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# Development and application of a recombinant M protein-based indirect ELISA for the detection of porcine deltacoronavirus IgG antibodies

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## Highlights

- Membrane protein of porcine deltacoronavirus was expressed in *E. coli*.
- A recombinant M protein based indirect ELISA was developed.
- The developed iELISA is specific and sensitive.
- This iELISA could be used for large-scale serological testing.

## Abstract

Porcine deltacoronavirus (PDCoV) is a recently identified coronavirus in the genus *Deltacoronavirus* that can cause enteric disease including diarrhea, vomiting,

dehydration and mortality in neonatal piglets. Serological assays to detect anti-PDCoV antibodies are presently limited to certain laboratories and geographic regions. In this study, a recombinant M protein-based indirect enzyme-linked immunosorbent assay (PDCoV-rM ELISA) was developed and utilized to determine the prevalence of anti-PDCoV IgG in Hebei province. The PDCoV-rM ELISA showed no cross-reaction with antisera against transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), porcine rotavirus (PRV), porcine circovirus 2 (PCV2), classical swine fever virus (CSFV) or porcine reproductive and respiratory syndrome virus (PRRSV). The diagnostic sensitivity was 90.6% and the diagnostic specificity was 93.3%. A total of 871 serum samples collected in Hebei from January 2015 to October 2016 were checked for presence of antibodies against PDCoV using the novel PDCoV-rM ELISA. Anti-PDCoV IgG antibodies were detected in 11% (96/871) of the samples and in 25% (10/40) of the investigated farms. The data suggest that PDCoV has a low seroprevalence in pig population in Hebei province, China.

**Key words:** Porcine deltacoronavirus, M protein, ELISA, serum epidemiology.

Porcine deltacoronavirus (PDCoV) is a recently identified coronavirus which has been associated with enteric infections in pigs of all ages (Marthaler et al., 2014; Li et al., 2014; Thachil et al., 2015). PDCoV has been reported initially in Hong Kong in 2012 (Woo et al., 2012). In early 2014, PDCoV was first detected in pigs in the U.S.

(Wang et al., 2014a). Since then, PDCoV has been identified in numerous U.S. farms, South Korea, Canada, Thailand and some provinces of China and the virus has been associated with substantial economic losses (Wang et al., 2014b; Sinha et al., 2015; Hu et al., 2015; Lee et al., 2015; Song et al., 2015; Zhai et al., 2016). However, little is known about the prevalence of PDCoV in Hebei, one of the major pig breeding provinces of China. Therefore, epidemiological studies based on serological and/or virological assays are valuable for the assessment of the PDCoV distribution to implement control strategies if needed.

Similar to other porcine enteropathogenic coronavirus such as porcine epidemic diarrhea virus (PEDV) and transmissible gastroenteritis virus(TGEV), four structural proteins including spike (S), membrane (M), envelop (E) and nucleocapsid (N) proteins (Song et al., 2015; Madapong et al., 2016; Lee and Lee., 2014) are encoded by the PDCoV genome. The M protein of coronaviruses plays an important role in the induction of protection and in mediating the course of the disease (Fleming et al, 1989; Vennema 1991). Cross-reactivity of PDCoV with antibodies to either PEDV or TGEV M protein has not been previously observed (Chen et al., 2015; Jung et al., 2015; Ma et al., 2015; Jung et al., 2016). Therefore, the M protein may be an ideal candidate for the detection of PDCoV specific antibodies and diagnosis of PDCoV infection. In this study, a recombinant M protein-based indirect enzyme-linked immunosorbent assay (ELISA) was developed and used to investigate the antibodies against PDCoV in porcine serum.

Thirty-two serum samples of PDCoV- infected pigs from two farms in Hebei

province were collected and used as positive controls and 30 serum samples obtained from a farm with no history of PDCoV infection and were used as negative controls. The infection status of the positive pigs was confirmed by detecting PDCoV RNA in fecal samples using a reverse transcription polymerase chain reaction (RT-PCR) assay (Wang et al., 2014a), while all fecal samples were negative for PEDV, TGEV and porcine rotavirus (PRV) RNA by a commercial real-time PCR (PEDV-TGEV-PRV PCR, DAAN GENE). The serum samples of PDCoV- infected pigs were collected four weeks after PDCoV RNA detection and were used to determine the cut-off value of the PDCoV-rM ELISA. All sera were confirmed to be negative for antibodies against PEDV, TGEV and PRCV by using a commercial PEDV antibody ELISA (Bionote) and a TGEV/PRCV antibody differential ELISA (Svanova).

Serum samples confirmed positive for antibodies against TGEV, PEDV, PRV, porcine circovirus 2 (PCV2), classical swine fever virus (CSFV) or porcine reproductive and respiratory syndrome virus (PRRSV) were obtained from the Hebei Center for Disease Prevention and Control. Antibodies against PDCoV were tested by both western blot and the PDCoV-rM ELISA.

A total of 871 serum samples were collected from 40 pig farms which had a history of diarrhea in Hebei province from January 2015 to October 2016. All the serum samples were tested using the indirect PDCoV-rM ELISA developed in this study.

The PDCoV HB-BD strain (M gene accession no.KY129985) was amplified from feces of piglets suffering from severe diarrhea in BaoDing, Hebei province, and

passaged in swine testicle (ST) cells as previously described (Hu et al., 2015). The 23rd passage of PDCoV in ST cells was used in this study.

The gene segment encoding the M region of PDCoV was amplified from viral RNA extracted directly from cell cultures by RT-PCR and cloned into the pET-32a plasmid DNA vector (TaKaRa Biotechnology (Dalian) Co., Ltd.). Positive recombinant plasmids were transformed into *E. coli* BL21 (TianGen Biotech Co., Ltd) for protein expression. Primers used for the amplification of the whole M gene sequence of PDCoV isolates were MF 5'- GAATTCACCAATTTCCTAGAAACA- 3' and MR 5' -CTCGAGTTACATATACTTATACAGGC -3'. Expressed M fusion protein was analyzed by SDS-PAGE and purified using a His·Bind® purification kit (Novagen). The recombinant M and His-tag fusion protein was successfully expressed (Fig 1) and the purified PDCoV-rM protein was identified by a Western blot (Fig. 1) as described below.

Polyclonal mouse anti-PDCoV sera for Western blot analysis was generated by inoculating 6-week-old healthy BALB/c mice intraperitoneally and orally with $3\times10^2$ 50% tissue culture infectious dose ($TCID_{50}$) of PDCoV three times in intervals of two weeks (Ethical approval number: SYXK, Hebei, 2015-0045). Serum was collected seven days after the last immunization and stored at -70°C until usage.

For western blot assay, the purified PDCoV-rM proteins were separated by SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes (GIBCO BRL). After blocking with 5% non-fat milk powder in a TBST (Tris-buffered

saline plus 0.1% Tween 20) buffer at 37°C for 2 h, the membranes were incubated first with mouse anti-PDCoV serum and then with a 1:1000 dilution of anti-mouse IgG conjugated with peroxidase (Sigma) at 37°C for 1 h, respectively. The protein band was visualized using DAB (3,3'-diaminobenzidine, Sigma).

The ELISA was carried out in 96-well microtiter plates (Nunc MaxiSorp). A checkerboard titration was used to determine the optimal dilutions of antigen and serum. The expressed protein antigen was diluted from 8 to 0.125μg/ml and the serum was diluted from 1:25 to 1:800. The optimal antigen concentration and serum sample dilution were set at 4μg/ml and 1:100, respectively. Microtitre plates were coated with 100 μl for 2 h at 37°C, followed by an incubation of 12h at 4°C overnight, then blocked with 100 μl 5% fetal bovine serum in PBST for 1 h at 37°C. After three washes with PBST (0.05% Tween-20 in PBS), 100 μl 1:100 diluted serum samples were added and incubated at 37°C for 1 h. The plate was washed three times and incubated with 100 μl 1:5000 diluted HRP-conjugated rabbit anti-pig IgG (Sigma) at 37°C for 45 min. After incubating the wells with a TMB (3,3,5,5,-tetramethyl-benzidine) substrate solution for 15 min at room temperature, the reaction was stopped by adding 50 μl of 2M sulphuric acid. The optical density (OD) was determined at 450 nm using an automated reader.

To establish a cutoff value for the ELISA, 30 serum samples from PDCoV negative farms and 32 serum samples from PDCoV positive farms previously classified as positive or negative by Western Blot, were tested in triplicate with the PDCoV-rM ELISA. The cut-off was calculated by receiver operator characteristic

(ROC) analysis for maximum diagnostic sensitivity and specificity using SPSS software (Version 22.0) (Fig. 2). The cut-off was set at 0.35, giving a sensitivity and specificity were 90.6% and 93.3% for a cut-off of 0.349, with an area under the curve (AUC) of 0.986 ± 0.01.

To evaluate the specificity of the developed ELISA, antibodies against PEDV, TGEV, PRV, PCV2, CSFV and PRRSV were used to analyze the cross-reaction with purified PDCoV-rM antigen. Three replicates of each sample were run on the same occasion. No cross-reaction between PDCoV-rM and any of these antisera was observed. The OD value (average ±SD) was 0.087±0.010 for PEDV, 0.093±0.014 for TGEV, 0.117±0.033 for PRV, 0.138±0.012 for PCV2, 0.173±0.020 for CSFV, and 0.204±0.017 for PRRSV.

From the 871 field serum samples, 11% (96/871) were positive for antibodies against PDCoV and the farm positive rate was 25% (10/40). Detection rates were similar in serum samples collected in 2015 (11.1%, 57/512) and 2016 (10.9%, 39/359). As for the pigs in different age groups, the detection positive rate was 16.9% (31/183) for sows, 12.1% (37/307) for suckling piglets (<28 days) and 7.4% (28/381) for weaning pigs (>28 days). As for the pig farms, the detection positive rate was 15.3% (51/334) for the farms less than 100 sows and 6.2% (16/258) for those more than 500 sows.

Since PDCoV was recently identified, serological assays available for the detection of antibody against PDCoV are limited. ELISA is suitable for testing a large

number of samples (Zhang, 2016). A recombinant PDCoV S1 polypeptide-based ELISA and a recombinant PDCoV N protein based ELISA have been developed to detect PDCoV IgG antibodies (Thachil et al., 2015; Su et al., 2015). Neither ELISA methods cross-reacted with antisera against PEDV, TGEV and other pig pathogens and these results were supported by other research studies using indirect immunofluorescence assays (Chen et al., 2015) and immunohistochemical staining assays (Jung et al., 2015; Ma et al., 2015). However, a recent research study reveals that the conserved or similar epitopes on the N proteins of PEDV and PDCoV could cause two-way antigenic cross-reactivity of the two viruses (Ma et al., 2016). No cross-reactivity was detected by virus neutralization, indirect immunofluorescence, and immunostaining assays using pig hyperimmune antisera to PEDV or PDCoV in that study (Ma et al., 2016) and the PEDV whole virus-based ELISA and the S1 protein-based ELISA showed no cross-reaction with pig antisera against PDCoV, PRCV or TGEV Purdue strain (Chen et al., 2016). While the S protein gene has a high degree of variability in the members of coronaviruses (Su et al., 2015), which could lead to decreased sensitivity, N protein gene is conserved, which could lead to cross-reaction to porcine coronaviruses (Ma et al., 2016).

In this study, a recombinant M protein based ELISA was developed. The coronavirus M protein is the most abundant protein in the virion envelope (Narayanan et al., 2000) and relatively conserved. Although there has been reported that the M protein of PEDV showed some cross-reactivity in pigs (1/12 pigs) inoculated with PDCoV (Gimenez-Lirola et al., 2017), there was no cross-reactivity was detected

between the M protein of PDCoV and the antisera against PEDV, TGEV PRV, PCV-2, CSFV and PRRSV in our study. Considering that there is possibility that antibodies produced by PDCoV infection may cross-react to other porcine coronaviruses antigen, and that it may affect the validation of the developed ELISA, serum samples used to establish the cutoff value were negative for antibodies against TGEV, PEDV and PRCV. The ROC curve assay showed that the relative sensitivity and specificity of the PDCoV-rM ELISA were 90.6% and 93.3%, respectively, indicating a potential diagnostic application compared to previously developed ELISAs (Thachil et al., 2015; Su et al., 2015) .

A total of 871 field serum samples collected from 40 farms which had a history of diarrhea in Hebei, China, from January 2015 to October 2016 were selected to determine the PDCoV antibody distribution. The detection results showed that 11% of samples were positive for antibodies against PDCoV. The total positive rate of the PDCoV antibodies in samples is similar to that reported in Heilongjiang province (11.6%) from January 2014 to June 2015 (Su et al., 2015). When compared with samples from farms with occurrence of diarrhea, the positive rate in Hebei province (11%) was much lower than that in Heilongjiang province (27.5%) (Su et al., 2015). In addition, the positive rate in weaning pigs (7.35%) was lower than that of suckling pigs (12.1%) and sows (16.9%) in our study. The relatively higher levels of antibodies in suckling pigs may be due to presence of maternally-derived antibody and are in agreement with the results that the PDCoV genome was absent in suckling piglet fecal samples in southern China (Zhai et al., 2016). Overall, the obtained data suggest that

PDCoV has been circulating in the Hebei province.

In conclusion, a recombinant M protein based ELISA with potential use for investigations of the epidemiology of PDCoV has been developed in this study. PDCoV showed a low prevalence rate in the Hebei pig population. The obtained anti-PDCoV IgG prevalence data will need to be further confirmed by other PDCoV serological tests in the future.

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**References**


1. Chen, Q., Gauger, P., Stafne, M., Thomas, J., Arruda, P., Burrough, E., Madson, D., Brodie, J., Magstadt, D., Derscheid, R., Welch, M., Zhang, J., 2015. Pathogenicity and pathogenesis of a United States porcine deltacoronavirus cell culture isolate in 5-day-old neonatal piglets. Virology 482, 51–59.
2. Chen, Q., Thomas, J.T., Gimenez-Lirola, L.G., Hardham, J.M., Gao, Q., Gerber, P.F., Opriessnig, T., Zheng, Y., Li, G., Gauger, P.C., Madson, D.M., Magstadt, D.R., Zhang, J., 2016. Evaluation of serological cross-reactivity and cross-neutralization between the United States porcine epidemic diarrhea virus prototype and S-INDEL-variant strains. BMC Vet. Res. 12 (1), 70.
3. Dong, N., Fang, L., Zeng, S., Sun, Q., Chen, H., Xiao, S., 2015. Porcine deltacoronavirus in mainland China. Emerg. Infect. Dis. 21 (12), 2254–2255
4. Fleming JO, Shubin RA, Sussman MA, Casteel N, Stohlman SA: Monoclonal antibodies to the matrix (El) glycoprotein of mouse hepatitis virus protect mice from encephalitis. Virology 1989, 168:162–167.
5. Gimenez-Lirola, L.G., Zhang, J., Carrillo-Avila, J.A., Chen, Q., Magtoto, R., Poonsuk, K., Baum, D.H., Piñeyro, P. and Zimmerman, J., 2017. Reactivity of Porcine Epidemic Diarrhea Virus Structural Proteins to Antibodies against Porcine Enteric Coronaviruses: Diagnostic Implications. Journal of Clinical Microbiology, 55(5), pp.1426-1436.
6. Hu H., Jung K., Vlasova AN., Chepngeno J., Lu Z., Wang Q., Saif LJ. 2015. Isolation and characterization of porcine deltacoronavirus from pigs with diarrhea in the United States. J.Clin. Microbiol. 53: 1537–1548.
7. Jung K, Hu H, Saif LJ. Porcine deltacoronavirus infection: Etiology, cell culture for virus isolation and propagation, molecular epidemiology and pathogenesis. Virus Res. 2016 Apr 13. pii: S0168-1702(16)30156-3. doi: 10.1016/j.virusres.2016.04.009.
8. Jung, K., Hu, H., Eyerly, B., Lu, Z., Chepngeno, J., Saif, L.J., 2015. Pathogenicity of 2 porcine deltacoronavirus strains in gnotobiotic pigs. Emerg. Infect. Dis. 21, 650–654.
9. Lee JH, Chung HC, Nguyen VG, Moon HJ, Kim HK, Park SJ, Lee CH, Lee GE, Park BK. Detection and Phylogenetic Analysis of Porcine Deltacoronavirus in Korean Swine Farms, 2015.Transbound Emerg Dis. 2016 Jun;63(3):248-252.
10. Lee, S. and Lee, C. 2014. Complete genome characterization of Korean porcine deltacoronavirus strain KOR/KNU14-04/2014. Genome Announc. 2: e01191–e14.
11. Li G, Chen Q, Harmon KM, Yoon KJ, Schwartz KJ, Hoogland MJ, et al. Full-length genome sequence of porcine deltacoronavirus strain USA/IA/2014/8734. Genome Announc. 2014; 2: pii: e00278–14.
12. Ma, Y., Zhang, Y., Liang, X., Lou, F., Oglesbee, M., Krakowka, S., Li, J., 2015. Origin, evolution, and virulence of porcine deltacoronaviruses in the United States.mBio 6, e00064.

13. Ma, Y., Zhang, Y., Liang, X., Oglesbee, M., Krakowka, S., Niehaus, A., Wang, G., Jia, A., Song, H., Li, J., 2016. Two-way antigenic cross-reactivity between porcine epidemic diarrhea virus and porcine deltacoronavirus. Vet. Microbiol. 186, 90–96.
14. Madapong A, Saeng-Chuto K, Lorsirigool A, Temeeyasen G, Srijangwad A, Tripipat T, Wegner M, Nilubol D. Complete Genome Sequence of Porcine Deltacoronavirus Isolated in Thailand in 2015.Genome Announc. 2016 May 26;4(3). pii: e00408-16. doi: 10.1128/genomeA.00408-16.
15. Marthaler D, Jiang Y, Collins J, Rossow K. Complete genome sequence of strain SDCV/USA /Illinois121/2014, a Porcine Deltacoronavirus from the United States. Genome Announc. 2014 , 2(2). pii: e00218-14. doi: 10.1128/genomeA.00218-14.
16. Narayanan K, Maeda A, Maeda J, Makino S. 2000. Characterization of the coronavirus M protein and nucleocapsid interaction in infected cells. J Virol 74:8127-8134.
17. Sinha, A., Gauger, P., Zhang, J., Yoon, K.J., Harmon, K., 2015. PCR-based retrospective evaluation of diagnostic samples for emergence of porcine deltacoronavirus in US swine. Vet. Microbiol. 179, 296–298.
18. Song, D., Zhou, X., Peng, Q., Chen, Y., Zhang, F., Huang, T., Zhang, T., Li, A., Huang, D., Wu, Q., He, H., Tang, Y., 2015. Newly emerged porcine deltacoronavirus associated with diarrhoea in swine in China: identification, prevalence and full-length genome sequence analysis. Transbound. Emerg. Dis. 62, 575–580.
19. Su, M., Li, C., Guo, D., Wei, S., Wang, X., Geng, Y., Yao, S., Gao, J., Wang, E., Zhao, X., Wang, Z., Wang, J., Wu, R., Feng, L., Sun, D., 2015. A recombinant nucleocapsid protein-based indirect enzyme-linked immunosorbent assay to detect antibodies against porcine deltacoronavirus. J. Vet. Med. Sci
20. Thachil A, Gerber PF, Xiao CT, Huang YW, Opriessnig T. Development and application of an ELISA for the detection of porcine deltacoronavirus IgG antibodies.PLoS One. 2015, 10(4):e0124363.
21. Vennema H, de Groot RJ, Harbour DA, Horzinek MC, Spaan WJM: Primary structure of the membrane and nucleocapsid protein genes of feline infectious peritonitis virus and immunogenicity of recombinant vaccinia viruses in kittens. Virology 1991, 181:327–335.
22. Wang, L., Byrum, B., Zhang, Y., 2014a. Detection and genetic characterization of deltacoronavirus in pigs, Ohio, USA, 2014. Emerg. Infect. Dis. 20, 1227–1230
23. Wang, L., Byrum, B., Zhang, Y., 2014b. Porcine coronavirus HKU15 detected in 9 US states, 2014. Emerg. Infect. Dis. 20, 1594–1595.
24. Woo, P.C., Lau, S.K., Lam, C.S., Lau, C.C., Tsang, A.K., Lau, J.H., Bai, R., Teng, J.L., Tsang, C.C., Wang, M., Zheng, B.J., Chan, K.H., Yuen, K.Y., 2012. Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of

alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. J. Virol. 86, 3995–4008.

25. Zhai SL, Wei WK, Li XP, Wen XH, Zhou X, Zhang H, Lv DH, Li F, Wang D. 2016. Occurrence and sequence analysis ofporcinedeltacoronaviruses in southern China.Virol. J. 13:136.
26. Zhang, J., 2016. Porcine deltacoronavirus: Overview of infection dynamics, diagnostic methods, prevalence and genetic evolution. Virus research, 226, pp.71-84.

**Figure legends**

Fig 1. Expression and identification of PDCoV-rM. A: Prokaryotic expression of the PDCoV-rM: Lane 1,Protein marker (14-100kDa); Lane 2, IPTG-induced recombinant bacteria with the pET-32a vector for 4h; Lane 3, Uninduced recombinant bacteria with PDCoV-rM; Lane 4, IPTG-induced recombinant bacteria with PDCoV-rM for 1 hours; Lane 5, IPTG-induced recombinant bacteria with PDCoV-rM for 2 hours; Lane 6, IPTG-induced recombinant bacteria with the PDCoV-rM vector for 3h. Lane 7, IPTG-induced recombinant bacteria with the PDCoV-rM vector for 4h. B: Western blot of the recombinant PDCoV-rM. Lane 1, IPTG-induced recombinant bacteria with the pET-32a vector for 4h; Lane 2, purified PDCoV-rM; Lane 3, Protein marker (14-100kDa).

**Fig.2.** Distribution of anti-PDCoV IgG antibodies in serum samples obtained from farms with known PDCoV infection. Serum samples were classified as negative or positive based on viral RNA detection on fecal samples of the pigs. Data presented as ELISA OD values±SEM. The assay cut-off (OD value of 0.35) is indicated by the dashed line.