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A recombinant nucleocapsid protein-based indirect enzyme-linked immunosorbent assay to detect antibodies against porcine deltacoronavirus

Mingjun SU[1)], Chunqiu LI[1)], Donghua GUO[1)], Shan WEI[1)], Xinyu WANG[1)], Yufei GENG[1)], Shuang YAO[1)], Jing GAO[1)], Enyu WANG[1)], Xiwen ZHAO[1)], Zhihui WANG[1)], Jianfa WANG[1)], Rui WU[1)], Li FENG[2)]* and Dongbo SUN[1)]*

[1)]*College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, No. 5 Xinfeng Road, Sartu District, Daqing 163319, P.R. China*
[2)]*Division of Swine Infectious Diseases, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, No. 427 Maduan Street, Nangang District, Harbin 150001, P.R. China*

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ABSTRACT. Recently, porcine deltacoronavirus (PDCoV) has been proven to be associated with enteric disease in piglets. Diagnostic tools for serological surveys of PDCoV remain in the developmental stage when compared with those for other porcine coronaviruses. In our study, an indirect enzyme-linked immunosorbent assay (ELISA) (rPDCoV-N-ELISA) was developed to detect antibodies against PDCoV using a histidine-tagged recombinant nucleocapsid (N) protein as an antigen. The rPDCoV-N-ELISA did not cross-react with antisera against porcine epidemic diarrhea virus, swine transmissible gastroenteritis virus, porcine group A rotavirus, classical swine fever virus, porcine circovirus-2, porcine pseudorabies virus, and porcine reproductive and respiratory syndrome virus; the receiver operating characteristic (ROC) curve analysis revealed 100% sensitivity and 90.4% specificity of the rPDCoV-N-ELISA based on samples of known status (n=62). Analyses of field samples (n=319) using the rPDCoV-N-ELISA indicated that 11.59% of samples were positive for antibodies against PDCoV. These data demonstrated that the rPDCoV-N-ELISA can be used for epidemiological investigations of PDCoV and that PDCoV had a low serum prevalence in pig population in Heilongjiang province, northeast China.

KEY WORDS: ELISA, porcine deltacoronavirus, serum epidemiology

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Porcine deltacoronavirus (PDCoV) was first identified in a rectal swab collected in 2009 from a pig in Hong Kong, China, and it is related to avian and Asian leopard deltacoronaviruses that have been identified in apparently healthy wild animals [6, 22, 23]. Since first reported in swine in the U.S.A. in February 2014, PDCoV rapidly spread to other states in the U.S.A., as well as to Canada, and it caused significant economic losses in the swine industry [14, 16, 19–21]. Recently, PDCoV has been reported in the pig population from China and South Korea [5, 12]. Hu *et al*. reported the cell culture isolation, serial propagation, and biologic and genetic characterizations of cell-adapted PDCoV strains [9]. Jung *et al*. reported that two PDCoV strains, OH-FD22 and OH-FD100, are enteropathogenic in gnotobiotic pigs [10]. Chen *et al*. revealed the pathogenicity of a plaque-purified PDCoV cell culture isolate and characterized PDCoV pathogenesis in neonatal piglets [3]. The accumulating reports demonstrated that PDCoV is associated with diarrhea in pigs.

Porcine deltacoronavirus is an enveloped, positive-sense, single-stranded RNA virus that belongs to the genus of the subfamily *Coronavirinae* of the family *Coronaviridae*. The genome of PDCoV is approximately twenty-five kilobases in length, which is similar to those of other porcine coronaviruses, and it encodes four similar major structural proteins: the spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins [13, 15]. Reports derived from other coronaviruses indicated that the N protein is highly conserved among different strains, and it is widely used as a diagnostic antigen for the development of serologic diagnostic tools [1, 8, 11, 17]. In the current study, a recombinant N protein-based indirect enzyme-linked immunosorbent assay (ELISA) (rPDCoV-N-ELISA) was established to detect antibodies against porcine deltacoronavirus. Furthermore, the serum prevalence of PDCoV was investigated and analyzed using the rPDCoV-N-ELISA in Heilongjiang province, northeast China. Our aim was to provide a potential serological diagnostic tool for PDCoV.

MATERIALS AND METHODS

Antibody: Antisera against porcine epidemic diarrhea virus (PEDV), swine transmissible gastroenteritis virus (TGEV), porcine group A rotavirus (pGARV), classical swine fever virus (CSFV), porcine circovirus-2 (PCV-2), porcine pseudorabies virus (PRV), and porcine reproductive and respiratory syndrome virus (PRRSV) were kindly provided

*CORRESPONDENCE TO: SUN, D., College of Animal Science and Veterinary Medicine, Heilongjiang Bayi Agricultural University, No. 5 Xinfeng Road, Sartu District, Daqing 163319, P.R. China. e-mail: dongbosun@126.com, FENG, L., Division of Swine Infectious Diseases, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, No. 427 Maduan Street, Nangang District, Harbin 150001, P.R. China. e-mail: fl@hvri.ac.cn

by the Division of Swine Infectious Diseases, National Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. Monoclonal antibodies against histidine (His) and glutathione S-transferase (GST) tags were obtained from Tiangen Biotech Co., Ltd. (Beijing, China).

Synthesis and expression of the PDCoV N gene: The nucleotide sequence of the entire N gene of PDCoV was obtained from the GenBank database at the National Center for Biotechnology Information website (accession no. JQ065043). The nucleotide sequence of the PDCoV N gene containing 5′ and 3′ *Bam*HI and *Xho*I restriction sites, respectively, was synthesized based on the codon usage bias of *Escherichia coli*. The synthesized PDCoV N gene was cloned into the prokaryotic expression vectors pET-32a and pGEX-6P-1 so that it was tagged with His and GST, respectively. Recombinant plasmids were transformed into *E. coli* BL21 (DE3) cells, and then, N gene expression was induced using 1.0 mM/*l* isopropyl β-D-thiogalactoside at 37°C for 4 hr. Protein expression was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Moreover, recombinant N proteins were purified according to the method described by Zhu *et al*. [24]. The recombinant N proteins of PDCoV were named rPDCoV-N.

Western blotting of the rPDCoV-N protein: Purified rPDCoV-N proteins with His or GST-tags were subjected to 12% SDS-PAGE and then transferred to a nitrocellulose (NC) membrane using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, U.S.A.). The NC membrane was blocked using 5% (w/v) nonfat dried milk in phosphate-buffered saline (PBS) at 37°C for 1 hr and then incubated with a mouse monoclonal antibody (mAb) against the His-tag (1:1,000 dilution in PBS) or a mouse monoclonal antibody (mAb) against the GST-tag (1:1,000 dilution in PBS) at 37°C for 1 hr. After washing three times with PBS, the membrane was incubated in PBS containing horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000 dilution) at 37°C for 1 hr. After washing three times with PBS containing 0.05% Tween 20 (PBST), the membrane was incubated with enhanced chemiluminescence detection reagents (Biotopped, Beijing, China) at room temperature for 3 min, and peroxidase-mediated luminescence was digitally captured using the Molecular Imager ChemiDoc XRS+ System (Bio-Rad) and Image Lab software (Bio-Rad).

Screening of PDCoV-positive and PDCoV-negative sera: To collect PDCoV-negative and PDCoV-positive serum samples, the reverse transcription polymerase chain reaction (RT-PCR) was used to detect PDCoV in pig farms in Heilongjiang province. Briefly, rectal swab samples were collected from sows and piglets in diarrhea-affected and non-affected pig farms in Heilongjiang province from September 2014 to December 2014. Viral RNA in each sample was extracted using the TIANamp Virus RNA Kit (Tiangen Biotech Co., Ltd.). The cDNA was synthesized by (RNase H-) Moloney murine leukemia virus reverse transcriptase (Novoprotein Scientific Inc., Shanghai, China) according to the manufacturer's instructions. The PCR amplifications were conducted using primers specific for the PDCoV M gene (67F, 5′-ATCCTCCAAGGAGGCTATGC-3′ and 560R, 5′-GCGAATTCTGGATCGTTGTT-3′) according to the protocol described by Wang *et al*. [20]. Moreover, the serum samples, which were collected from PDCoV-positive and PDCoV-negative farms, were tested by western blot using rPDCoV-N proteins with His-tag and GST-tag, respectively. Briefly, purified rPDCoV-N proteins with a His-tag and GST-tag were subjected to 12% SDS-PAGE, respectively, and then transferred to an NC membrane. After blocking with 5% (w/v) nonfat dried milk, the NC membrane was incubated with pig serum samples (1:200 dilution in PBS) at 37°C for 1 hr. After washing three times with PBS, the membrane was incubated in PBS containing HRP-conjugated rabbit anti-pig IgG (1:5,000 dilution) at 37°C for 1 hr. The peroxidase-mediated luminescence was digitally captured using the Molecular Imager ChemiDoc XRS+ System (Bio-Rad) and Image Lab software (Bio-Rad). The serum sample, which was collected from a PDCoV-positive and diarrhea-affected farm and can recognize purified rPDCoV-N proteins with either a His-tag and GST-tag, was used as a positive control in the ELISAs. In addition, a total of 56 serum samples, which were collected from piglets before eating colostra in a PDCoV-negative and diarrhea-unaffected pig farm and can not recognize purified rPDCoV-N proteins with either a His-tag and GST-tag, were used as negative sera in ELISAs.

rPDCoV-N-ELISA procedures: The conditions of the rPDCoV-N-ELISA, including the concentrations of coated antigen, blocking solution, sera and HRP-conjugated rabbit anti-pig IgG, as well as their incubation times, were optimized according to the P/N value (the OD_{450} value of the PDCoV-positive serum/the OD_{450} value of the PDCoV-negative serum). The best reaction conditions for the rPDCoV-N-ELISA were as follows. ELISA plate (Costar, Corning, NY, U.S.A.) wells were coated with 1 μg/m*l* of purified His-tagged rPDCoV-N in 0.05 mol/*l* carbonate buffer (pH 9.6) at 4°C for 12 hr and blocked with 5% skimmed milk at 37°C for 2 hr. After washing four times with PBST, 100 μl of the test antisera, diluted 1:200, was added to the wells and incubated at 37°C for 1 hr. The plates were washed four times and incubated with 100 μl of HRP-conjugated rabbit anti-pig IgG diluted 1:5,000 in PBST at 37°C for 1 hr. After adding 100 μl of a 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution and incubating at room temperature for 10 min, the reaction was stopped by adding 50 μl of 2 M H_2SO_4, and the absorbance at 450 nm was measured.

Determination of the cut-off value for the rPDCoV-N-ELISA: The 56 PDCoV-negative serum samples were tested using the rPDCoV-N-ELISA. The reaction conditions were the same as those described for the rPDCoV-N-ELISA procedures. Each sample was tested three times, and the mean OD_{450} value was used for analysis. The result for each sample was converted into a percent reactivity (PR) value based on the formula: PR value=[(the |OD_{450} value of the tested sample−the OD_{450} value of the negative control)|/(the OD_{450} value of the positive control−the OD_{450} value of the negative control)] × 100%. The PR cut-off value was determined as the mean PR value of the 56 PDCoV-negative sera + 2 × the

standard deviation.

Specificity test: To evaluate the specificity of the rPDCoV-N-ELISA, antisera against PEDV, TGEV, PRoV, CSFV, PCV-2, PRV and PRRSV were tested using the rPDCoV-N-ELISA. The reaction conditions were the same as those used for the rPDCoV-N-ELISA procedures. The PR value of the test samples was calculated. Each sample was tested three times, and the mean PR value was used to determine whether the sample was positive or negative.

Validation of the rPDCoV-N-ELISA: To evaluate the feasibility of the rPDCoV-N-ELISA method, a total of 62 serum samples were randomly selected from six pig farms in Heilongjiang Province; 30 serum samples were collected from three diarrhea-free pig farms, and 32 serum samples were collected from three diarrhea-affected farms. These serum samples were tested using the rPDCoV-N-ELISA. Each sample was tested three times, and the mean PR value was used to determine whether samples were positive or negative. Meanwhile, 62 serum samples were subjected to western blotting using purified, GST-tagged rPDCoV-N. The western blotting procedure was the same as described above. To evaluate the cut-off value, sensitivity and specificity of the rPDCoV-N-ELISA, the receiver operating characteristic (ROC) curve was generated using the results of the western blotting as the standard for negative and positive determination. The statistical analysis was carried out by using SPSS software (Version 11.5 for windows, SPSS Inc., Chicago, IL, U.S.A.).

Detection of PDCoV in field samples by the PDCoV-N-ELISA: A total of 319 serum samples were collected from sows in 15 farms in Heilongjiang province from January 2014 to June 2015, of which 210 serum samples were collected from 9 farms without occurrence of diarrhea, and 109 serum samples were collected from 6 farms with occurrence of diarrhea. All serum samples were tested using the rPDCoV-N-ELISA. The reaction conditions were the same as those used for the rPDCoV-N-ELISA procedures. In the rPDCoV-N-ELISA, each sample was tested three times, and the mean PR value was used to determine whether a sample was positive or negative.

RESULTS

Expression, purification and identification of rPDCoV-N: Prokaryotic expression of the synthesized N gene of PDCoV was conducted in the *E. coli* BL21 (DE3) strain using the vectors pET-32a (with a His-tag) and pGEX-6p-1 with a GST-tag. The results indicated that the recombinant N protein of PDCoV (rPDCoV-N) was successfully expressed in the vectors pET-32a with a His-tag (~56 kDa) (Fig. 1A) and pGEX-6p-1 with a GST-tag (~64 kDa) (Fig. 1B). Furthermore, the rPDCoV-N protein was verified using anti-His-tag and anti-GST mAbs by western blotting (Fig. 1C).

Screening for PDCoV-positive and PDCoV-negative sera: Screening of PDCoV-positive sera was conducted by western blotting using the rPDCoV-N protein with a His-tag and GST-tag as an antigen. The results revealed that one serum sample, named pZD-166, which was collected from one sow in a PDCoV-positive and diarrhea-affected farm in Heilongjiang province, exhibited a specific reaction with His-tagged and GST-tagged rPDCoV-N proteins (Fig. 2). The pZD-166 serum sample was used as a positive control when establishing the rPDCoV-N-ELISA procedures. Moreover, a total of 56 serum samples were collected from piglets, prior to eating colostra, in one diarrhea-unaffected farm in the Daqing area of Heilongjiang province, which was determined to be PDCoV-negative by RT-PCR (data not shown). All 56 serum samples were used to determine the cut-off value of the rPDCoV-N-ELISA, and the nSJZ-15 sample was used as a negative control when establishing the rPDCoV-N-ELISA procedures.

Cut-off value of the rPDCoV-N-ELISA: The 56 PDCoV-negative serum samples were tested using the rPDCoV-N-ELISA according to previously established ELISA procedures. All test results were converted into the PR value according to the formula described previously (Fig. 3). The PR cut-off value of 48.5 (mean + 2× SD, 20.9+27.6) was used as the positive standard in the rPDCoV-N-ELISA.

Specificity of the rPDCoV-N-ELISA: In specific experiments, antisera against PEDV, TGEV, pGARV, CSFV, PCV-2, PRV and PRRSV were used to test the specificity of the rPDCoV-N-ELISA. The results showed that the rPDCoV-N-ELISA did not cross-react with these antisera (PR value <48.5) (Fig. 4).

Validation of the rPDCoV-N-ELISA: To validate the rPDCoV-N-ELISA, a total of 62 serum samples were tested by western blotting using purified, GST-tagged rPDCoV-N and the rPDCoV-N-ELISA. The ROC curve indicated that sensitivity and specificity of the rPDCoV-N-ELISA were 100% and 90.4%, respectively, when the optimized PR cut-off value was 48.9; the optimized PR cut-off value generated from the ROC curve was nearly in line with the PR cut-off value of 48.5 determined by negative sera (Fig. 5). Meanwhile, the area under ROC curve (ROC AUC) was 0.962, which further supported the effectiveness of the rPDCoV-N-ELISA (Table 1).

Detection of PDCoV in field samples using the rPDCoV-N-ELISA: A total of 319 serum samples from 15 pig farms were tested by the rPDCoV-N-ELISA. The results indicated that 11.59% of samples (37/319) were positive for antibodies against PDCoV; for the farms without diarrhea, 3.33% of samples (7/210) were positive for antibodies against PDCoV; for the farms with diarrhea, 27.52% of samples (30/109) were positive for antibodies against PDCoV (Table 2).

DISCUSSION

In the current study, an indirect ELISA, rPDCoV-N-ELISA, which used recombinant N protein that was expressed from *E. coli*, was developed to detect antibodies against PDCoV. The PDCoV N protein shared 22.2%, 28.2% and 18.4% amino acid identities with PEDV (strain CV777, accession no. DQ355221), TGEV (strain H, accession no. EU074218) and porcine respiratory coronavirus (PRCV) N proteins (strain ISU-1, accession no. DQ811787), respectively. Therefore, a cross-reaction between the N protein

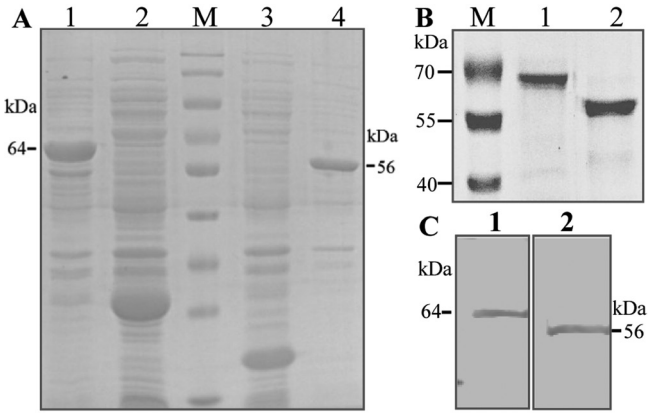

Fig. 1. Expression, purification and identification of the rPDCoV-N. A, Prokaryotic expression of the rPDCoV-N: Lane 1, the IPTG-induced recombinant bacteria of the rPDCoV-N with GST tag; Lane 2, the IPTG-induced recombinant bacteria of the pGEX-6p-1 vector; Lane M, PageRuler™ Prestained Protein Ladder (10k Da-170k Da); Lane 3, the IPTG-induced recombinant bacteria of the pET-32a vector; Lane 4, the IPTG-induced recombinant bacteria of the rPDCoV-N with His tag. B, Purification of the recombinant rPDCoV-N proteins: Lane M, PageRuler™ Prestained Protein Ladder (10k Da-170k Da); Lane 1, purified rPDCoV-N protein with GST tag; Lane 2, purified rPDCoV-N protein with His tag. C, Western blot of the recombinant rPDCoV-N protein: Lane 1, purified rPDCoV-N protein with GST tag; Lane 2, purified rPDCoV-N protein with His tag.

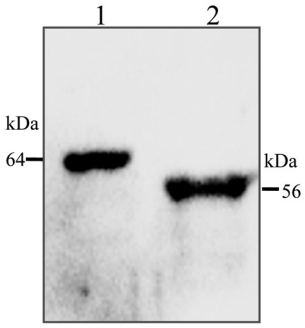

Fig. 2. Screening of positive and negative sera against the PDCoV.

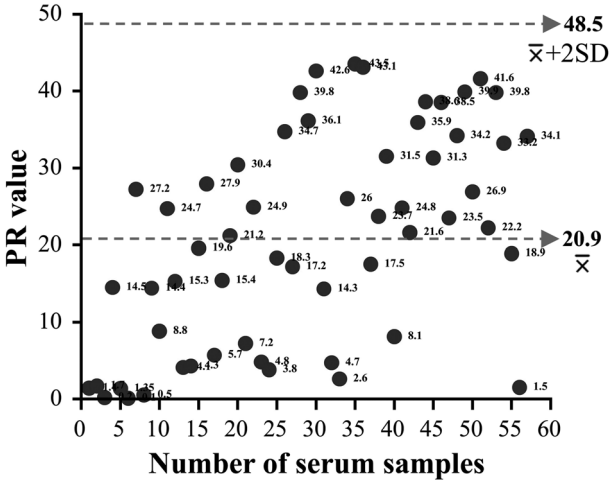

Fig. 3. Determination of the cut-off value of the rPDCoV-N-ELISA.

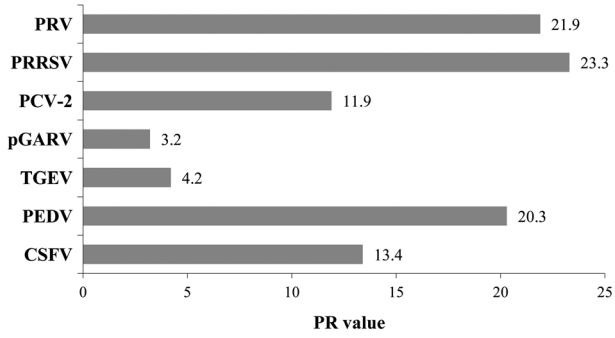

Fig. 4. Specificity test of the rPDCoV-N-ELISA.

of the PDCoV and the N proteins from TGEV, PEDV and PRCV is unlikely. As expected, the PDCoV N protein did not cross-react with TGEV, PEDV, PRCV, PRoV, CSFV, PCV-2, PRV and PRRSV in the specificity assay. Our results are in line with those of an indirect anti-PDCoV IgG ELISA based on the putative S1 portion of the S protein, as described by Thachil *et al*. [17]. In addition, the N protein exhibited a high degree of conservation, 98.8–100% identities, among different PDCoV strains. Antón *et al*. reported that high levels of TGEV-specific antibodies could be induced by a combination of S-rosettes and the N protein [2]. These properties made the N protein suitable as a diagnostic antigen of PDCoV in an indirect ELISA, which was supported by other coronavirus studies [1, 7, 10, 16].

Porcine deltacoronavirus is an emerging coronavirus; thus, a positive standard serum against PDCoV was not available in our study. However, positive serum against PDCoV is a key factor needed for the establishment of a rPDCoV-N-ELISA. In our study, to screen for PDCoV-positive serum, the PDCoV N gene was expressed using two prokaryotic expression vectors with different fusion tags: pGEX-6P-1 with a GST-tag and pET-32a with a His-tag. The PDCoV-positive sera from farms were validated when the samples showed a specific reaction with purified His-tagged and GST-tagged rPDCoV-N proteins, thereby eliminating all false-positive serum samples. Additionally, the PDCoV-negative serum samples were also strictly identified using a combination of the RT-PCR and western blotting using GST-tagged rPDCoV-N as an antigen. Moreover, the PR cut-off value of the rPDCoV-N-ELISA was determined by using PDCoV-negative serum samples. In our study, the introduction of

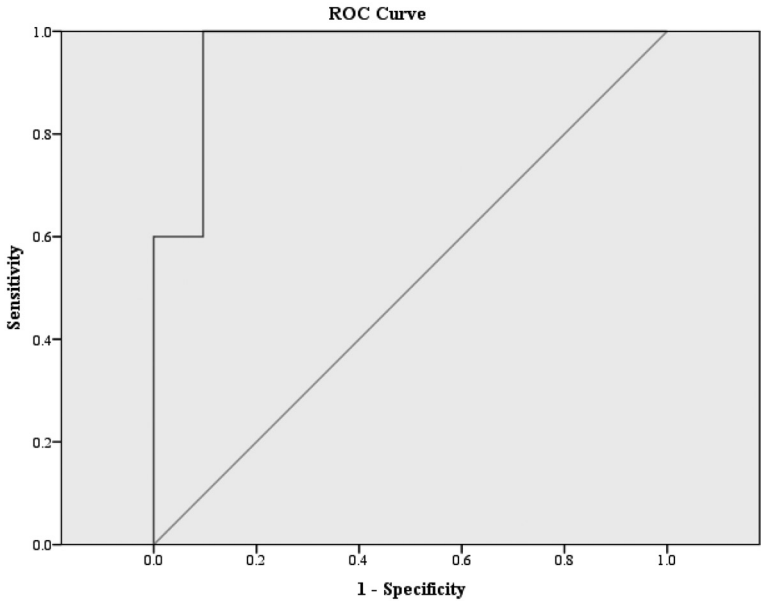

Fig. 5. The receiver operating characteristic (ROC) curve using western blotting as diagnostic standard.

Table 1. The area under the ROC curve

Area	Std. Error[a)]	Asymptotic Sig[b)]	Asymptotic 95% confidence interval	
			Lower bound	Upper bound
0.962	0.023	0.000	0.916	1.000

a) Under the nonparametric assumption, b) Null hypothesis: true area=0.5.

Table 2. Detection of the rPDCoV-N-ELISA in field samples

	Positive rate of PDCoV antibodies
Non-diarrhea	3.33% (7/210)
Diarrhea	27.52% (30/109)
Total	11.59% (37/319)

the PR value to the rPDCoV-N-ELISA reduced errors that resulted from different operation conditions, as was reported in a similar study [6].

Generally, a novel ELISA diagnostic method requires validation via a comparison with the same, or a similar, commercial ELISA kit. Although an indirect anti-PDCoV IgG ELISA based on the putative S1 portion of the spike protein has recently been reported by Thachil *et al*. [17], no ELISA kit was available for evaluation of the rPDCoV-N-ELISA that was established in our study. To evaluate the rPDCoV-N-ELISA, comparison with the western blotting using GST-tagged rPDCoV-N as an antigen was carried by analysis of the receiver operating characteristic (ROC) curve. Compared with the western blotting, the rPDCoV-N-ELISA exhibited a relative specificity of 90.4% and a relative sensitivity of 100%. Accumulating reports indicate that the S protein has a high degree of variability in the members of coronaviruses [4, 18]. Therefore, compared with S protein-based ELISAs, the rPDCoV-N-ELISA still has potential application value as a diagnostic antigen, because of the highly conserved nature. The rPDCoV-N-ELISA results of field samples indicated that 11.59% of samples were positive for antibodies against PDCoV. In our study, positive rate of the PDCoV antibodies in samples is similar to that reported by Thachil *et al*. [17]. It is suggested that an extensive serological investigation of the epidemiology of PDCoV in China should be performed in a future study.

In conclusion, the rPDCoV-N-ELISA has potential use for investigations of the epidemiology of PDCoV. Porcine deltacoronavirus has shown a low prevalence in limited serological investigations in Heilongjiang province, northeast China, and in the future, these results will need to be confirmed through more extensive serological investigation of PDCoV epidemiology.

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