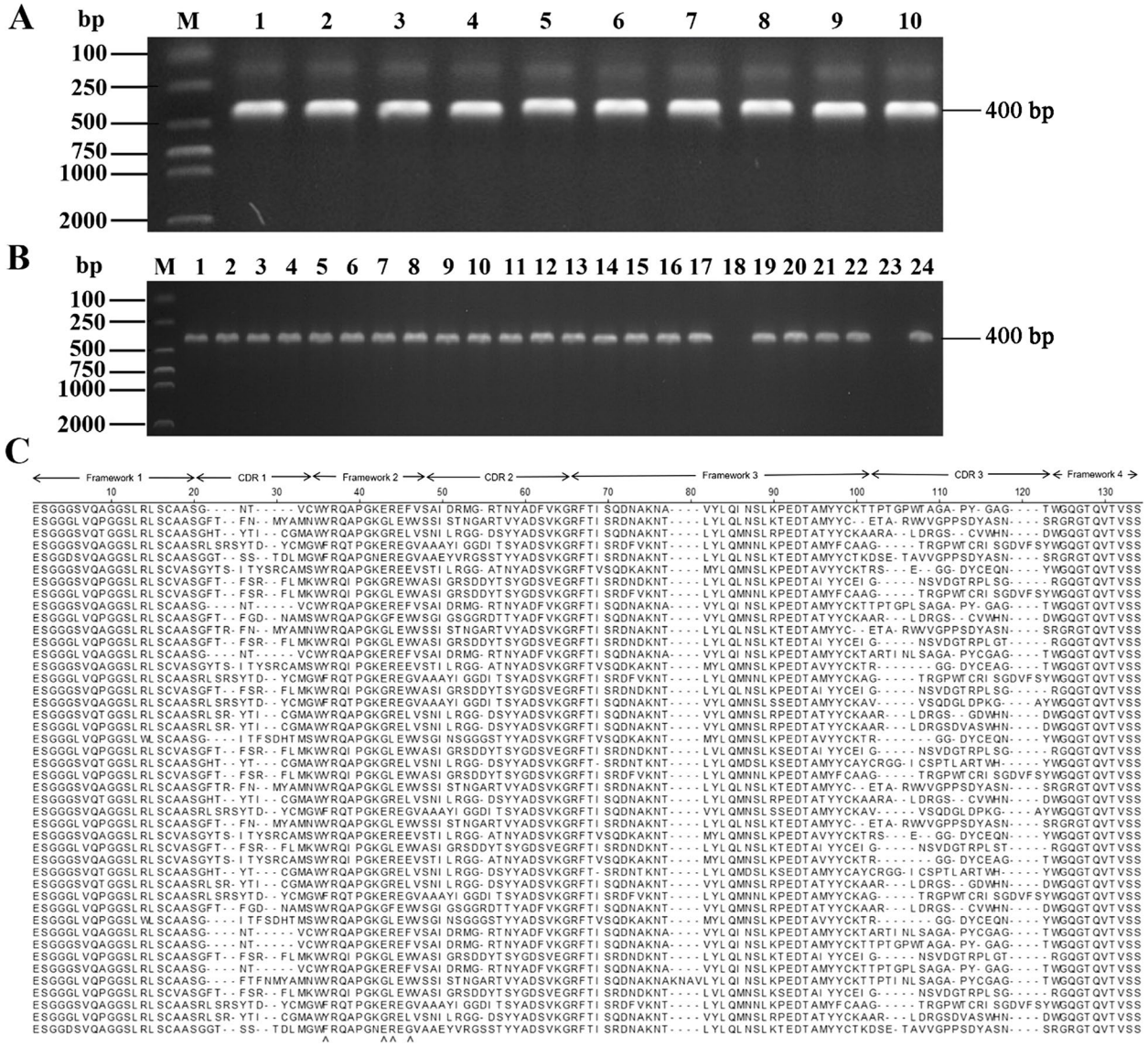

Fig. 2 Construction and identification of a phage display VHH library. **A** The VHH genes (approximately 400 bp) were amplified via PCR using the total RNA extracted from the PBMCs as a template. M, DNA Marker 2000; 1–10, different RNA extracted from the PBMC samples. **B** Evaluation of the correct insertion rate of VHH genes into the pMECS vector via PCR. M, DNA Marker 2000; 1–24, different clones. **C** The VHH library presented a distinct VHH sequence and good diversity. The residues at positions 37, 44, 45 and 47 are indicated by red arrows, and hydrophilic amino acids are features of nanobodies

Table 1 Enrichment of specific phage particles against PEDV-S protein during three rounds of panning

Round of panning	Input (PFU/well)	P output (PFU/well)	N output (PFU/well)	Recovery (P/input)	P/N
1st round	5×10^{10}	3×10^{2}	7×10^{2}	6×10^{-9}	0.42
2nd round	5×10^{10}	1.1×10^{6}	5×10^{3}	2.2×10^{-5}	2.2×10^{2}
3rd round	5×10^{10}	1×10^{7}	1.2×10^{4}	2×10^{-4}	8.3×10^{2}

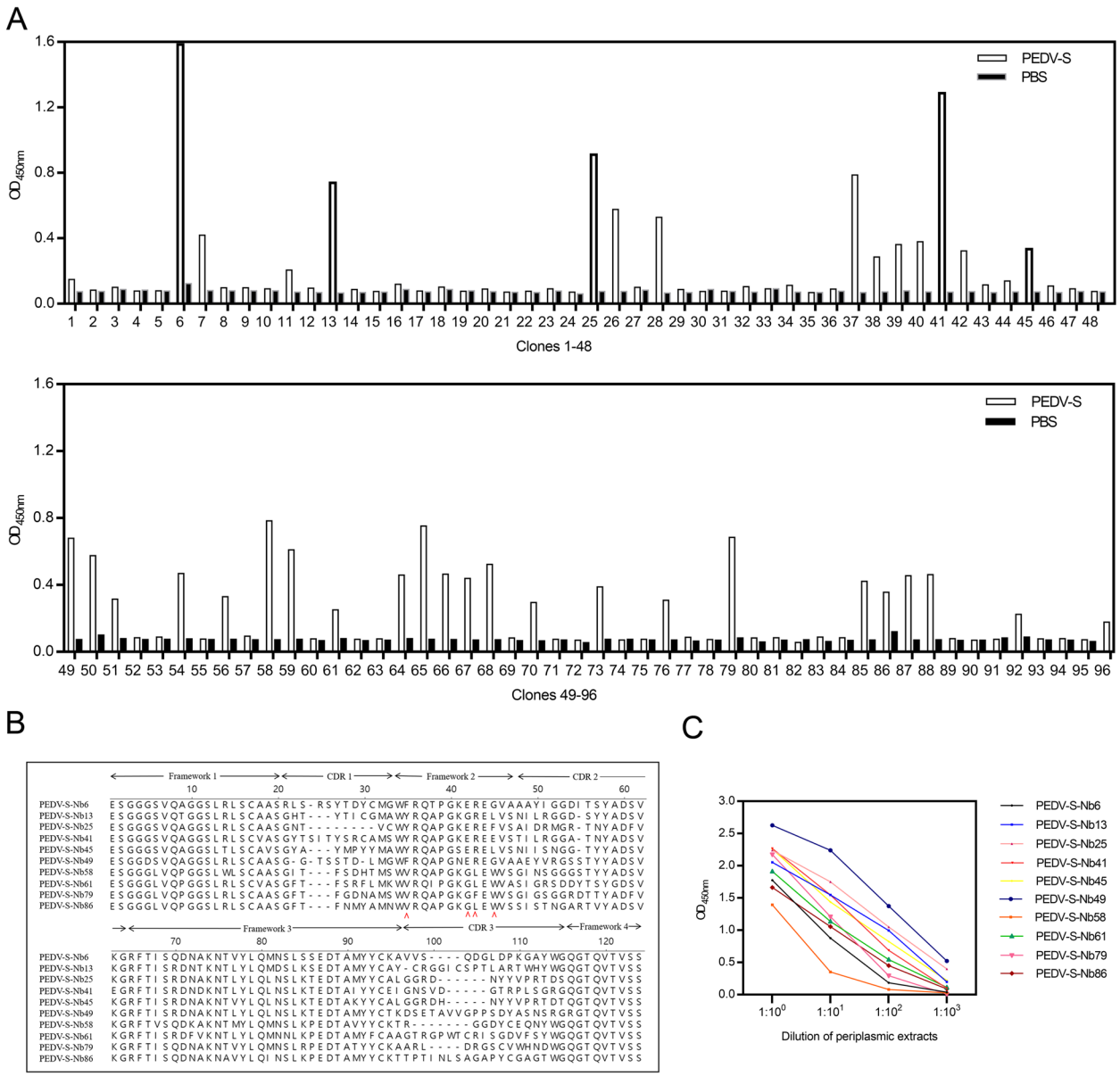

Fig. 3 Screening and identification of nanobodies (Nbs) against PEDV-S. **A** Detection of the periplasmic extracts from the specific clones that bind to PEDV-S via indirect ELISA with PEDV-S as the coating antigen. **B** Amino acid alignment of 10 screened (Nbs). The residues at positions 37, 44, 45 and 47 are indicated by red arrows, and hydrophilic amino acids are features of (Nbs). **C** Titers of the 10 (Nbs) in the periplasmic extracts that bind to PEDV-S

Specificity, reproducibility and stability of the developed cELISA

Based on the results of the established cELISA, the PI values of the positive pig sera for antibodies against other porcine viruses ranged from 2–24% (Fig. 5A), indicating high specificity of the assay. Additionally, after positive pig sera from pigs challenged with PEDV G1 and PEDV G2 were used for detection via cELISA, the results showed that the developed cELISA could detect antibodies against different PEDV genotypes (Fig. 5A). In addition, detection result of 55 positive pig sera containing nAbs and 22 positive pig sera without nAbs were positive, with PI values ranging from 42–80%, whereas 22 positive pig sera without nAbs were negative (Fig. 5A). Thus, the specificity of detecting nAbs via cELISA was 100%.

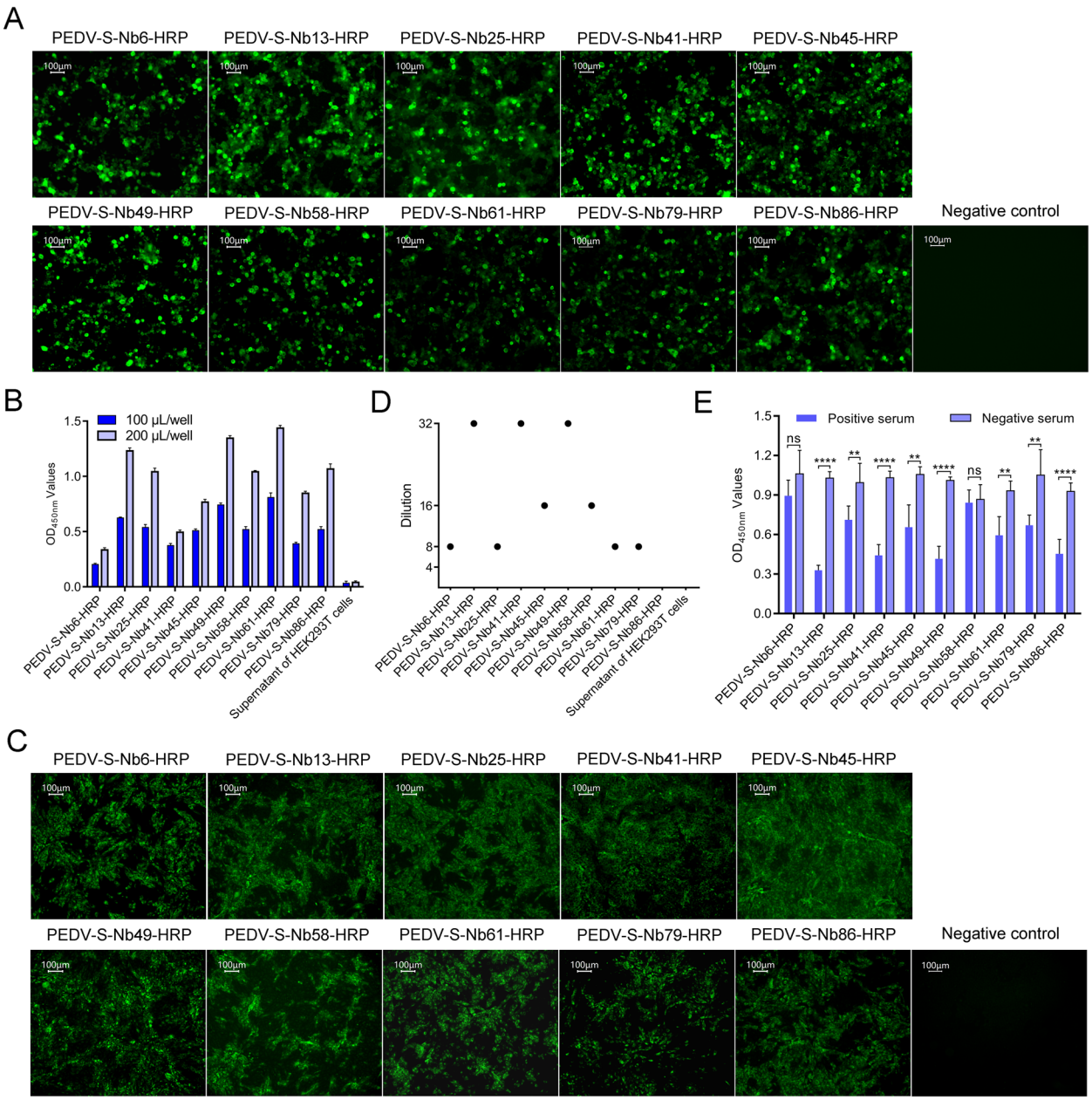

Fig. 4 Characterization of the 10 PEDV-S-Nb-HRP fusion proteins expressed in HEK293T cells. **A** Identification of 10 PEDV-S-Nb-HRP fusion proteins expressed in HEK293T cells by IFA using an anti-HA mAb as the primary antibody and a FITC-labeled goat anti-mouse as the secondary antibody. **B** The 10 PEDV-S-Nb-HRP fusion proteins in the culture medium of HEK293T cells directly reacting with TMB, as detected by direct ELISA. **C** The binding of the 10 PEDV-S-Nb-HRP fusion proteins to the PEDV-infected Vero cells, as detected by IFA using the fusion proteins as the primary antibodies. **D** The 10 PEDV-S-Nb-HRP fusion proteins, with the exception of PEDV-S-Nb86-HRP, had neutralizing activity, as detected by a nanobody neutralization test. **E** The 10 PEDV-S-Nb-HRP fusion proteins were blocked from binding to PEDV-S by positive pig sera for the anti-PEDV antibody. Significant differences were analyzed with a *t* test and are marked with asterisks and "ns": ****$P < 0.0001$; ** $P < 0.01$

The five positive and five negative pig serum samples for the anti-PEDV antibody were tested three times within a plate, and the intra-assay coefficient of variation (CV) of the PI values ranged from 1.01 to 5.79%, with a mean value of 3.13%. When these samples were tested on three plates at different times, the interbatch CV of the PI values ranged from 1.12 to 4.23%, with a mean value of 3.05%. These results indicated good reproducibility of the cELISA.

Table 2 Optimized amount of PEDV-S protein and dilution of PEDV-S-Nb13-HRP fusion protein

PEDV-S protein (ng/well)	Dilutions of PEDV-S-Nb13-HRP			
	1:1	1:10	1:100	1:200
80	1.99	1.852	1.272	**1.012**
100	2.286	2.213	1.512	1.111
200	2.456	1.952	1.585	1.344
400	2.123	2.203	1.799	1.346

To evaluate the stability of the cELISA, vacuum-dried plates and Nb-HRP fusion solutions from different storage times were used to perform direct ELISA and cELISA. The results of the two ELISAs revealed that the OD_{450nm} values at different months ranged from 0.89 to 1.13 (CV = 8.60%) (Fig. 5B), with a good competitive effect for 6 months (CV = 9.31%) (Fig. 5C), indicating that the PEDV-S-coated plates and PEDV-S-Nb13-HRP solutions stored at 4°C.

Comparison between the developed cELISA and commercial ELISA kits

A total of 2,238 clinical pig serum samples were tested via the developed cELISA and two commercial ELISA kits, and the positive rates were 41.8% (936/2,238) and 48.3% (1,080/2,238), respectively. The results of the two commercial kits were in 100% agreement. The agreement between the cELISA and commercial ELISA kits was 93.6%. In addition, the statistical analysis revealed that there were no significant differences (Kappa value > 0.4) between the cELISA and the two commercial ELISA kits (Kappa value = 0.874).

For the 232 oral and 132 milk samples, the results of cELISA revealed that the positive rates were 22.0% (51/232) and 24.2% (32/132), respectively. The results of the IDEXX PEDV IgA kit revealed that the positive rates were independently 20.3% (47/232) and 18.9% (25/132). The agreement between the developed cELISA and the IDEXX PEDV IgA kit was 96.6% for the oral samples, and 89.9% for the milk samples.

Correlation of the developed cELISA with an SNT

A total of 164 pig serum samples were positive for SNT, and 85 were positive for anti-PEDV nAbs, with titers ranging from 1:4 to 1:32 (Table 5). When detected with the cELISA, 79 samples were also positive, and the five negative samples that were positive according to the SNT

Table 4 Optimized incubation and color reaction time of the pig sera and PEDV-S-Nb13-HRP fusion protein

Times of color reaction (min)	Sera type	Incubation times of pig sera, ASFV-Nb75-HRP fusions and coated antigens (min)		
		30	40	60
10	Positive	0.589	0.650	0.418
	Negative	1.300	1.489	1.226
	P/N	0.453	0.437	0.341
15	Positive	0.554	0.594	0.220
	Negative	1.300	1.489	1.226
	P/N	0.426	0.399	**0.179**

Table 3 Optimized dilution of pig sera tested by the developed cELISA

No. serum	Sera type	Different dilutions of pig sera				
		1:10	1:20	1:40	1:80	1:160
1	Positive	0.128	0.199	0.320	0.562	0.689
	Negative	1.134	1.123	1.12	1.119	1.115
	P/N	**0.113**	0.177	0.286	0.502	0.618
2	Positive	0.323	0.413	0.687	0.907	1.005
	Negative	1.231	1.229	1.204	1.194	1.191
	P/N	0.262	0.336	0.571	0.760	0.844
3	Positive	0.135	0.165	0.257	0.443	0.566
	Negative	1.031	1.024	1.018	1.021	1.014
	P/N	0.131	0.161	0.252	0.434	0.558
4	Positive	0.264	0.278	0.397	0.523	0.704
	Negative	1.065	1.054	1.058	1.061	1.058
	P/N	0.248	0.264	0.375	0.493	0.665
5	Positive	0.424	0.528	0.653	0.928	1.070
	Negative	1.047	1.134	1.134	1.134	1.134
	P/N	0.405	0.466	0.576	0.818	0.944

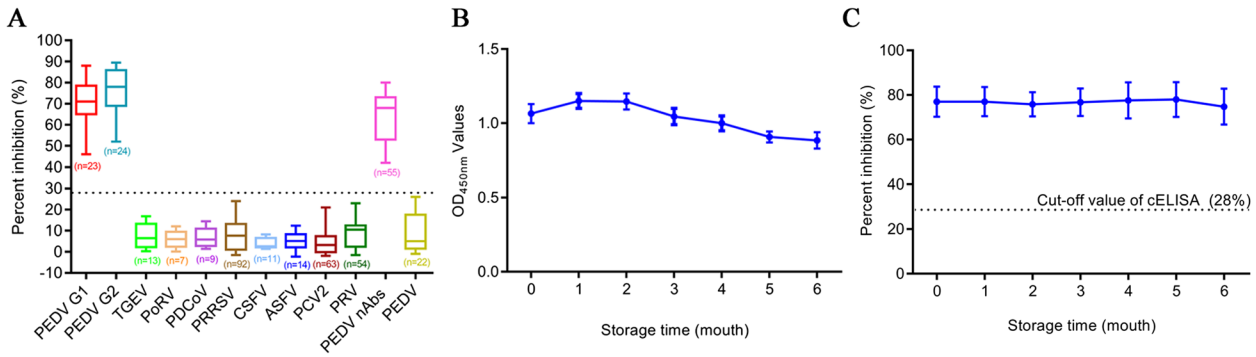

Fig. 5 Specificity and stability of the developed cELISA. **A** Detection of antibodies against pig viruses, including PEDV G1, PEDV G2, TGEV, PoRV, PDCoV, CSFV, ASFV, PRRSV, PCV2 and PRV, and pig sera containing and not containing nAbs against PEDV via the developed cELISA. **B** Stability analysis of the PEDV-S-Nb13-HRP fusion protein and coated plates stored at 4°C for different durations via direct ELISA. **C** Stability analysis of the developed cELISA to detect anti-PEDV antibodies in pig sera

Table 5 Agreement between serum neutralization test (SNT) and the developed cELISA

Serum neutralization test	Number	cELISA		Agreement (%)	Kappa value
		+	-		
-	79	0	79	96.34	0.883
1:4	11	6	5		
1:8	30	29	1		
1:16	24	24	0		
1:32	20	20	0		

The Kappa value > 0.4 was regarded as no significant difference

had low SN titers of 1:4 and 1:8. The agreement between the cELISA and the SNT was 96.34% (Table 5), suggesting that the cELISA might be used to evaluate PEDV nAbs in pig sera.

Discussion

Nbs exhibit distinct advantages over traditional polyclonal and monoclonal antibodies, including small molecules genetically easy to engineer and express in vitro (Arbabi-Ghahroudi 2017; Greenberg et al. 1995). Nbs have been widely applied as tools in the research of protein function, diagnostic reagents and pharmaceutical drugs in place of conventional antibodies (Beghein et al. 2017; Wilken et al. 2018). For example, Nbs with enzymatic molecules as tags, such as HRP, can reduce the complex processes needed to couple two biological molecules together in vitro and can reduce the risk of exposure to dangerous chemicals. Therefore, many Nbs against different antigens, including bacteria, viruses and host proteins, have been produced (Abbady et al. 2011; Qasemi et al. 2016; Sheng et al. 2019). In this study, 10 Nbs against PEDV-S were first screened and shown to specifically recognize PEDV-infected Vero cells via IFA (Fig. 4). These Nbs may be ideal tools for investigating the biological function of PEDV-S.

Monitoring antibodies in sow herds is considered useful for developing preventative strategies against PEDV infection in piglets (Wang et al. 2022). SNTs are widely used to detect anti-PEDV nAbs in pig sera. However, the operating processes of SNT are laborious, complicated, expensive, and time-consuming (Oh et al. 2005). Therefore, many attempts have been made to develop simpler, faster, and less expensive methods that may replace SNTs. For example, indirect ELISAs using complete and truncated S proteins of PEDV as a coating antigen were developed to detect anti-PEDV nAbs in pig sera (Wang et al. 2022). However, owing to the specificity and sensitivity of these indirect ELISAs, none of these methods have been commercialized. However, the PEDV S protein plays a crucial role in specific receptor binding and cell membrane fusion (Kang et al. 2021; Li et al. 2021). The S protein can recognize cellular receptors through precise interactions and mediate the production of virus-neutralizing antibodies (Bosch et al. 2003). Therefore, it represents an ideal target for the development of vaccines and an ideal antigen for nAb detection (Huang et al. 2022). In this study, the S protein was also selected as the coating antigen to establish the cELISA, which was the same as the indirect ELISA for detecting the nAb in pig sera. Recently, several cELISAs using Nb-HRP fusion proteins as reagents have been developed to detect antibodies in the serum, which are apparently better than indirect ELISAs (Duan et al. 2021; Ji et al. 2020; Lu et al. 2020; Zhao et al. 2022). To date, few reports exist regarding the use of Nb-HRP-based cELISAs to detect anti-PEDV nAbs in pig sera. In the present study, we developed and evaluated a Nb-HRP fusion protein-based cELISA for detecting anti-PEDV antibodies in pig sera. Compared with SNT,

cELISA showed an agreement of 96.34% and may have the potential to replace SNTs (Table 5). However, further analysis suggested that the results of the cELISA were not fully correlated with the SNT when the SN titers were low. Further work is needed to improve the correlation between the two methods.

In this study, when positive pig sera were used as competitive antibodies, 8 of the 10 Nbs were found to competitively bind to the recombinant PEDV-S. Among them, PEDV-S-Nb13 was selected as the best competing antibody for developing a cELISA to detect the anti-PEDV antibody. The developed nanobody-based cELISA was evaluated for its specificity, sensitivity, and cross-reactivity. The assay demonstrated exceptional specificity and sensitivity, with no cross-reactivity observed against pig sera positive for TGEV, PoRV, PDCoV, PRRSV, CSFV, ASFV, PCV2 or PRV. Additionally, intra-assay comparison and stability tests confirmed the high reproducibility and stability of the cELISA. Importantly, the results of the cELISA were in reasonable correlation with those of two commercial ELISA kits for detecting anti-PEDV IgG and in reasonable agreement in detecting anti-PEDV nAbs with an SNT. Notably, the results of the cELISA also agreed well with those of the IDEXX PEDV IgA kit for detecting anti-PEDV IgA in oral and milk samples. However, the results of the present study also showed that cELISA failed to quantify SN titers. Given the neutralization effect of serum antibodies, which may be the result of these antibodies binding to different epitopes of the S protein, it is not surprising that a cELISA using a single Nb-HRP molecule was not completely correlated with the SNT to titrate the nAbs. Multiple Nb–HRP fusion proteins may be required in a cELISA to establish such a correlation with an SNT. However, cELISA has the potential to be easily adapted for the surveillance of PEDV infection.

At present, many Nb-HRP fusion protein-based cELISAs have been established for detecting antibodies against different viruses, including influenza virus, African swine fever virus (ASFV), porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), porcine parvovirus (PPV), and Newcastle disease virus (NDV) (Duan et al. 2021; Ji et al. 2020; Lu et al. 2020; Zhao et al. 2022). These cELISAs can be performed in only one step, in which the tested serum samples are mixed with the fusion proteins in the coated plates. In the present study, the developed cELISA for detecting anti-PEDV nAbs was also a one-step operation after the plates were coated and blocked. Additionally, the stored coated plates and Nb-HRP fusion protein were shown to have good stability for 6 months (Fig. 5). These results indicate that cELISA may be a promising candidate for further development of a commercial ELISA kit.

Although the developed cELISA is simple to perform, has a low cost of production, and has good sensitivity and specificity, the established process of cELISA is highly complex, and a dedicated technician is required for its operation. However, notably, after the cELISA was successfully established, its production and clinical application were convenient. In addition, nanobody-HRP fusion proteins can be produced on a large scale in vitro, and cELISA does not require enzyme-labeled secondary antibodies; it is simple to perform, less time-consuming, low-cost, and easy to use in clinical practice.

Conclusion

In conclusion, 10 Nbs against PEDV-S were screened in this study, and the 8 Nbs were found to competitively bind to the recombinant PEDV-S protein when positive pig sera were used as competition antibodies. Using PEDV-S-Nb13-HRP as a competing reagent, a cELISA was developed for testing anti-PEDV nAbs in pig sera. The cELISA results were highly specific and consistent with those of the SNT, commercial ELISA and IDEXX PEDV IgA kits, which suggested that the cELISA may be an ideal method for detecting anti-PEDV nAbs and evaluating the immune status of sows.

Methods

Cells, viruses, proteins, and plasmids

HEK293T and Vero cells from the American Type Culture Collection (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Life Technologies Corp., USA) supplemented with 10% fetal bovine serum (Gibco, USA). The PEDV variant strain HNXP, which belongs to the genotype 2 group (GenBank No. MT787025), was propagated in Vero cells. The full-length gene encoding PEDV-S was amplified by PCR from the HNXP strain and subsequently inserted into a baculovirus expression vector (pFastBac1, Thermo Fisher Scientific, USA) using primers pairs (PEDV-S-Forward: 5'-TGGTAAGTTGCTAGTGCGTAATA-3' and PEDV-S-Reverse: 5'-AGCACAGCAGCAGCCGCAGC-3') (Li et al. 2022). Then, the PEDV-S with His tag was expressed via the baculovirus expression vector system (Bac-to-Bac Baculovirus Expression System, Thermo Fisher Scientific, USA) and performed by Guangzhou Qianxun Biotechnology Co., Ltd., China. The proteins were purified with Ni NTA Beads 6FF (SMART, Changzhou, China) via His tag. The purity of the protein was confirmed by SDS–PAGE. The pMECS vector with HA tags for constructing the VHH library was kindly provided by Prof. Muyldermans (Vincke et al. 2012). The plasmid for expressing Nb-HRP fusion proteins was constructed with the pCMV-N1-HRP vector (Clontech, Japan) as previously described (Sheng et al. 2019).

Serum samples

A total of 176 pig serum samples negative for anti-PEDV antibody and pig serum samples positive for antibodies against transmissible gastroenteritis virus (TGEV; $n=13$), porcine reproductive and respiratory syndrome virus (PRRSV; $n=92$), porcine rotavirus (PoRV; $n=7$), classical swine fever virus (CSFV; $n=11$), porcine delta coronavirus (PDCoV; $n=9$), African swine fever virus (ASFV; $n=14$), porcine circovirus type 2 (PCV2; $n=63$) and pseudorabies virus (PRV; $n=54$) were confirmed by commercial ELISA kits, western blot analysis or indirect immunofluorescence assay (IFA), and stored in our laboratory. Additionally, 23 positive pig serum samples from pigs challenged with genotype I PEDV (PEDV G1) and 24 positive pig serum samples from pigs challenged with genotype 2 PEDV (PEDV G2) were also stored in our laboratory and used to determine the ability of cELISA to detect antibodies against different PEDV genotypes. A total of 55 positive pig sera for antibodies against PEDV were confirmed to contain nAbs by SNT and were used to evaluate nAb detection via cELISA (Fig. S1). Moreover, 22 positive pig sera samples for antibodies against PEDV without nAbs were also used to evaluate the specificity of cELISA (Fig. S1). These pig sera were also confirmed by SNT and were first attached to the PEDV to ensure that there were no nAbs in these pig sera. To evaluate the consistency of the three methods, a total of 2,238 pig serum samples from 12 pig farms were tested via the developed cELISA and two commercial ELISA kits (NECVB, Heilongjiang, China; ID. VET, France). Additionally, 232 oral fluid samples and 132 milk samples from sows from three pig farms were tested via cELISA and an IDEXX PEDV IgA kit (IDEXX Laboratories, Inc., Maine, United States) to evaluate the ability of the assay to detect anti-PEDV IgA. To evaluate whether the assay could detect nAbs, 164 pig serum samples were also tested with the developed cELISA and an SNT.

Bactrian camel immunization and library construction

The Bactrian camel was immunized as previously described with some modifications (Zhao et al. 2022). Briefly, a camel was subcutaneously immunized with an inactivated PEDV vaccine (TECON Biopharmaceutical Co. Ltd., China). The titer of antibodies against PEDV-S in the immunized camel was detected via indirect ELISA with purified PEDV-S as the coating antigen. Anticoagulated blood samples were collected, and the PBMCs were separated. Total RNA was extracted from lymphocytes via the Neasy® Plus Mini RNA extraction kit (QIAGEN, Germany) according to the manufacturer's instructions. cDNA was synthesized, and VHH genes were amplified via nested PCR with paired primers (Table 6). The amplified genes were digested, ligated into the pMECS vector, and electrotransformed into *Escherichia coli* TG1 competent cells. Colonies were scraped from the plates, and the insertion rate was confirmed via PCR via primers (Table 6). The positive colonies were then randomly selected and sequenced to evaluate the positive rate and diversity of the library as previously reported (Zhao et al. 2022).

Screening and identification of Nbs against PEDV-S

To select Nbs targeting PEDV-S from the library, three rounds of phage rescue and biological screening were performed as previously described (Zhao et al. 2022). All of the positive colonies containing VHH genes were sequenced and grouped according to their complementary determining region 3 sequences.

Expression of the Nb-HRP fusion protein in HEK293T cells

Some previous studies have shown that Nb-HRP fusion proteins can reduce complex processes by coupling the two molecules together in vitro (Yamagata et al. 2018). In addition, the use of Nb-HRP fusion protein-based immunoassays can eliminate the use of secondary antibodies. Therefore, Nb-HRP fusion proteins were produced to develop a cELISA to detect anti-PEDV antibodies on the basis of previous methods (Duan et al. 2021; Ji et al. 2020; Lu et al. 2020; Zhao et al. 2022). Briefly, the VHH genes were inserted into the pCMV-N1-HRP vector. After sequencing, the positive plasmids were transfected into HEK293T cells. The expression of the Nb-HRP fusion protein against PEDV-S was detected by IFA and direct ELISA with PEDV-S as the coating antigen. In addition, IFA was performed to demonstrate that the Nb-HRP fusion protein could bind to the virus in PEDV-infected cells.

Nanobody neutralization test

The nanobody neutralization test was performed as described in a previous study with some modifications (Paudel et al. 2014). Briefly, the Nb-HRP fusion proteins were serially diluted twofold, and the neutralization titer was defined as the reciprocal of the highest dilution that completely neutralized the virus. The positive threshold was set at 1:4.

Table 6 Primers used in this study

Primers	Sequences (5′–3′)
CALL001	GTCCTGGCTGCTCTTCTACAAGG
CALL002	GGTACGTGCTGTTGAACTGTTCC
VHH-F	CATGTGCAGCTGCAGGAGTCTGGRGGAGG
VHH-R	CTAGTGCGGCCGCTGAGGAGACGGTGACCTGGGT
MP57	TTATGCTTCCGGCTCGTATG
GIII	CCACAGACAGCCCTCATAG

Restriction sites are underlined. VHH, heavy chain antibody variable region

Optimization conditions of the developed cELISA

To select the optimal Nb-HRP fusion protein for developing the cELISA, five positive and five negative pig serum samples for anti-PEDV antibodies were used to block the different Nb-HRP fusion proteins from binding to the PEDV-S protein in the cELISA.

Direct ELISA was used to optimize the amount of coating antigen and the dilution of the Nb-HRP fusion protein for cELISA by checkerboard titration. The amounts of PEDV-S used as coating antigens were 80, 100, 200 and 400 ng/well, and the dilutions of the Nb-HRP fusion protein were 1:1, 1:10, 1:100 and 1:200. The best conditions were determined when the OD_{450nm} of direct ELISA was approximately 1.0.

To select the best dilution of pig serum for testing, five positive and five negative samples were detected via cELISA. The dilutions of pig sera used were 1:10, 1:20, 1:40, 1:80, 1:160 and 1:320. Notably, the optimal dilution was determined when the P/N value of cELISA was the smallest.

The incubation time of the mixture, including the pig sera and Nb-HRP fusion protein with the coating antigen, and the time of the colorimetric assay following the addition of tetramethylbenzidine (TMB) were also optimized by checkerboard titration via cELISA. The optimized times were also determined when the P/N was at its minimum.

After the aforementioned optimization, the cELISA procedure was performed as follows. A 96-well ELISA plate (Nunc-Immunol Plate Master, Inc.) was coated with the optimized amount of PEDV-S in 0.01 M PBS (pH 7.4; 100 μL/well) and incubated overnight at 4 °C. After washing five times with PBS-0.05% Tween-20 (PBST, 300 μL/well), the plate was blocked with blocking buffer (PBST containing 2.5% nonfat dry milk) for 1 h at room temperature (RT). After washing five times, the mixture (100 μL/well) containing the diluted test pig sera and Nb-HRP fusion protein was added to the plate. After incubation for 1 h at 37°C, the plate was washed five times, and TMB (100 μL/well) was added and incubated in the dark for the optimized time at RT. The color reaction was stopped by the addition of 50 μL 3 M H_2SO_4, and the OD_{450nm} value was tested via an automated ELISA plate reader (Bio-Rad, USA).

Determination of the cutoff value for the cELISA

A total of 176 pig serum samples negative for the anti-PEDV antibody were tested to determine the cutoff value of the developed cELISA. The percentage inhibition (PI) of the cELISA was calculated as previously described (Zhao et al. 2022).

Evaluation of the specificity, reproducibility and stability of the cELISA

The specificity of the cELISA was evaluated by detecting sera from pigs infected with other pathogens, including TGEV, PoRV, PDCoV, CSFV, ASFV, PRRSV, PCV2 and PRV. Additionally, positive pig sera for antibodies against PEDV G1 and G2 were used to determine the ability of cELISA to detect antibodies against different PEDV genotypes. Furthermore, to assess the specificity of detecting anti-PEDV nAbs via cELISA, 55 pig sera containing nAbs against PEDV and 22 positive pig sera containing nAbs against PEDV without nAbs were also tested via the developed cELISA.

Each of the five pig serum samples that were positive and negative for the anti-PEDV antibody were detected to evaluate the reproducibility of the developed cELISA. Each sample was tested within a plate for three replicates to determine the intra-assay variation and among the different plates to determine the interassay variation. The coefficient of variation (CV) was subsequently calculated to analyze the reproducibility of the cELISA.

To evaluate the stability of the developed cELISA, 96-well plates were coated with the purified PEDV-S protein and blocked with blocking buffer. The samples were subsequently dried, vacuum-packed and stored at 4°C. Additionally, the Nb-HRP fusion proteins were stored at 4°C. The plates and fusion proteins were used at 0, 1, 2, 3, 4, 5 and 6 months poststorage to perform cELISA and direct ELISA.

Comparisons of the developed cELISA with commercial ELISA kits

A total of 2,238 clinical pig serum samples were tested via the developed cELISA and two commercial ELISA kits, including the NEVCB PEDV IgG kit (NEVCB, Heilongjiang, China) and the ID Screen® PEDV Indirect ELISA (ID. VET, France). Additionally, 232 oral fluid samples and 132 milk samples were tested for anti-PEDV IgA with the developed cELISA and the IDEXX PEDV IgA kit (IDEXX, USA). The agreement rates between the cELISA and commercial ELISA kits were subsequently calculated via Microsoft Excel 2019 (Microsoft, Redmond, Washington, USA).

Consistency between the developed cELISA and SNT

A total of 164 pig serum samples were tested with the developed cELISA and an SNT. On the basis of the PI values of the developed cELISA and the titers of the SNTs, the agreement of the two assays was calculated via the Microsoft Excel program.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-025-00161-2.

Supplementary Material 1: Fig. S1. The 55 positive and 22 negative pig sera for neutralizing antibodies against PEDV were confirmed by SNT.

Authors' contributions

Jiakai Zhao: Data curation, Investigation, Writing-original draft. Lu Zhang: Validation, Investigation. Yibo Kong: Investigation, Formal analysis, Methodology. Miao Dan: Validation, Investigation. Yangzong Xiri: Formal analysis, methodology. Pinpin Ji: Data curation, Software. Shijin Jiang: Writing-review & editing. Yani Sun: Methodology, Validation, Writing-review & editing. Qin Zhao: Conceptualization, project administration, supervision, and resources.

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Data availability

All of the pig sera were tested three times with the developed cELISA. GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) was used to perform the statistical analysis. SPSS software (version 20, http://www.spss.com.cn) was used to calculate kappa index values and estimate the consistency between the cELISA and commercial ELISA kits.

Declarations

Ethics approval and consent to participate

Animal experiments were performed in accordance with the Guidelines for Experimental Animal Welfare and Ethical Treatment of the Ministry of Science and Technology of China. The protocols of the experimental animal procedures were carried out following the guidelines of the Northwest A&F University Institutional Committee for the Care and Use of Laboratory Animals and were approved by the Committee on Ethical Use of Animals of Northwest A&F University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Author Qin Zhao was not involved in the journal's review or decisions related to this manuscript.

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