Wang *et al. Animal Diseases* (2024) 4:30
https://doi.org/10.1186/s44149-024-00137-8

Animal Diseases

SHORT COMMUNICATION

Open Access

Development and evaluation of a monoclonal antibody-based competitive ELISA for detecting porcine deltacoronavirus antibodies

Wei Wang[1,2,3,4,6], Baochao Fan[1,3,4,6], Xuehan Zhang[1,3,4,6], Shanshan Yang[1,3,4,6], Junming Zhou[1,3,4,6], Rongli Guo[1,3,4,6], Yongxiang Zhao[1,3,4,6], Jinzhu Zhou[1,3,4,6], Jizong Li[1,3,4,5,6*] and Bin Li[1,3,4,6*]

Abstract

Porcine deltacoronavirus (PDCoV) is an emerging swine enteropathogenic coronavirus that can cause acute diarrhea and vomiting in newborn piglets and poses a potential risk for cross-species transmission. It is necessary to develop an effective serological diagnostic tool for the surveillance of PDCoV infection and vaccine immunity effects. In this study, we developed a monoclonal antibody-based competitive ELISA (cELISA) that selected the purified recombinant PDCoV nucleocapsid (N) protein as the coating antigen to detect PDCoV antibodies. To evaluate the diagnostic performance of the cELISA, 122 swine serum samples (39 positive and 83 negative) were tested and the results were compared with an indirect immunofluorescence assay (IFA) as the reference method. By receiver operating characteristic (ROC) curve analysis, the optimum cutoff value of percent inhibition (PI) was determined to be 26.8%, which showed excellent diagnostic performance, with an area under the curve (AUC) of 0.9919, a diagnostic sensitivity of 97.44% and a diagnostic specificity of 96.34%. Furthermore, there was good agreement between the cELISA and virus neutralization test (VNT) for the detection of PDCoV antibodies, with a coincidence rate of 92.7%, and the κ analysis showed almost perfect agreement (κ = 0.851). Overall, the established cELISA showed good diagnostic performance, including sensitivity, specificity and repeatability, and can be used for diagnostic assistance, evaluating the response to vaccination and assessing swine herd immunity.

Keywords Porcine deltacoronavirus (PDCoV), Competitive ELISA (cELISA), Antibody detection, Monoclonal antibody, Nucleocapsid (N) protein

Handling Editor: Wentao Li.

*Correspondence:
Jizong Li
lijizong22@sina.com
Bin Li
libinana@126.com
[1] Institute of Veterinary Medicine, Jiangsu Academy of Agricultural Sciences, Key Laboratory of Veterinary Biological Engineering and Technology Ministry of Agriculture and Rural Affairs, Nanjing 210014, China
[2] Shaoxing Academy of Biomedicine of Zhejiang Sci-Tech University, Shaoxing 312000, China
[3] Jiangsu Key Laboratory for Food Quality and Safety, State Key Laboratory Cultivation Base of Ministry of Science and Technology, Nanjing 210014, China
[4] Jiangsu Coinnovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou University, Yangzhou 225009, China
[5] Key Laboratory for Prevention and Control of Avian Influenza and Other Major Poultry Diseases, Ministry of Agriculture and Rural Affairs, Guangzhou 510640, China
[6] GuoTai (Taizhou) Center of Technology Innovation for Veterinary Biologicals, Taizhou 225300, China

Introduction

Porcine deltacoronavirus (PDCoV) is an emerging swine enteropathogenic coronavirus known to induce acute diarrhea, vomiting and dehydration in newborn piglets (Wang et al. 2022a; Xia et al. 2023). It was first identified in Hong Kong in 2012 (Woo et al. 2012) and has since spread rapidly to many countries, such as the United States (Wang et al. 2014a, b), Canada (Marthaler et al. 2014), Thailand (Lorsirigool et al. 2017), Vietnam (Saeng-Chuto et al. 2017), Lao PDR (Lorsirigool et al. 2016), Japan (Suzuki et al. 2018) and Peru (More-Bayona et al. 2022). PDCoV was first reported in pigs in mainland China in 2014 (Zhao et al. 2017). Experiments have shown that PDCoV can infect calves, chickens, turkey poults and mice, suggesting a potential risk of cross-species transmission (Duan 2021; Xia et al. 2023). Moreover, three children were infected with PDCoV in Haiti (Lednicky et al. 2021). The global spread of the PDCoV outbreak has inflicted, devastating economic losses, with mortality rates ranging from 30%-40% in the pig industry (Wang et al. 2023). Unfortunately, there are currently no effective drugs or commercial vaccines available to prevent or control PDCoV infection (He et al. 2023; Tang et al. 2021).

Currently, serological methods for detecting PDCoV antibodies have been established, including a virus neutralization test (VNT), an indirect immunofluorescence assay (IFA) (Okda et al. 2016), a fluorescent microsphere immunoassay (FMIA) (Okda et al. 2016), and various enzyme-linked immunosorbent assays (ELISAs) (Luo et al. 2017; Thachil et al. 2015; Wang et al. 2022b). ELISAs, which are commonly used for the serological diagnosis of many pathogens, are divided into indirect ELISA, sandwich ELISA, blocking ELISA, and competitive ELISA (cELISA). The potential for cross-species transmission of PDCoV signifies its capacity to infect various animals, including humans. If indirect ELISAs are used to detect PDCoV antibodies in animal species other than pigs, horseradish peroxidase (HRP)-conjugated secondary antibodies must be matched to the species being tested. In addition, VNT, IFA and FMIA require cell culture and live virus for testing, thus limiting their application in PDCoV serological investigations owing to their inability to process large-scale samples.

The main advantage of cELISA over indirect ELISA is that it does not necessitate the use of species-specific enzyme-conjugated antibodies, which is a requirement of indirect ELISA, underlining cELISA as an optimal tool for detecting antibodies from various species (Moreno et al. 2019). This cELISA relies on the use of a specific monoclonal antibody (mAb) against the targeted antigen so that only serum antibodies against the targeted antigen can be recognized by the selected competitor mAb, specifically blocking the reaction between the targeted antigen and mAb. This study aimed to develop and validate a monoclonal antibody (mAb)-based cELISA for the detection of anti-PDCoV antibodies in porcine serum to offer a convenient serological diagnostic tool for the surveillance of PDCoV infection and vaccine immunity effects.

Confirmation of the rPDCoV-N protein, positive/negative reference serum and monoclonal antibody No.2

Purified rPDCoV-N proteins were acquired and identified by SDS–PAGE, and the purity of the target protein exceeded 90% (data not shown). The IFA results revealed that the positive reference serum (1:800 dilution) and monoclonal antibody No.2 (mAb-2$^{\#}$) were specifically reactive to PDCoV (Fig. 1B&F), whereas the negative reference serum (1:20 dilution) did not react (Fig. 1D). In addition, our IFA and western blot results demonstrated that PDCoV mAb-2$^{\#}$ does not cross-react with PEDV or TGEV (data not shown). These results indicated that positive/negative reference sera and mAb-2$^{\#}$ are applicable for developing a diagnostic method to identify PDCoV antibodies.

Research has indicated that the S protein of SARS-CoV-2 has a greater correlation with virus neutralization assays than the N protein does (Jung et al. 2021; Muecksch et al. 2021). Similar results have been reported in antibody detection assays for PEDV (Song et al. 2023) and SARS-CoV (Qiu et al. 2005). As the N protein is highly conserved among different PDCoV isolates with few base mutations (Okda et al. 2016; Sun et al. 2022) and abundance in PDCoV-infected host cells (Lee and Lee 2015; Okda et al. 2016), it was chosen as the diagnostic target for cELISA. Future studies will endeavor to express the S protein and develop a mAb-S-based competitive ELISA for detecting PDCoV antibodies.

Establishment of the cutoff value, diagnostic specificity and diagnostic sensitivity

The cELISA was optimized, and a panel of 122 pig serum samples (39 positive and 83 negative sera) was tested, with the PI values of each sample being calculated. ROC analysis was used to determine the cutoff value for defining positive and negative test outcomes, resulting in maximal diagnostic sensitivity and specificity of the assay (Fig. 2A). An interactive dot plot diagram displaying the PI values of the serum samples was also produced (Fig. 2B). The AUC of the established cELISA was 0.9919 (95% CI: 0.9811–1.003), indicating its high accuracy. In addition, a diagnostic sensitivity of 97.44% (95% CI: 86.52% to 99.94%) and a diagnostic specificity of 96.34% (95% CI: 89.68% to 99.24%) were achieved when the cutoff value was set to 26.8%. Therefore, samples with a PI value less than 26.8% were considered

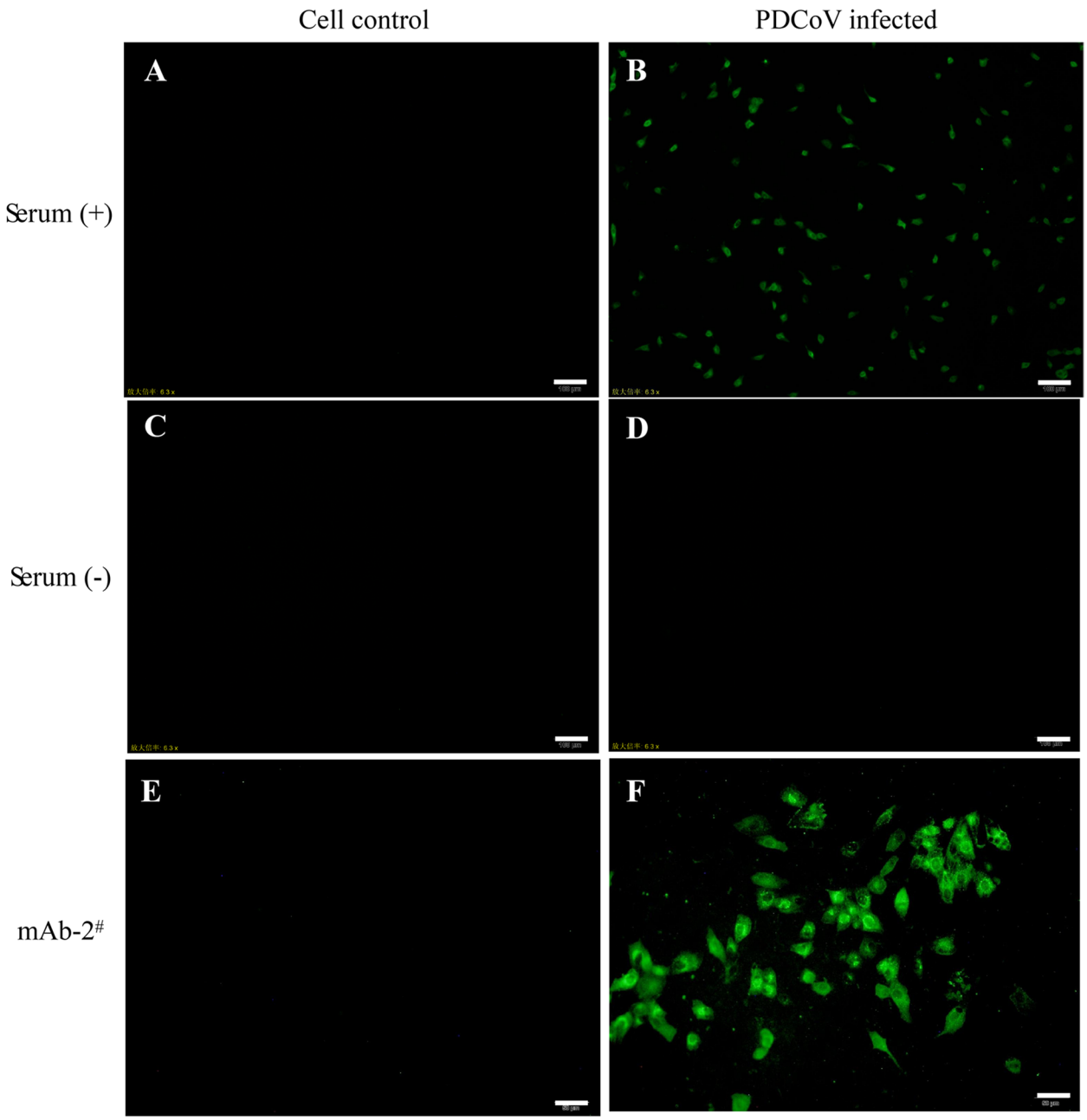

Fig. 1 IFA analysis of positive/negative reference serum and monoclonal antibody No.2. Positive reference serum (**B**) and monoclonal antibody No.2 (**F**) recognized the nucleocapsid protein in PDCoV-infected LLC-PK1 cells, while negative reference serum (D) did not react. Uninfected LLC-PK1 cells (**A**, **C** and **E**) were used as a negative control. Serum +: positive reference serum; serum -:negative reference serum. The bars in **A**, **B**, **C** and **D** represent 100 μm, and the bars in **E** and **F** represent 50 μm

negative, whereas samples with a PI value of 26.8% or greater were considered positive.

In the diagnosis of the 122 serum samples, cELISA displayed a diagnostic sensitivity of 97.44% (38/39) and a diagnostic specificity of 96.34% (80/83), which were slightly greater than those of the reference method (IFA). Thus, it is more suitable for wide use in PDCoV clinical diagnosis, especially in resource-limited diagnostic laboratories.

Determination of analytic sensitivity, specificity and repeatability of the competitive ELISA

Following the optimization of the cELISA, the analytic sensitivity was evaluated by the positive reference

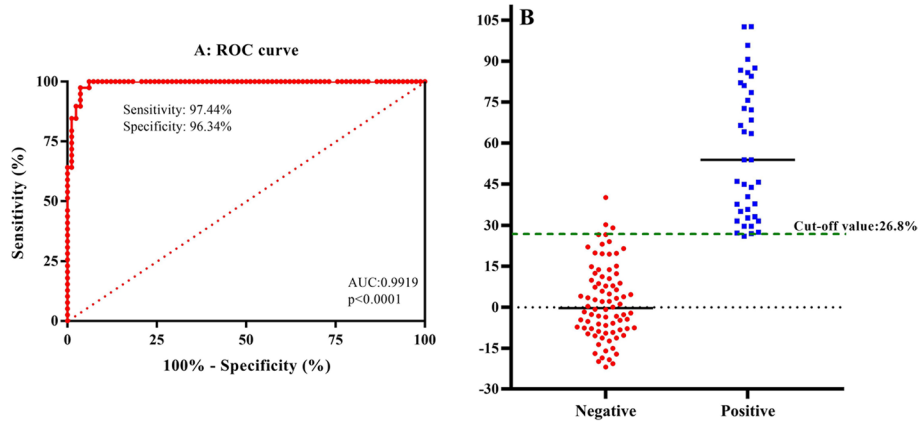

Fig. 2 Receiver operating characteristic (ROC) analysis for competitive ELISA. The assay was conducted with PDCoV-positive sera (n = 39) and PDCoV-negative sera (n = 83). **A** ROC analysis of the competitive ELISA results; the area under the curve (AUC) of the test was 0.9919. **B** Interactive dot plot diagram displaying the PI value of each serum sample when the cutoff value was set to 26.8%. PDCoV (porcine deltacoronavirus), ROC (receiver operating characteristic), AUC (area under the curve)

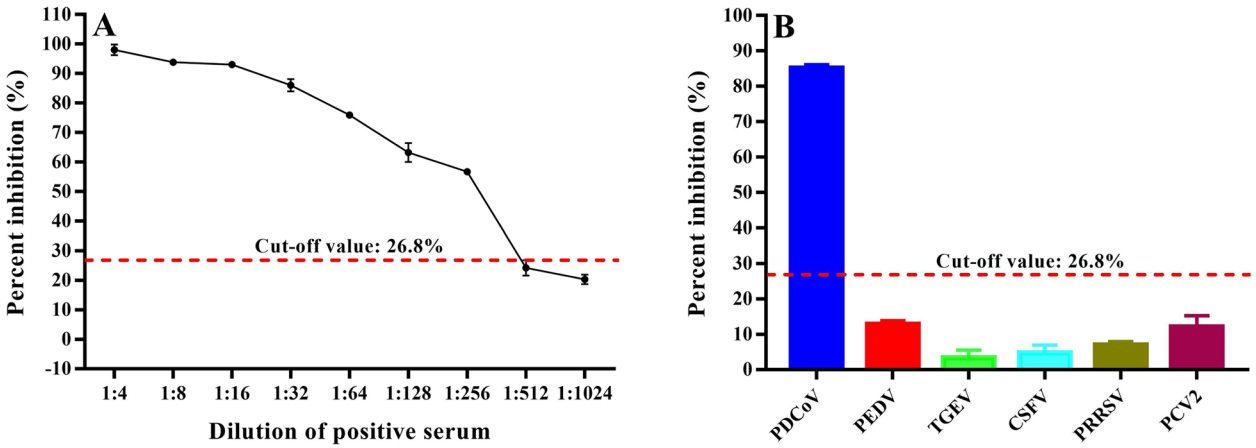

Fig. 3 Analytic sensitivity and specificity of the competitive ELISA. **A** Twofold serially diluted PDCoV-positive reference serum samples ranging from 1:4 to 1:1024 were detected to evaluate analytic sensitivity. **B** Sera positive for PDCoV, PEDV, TGEV, CSFV, PRRSV and PCV2 were tested to evaluate analytic specificity. The PI cutoff value of 26.8% is marked with a dashed line. PDCoV (porcine deltacoronavirus), PEDV (porcine epidemic diarrhea virus), TGEV (transmissible gastroenteritis virus), CSFV (classical swine fever virus), PRRSV (porcine reproductive and respiratory syndrome virus), PCV2 (porcine circovirus type 2)

serum, which had the highest serum dilution of 1:256 (1:2^8) (Fig. 3A). Although this titer was slightly lower than the VNT titer of 2^9, cELISA proved to be more convenient as the VNT was excessively labor-intensive and impractical for the analysis of a large quantity of samples.

To evaluate the analytic specificity of the established cELISA, PDCoV-, PEDV-, TGEV-, CSFV-, PRRSV-, and PCV2-positive sera were tested. All nonspecific positive sera were determined to be PDCoV-negative, with PI values that were lower than the cutoff value. The PI value for PDCoV-positive serum was approximately 85.1%, whereas the PI values for other virus-positive sera ranged from 3.4% to 12.85%, indicating that the cELISA had good analytical specificity (Fig. 3B).

The intra-batch assay CV of the developed cELISA ranged from 1.32% to 3.22%, whereas the interassay CV ranged from 4.51% to 7.54%, indicating that the cELISA results were reproducible and reliable (Table 1). These findings indicate that cELISA is suitable for the serological diagnosis of PDCoV.

ELISAs are widely used serological tests that can detect exposure to pathogens and evaluate the efficacy of vaccines. ELISAs are easy to standardize and validate, and they can also analyze samples in a high-throughput manner (Riepler et al. 2020). Although several indirect

Table 1 Repeatability analysis of the developed competitive ELISA

PDCoV sera	Intrabatch assay			Interbatch assay		
	Mean	SD	CV	Mean	SD	CV
Strongly positive	99.4%	0.0320	3.22%	93.6%	0.0698	7.46%
Moderately positive	54.7%	0.0072	1.32%	56.9%	0.0428	7.54%
Weakly positive	31.8%	0.0061	1.91%	30.1%	0.0136	4.51%

Mean: average PI values from three repeated competitive ELISAs; CV: coefficient of variation; SD: standard deviation

Table 2 Comparison of competitive ELISA and virus neutralization tests for detecting PDCoV antibodies in serum samples

Serum samples		cELISA		Total	Kappa value
		Positive	Negative		
VNT	Positive	80	10	90	0.851
	Negative	5	111	116	
	Total	85	121	206	

ELISAs based on S1, N and M proteins (Luo et al. 2017; Su et al. 2016; Thachil et al. 2015), as well as a blocking ELISA based on N proteins (Wang et al. 2022b), have been developed for the detection of PDCoV antibodies in swine, there are currently no commercial ELISA kits available to compare the diagnostic correlation between IgG antibodies detected via ELISA and neutralizing antibodies detected via VNT. However, owing to the high level of genetic diversity among different strains of the same coronavirus, the application of S proteins as an antibody detection platform in field applications is limited. Bahoussi reported that the PDCoV S genome exhibited the lowest similarity levels (< 93%), with the N genome sequence demonstrating greater similarity (> 95%) (Bahoussi et al. 2022).

Comparison of clinical field serum sample detection methods

The results of the competitive ELISA and virus neutralization test (VNT) on 206 serum samples showed almost perfect agreement (Kappa value of 0.851), with a coincidence rate of 92.7%. In addition, 41.3% of the PDCoV antibodies were positive by cELISA, whereas 43.7% of the PDCoV antibodies were positive by VNT (Table 2). These findings demonstrate that cELISA can be used as an efficient alternative to VNT for the detection of PDCoV antibodies.

The presence of neutralizing antibodies is essential for providing protection and clearing enteropathogenic coronavirus from an infected individual (Liu and Arase 2022). Vaccine candidates should be able to effectively induce neutralizing antibodies to provide immunity against the virus. Neutralizing antibodies are a key factor in providing immunity and clearing the virus, and virus neutralization tests are labor-intensive and time-consuming, which requirs live viruses and complicates their standardization process in comparison to ELISAs. Therefore, the repeatability of the virus neutralization test is crucial. In this study, we compared the correlations between ELISA-detected antibodies and neutralizing antibodies, and our results were ideal, with a coincidence rate of 92.7% and a kappa value of 0.851, indicating almost perfect agreement between the two detection methods (Li et al. 2021; Moreno et al. 2019).

Conclusions

In summary, this research developed a monoclonal antibody-based competitive ELISA that uses the purified recombinant PDCoV N protein as a coating antigen to detect PDCoV antibodies. This assay exhibits excellent diagnostic performance, including sensitivity, specificity and repeatability. In addition, cELISA has a strong correlation with the virus neutralization test, proving its utility for diagnostic assistance, evaluating the response to vaccination, and assessing swine herd immunity in the future.

Methods

Virus, cell and serum samples

The PDCoV CZ2020 strain (GenBank accession number: OK546242) was isolated and maintained in our laboratory. The LLC-PK1 cell line was purchased from the China Institute of Veterinary Drug Control.

Sera positive for PEDV, TGEV, CSFV, PRRSV and PCV2 were preserved in our laboratory. PDCoV-, PEDV-, and TGEV-positive sera were collected during our previous pig challenge experiments and identified by IFA and VNT. CSFV-, PRRSV-, and PCV2-positive sera were purchased from commercial kits. PDCoV sera were collected during our previous pig challenge experiments (Li et al. 2022; Zhang et al. 2022).

Preparation of recombinant PDCoV N protein and indirect immunofluorescence assay for identifying swine sera and monoclonal antibodies

The recombinant PDCoV N (rPDCoV-N) protein and monoclonal antibody No.2 (mAb-2$^{\#}$) against the N protein were prepared according to our previous study (Wang et al. 2021). IFA was performed as described previously (Okda et al. 2016; Wang et al. 2022a). Briefly, $10^{4.0}$ $TCID_{50}$/mL PDCoV was inoculated into LLC-PK1 cells, fixed with methyl alcohol, and then blocked with 5% skim milk. Next, the cells were incubated with swine sera (dilutions of 1:20, 1:40, 1:200, 1:400 and 1:800) or mAb-2$^{\#}$ at 37℃ for 60 min and incubated with goat anti-pig IgG conjugated with FITC (Abcam, UK) (1:1000) or goat anti-mouse IgG conjugated with FITC (Boster, China) (1:500) for an additional 60 min. Finally, the cells were observed under a fluorescence microscope (Olympus IX-51, Japan). Uninfected cells served as a negative control. Serum samples were considered positive if PDCoV-specific fluorescence was observed at a 1:20 serum dilution (Okda et al. 2016).

Virus neutralization test

A virus neutralization test was performed via a method described previously with some modifications (Song et al. 2023). The CPE was examined, and the neutralizing antibody titer was expressed as the $\log_2$ transformation of the reciprocal of the highest serum dilution that completely inhibited the CPEs.

Establishment and optimization of the competitive ELISA

The optimal conditions of cELISA were established, including the optimal antigen-coating concentration, working concentration of mAb-2$^{\#}$ and concentration of HRP-conjugated goat anti-mouse IgG. To begin, microplates were coated with the rPDCoV-N protein at a concentration of 0.8 μg/mL at 4°C overnight. Then, the cells were blocked with 5% skim milk for 2 h at 37°C. Two-fold dilutions of positive or negative reference serum or an unknown serum mixture were separately added to the plates and incubated for 60 min. mAb-2$^{\#}$ at a concentration of 1.0 μg/mL was added to each well and then incubated for 60 min. HRP-conjugated goat anti-mouse IgG at a 1:14,000 dilution was subsequently added to each well, and the mixture was incubated for 60 min. Then, TMB substrate was added to each well, followed by incubation at room temperature in the dark for 13 min. Finally, the reaction was stopped by adding sulfuric acid to each well. The OD values of the samples were read at 450 nm via a microplate spectrophotometer.

Determination of the cutoff value, diagnostic specificity and diagnostic sensitivity of competitive ELISA

The cutoff value of the developed cELISA was determined by analyzing 122 swine serum samples (39 positive and 83 negative) collected from our previous pig challenge experiments, which had a known PDCoV antibody status confirmed by IFA. These 122 swine sera were then detected via the optimized cELISA.

The cELISA and IFA results of the serum samples were compared using receiver operating characteristic (ROC) curves to determine the optimal cutoff value that maximized the diagnostic specificity and sensitivity of the assay. The PI values of each serum sample were analyzed using GraphPad Prism software (version 7.0; USA) to determine the area under the curve (AUC) at the 95% confidence interval (CI). The IFA results and their corresponding PI values were also analyzed by receiver operating characteristic (ROC) curve analysis to determine diagnostic sensitivity and specificity values (Swets 1988).

Assessment of analytic sensitivity, specificity and repeatability of the competitive ELISA

After the cutoff value criteria were determined, the analytic sensitivity of the cELISA was assessed via a PDCoV-positive reference serum that was serially diluted twofold from 1:4–1024. Furthermore, the analytic specificity of the cELISA was evaluated via six different virus-positive serum samples, namely, PDCoV, PEDV, TGEV, CSFV, PRRSV and PCV2.

To evaluate the intra-batch and inter-batch assay repeatability of the developed cELISA, three serum samples were tested and showed strongly, moderately and weakly positive results against PDCoV. The cELISA was conducted on one plate in one run (intra-assay) or on three distinct plates in three independent runs (inter-assay), with each serum being detected in triplicate. The CV was used to evaluate the degree of variation of the cELISA, which was calculated by dividing the standard deviation (SD) by the mean PI value of each serum sample.

Comparison of competitive ELISA and virus neutralization tests in clinical field serum samples for PDCoV antibody detection

To evaluate the validity of the developed cELISA, a total of 206 serum samples were separately collected from vaccinated and unvaccinated control pigs 14–28 days post-vaccination. The "Porcine Deltacoronavirus Vaccine, Inactivated (Strain LYG/14)" was used in the immunization trial and is a pilot plant product that is not commercially available. The sera were tested by VNT to

determine the neutralizing antibody titer and then further detected by cELISA. The consistency was calculated via the following formula: (true positive + true negative)/(true positive + false positive + true negative + false negative) × 100%. The agreement between the cELISA and VNT was measured by calculating the Cohen's kappa (κ) statistic value. The κ values were interpreted according to the Landis and Koch descriptors (Landis and Koch 1977).

Abbreviations

PDCoV	Porcine deltacoronavirus
ELISA	Enzyme-linked immunosorbent assay
cELISA	Competitive ELISA
VNT	Virus neutralization test
IFA	Immunofluorescence assay
HRP	Horseradish peroxidase
mAb	Monoclonal antibody
PEDV	Porcine epidemic diarrhea virus
TGEV	Transmissible gastroenteritis virus
CSFV	Classical swine fever virus
PRRSV	Porcine reproductive and respiratory syndrome virus
PCV2	Porcine circovirus type 2
ECL	Enhanced chemiluminescence
CPE	Cytopathic effect
TMB	3,3′,5,5′-Tetramethylbenzidine
OD	Optical density
AUC	Area under the curve
ROC	Receiver operating characteristic
PI	Percent inhibition
CI	Confidence interval
CV	Coefficient of variation
SD	Standard deviation

Acknowledgements
Not applicable.

Authors' contributions
W.W. participated in all the experiments and wrote the manuscript. B.F. helped design the entire project and draft the manuscript. R.G., Y.Z., and J.Z. (Jinzhu Zhou) conducted the cell culture, virus proliferation, IFA and VNT detection. J.Z. (Junming Zhou) conducted the data analysis. X.Z. and S.Y. revised the English language of this article. B.L. and J.L. contributed essential ideas and discussion. All the authors have read and approved the final version of the manuscript.

Funding
This study was supported by the National Key Research and Development Program (2023YFD1800501), the National Natural Science Foundation of China (32373030 and 32202787), the S&T Program of Hebei (21322401D), the Jiangsu Province Natural Sciences Foundation (BK20221432 and BK20210158), the Jiangsu Agricultural Science and Technology Innovation Fund (CX(22)3028), the Special Project of Northern Jiangsu (SZ-LYG202109), the Open Fund of Shaoxing Academy of Biomedicine of Zhejiang Sci-Tech University (SXAB202215), and the Open Fund of Key Laboratory for Prevention and Control of Avian Influenza and Other Major Poultry Diseases, Ministry of Agriculture and Rural Affairs (YDWS202213).

Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
The experimental protocol was previously approved by the Jiangsu Academy of Agricultural Sciences Experimental Animal Ethics Committee (IACUC-LE-2023–08-010) and was performed in accordance with relevant guidelines and regulations.

Consent for publication
The authors approved the publication of the manuscript.

Competing interests
The authors declare that they have no competing interests. Author Bin Li was not involved in the journal's review or decisions related to this manuscript.

Received: 2 April 2024 Accepted: 26 August 2024
Published online: 13 September 2024

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