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Identification of novel serological agents for porcine deltacoronavirus infection based on the immunogenic accessory protein NS6

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Abstract

Porcine deltacoronavirus (PDCoV) is a swine enteropathogenic CoV that causes severe vomiting, diarrhea and dehydration in suckling piglets, leading to economic losses in the swine industry. There is a great need for a convenient method to detect circulating antibodies and help in accurate diagnosis and disease control. Previously, we demonstrated that a unique PDCoV accessory protein, NS6, is expressed during PDCoV infection in pigs and is incorporated into PDCoV virions; thus, we deduced that NS6 is likely an immunogenic target that can be used for the diagnosis of PDCoV infection. In this study, we first confirmed that NS6 is immunogenic in PDCoV-infected pigs by performing a serum western blot. Furthermore, we developed a novel NS6-based indirect enzyme-linked immunosorbent assay (iELISA) method and compared it to an established S1-based iELISA for the survey of anti-PDCoV IgG or IgA in pigs of different ages in China. The NS6-iELISA has high specificity for the detection of IgG antibodies and no cross-reactivity with other porcine enteric CoVs (transmissible gastroenteritis coronavirus, porcine epidemic diarrhea virus, or swine acute diarrhea syndrome coronavirus). This NS6 serology-based method has great sensitivity and good repeatability, making it a new and cost-saving option for the rapid diagnosis and immunosurveillance of PDCoV, which may also be important for the prevention and control of deltacoronavirus-related infection in pigs and other animals.

Keywords Coronavirus, Porcine deltacoronavirus (PDCoV), NS6, ELISA, Serology, IgG

Introduction

Porcine deltacoronavirus (PDCoV), which belongs to the genus *Deltacoronavirus* of the family *Coronaviridae*, is a porcine intestinal coronavirus that has emerged in recent years (Jung et al. 2016; Liang et al. 2019). PDCoV was first detected in swine rectal swabs in Hong Kong during a molecular surveillance study in 2009 (Y et al. 2012). However, at that time, no corresponding virus was isolated, and no epidemiological investigation was conducted on PDCoV. In early 2014, an outbreak of PDCoV in the United States and its rapid spread to other countries aroused widespread attention (Li et al. 2014; Marthaler et al. 2014; Wang et al. 2014). To date, PDCoV has been identified in Canada, China, South Korea, Thailand, Laos, Vietnam and Japan (Dong et al. 2015; Lee and Lee 2014; Lorsirigool et al. 2016; Suzuki et al. 2017; Wang et al. 2015), and it has resulted in moderate economic losses in the swine industry (Chen et al. 2015; Jung et al. 2016). Clinical symptoms caused by PDCoV are similar

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to those caused by other porcine enteric pathogens, such as transmissible gastroenteritis coronavirus (TGEV), porcine epidemic diarrhea virus (PEDV) and swine acute diarrhea syndrome coronavirus (SADS-CoV), which are characterized by vomiting, moderate or severe diarrhea and dehydration (Huang et al. 2013; Ma et al. 2015; Pan et al. 2017).

PDCoV is an enveloped virus with a single-stranded, positive-sense RNA genome that is approximately 25 kb in length and is arranged in the order 5' UTR-ORF1a/1b-S-E-M-NS6-N-NS7-NS7a-3' UTR, encoding 15 mature nonstructural proteins, four structural proteins and three species-specific accessory proteins (Fang et al. 2016; Liu et al. 2021; Ma et al. 2015; Wang et al. 2018; Zhang 2016). The accessory protein genes are interspersed among the structural genes: NS6, located between the M and N genes (Fang et al. 2016; Qin et al. 2021a); NS7, embedded in the N gene; and NS7a, consistent with a sequence of 100 amino acids overlapping the C-terminus of NS7 (Fang et al. 2017). There is evidence that NS6 contributes to viral pathogenesis *in vivo* (Zhang et al. 2020). It is expressed in virus-infected cells, in target tissues of infected animals and participates in the assembly of progeny virions (Qin et al. 2021a), indicating its important role in the replication cycle of PDCoV. Furthermore, studies have shown that NS6 antagonizes the production of interferon beta by preventing the binding of RIG-I/MDA5 to double-stranded RNA (Fang et al. 2018), and the deletion of NS6 strongly impairs viral replication, resulting in marked attenuation of the virus in piglets (Zhang et al. 2020). Thus, we deduce that NS6 is likely an immunogenic target and may be a new therapeutic target; further studies of this topic may lead to the development of novel diagnostic assays and more effective vaccines against PDCoV infection.

Since its first detection, various methods, including a eukaryote-expressed PDCoV S1 protein-based enzyme-linked immunosorbent assay (ELISA) (Thachil et al. 2015), a prokaryote-expressed PDCoV N protein-based ELISA (Su et al. 2016), and a PDCoV whole virus-based ELISA (Hu et al. 2016; Ma et al. 2016), have been developed to detect antibodies against PDCoV. The minor antigenic cross-reactivity between PEDV and PDCoV (Ma et al. 2016) and between TGEV and PEDV (Lin et al. 2015) prompted us to develop more specific serological diagnostic assays to help monitor and control PDCoV outbreaks. In this study, we established a novel in-house indirect ELISA (iELISA) method based on the unique PDCoV accessory protein NS6, which does not exist in PEDV, TGEV or SADS-CoV, and validated this method in a large-scale serological survey in China.

Results

NS6 protein is immunogenic in PDCoV-infected pigs

The His-tagged recombinant NS6 protein was expressed in a prokaryotic system as an insoluble protein, and Ni–NTA purification resulted in a concentrated single band, as detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis (Fig. 1a). To confirm the identity and antigenicity of the proteins, the purified proteins were subjected to western blot (WB) analysis with a mouse anti-His mAb and mouse anti-NS6 pAb generated previously (Qin et al. 2021a). Specific bands of approximately 13 kDa indicated correct NS6 expression and specific antigenicity (Fig. 1b). In addition, recombinant PDCoV S1 protein was prepared for use in

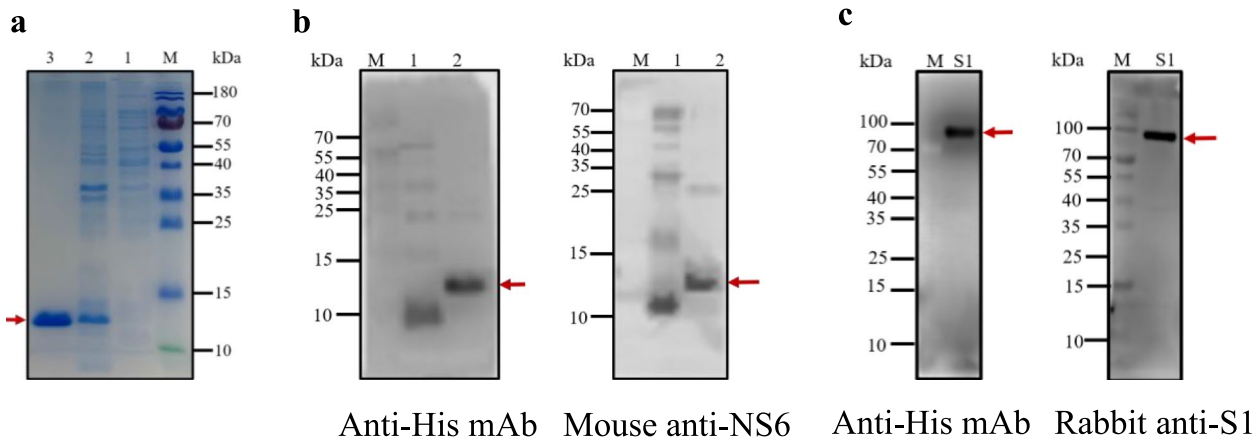

Fig. 1 Expression and purification of PDCoV NS6 and S1 proteins. **a** SDS–PAGE analysis of purified recombinant PDCoV NS6 protein. M: protein marker, empty vector pCold I (lane 1), unpurified NS6 protein (lane 2), purified NS6 protein (lane 3). **b** WB analysis of empty vector pCold I (lane 1) and purified NS6 protein (lane 2) using anti-His monoclonal antibody (mAb) and mouse anti-NS6 pAb, respectively; M: marker. **c** WB analysis of recombinant S1 protein. An 85-kDa band was detected by an anti-His mAb or a rabbit anti-S1 pAb; M: protein marker. Red arrows indicate the target protein bands

a standard S1-based ELISA (developed previously in our laboratory) (Thachil et al. 2015; Wang et al. 2017) to compare and evaluate the reliability of the novel NS6-based ELISA. The S1 protein was expressed using an insect cell expression system as described previously (Qin et al. 2021b; Wang et al. 2017). WB analysis with an anti-His mAb or an anti-S1 pAb revealed a band of ~ 85 kDa in size, which was larger than the predicted molecular weight of PDCoV-S1, likely due to glycosylation (Fig. 1c).

In our previous study, we demonstrated that NS6 is expressed in the intestinal tissue of infected newborn piglets (Qin et al. 2021a). However, when and what kind of immune response are elicited by NS6 are unknown. To test whether NS6 is immunogenic in PDCoV-infected pigs and to identify NS6-negative and NS6-positive sera as reference controls, serum samples from postweaned pigs with or without PDCoV infection (Qin et al. 2021a) were tested by WB for the presence of anti-NS6 and anti-S1 IgG antibodies (as a positive control) using purified PDCoV NS6, S1 and PEDV S1 (as a negative control) proteins as the antigens. The criterion for determining the seropositivity of a sample was whether the expected NS6 or S1 protein band was present on the membrane. The appearance of the expected 13- and 85-kDa bands confirmed the seropositivity of the sample (Fig. 2a), indicating the presence of anti-NS6 and anti-S1 antibodies in PDCoV-infected pigs. In contrast, no specific bands were observed in a negative serum sample from a PDCoV-uninfected pig (Fig. 2b). This result suggested that NS6 is immunogenic and elicits an antibody response in PDCoV-infected pigs.

Development of iELISAs based on the PDCoV NS6 protein

Having confirmed the immunogenicity of NS6 in PDCoV-positive pigs by serum WB, we next wanted to develop an NS6-based iELISA method for detecting PDCoV IgG or potential IgA antibodies. A checkerboard titration was used to determine the optional antigen coating concentration and dilution ratio of the secondary antibody for the NS6-iELISA based on the following standards: when the OD_{450nm} value of the positive serum was close to 1.0 and the OD_{450nm} value of the negative serum was less than 0.3, the P/N value reached the maximum (Lei et al. 2019; Peng et al. 2021). The optimal coating concentration of the NS6 protein for the detection of the IgG antibody was 50 ng/well, and the optimal secondary antibody dilution was 1:10,000 (Supplemental Fig. S1a and b). Furthermore, 5% skim milk diluted in 1 × PBST was the best blocking solution (Fig. S1c), and the most effective and time-efficient blocking duration was 2 h at 37°C (Supplemental Fig. S1d). The optimal test sample dilution was determined to be 1:100 (Supplemental Fig. S1e). In addition, the optimal incubation time for both the test sera and secondary antibodies was determined to be 30 min (Supplemental Fig. S1f and g). Finally, the optimal length of time for incubation with horseradish peroxidase (HRP) substrate was determined to be 15 min (Supplemental Fig. S1h). The optimal coating concentrations for the NS6 and S1 proteins for the detection of IgA antibodies were 400 ng and 40 ng per well, respectively (Supplemental Fig. S1i and k), and the optimal dilution of the anti-IgA secondary antibody for both the NS6 and S1 proteins was 1:10 000 (Supplemental Fig. S1j-l).

For determining the positive cutoff value of the NS6-based iELISA, statistical analysis showed that the mean OD_{450nm} and SD of the PDCoV-negative sera were 0.248 and 0.043, respectively (Fig. 3a). Therefore, the cutoff value of 0.377 was calculated as the mean OD value of the negative control serum plus 3 × SD. Samples with an OD value above this cutoff were considered positive. Moreover, the suspicion interval was determined; that is, when the OD_{450nm} was suspected to be positive between 0.334 and 0.377, the result needed to be retested; when the retest value was still greater than 0.377, the result was

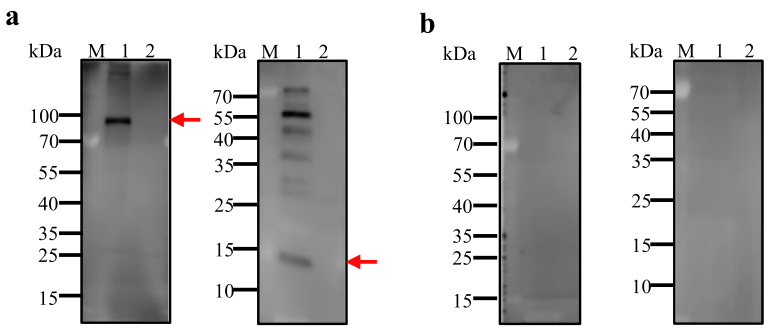

Fig. 2 WB analysis of serum samples from pigs with or without PDCoV infection (PDCoV-positive or -negative sera). **a** Detection of serum anti-S1 or anti-NS6 IgG antibody in PDCoV-infected pigs using purified recombinant PDCoV S1 (lane 1), NS6 (lane 1) and PEDV S1 (lane 2; as a negative control) protein as the antigens. M: protein marker. Red arrows indicate the target protein bands. **b** Serum anti-S1 or anti-NS6 IgG antibody was undetectable in PDCoV-negative pigs. No target bands were displayed

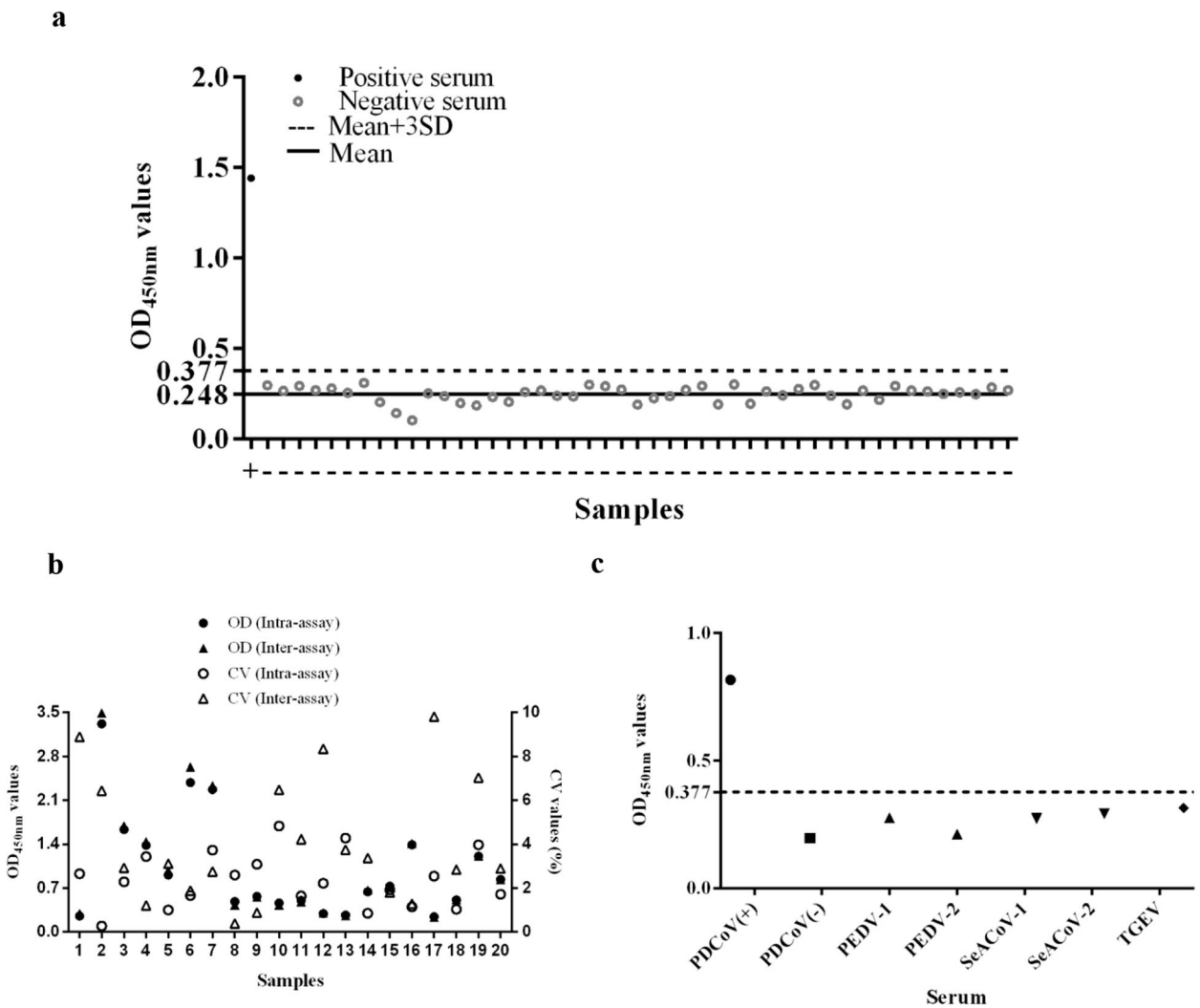

Fig. 3 Determination of the cutoff value, specificity and reproducibility of NS6-iELISA. **a** PDCoV iELISA cutoff value determination. PDCoV-negative serum samples were tested using the optimized NS6-based ELISA and the cutoff value (dashed line) was calculated as the mean OD_{450nm} value of PDCoV-negative serum samples plus three standard deviations (SD). A representative PDCoV-positive serum sample is shown as control. **b** Reproducibility of the PDCoV iELISA. 20 control serum samples (16 positive and four negative) were tested repeatedly by the NS6-based iELISA, and the OD_{450nm} values of the serum samples were used to calculate the coefficient of variation (CV), showing the intra- and interassay reproducibility. **c** PDCoV iELISA specificity. Positive control serum samples for PEDV, SADS-CoV and TGEV were tested by the NS6-based iELISA method; PDCoV positive and negative sera were included as controls

considered positive; otherwise, the result was considered negative.

Reproducibility and specificity of the PDCoV NS6 IgG ELISA

To validate the precision and accuracy of the NS6 protein-based ELISA, the reproducibility and specificity of the method were evaluated. Sixteen PDCoV-positive serum samples and four PDCoV-negative serum samples were used to empirically determine the intra- and interassay coefficients of variation (CVs) of the NS6-iELISA (Fig. 3b), which were found to be 0.27–4.83% (Table 1) and 0.39–9.82% (Table 2), respectively. To check for cross-reactivity of the NS6-based ELISA, known positive antisera from other common swine enteric viruses were examined. The average OD_{450nm} values of the positive control serum samples for PEDV (0.2765, 0.2078), TGEV (0.2080), and SADS-CoV (0.2552, 0.2952) were all below the cutoff value (Fig. 3c), indicating that the NS6-based ELISA did not cross-react with these samples.

Validation of the NS6-iELISA

To validate the sensitivity and specificity of the PDCoV NS6 IgG ELISA, a total of 39 serum samples were tested by serum WB (Fig. 4a) and NS6-iELISA. As shown in

Table 1 Intrabatch reproducibility of the ELISA

Samples	Intrabatch repetitions			Mean	SD[a]	CV/%[a]
	1	2	3			
1	3.3208	3.3098	3.3318	3.3208	0.008981	0.27
2	1.5826	1.6589	1.6649	1.6355	0.037463	2.29
3	1.3806	1.3282	1.4448	1.3845	0.047683	3.44
4	0.9048	0.8991	0.9207	0.9082	0.00914	1.00
5	2.4247	2.3300	2.3955	2.3834	0.039597	1.66
6	2.3389	2.1529	2.3261	2.2726	0.084825	3.73
7	0.4452	0.4180	0.4282	0.4305	0.011219	2.60
8	0.2631	0.2466	0.2561	0.2553	0.006762	2.65
9	0.5445	0.5659	0.5874	0.5659	0.017514	3.09
10	0.4591	0.4918	0.4374	0.4628	0.02236	4.83
11	0.4944	0.4984	0.5136	0.5021	0.008271	1.65
12	0.3006	0.2948	0.2848	0.2934	0.006526	2.22
13	0.2854	0.2702	0.2570	0.2709	0.011604	4.28
14	0.6397	0.6510	0.6391	0.6433	0.005474	0.85
15	0.7124	0.7468	0.7301	0.7298	0.014046	1.92
16	1.4151	1.3844	1.3784	1.3926	0.016074	1.15
17	0.2499	0.2349	0.2445	0.2431	0.006203	2.55
18	0.5048	0.5157	0.5165	0.5123	0.005337	1.04
19	1.1418	1.2364	1.2496	1.2093	0.04801	3.97
20	0.8588	0.8305	0.8264	0.8386	0.014405	1.72

[a] *SD* standard deviation, CV% was calculated as SD/mean × 100%

Table 2 Interbatch reproducibility of the ELISA

Samples	Interbatch repetitions			Mean	SD[a]	CV/%[a]
	1	2	3			
1	3.2655	3.4112	3.7987	3.4918	0.225015	6.44
2	1.7228	1.7333	1.6231	1.6931	0.049659	2.93
3	1.4499	1.4525	1.4144	1.4389	0.01738	1.21
4	0.9421	1.0163	0.9748	0.9777	0.030363	3.11
5	2.5685	2.6338	2.6906	2.6310	0.049887	1.89
6	2.2507	2.3222	2.4312	2.3347	0.064274	2.75
7	0.4813	0.4864	0.4851	0.4843	0.001874	0.39
8	0.2560	0.3106	0.3128	0.2931	0.026273	8.90
9	0.5545	0.5612	0.5492	0.5550	0.00491	0.88
10	0.4589	0.3920	0.4374	0.4294	0.027887	6.49
11	0.4524	0.5015	0.4836	0.4792	0.020289	4.23
12	0.3324	0.3045	0.2707	0.3025	0.025227	8.34
13	0.2552	0.2719	0.2489	0.2587	0.009704	3.75
14	0.6573	0.6987	0.6466	0.6675	0.022467	3.37
15	0.7065	0.6853	0.7155	0.7024	0.01266	1.80
16	1.4362	1.3987	1.3976	1.4108	0.017943	1.27
17	0.2375	0.2022	0.2574	0.2324	0.022826	9.82
18	0.5317	0.5095	0.4964	0.5125	0.01457	2.84
19	1.1735	1.3328	1.1355	1.2139	0.085471	7.04
20	0.8648	0.8058	0.8375	0.8360	0.02413	2.89

[a] *SD* standard deviation, CV% was calculated as SD/mean × 100%

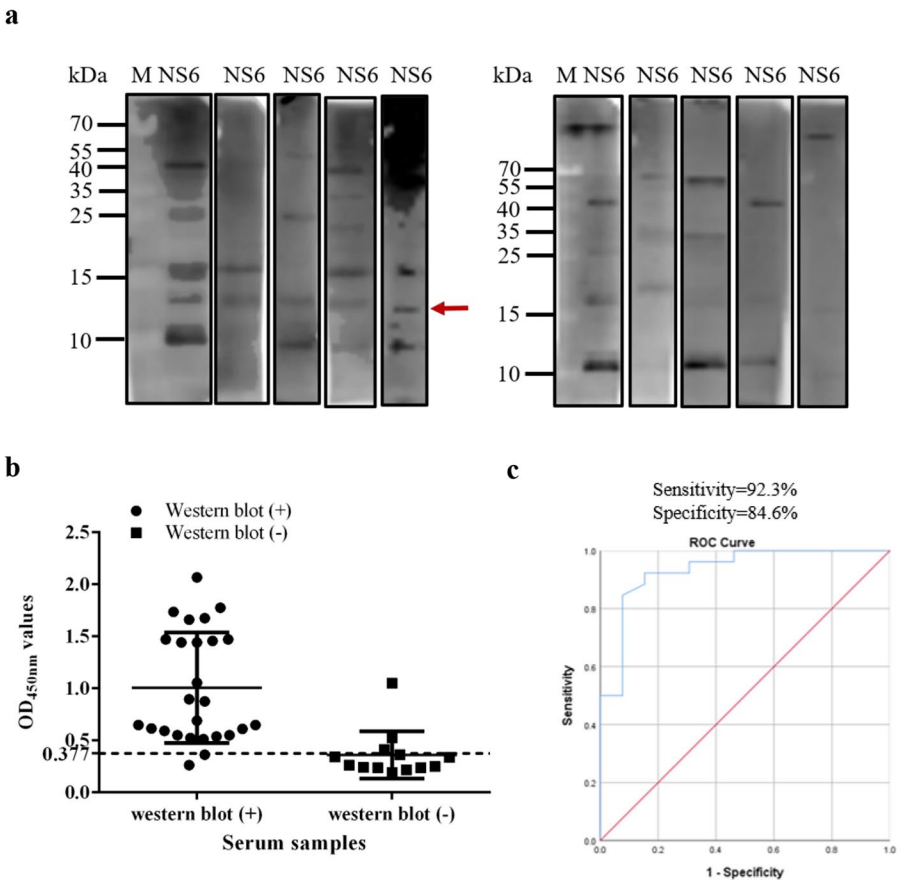

Fig. 4 Validation of the NS6-iELISA by western blot. **a** Identification of PDCoV-negative and positive sera by western blot (WB). Bands appeared at approximately 13 kDa in positive serum samples and no target bands in negative samples. M: protein marker. Red arrows indicate the target protein bands. **b**, **c** ROC curve using the WB as the diagnostic standard. Serum samples were classified as negative or positive based on the results of WB. Data presented as ELISA OD values ± SD

Fig. 4b, WB detected 26 PDCoV-positive samples, 24 of which tested PDCoV-positive by NS6-iELISA. Of the remaining 13 samples that tested PDCoV negative by WB, 11 tested PDCoV negative by NS6-iELISA (Fig. 4b). Additionally, the receiver operating characteristic (ROC) curve (Fig. 4c) showed that the specificity and sensitivity of the PDCoV NS6 IgG ELISA were 92.3% and 84.6%, respectively.

Detection of PDCoV in field samples

A total of 673 serum samples were collected from diarrheic pigs in seven provinces of China between 2015 and 2016 and tested using NS6- or S1-based IgG ELISAs. The results showed that 85.3% (574/673) of the serum samples were positive for anti-NS6 IgG antibodies and that 96.9% (652/673) of the serum samples were positive for anti-S1 IgG antibodies (Table 3). Among these, there was a 100% positive rate in gilts and multiparous sows according to both detection methods. The positivity rate for NS6-based IgG ELISA in growing pigs was highest in newborn

Table 3 Summary of PDCoV seroprevalence in swine of different ages

Age	No. tested	No. positive	
		Anti-NS6	Anti-S1
Gilts	69	69 (100%)	69 (100%)
Sows (primiparous)	58	57 (98.3%)	58 (100%)
Sows (multiparous)	117	117 (100%)	117 (100%)
< 1-week-olds	45	42 (93.3%)	45 (100%)
1-week-olds	83	81 (97.6%)	82 (98.8%)
3-week-olds	106	81 (76.4%)	91 (85.8%)
5-week-olds	61	35 (57.4%)	61 (100%)
11-week-olds	65	34 (52.3%)	61 (93.8%)
17-week-olds	24	21 (87.5%)	24 (100%)
20-week-olds	45	37 (82.2%)	44 (97.8%)
Total	673	574 (85.3%)	652 (96.9%)

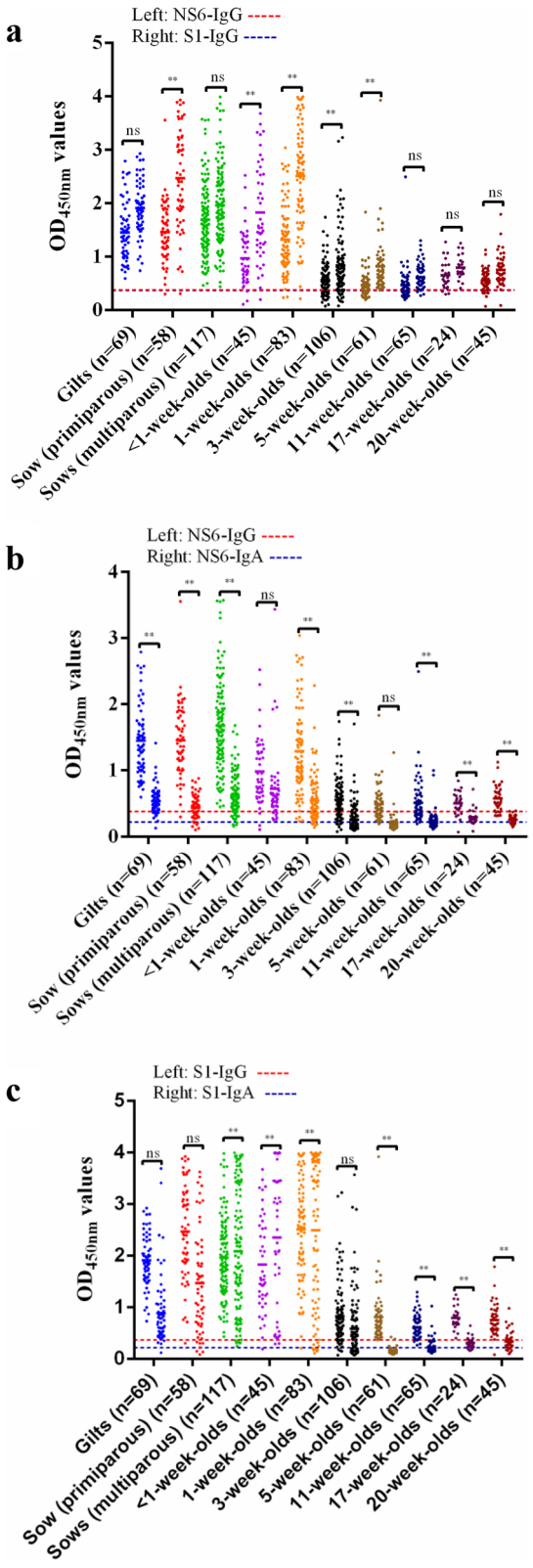

Fig. 5 Serological profiles of serum anti-PDCoV IgG and IgA collected from diarrheic sows and piglets at different ages by using NS6-based and S1-based iELISA. **a** Distribution of anti-NS6 and anti-S1 IgG antibodies among serum samples. Left and right in each group represent NS6 and S1 IgG antibody levels, respectively. The positive cutoff value for NS6-iELISA and S1-iELISA is indicated by red dashed lines and blue dashed lines, respectively. The "ns" denoted no statistical differences. **b**, **c** Distribution of anti-NS6 IgG and IgA antibodies and anti-S1 IgG and IgA anti-bodies among serum samples. Left and right in each group represent IgG and IgA antibody levels, respectively. The positive ELISA cutoff values are indicated by dashed lines. The "ns" denoted no statistical differences

piglets (< 1-week-old), decreased thereafter to a minimum in 11-week-old piglets, and then gradually increased and stabilized. The trend in the IgG response relative to all ages was similar regardless of the ELISA antigen (NS6 or S1), with a greater prevalence in gilts, sows and 1-week-old piglets (Fig. 5a).

It is critical to measure IgA levels to assess the protection of the gastrointestinal tract from viral infection (Lu et al. 2020). Here, serological assays for IgA detection based on the PDCoV NS6 and S1 proteins were also developed. However, the seroprevalence of anti-NS6 IgA antibodies was much lower than that of anti-NS6 IgG antibodies (Fig. 5b). The level of IgA antibodies was significantly lower than that of IgG antibodies, except for samples from 1- and 5-week-old piglets. The trend in the IgA antibody level response to PDCoV NS6 from gilts to 20-week-old pigs was similar to that of IgG, with both levels decreasing significantly after 3 weeks of age. Similarly, IgG and IgA antibody levels against the S1 protein were detected in gilts, primiparous sows and 3-week-old pigs; however, in the other groups, the level of IgA that reacted to S1 was significantly lower than that of anti-S1 IgG (Fig. 5c).

Correlation between the iELISAs based on the PDCoV NS6 and S1 proteins for the detection of IgG antibodies

To determine the correlation between the two different serological detection methods, we used both NS6- and S1-based ELISA methods to analyze IgG antibody levels in serum samples from 11-week-old, 17-week-old, and 20-week-old piglets. We found good linear correlations between the two ELISA methods (Fig. 6). The result was supported by the Pearson correlation [coefficients were 0.901 ($p < 0.01$), 0.875 ($p < 0.01$) and 0.907 ($p < 0.01$], indicating a significant correlation and reliability based on the NS6 iELISA method.

Discussion

Several different diagnostic methods for PDCoV infection have been established, including molecular and serological methods. Among the molecular methods,

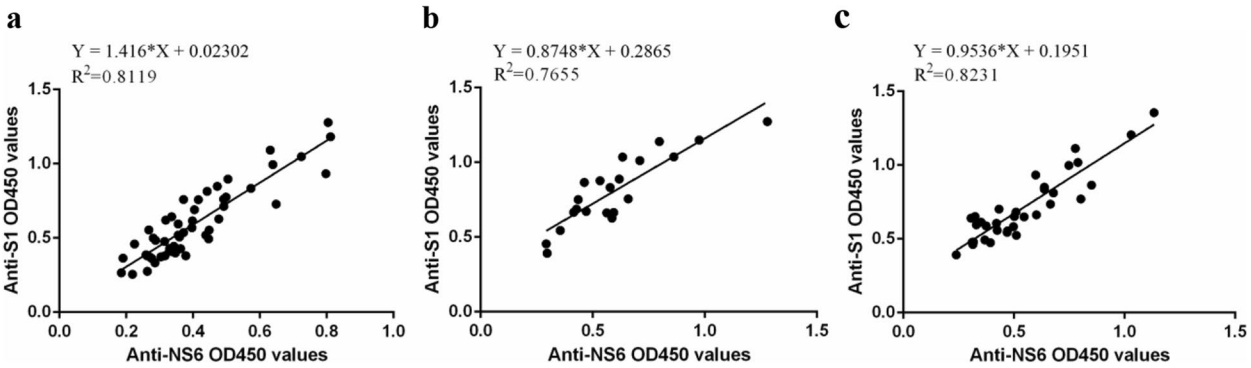

Fig. 6 Correlation analysis of the NS6- and S1-based ELISA methods. Scatter plots show a correlation between the anti-NS6 and anti-S1 antibodies in sera from **a** 11-week-old piglets ($n = 54$; R2 = 0.8119), **b** 17-week-old piglets ($n = 21$; R2 = 0.7655) and **c** 20-week-old piglets ($n = 35$; R2 = 0.8231)

RT–PCR is still preferred for initial detection due to its great specificity and sensitivity (Zhang 2016). Serological assays can reveal previous exposure to the virus, determine the kinetics of antibody responses to viral infection, and evaluate vaccine efficacy. Given its simplicity, several iELISAs, including those based on eukaryote-expressed S1 protein (Thachil et al. 2015), prokaryote-expressed N or M proteins (Luo et al. 2017; Su et al. 2016), and whole virus (Ma et al. 2016), have been used to detect PDCoV IgG antibodies. In particular, S1-based ELISAs have been used to screen large numbers of serum samples, detecting the prevalence of PDCoV (Qin et al. 2021b; Thachil et al. 2015; Wang et al. 2017).

Our previous study revealed the presence of NS6 in the intestinal tissue of infected newborn piglets (Qin et al. 2021a). We deduce that antibodies against the NS6 protein may be present in PDCoV-infected animals. In the present study, we first demonstrated that the NS6 accessory protein is immunogenic in PDCoV-infected pigs by serum WB (Fig. 2); based on this, we developed a novel NS6-based iELISA method and compared it to an established S1-based ELISA for the survey of anti-PDCoV IgG or IgA in pigs of different ages. As expected, IgG levels in newborn piglets (< 1-week-old and 1-week-old) increased and decreased with age, consistent with the pattern of maternal antibodies received by suckling from sows (Fig. 5). After weaning, the piglets began to produce their own antibodies against PDCoV infection. Similar findings were demonstrated in a serological assay for PEDV and porcine torovirus based on the respective S1 protein (Lei et al. 2019; Qin et al. 2021b). The S1-based and NS6-based ELISA methods for IgG detection showed good linear correlations (Fig. 6), supporting the reliability of the NS6-based method.

When designing serological assays for clinical use, it is important to choose the coating antigen carefully. The most commonly targeted protein for CoV diagnostics is the full-length spike protein or the S1 subunit produced in eukaryotic expression systems. However, the relatively low expression of this gene in eukaryotic systems makes this process laborious and costly. Considering that the NS6 protein is unique to PDCoV and has no significant homology with viral proteins of other known swine CoVs, it is unlikely to cross-react with PEDV, TGEV, or SADS-CoV/SeACoV, which was supported by our data (Fig. 3c). Compared to that in S1, the expression of PDCoV NS6 in a prokaryotic system resulted in a relatively greater yield, quick turnaround and cost savings.

Given the obvious intestinal tropism of PDCoV, the development of IgA antibodies is critical to mucosal immunity and involves aspects of both humoral and cellular immunity (Lu et al. 2020; Zhang 2016). To evaluate the incidence of IgA in PDCoV, further development of ELISA methods based on the NS6 or S1 protein was performed. In our study, a few anti-NS6 IgG-positive serum samples tested negative for IgA, indicating that the concentration of anti-NS6 IgA antibodies in the serum was relatively low or that the NS6 protein was not suitable for the detection of IgA antibodies in the serum. Since the NS6 protein is an accessory protein with a small molecular weight, it does not cause as strong an immune response (in particular, neutralizing antibodies) as structural proteins, such as S1 (Qin et al. 2021a). In addition, the relatively low levels of IgA that circulate in the serum also likely increase the difficulty of detection. Interestingly, a previous study showed that an S1-based ELISA effectively detected IgA in milk but not in serum (Lu et al. 2020). Nevertheless, in our study, although the IgA titers were lower overall, the trend of IgA detected by the NS6 antigen was similar to that detected by the S1 protein.

The genus *Deltacoronavirus* has three subgenera (*Andecovirus*, *Buldecovirus*, and *Herdecovirus*), of which *Buldecovirus* mainly infects birds, including bulbul, thrush, munia, white eye, sparrow, magpie robin, wigeon,

common moorhen, falcon, houbara, pigeon and quail (Zhou et al. 2021). PDCoV is a member of the *Buldecovirus family*, and phylogenetic comparisons with other members have shown that they are closely related(Liang et al. 2023), especially with the sparrow CoV HKU17 and quail CoV HKU30 (Lau et al. 2018). To demonstrate their genetic diversity, phylogenetic trees were constructed based on the ORF1ab RNA-dependent RNA polymerase (RdRp) and NS6 amino acid sequences of PDCoV and 10 avian deltacoronaviruses taken from GenBank and using SARS-CoV-2 (NC_045512.2) as the outgroup (Supplemental Fig. S2). In both trees, falcon CoV UAE-HKU27, pigeon CoV UAE-HKU29 and Houbara CoV UAE-HKU28 were clustered together, whereas PDCoV was clustered with quail CoV UAE-HKU30 and sparrow CoV HKU17. This result may indicate the possibility of future cross-species transmission of deltacoronaviruses from avian species to pigs or other animals. Ongoing surveillance of PDCoV across different regions will contribute to a better understanding of viral and host diversity and of the potential for other mammalian species to harbor deltacoronaviruses. Since NS6 is unique to the *Deltacoronavirus* genus, our novel NS6-based ELISA may be useful as a general tool for detecting potential transmission of primarily avian CoVs to pigs or other animals, helping to identify and prevent future epidemics.

Conclusions

In summary, we validated the accuracy and reliability of a new iELISA method based on the PDCoV NS6 protein, which exhibited high sensitivity and specificity and no cross-reactivity with other key swine CoVs. To our knowledge, this is the first large seroepidemiological investigation based on the NS6 accessory protein to be performed in Chinese swine herds. PDCoV infection occurred on 20 pig farms in seven provinces in Eastern China, indicating that PDCoV has become widespread. Our novel ELISA is useful for the differential diagnosis of PDCoV and other swine enteric viruses and may provide greater insights into swine diarrhea outbreaks. This study also lays the foundation for future clinical studies of IgG antibody responses against PDCoV infection and for evaluating the effectiveness of future vaccines.

Methods

Construction, expression and purification of the recombinant NS6 plasmid

The nucleotide sequence of the NS6 gene was amplified from an available recombinant plasmid constructed in our laboratory previously (Qin et al. 2021a) and subsequently cloned and inserted into the prokaryotic expression vector pCold I (Takara) with a polyhistidine tag at the N-terminus between the *Eco*RI and *Xba*I restriction sites, yielding the recombinant plasmid pCold I-PDCoV-NS6.

The plasmid pCold I-PDCoV-NS6 was transformed into *E. coli* BL21 (DE3) competent cells. After shaking at 37°C and 220 rpm for 1 h, the transformation mixture was plated onto LB agar supplemented with 50 mM ampicillin and incubated at 37°C for 12 h. Single colonies were selected and inoculated into LB media supplemented with 50 mM ampicillin overnight at 37°C with shaking at 220 rpm. The broth was cultured to the middle of logarithmic growth, i.e., when the OD_{600nm} reached ~ 0.6, isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 1 mM for induction of expression. Following overnight culture at 16°C with 180 rpm shaking, the bacteria were collected by centrifugation (12,000 × *g*, 15 min), lysed via ultrasonication and analyzed by 15% SDS–PAGE to determine whether the expressed protein was present in the supernatant or pellet. The PDCoV-NS6 protein expressed in the pellet was purified with a Ni–NTA His-Tagged kit (TransGen Biotech, China) according to the manufacturer's protocol.

Expression and purification of the recombinant S1 protein

The recombinant baculovirus harboring PDCoV-S1, which was produced in our laboratory previously (Wang et al. 2017), was amplified in Sf21 insect cells supplemented with 10 μg/mL gentamicin, 0.25 μg/mL amphotericin B, 100 U/mL penicillin and 100 μg/mL streptomycin. Briefly, monolayers of Sf21 cells were grown to log phase at a density of 1.0×10^7/mL and infected with the recombinant baculovirus. After incubating for 72–96 h at 27°C, the supernatant containing the recombinant baculovirus was harvested by centrifugation at 1000 × *g* for 5 min until signs of viral infection appeared, after which the mixture was stored at 4°C in the dark.

To express the recombinant S1 protein, Hi5 insect cells cultured to the middle of logarithmic growth (density of 2×10^6/mL) were infected with the prepared recombinant baculovirus at a multiplicity of infection (MOI) of 1. The cells were incubated at 27°C for 60 h until signs of viral infection appeared, after which the supernatant was centrifuged at 3500 × *g* for 25 min to remove cell debris. The S1 protein secreted in the supernatant was purified by the Ni–NTA kit as described above.

SDS–PAGE and WB analysis

To detect recombinant PDCoV S1 or NS6 proteins, the purified products were resuspended in 80 μL of 1 × SDS–PAGE loading buffer and denatured at 100°C for 10 min. Subsequently, the proteins were resolved on 10% (for S1) or 15% (for NS6) polyacrylamide gels by SDS–PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with Tris-buffered saline

with Tween 20 (TBST) containing 5% nonfat milk. Proteins were detected using a mouse anti-His mAb (1:5000 dilution; Protein-tech, Wuhan, China) and a rabbit anti-S1 pAb (1:200 dilution) in TBST supplemented with 3% bovine serum albumin (BSA) overnight at 4°C and then incubated with HRP-conjugated goat anti-mouse IgG (1:5000 dilution; Thermo Fisher Scientific, United States) at room temperature for 1 h. For serum WB analysis, purified PDCoV S1, NS6 and PEDV S1 (as a negative control) proteins were incubated with swine sera at a 1:100 dilution and with HRP-conjugated goat anti-pig IgG (Thermo Fisher Scientific, United States) at a 1:5000 dilution.

PDCoV NS6 protein-based indirect ELISA

Using the recombinant and purified PDCoV NS6 proteins as coating antigens, a novel NS6-based iELISA for the detection of serum IgG antibodies against PDCoV was optimized by checkerboard titration as described previously (Lei et al. 2019; Qin et al. 2017). Briefly, for the detection of IgG or possible IgA antibodies in serum samples, the concentration of the NS6 protein used to coat 96-well microtiter plates was gradually reduced from 4 μg/mL to 0.125 μg/mL or from 4 μg/mL to 0.25 μg/mL, after which the protein was tested against PDCoV-positive and -negative sera diluted to 1:100. To determine the optimal dilution of the secondary antibody, HRP-labeled goat anti-pig IgG or anti-pig IgA was tested in the range from 1:2000 to 1:20,000 or from 1:2000 to 1:10,000, respectively. Reaction conditions were considered optimal when the ratio of positive to negative serum OD_{450nm} values was the largest, the positive serum OD_{450nm} value was close to 1.0, and the negative serum OD_{450nm} value was less than 0.3.

After the above conditions were optimized, the best incubation time was determined with blocking buffer (5% nonfat powdered milk, 5% fetal bovine serum and 1% BSA), and the samples were incubated for 1, 2, 3 and 4 h at 37°C. The incubation times for the serum samples (0.5, 1, 1.5 and 2 h) and HRP-conjugated goat anti-pig (15, 30, 45 and 60 min) were optimized in the same way. After that, TMB (3,3',5,5' tetramethylbenzidine) one-component color liquid (Solarbio, Beijing, China) was added to carry out the color reaction, and the color was stopped after 10, 15, 20 and 25 min with 2 M sulfuric acid.

For comparison, an iELISA for the detection of IgG antibodies using the PDCoV S1 protein as an antigen established previously in our laboratory was performed (Thachil et al. 2015; Wang et al. 2017). Briefly, a 96-well (Costar, USA) ELISA plate was coated with 100 μL/well of purified recombinant S1 protein in carbonate coating buffer (pH 9.6). After incubation overnight at 4°C, each well was washed 3 times with 250 μL of PBST wash buffer (0.01 M phosphate-buffered saline [PBS], 0.05% Tween 20, pH 7.4) and then blocked with 5% nonfat powdered milk in 1 × PBST at 37°C for 2 h. The diluted serum samples were subsequently transferred to ELISA plates and incubated at 37°C for 1 h. After washing with PBST three times, the plates were incubated with 100 μL of diluted secondary antibody (HRP-goat anti-pig IgG, Thermo Fisher Scientific, United States) for 45 min at 37°C. After the same washing step, 100 μL of TMB Color liquid (Solarbio, Beijing, China) was added to each well, and the mixture was incubated for 15 min at 37°C. The reactions were subsequently stopped by adding 50 μL of 2 M sulfuric acid. Finally, the plates were read at OD_{450nm} using a spectrophotometer. Furthermore, a similar S1-based iELISA for the detection of IgA antibodies was developed by checkerboard titration. The concentration of the coated S1 protein was gradually decreased from 1.6 μg/mL to 0.4 μg/mL, and the concentration of the HRP-labeled goat anti-pig IgA was tested in the range from 1:2000 to 1:10,000.

Determination of the cutoff value of the NS6-iELISA

Forty-eight PDCoV-seronegative swine serum samples were previously verified by WB (Thachil et al. 2015; Wang et al. 2017) and tested using the optimized NS6-based iELISA method to determine the cutoff value. The optimal coating concentration of the NS6 antigen was 50 ng/well. The reaction conditions were described above, with each sample tested three times to determine the average OD_{450nm}. The positive cutoff value was calculated as the mean OD_{450nm} value of PDCoV-negative serum samples plus 3 × standard deviation (SD). A test sample was considered to be positive when its OD_{450nm} was equal to or greater than the cutoff value. A value of OD450 nm less than the mean plus 3 × the standard deviation (SD) was regarded as a true negative. The interval between the mean plus 2 × SD and the mean plus 3 × SD was considered to indicate "suspected positive", and samples in this range were retested. If the retested value was still greater than the mean plus 3 × SD, it was considered positive; otherwise, it was considered negative.

Determination of the reproducibility of the NS6-iELISA

To determine the reproducibility of the NS6-based ELISA, 16 serum samples that were positive and four serum samples that were negative for PDCoV IgG were chosen. To determine the intra-assay reproducibility, each serum sample was tested in three replicates on the same day. For interassay reproducibility, the samples were tested three times (three replicates per time) on different

days. A coefficient of variation (CV; ratio of the SD to the average OD_{450nm} value of each group) of less than 10% was considered to be ideal, thus meeting the repeatability requirements of the test.

Cross-reactivity of NS6-iELISA with antisera to other swine pathogens

To confirm that our assay is specific to PDCoV NS6, two standard positive serum samples from PEDV, one from TGEV, and two from SADS-CoV patients maintained in our laboratory were tested, with PDCoV-positive and PDCoV-negative sera included as controls. Each sample was tested in triplicate, and the average OD_{450nm} was calculated to determine whether the sample was negative or positive.

Validation of the PDCoV NS6 IgG ELISA

To evaluate the specificity and sensitivity of the NS6-iELISA, a total of 39 pig serum samples were tested in triplicate using the PDCoV NS6 IgG ELISA. Moreover, a serum WB was performed on each sample. The results of the serum WB were used as the standard for determining the sensitivity and specificity of the NS6-iELISA as described previously (Peng et al. 2021). Briefly, sensitivity was defined as the ratio of positive samples from the NS6-iELISA to those positive according to the reference serum WB. Specificity was defined as the ratio of negative test results from the NS6-iELISA to those negative from the reference serum WB. A ROC curve was generated using the results of the WB as criteria for negative and positive determinations.

Detection of PDCoV IgG and IgA in field serum samples by NS6-iELISA or S1-iELISA

A total of 673 archived serum samples from pigs with diarrhea were collected from 20 commercial pig farms in Eastern China's Shandong, Henan, Zhejiang, Jiangxi, Jiangsu, Hunan and Heilongjiang provinces between 2015 and 2016. Of these, 69 were from gilts, 58 were from primiparous sows, 117 were from multiparous sows, 45 were from < 1-week-old piglets, 83 were from 1-week-old piglets, 106 were from 3-week-old piglets, 61 were from 5-week-old piglets, 65 were from 11-week-old piglets, 24 were from 17-week-old piglets, and 45 were from 20-week-old piglets. All the serum samples were tested for PDCoV IgG or IgA using PDCoV NS6-based or S1-based ELISA. The reaction conditions were established and optimized above, and each sample was tested in triplicate.

Statistical analyses

All the data were analyzed using SPSS software (version 25.0) and GraphPad Prism software, and Student's t test was used to evaluate the statistical significance of the OD_{450nm} values calculated via ELISA; a p value of 0.05 indicated statistical significance.

Abbreviations

PDCoV	Porcine deltacoronavirus
iELISA	Indirect enzyme-linked immunosorbent assay
IgG	Immunoglobulin G
IgA	Immunoglobulin A
TGEV	Transmissible gastroenteritis coronavirus
PEDV	Porcine epidemic diarrhea virus
SADS-CoV	Swine acute diarrhea syndrome coronavirus
UTR	Untranslated region
ORF	Open reading frame
LB	Luria broth
IPTG	Isopropylthio-β-D-galactoside
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
MOI	Multiplicity of infection
TMB	3,3′,5,5′Tetramethylbenzidine
mAb	Monoclonal antibody
pAb	Polyclonal antibody
BSA	Bovine serum albumin
HRP	Horseradish peroxidase
PBS	Phosphate-buffered saline
WB	Western blot
OD	Optical density
SD	Standard deviation
CV	Coefficient of variation
ROC	Receiver operating characteristic
RdRp	RNA-dependent RNA polymerase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44149-023-00109-4.

Additional file 1: Supplemental Figure S1. Optimization of indirect ELISA methods. **Supplemental Figure S2.** Phylogenetic analysis between different aviancoronaviruses and PDCoV.

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Authors' contributions

Y.-W. H. designed the experiments; Y.-L. L., F.-S. S., L.-X. C., Q. Z., Y. F. and B. W. performed the experiments; Y-L. L. and Y.-W. H. analyzed the data and wrote the manuscript.

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Availability of data and materials

The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Competing interests

The authors declare no conflicts of interest.

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