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Reactivity of anti-PEDV structural protein antibodies to porcine enteric coronaviruses: diagnostic implications

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Running title: **Antibody response against PEDV structural proteins**

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Abstract

The development of porcine epidemic diarrhea virus (PEDV) antibody-based assays is important for detecting infected animals, confirming previous virus exposure, and monitoring sow herd immunity. However, the potential cross-reactivity among porcine coronaviruses is a major concern for the development of pathogen-specific assays. In this study, we used serum samples (n = 792) from pigs of precisely known infection status and a multiplex fluorescent microbead-based immunoassay and/or enzyme-linked immunoassay platform to characterize the antibody response against PEDV whole-virus (WV) particles and recombinant polypeptides derived from the four PEDV structural proteins, i.e., spike (S), nucleocapsid (N), membrane (M), and envelope (E). Antibody assay cut-off values were selected to provide 100% diagnostic specificity for each target. The earliest IgG antibody response was observed at days 7–10 post-infection, mainly directed against S1 polypeptides. With the exception of non-reactive protein E, we observed a similar antibody ontogeny and pattern of seroconversion for S1, N, M, and WV antigens. Recombinant S1 provided the best diagnostic sensitivity, regardless of PEDV strain, with no cross-reactivity detected against transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), or porcine deltacoronavirus (PDCoV) pig antisera. The WV particles showed some cross-reactivity against TGEV Miller and TGEV Purdue antisera, while N protein presented some cross-reactivity against TGEV Miller. The M protein was highly cross-reactive against TGEV and PRCV antisera. Differences in the antibody response against specific PEDV structural proteins have important implications in the development and performance of antibody assays for the diagnosis of PEDV enteric disease.

Key words: PEDV; recombinant structural proteins; whole virus; multiplex FMIA; ELISA,

46 antibody response; cross-reactivity.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is an enveloped, singled-stranded, positive-sense RNA virus that belongs to the order *Nidovirales*, family *Coronaviridae*, subfamily *Coronavirinae*, and genus *Alphacoronavirus* (1). The PEDV genome (~28 kb) consists of seven open reading frames (ORFs) (2). The 5' two-thirds contain the replicase-transcriptase ORF1 (overlapping ORF1a and ORF1b), followed by five ORFs encoding four structural proteins and one strain-specific accessory protein in the following order: spike (S), ORF3 (accessory), envelope (E), membrane (M), and nucleocapsid (N) (3).

PEDV was first reported in Europe in the early 1970s as the causative agent of PED (7). PEDV classical CV777-like strains were subsequently reported in Europe and Asia (8), but PEDV was absent from the Americas, Africa, and Oceania prior to 2013 (9). The emergence of high-virulence PEDV strains was first recognized in late 2010 in China, with outbreaks reported in April 2013 in U.S. (5). Subsequently, high-virulence PEDV strains have been the cause of major economic loss in the swine industry worldwide, producing high mortality in neonatal piglets and high morbidity, but moderate mortality, in weaned pigs (4-6). The emergent PEDV strains are genetically distinct from the classical CV777-likes strains that continue to circulate in the field (5, 10, 11). Based on differences in the S gene and virulence, emerging PEDV strains can be divided into non S-INDEL (S gene insertions and deletions) and S-INDEL strains (9, 12). Overall, S-INDEL strains cause lower mortality than the high-virulence non-S INDEL strains (13, 14).

In addition to PEDV, three other porcine enteric coronaviruses have been described: transmissible gastroenteritis coronavirus (TGEV) (15), porcine deltacoronavirus (PDCoV) (16), and a recently described swine enteric coronavirus (SeCoV) that emerged by recombination

between TGEV and PEDV (17). Enteric coronaviruses primarily infect villous enterocytes, causing atrophic enteritis that leads to malabsorptive diarrhea (4, 5, 18). In general, PEDV and TGEV are considered more virulent than PDCoV, but the three pathogens are clinically and histopathologically indistinguishable (5, 14, 19). Porcine respiratory coronavirus (PRCV) has a predilection for the respiratory tract, but PRCV is an S gene deletion mutant of TGEV and remains on the list of enteric coronavirus differentials.

The differential diagnosis of porcine enteric coronaviruses relies on laboratory direct-detection methods, e.g., polymerase chain reaction (PCR) methods, immunohistochemistry, fluorescent *in situ* hybridization, and direct immunofluorescence in tissues (20-23). Antibody-based assays play an important role in detecting infection and evaluating immunity, but antibody cross-reactivity between porcine enteric coronaviruses is a major concern. As part of the process of developing PEDV-specific antibody assays, we experimentally inoculated pigs with each of the porcine coronaviruses (PEDV, TGEV, PRCV, and PDCoV) and characterized the antibody response to recombinant polypeptides derived from PEDV structural proteins, and to the intact PEDV virion using a multiplex fluorescent microbead-based immunoassay (FMIA) and a whole-virus (WV) ELISA. The final aim of this project was to identify highly sensitive and specific PEDV antigen targets for the antibody-based differential diagnosis of coronavirus-related enteric disease.

2. Material and Methods

2.1. Experimental design

The study was conducted under the approval of the Iowa State University Office for Responsible Research. Seven-week-old pigs (n = 72) were purchased from a conventional wean-

to-finish farm with no previous history of porcine coronavirus infections. The pigs were prescreened for evidence of infection with PEDV, TGEV, PRCV, and PDCoV. Pig fecal swabs were tested by a PEDV N gene-based rRT-PCR (21) and PDCoV M gene-based rRT-PCR (14), while pig fecal and nasal swabs were tested by TGEV (S gene)/PRCV (N gene)-based differential rRT-PCR (20). The pigs' serum samples were tested with the PEDV immunofluorescence assay (IFA) (21), PEDV WV ELISA (24), TGEV/PRCV differential ELISA (Svanova, Sweden), and PDCoV IFA (14). Animals (n = 72) were randomized into six groups; each group consisted of 12 pigs in one room, with 6 pens per room and 2 pigs per pen. Details related to virus strains and the routes of experimental inoculation are presented in Table 1. The age-related infectious dose for each virus was previously determined in a pilot study (data not shown). The pigs were closely observed twice daily for clinical signs throughout the study. A total of 792 serum samples were collected from each group on day post-infection (DPI) –7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42. Virus shedding within groups and absence of cross-contamination between groups during the observation period (–7 to 42 DPI) was confirmed by rRT-PCR, whereas seroconversion within inoculation groups was confirmed by ELISA or IFA (data not shown). At 42 DPI, all pigs were humanely euthanized by penetrating captive bolt (Accles and Shelvoke, Ltd., Sutton Coldfield, UK) followed by exsanguination.

Five different polypeptides corresponding to the four PEDV structural proteins (S, N, M, and E) were recombinantly generated; PEDV WV particles were purified from cell culture by ultracentrifugation, as described elsewhere (24). The PEDV recombinant and WV target antigens were used to develop a multiplex (6-plex) FMIA platform to analyze the antibody response to each target using sera from pigs experimentally inoculated with PEDV, TGEV Miller and Purdue strains, PRCV, PDCoV, or sham control. The diagnostic sensitivity and specificity and the

analytical specificity were evaluated for each individual antigen.

2.2. Generation of PEDV recombinant spike (rS)-derived proteins

The coding region of the amino-terminal receptor-binding (S1) domain derived from a consensus sequence (Figure 1) based on ten PEDV non S-INDEL strains (2,151 nt) or five S-INDEL strains (2,142 nt) (Table 2), respectively, were codon-optimized for expression in mammalian cells and synthetically produced (Shanghai Genery Biotech Co., Ltd.) with the addition of a 5' terminal eukaryotic native signal (encoding MKSLTYFWLFLP VLSTLSLP) and a 3' terminal Tobacco Etch Virus (TEV) cysteine protease site (encoding ENLYFQS), followed by the Fc portion of human IgG1 (Genbank #JX292764.2). The genes were amplified or manipulated using the forward primers F1-(5'-TAA ACG GAT CTC TAG CGA ATT CGC CGC CAC CAT GAA GAG CCTG-3') and F2-(5'-CTT CCA GAG CGG CTC CGA CAA GAC CCA CAC CGT CGA GTG CCC ACC GTG CCC AG-3'), and reverse primers R1-(5'-CGA GCG GCC GCT AGA AGC TTT CAT TTA CCC GGA GAC AGG GAG-3') and R2-(5'-GTC GGA GCC GCT CTG GAA GTA CAG GTT CTC GTG ATA GAA GAA TCC GGG CAG-3'). Amplicons were cloned into pNPM5 eukaryotic expression vector (Novoprotein, Short Hills, NJ, USA) and the recombinant plasmids were transiently transfected into human embryonic kidney (HEK) 293 cells (1×10^6 cells/ml) (Invitrogen, Thermo Fisher Scientific, Grand Island, NY, USA) using polyethylenimine (PEI) (Thermo Fisher Scientific) at an optimal 1:4 ratio (plasmid:PEI, w/w). Transfected HEK293 cells were grown in serum-free FreeStyle™ 293 Expression Medium (Gibco®, Life Technologies, Carlsbad, CA, USA) at 37°C with 5% CO_2 by orbital shaking at 120 rpm. At day 5 post-transfection, culture supernatants were harvested by centrifugation at 3,500 ×g for 20 min, and filter-sterilized (0.45 μm filter). The soluble expression of Fc-tag fused S1 proteins (107.5 kDa) was confirmed by 12% dodecyl sulfate-polyacrylamide gel

139 electrophoresis (SDS-PAGE). PEDV S1-Fc proteins were enzymatically cleaved by incubation
140 with TEV (20 IU/mg sample) for 3 h at 25°C under endotoxin control, and purified from culture
141 supernatant by protein A chromatography (GE Healthcare, Pittsburgh, PA, USA), and nickel
142 (Ni)-chelating Sepharose Fast Flow (SFF) affinity chromatography (GE Healthcare), according
143 to the manufacturer's instructions. Purified rS1 non S-INDEL (717 aa) and rS1 S-INDEL (714
144 aa) proteins were dialyzed against phosphate-buffered saline (PBS) (10 mM phosphate and 150
145 mM NaCl, pH 7.4) and analyzed by SDS-PAGE and Western blot.

146 **2.3. Generation of PEDV recombinant nucleocapsid (rN) protein**

147 An *Escherichia coli* (*E. coli*)-codon optimized consensus version (Figure 1) obtained from a
148 multiple sequence alignment (non S-INDEL, S-INDEL, and CV777 strains) of the full-length
149 PEDV N (1,356 nt) gene (Table 2) was synthesized *in vitro* (Shanghai Genery Biotech Co., Ltd.,
150 Shanghai, China). The gene was amplified using the forward primer (5'-CAT CAT CAT CAT
151 CAT CAT ATG GCA TCT GTT AGC TTT CAG GAT CG-3') and reverse primer (5'-AGA CTG
152 CAG GTC GAC AAG CTT TTA ATT GCC GGT ATC GAA GAT C-3'). The amplicon was
153 cloned into pCold II expression plasmid (Novoprotein Scientific Inc., Shanghai, China),
154 confirmed by sequencing (Genewiz Inc., Suzhou, China), and then transformed into the *E. coli*
155 BL21 (DE3) host strain (Invitrogen™, Carlsbad, CA, USA). The transformants were
156 resuspended and grown in 1 liter of Luria-Bertani (LB) medium (Invitrogen™) containing 100
157 µg/ml ampicillin, at 16°C by shaking at 250 rpm. When an A_{600} of 0.9 was reached, 0.1 mM
158 isopropyl--thio-D galactopyranoside (IPTG) was added, and cultures were grown for an
159 additional 16 h at 16°C. Cells were chilled at 4°C and harvested by centrifugation at 3,500 ×g for
160 15 min, resuspended in 150 ml of lysis buffer (20 mM Tris-HCl and 500 mM NaCl, pH 8.0), and
161 disrupted by ultrasonication (Vibra-Cell™ sonicator, Sonics & Materials, New Town, CT, USA).

The crude extracts were centrifuged at 15,000 ×g for 30 min at 4°C and the soluble expression of histidine (his)-tag fused N-PEDV protein (50.4 kDa) was confirmed by SDS-PAGE analysis. The rN protein (452 aa) was purified from the soluble fraction by Ni-chelating SFF affinity chromatography (GE Healthcare) according to the manufacturer's instructions. After separation of thrombin cleavage products on the Ni^{2+} column, HiTrap™ Phenyl High Performance (HP) (GE Healthcare) hydrophobic interaction chromatography followed by a HiTrap™ SulfoPropyl (SP) HP strong cation exchange chromatography (GE Healthcare) were consecutively applied according to the manufacturer's instructions. Protein elutions were pooled, dialyzed against PBS, pH 7.4, at 4 °C, and analyzed by SDS-PAGE and Western blot.

2.4. Generation of PEDV recombinant membrane (rM) protein

An *E. coli*-codon optimized consensus version (Figure 1) from multiple alignment (non S-INDEL, S-INDEL, and CV777 strains) of the PEDV M (429 nt) gene encoding a truncated fragment corresponding to the C-terminal intra-virion topological domain of the M protein (Table 2) was synthetically generated (Shanghai Genery Biotech Co., Ltd.). The gene was amplified using the forward primer (5'-CAT CAT CAT CAT CAT CAT ATG TTT GTG AAT AGT ATT CGC TTA TGG-3') and reverse primer (5'-AGA CTG CAG GTC GAC AAG CTT TTA AAC CAG ATG CAG AAC TTT TTC G-3'). PCR products were cloned, sequenced, and transformed into BL21 (DE3) cells as described above for the N protein. The truncated his-tagged fusion M-PEDV polypeptide (15.7 kDa; 143 aa), expressed in the precipitate of cell lysate as an inclusion body, was solubilized using a denaturing buffer (20 mM Tris-HCl, 300 mM NaCl, 8 M urea, and 5 mM β-mercaptoethanol; pH 8.0), and purified by Ni-chelating chromatography (GE Healthcare). Elutions were pooled, dialyzed against 20 mM phosphate buffer, 150 mM NaCl, and 6 M urea, pH 7.4, at 4 °C, and analyzed by SDS-PAGE and Western blot.

185 **2.5. Generation of recombinant envelope (rE) small membrane protein**

186 A commercial *E. coli*-expressed PEDV (strain CV777) recombinant his-tagged E protein (76
187 aa) was purchased from Cusabio (CSB-EP771125PPW; Cusabio Biotech Co., MD, USA) and it
188 sequence is presented in Figure 1.

189 **2.6. PEDV multiplex fluorescent microbead-based immunoassay (FMIA)**

190 The covalent coupling of purified PEDV WV antigen and recombinant polypeptides to
191 microbead sets was performed as previously described (25). A total of 25 µg of each protein, i.e.,
192 rS1 non S-INDEL (0.6 mg/ml; bead region 53), rS1 S-INDEL (0.3 mg/ml; bead region 45), rN
193 (0.44 mg/ml; bead region 64), rM (1 mg/ml; bead region 12), and rE (1 mg/ml; bead region 54),
194 and 60 µl of purified WV antigen were coupled to 5×10^6 carboxylated magnetic microspheres
195 (MagPlex®-C Microspheres, Luminex Corp., Austin, TX, USA). Each set of coupled beads were
196 individually evaluated using different assay buffers to exclude matrix inhibitory effects, and to
197 select optimum buffer combination working for all bead sets. After verified that each bead sets
198 worked properly in a single-analyte assay, they were sequentially added into progressively larger
199 multiplex assay to exclude interference between bead regions. Serum samples were diluted to
200 1/50 in assay buffer (Sea Block Blocking Buffer, Thermo Fisher Scientific) and mixed with 50 µl
201 of the bead suspension (~2,500 beads per well for each target) in each well (Bio-Plex Pro™ flat
202 bottom plates, Bio-Rad Laboratories Inc., Hercules, CA, USA). The plates were incubated for 30
203 min on a microplate shaker (VWR®, Radnor, PA, USA) at 400 rpm, and washed three times
204 with 200 µl of 0.1 M PBS, pH 7.4, containing 0.1% Tween-20 (PBST). All incubations were
205 performed at ~22°C in a dark environment. Next, 50 µl of biotin-labelled goat anti-pig IgG (Fc)
206 (Bethyl Laboratories Inc., Montgomery, TX, USA) at 1/3,000 in assay buffer was added to each
207 well, followed by a 30 min incubation. After a washing step, 50 µl streptavidin phycoerythrin
208 (SAPE; Moss Inc., Pasadena, MD, USA) at 1/100 in assay buffer was added to each well and the

plates were incubated for 30 min. After an additional wash step, the microspheres were resuspended in 100 µl of assay buffer and analyzed using a dual-laser Bio-Plex® 200 instrument (Bio-Rad Laboratories, Inc.). The events were gated to exclude doubles and other aggregates (Bio-Plex Manager™ software 6.0, Bio-Rad Laboratories, Inc.). Antibody response, reported as median fluorescent intensity (MFI), was expressed as the sample-to-positive (S/P) ratio:

$$\text{S/P ratio} = \frac{\text{(sample FI – background control mean FI)}}{\text{(positive control mean FI – background mean FI)}}$$

2.7. Data analysis

The cutoff and diagnostic performance of each individual PEDV antigen was determined by receiver operating characteristic (ROC) analysis (SAS® Version 9.4, SAS® Institute, Inc., Cary, NC, USA) using 6-plex FMIA and/or WV ELISA test results. Porcine coronavirus-negative samples (n = 252) were used to estimate diagnostic specificity and PEDV-positive samples (n = 72) collected from the 12 PEDV-inoculated pigs between DPI 14 to 42 were used to estimate diagnostic sensitivity, time of detection, and over-time detection through the observational period. The analytical specificity (cross-reactivity) of each PEDV antigen was evaluated using samples (n = 384) collected between DPI 7 and 42 from animals inoculated with TGEV Miller and Purdue strains, PRCV, and PDCoV.

Pearson's chi-square test was used to detect differences in the number of seropositive animals over time (by DPI) among the individual antigens. Fisher's exact test was used when 25% of the cells in a contingency table contained counts of <5 (SAS® Version 9.4, SAS® Institute, Inc., Cary, NC, USA). One-way ANOVA with Tukey's correction was used for multiple comparisons with alpha=0.05 (GraphPad Prism® 6). Specifically, we compare the antibody response between inoculation groups by day post inoculation for each antigen target.

3. Results

3.1. Dynamics of antibody responses to different PEDV antigens after experimental inoculation

The IgG serum antibody responses to individual PEDV antigens (rS1s [S-INDEL and non S-INDEL], rN, rM, rE, and WV) were evaluated over time (DPI –7 to 42) in pigs inoculated with PEDV, TGEV Miller, TGEV Purdue, PRCV, PDCoV, or negative control by 6-plex FMIA (Figure 2) or PEDV WV ELISA (Figure 4A). In the PEDV-inoculated group, similar antibody dynamics against rS1s, rN, rM, and WV antigens were observed, with significantly higher ($p<0.05$) antibody levels than the negative control group at DPI ≥10. Likewise, antibody responses against rS1s (FMIA), rN (FMIA), and WV (ELISA) in the PEDV-inoculated group were significantly higher ($p < 0.05$) at DPI ≥10 than the TGEV, PRCV, and PDCoV inoculation groups. For rM protein, there were significant differences ($p < 0.05$) between the PEDV and PDCoV inoculation groups; however, no significant differences in rM response were found in pairwise comparisons between the following treatment groups: PEDV vs TGEV Miller (over time), PEDV vs TGEV Purdue (DPI –7 to 10 and DPI 28 to 42), and PEDV vs PRCV (DPI –7 to 10 and DPI 42). No differences in the FMIA IgG S/P ratio antibody response to S1 from the S-INDEL strain compared to S1 from the non S-INDEL strain were observed over time in any inoculation group.

3.2. Diagnostic sensitivity and specificity of PEDV antigens

The optimal S/P cut-off points were selected to ensure 100% diagnostic specificity. Selected cut-off values and diagnostic sensitivity associated for each target tested by FMIA (rS1 non S-INDEL, rS1 S-INDEL, rN, rM, rE, and WV) or ELISA (WV) is presented in Table 1.

The time of detection and the over-time detection of PEDV antibodies to different PEDV antigens is presented in Table 2. The first PEDV antibody-positive pigs were observed on DPI 7

255 for all PEDV antigens, except rE protein. Recombinant E protein showed poor antigenicity,
256 inducing seroconversion in only one animal infected with PEDV (1/12) at DPI 35. With the
257 exception of the non-reactive rE protein, no significant differences ($p > 0.05$) in the number of
258 seropositive animals were observed over time among the PEDV antigens. Likewise, no
259 difference was observed over time in the proportion of seropositive animals detected by both
260 rS1s derived from the PEDV non S-INDEL and S-INDEL strains. Interestingly, the rate of
261 seropositivity using the WV antigen was higher when it was used on the ELISA platform as
262 opposed to the FMIA platform.

263 **3.3. Cross-reactivity of PEDV antigens against PEDV-related porcine enteric coronaviruses**

264 The cumulative distribution of serum antibody responses (S/P) against specific PEDV
265 markers is given in Figure 3 (6-plex FMIA) and Figure 4B (WV ELISA) for each inoculation
266 group. Based on the selected cut-off points, the overall analytical specificity of individual PEDV
267 antigens was 100% for both rS1 polypeptides and rE protein, 98.7% for rN protein, 51.6% for rM
268 polypeptide, 95.6% for WV in the 6-plex FMIA, and 96.3% for WV ELISA. The cross-reactivity
269 of individual antigens against antiserum obtained over time from pigs inoculated with PEDV-
270 related porcine coronaviruses (TGEV Miller, TGEV Purdue, PRCV, and PDCoV) tested by the 6-
271 plex FMIA and/or WV ELISA is presented in Table 2. PEDV rN protein cross-reacted against
272 TGEV Miller antisera (2/12 pigs) at DPI 7 and 14. Similarly, WV FMIA detected one positive
273 pig from both the TGEV Miller and PRCV infected groups, and WV ELISA detected one
274 positive animal from the TGEV Purdue infected group. The WV antibody response against
275 PEDV was higher and discriminated better between groups with ELISA (Figure 4A) compared to
276 FMIA (Figure 2). The rM protein was highly reactive against pigs experimentally inoculated
277 with TGEV Miller (12/12), TGEV Purdue (9/12), and PRCV (7/12) between DPI 7–42.

Moreover, rM protein was the only antigen showing some cross-reactivity in pigs (1/12 pigs at DPI 35) inoculated with PDCoV.

The comparison of the amino acid sequence homology of the 5 purified PEDV polypeptides recombinantly generated (i.e., S1 non S-INDEL and S-INDEL, N, M, and E) with the homologous regions corresponded to porcine coronaviruses PEDV, TGEV Miller, TGEV Purdue, PRCV, and PDCoV used during experimental inoculation is presented in Table 3.

4. Discussion

The pig immune system has the ability to recognize specific PEDV proteins and to respond by producing specific antibodies (21, 24, 26-28) and the development of PEDV antibody assays is important for detecting infection, confirming previous virus exposure, and monitoring levels of immunity. To characterize the antibody response against primary PEDV infection, five recombinant polypeptides derived from the four PEDV structural proteins (S, M, N, and E) and a WV antigen were generated. Proteins S and M were truncated based on gene size, hydrophobicity, and antigenic features to facilitate overexpression and efficient purification. A C-terminal intravirion topological domain of the M protein, previously identified as the major immunodominant region in the M protein, was selected (29). PEDV S protein can be structurally or functionally divided into two subunits: S1 (N-terminal globular head), which binds to the host cell receptors, and S2 (C-terminal membrane-bound stalk), which is responsible for membrane fusion (30, 31). The S1 subunit was selected because it contains major antigenic sites and antiviral neutralizing determinants in many animal coronaviruses, including TGEV (32, 33). However, S1 diverges in sequence even among species of a single coronavirus, thereby contributing to the broad host range of coronaviruses, whereas the S2 subunit is the most

301 conserved region of the protein (34, 35). Therefore, two recombinant S1 proteins derived from
302 PEDV non S-INDEL and S-INDEL strains, respectively, were generated and comparatively
303 evaluated.

304 The analysis showed that serum antibody from PEDV-inoculated pigs reacted to all
305 proteins evaluated, except rE. These data are in agreement with previous results on the utility of
306 N (26), S (28), M (27), and WV antigen (24) for PEDV antibody detection. In general, the
307 magnitude of an antibody response is dependent upon the amount of antigen presented to the
308 immune system. The M protein is the most abundant protein in the virion envelope (36), the N
309 protein is the most abundant coronavirus antigen produced throughout infection (37), and the S
310 protein forms the crown-like projection of the viral surface (38). In contrast, E protein is present
311 only in small amounts in infected cells and the viral envelope (39); which may explain its poor
312 antigenicity.

313 A difference in the WV antibody response was noted between the FMIA and ELISA
314 formats. The lower response in the FMIA assay can be attributed to the fact that the Luminex®
315 carboxylated magnetic microbeads bind WV (intact) PEDV particles less efficiently than they
316 bind proteins or polypeptides with free amino groups. Likewise, the highly charged polystyrene
317 microwells utilized on the ELISA platform bind WV PEDV particles most efficiently, providing
318 better detection.

319 PEDV IgG antibodies against S1 (S-INDEL and non S-INDEL), N, M, and WV were
320 first detected between 7 to 10 DPI and thereafter for the duration of the study. However, our data
321 confirmed the antibody response to S1 proteins as the most sensitive marker of early PEDV
322 infection and the best choice for reliable detection of weak seroresponders (Table 2). Notably,
323 antibodies produced against the PEDV non S-INDEL (USA/IN/2013/19338E) strain used for

experimental inoculation reacted similarly against the two S1 proteins generated from a consensus between different S-INDEL and non S-INDEL PEDV strains, respectively. Previously, Chen et al. (2016) demonstrated that the antibodies against U.S. PEDV S-INDEL and non S-INDEL strains cross-reacted and cross-neutralized both strains *in vitro* (40). Our results confirmed that the high percentage of homology in the amino acid sequence of S1 protein among PEDV non-S INDEL and S-INDEL strains (>90%) assures that S1 could be used as a diagnostic marker for a wide range of PEDV strains.

In the field, pigs are exposed to different coronaviruses that are known to share genetic and antigenic traits that may contribute to false-positive results. Recent evidence suggesting antibody cross-reactivity between PEDV and TGEV (13) and between PEDV and PDCoV (41) raised concerns about the specificity of PEDV serologic testing using assays based on targets containing the most conserved regions (e.g., N, M, and WV). Therefore, the serologic cross-reactivity between individual PEDV antigens and other swine coronaviruses was evaluated by testing antisera from pigs experimentally inoculated with TGEV (Miller and Purdue strains), PDCoV, and PRCV using 6-plex FMIA and WV ELISA.

The S1-derived antigens proved to be the best candidate for antibody-based differential testing of porcine coronaviruses based on a complete absence of detectable cross reactivity (100% analytical specificity). Protein N showed some cross-reactivity (2/12 pigs) against TGEV Miller, but not against TGEV Purdue antisera, and only at the early stages post-infection (DPI 7 to 14). This “transient” cross-reactivity may explain why it has been reported infrequently. In contrast, PEDV M protein showed the highest percentage of homology with other porcine coronaviruses (Table 4) and should be ruled out as a marker for differential diagnosis, as it was highly cross-reactive against TGEV strains and PRCV.

Differences in cross-reactivity among TGEV strains cannot be explained simply on the basis of amino acid sequence homology (Table 3). Lin et al. (2015) reported one-way cross-reactivity between different PEDV strains and TGEV Miller antisera, but not TGEV Purdue antisera (42). Further analysis identified at least one epitope on the N-terminal region of the PEDV/TGEV N protein that contributed to this cross-reactivity (13). In the present study, a full-length N protein was produced. Truncation of the N-terminal region could help avoid possible cross-reactivity (42, 43). Alternatively, cross-reactivity could be corrected by increasing the cut-off from S/P ≥0.59 to S/P ≥0.80, but this would reduce the diagnostic sensitivity from to 83.3% to 76.3%. Similarly, the cross-reactivity of the WV antigen against TGEV Miller (1/12 pigs) and TGEV Purdue (1/12) antisera revealed by ELISA (Table 2) could be corrected using a more conservative cut-off (S/P ≥1.3 instead of S/P ≥0.7) without severely impacting diagnostic sensitivity (from 88.8% to 83.3%).

This study demonstrated that variations in the antibody response against different PEDV structural proteins may have important implications in the diagnosis of PEDV enteric disease. We also successfully identified targets of interest (e.g., S1) for the diagnosis of PEDV, providing a truly molecular immunological view of antigenic distribution and a complete antibody cross-reactivity profile between PEDV and other porcine enteric coronaviruses.

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Declaration of conflicting interest

The author(s) declare no conflicts of interest with respect to the research authorship, and/or

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512 **Tables**

513

514 Table 1. **Optimal S/P cut-off points and diagnostic sensitivity of serum IgG antibody**
515 **response to each PEDV antigen by 6-plex FMIA or WV ELISA.**

516

PEDV marker	Assay	Cut-off (S/P)*	No samples tested positive/Total No positive samples (%); 95% CI
S1 (non S-INDEL)		0.10	69/72 (95.8); 88.4-98.6
S1 (S-INDEL)		0.11	68/72 (94.4); 86.5-97.8
N	6-plex	0.59	59/72 (81.9); 71.5-89.1
M	FMIA	0.21	58/72 (80.6); 69.9-88.0
E		0.90	1/72 (1.4); 0.25-7.5
WV particles		0.67	48/72 (66.7); 55.2-76.5
WV particles	ELISA	0.70	63/72 (87.5); 77.9-93.3

517 *The optimal S/P cut-off points were selected to ensure 100% diagnostic specificity

518

519 Table 2. **Detection of serum IgG antibody response among inoculation groups by day post**
520 **inoculation. Data are presented as number of positive samples (above selected S/P cut-off**
521 **values) to each PEDV antigen by 6-plex FMIA or WV ELISA. Dark grey shaded boxes**
522 **indicate true positive results and light grey shaded boxes indicate false positive results**
523 **(cross-reactivity).**

Inoculation group	No pigs	PEDV marker	Assay	Number pigs positive by day post-inoculation									
				-7	0	3	7	10	14	17	21	28	35	42
PEDV	12	S1 (non S-INDEL)	6-plex FMIA	0	0	0	6	11	11	11	12	12	12	11
		S1 (S-INDEL)		0	0	0	5	10	11	11	12	12	11	11
		N		0	0	0	2	6	10	10	10	10	10	9
		M		0	0	0	1	8	10	10	10	10	9	9
		E		0	0	0	0	0	0	0	0	0	1	0
		WV particles		0	0	0	0	3	7	9	9	8	8	7
		WV particles	Single ELISA	0	0	0	2	7	11	10	11	11	10	10
TGEV Purdue	12	S1 (non S-INDEL)	6-plex FMIA	0	0	0	0	0	0	0	0	0	0	0
		S1 (S-INDEL)		0	0	0	0	0	0	0	0	0	0	0
		N		0	0	0	0	0	0	0	0	0	0	0
		M		0	0	0	3	9	9	9	9	8	8	8
		E		0	0	0	0	0	0	0	0	0	0	0
		WV particles		0	0	0	1	1	1	1	1	1	1	1
		WV particles	Single ELISA	0	0	0	1	1	1	1	1	1	0	1
TGEV Miller	12	S1 (non S-INDEL)	6-plex FMIA	0	0	0	0	0	0	0	0	0	0	0
		S1 (S-INDEL)		0	0	0	0	0	0	0	0	0	0	0

		N		0	0	0	2	2	1	0	0	0	0	0
		M		0	0	0	2	10	11	11	12	11	11	11
		E		0	0	0	0	0	0	0	0	0	0	0
		WV particles		0	0	0	1	1	1	1	1	1	1	1
		WV particles	Single ELISA	0	0	0	1	1	1	1	1	1	1	0
PRCV	12	S1 (non S-INDEL)	6-plex FMIA	0	0	0	0	0	0	0	0	0	0	0
		S1 (S-INDEL)		0	0	0	0	0	0	0	0	0	0	0
		N		0	0	0	0	0	0	0	0	0	0	0
		M		0	0	0	3	5	6	5	7	7	3	7
		E		0	0	0	0	0	0	0	0	0	0	0
		WV particles		0	0	0	0	1	0	0	0	0	0	0
		WV particles	Single ELISA	0	0	0	0	0	0	0	0	0	0	0
PDCoV	12	S1 (non S-INDEL)	6-plex FMIA	0	0	0	0	0	0	0	0	0	0	0
		S1 (S-INDEL)		0	0	0	0	0	0	0	0	0	0	0
		N		0	0	0	0	0	0	0	0	0	0	0
		M		0	0	0	0	0	1	0	0	0	0	0
		E		0	0	0	0	0	0	0	0	0	0	0
		WV particles		0	0	0	0	0	0	0	0	0	0	0
		WV particles	Single ELISA	0	0	0	0	0	0	0	0	0	0	0

Table 3. **Amino acid sequence homology of PEDV recombinant polypeptides Spike (S1 globular head), Nucleocapsid (N; full length), Membrane (M; C-terminal intravirion topological domain), and Envelope (E; full length) compared to homologous regions of PEDV-related porcine coronaviruses TGEV Miller (ATCC VR-1740), TGEV Purdue (ATCC VR-763), PRCV (ATCC VR-2384), PDCoV (USA/IL/2014) used during experimental inoculation. Sequence analysis was performed using Unipro UGene multiplatform software (Version 1.25, Novosibirsk, Russia).**

Strains used for experimental inoculation	**% homology amino acid sequence of PEDV recombinant polypeptides**				
	S1 PEDV non S-INDEL (consensus)	**S1 PEDV S-INDEL (consensus)**	**N PEDV (consensus)**	**M PEDV (consensus)**	**E PEDV (CV777 strain)**
PEDV non S-INDEL (USA/IN/2013/19338E)	100%	92%	100%	100%	98%
TGEV Purdue (ATCC VR-763)	28%	28%	31%	55%	28%
TGEV Miller (ATCC VR-1740)	28%	28%	31%	55%	28%
PRCV (ATCC VR-2384)	29%	29%	31%	55%	30%

PDCoV (USA/IL/2014)	23%	23%	20%	20%	19%

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Figure legends

Figure 1. Amino acid sequences of 5 recombinant polypeptides derived from the 4 PEDV structural proteins named: 1) Envelope (E) full length protein (CV777 PEDV strain); 2) Full length nucleocapsid (N) protein from a consensus among PEDV strains (CV777, non S-INDEL, and S-INDEL); 3) Truncated (C-terminal intravirion topological domain) membrane (M) protein from a consensus among PEDV strains (CV777, S-INDEL, non S-INDEL); 4) Truncated spike protein (globular head; S1) from a consensus among PEDV non S-INDEL strains, 5) S1 from a consensus among PEDV S-INDEL strains.

Figure 2. PEDV multiplex (6-plex) fluorescent microbead-based immunoassay (FMIA) sample-to-positive (S/P) ratios of serum antibody (IgG) response (mean, SE) over time against the 5 recombinant polypeptides (S1 non S-INDEL, S1 S-INDEL, N, M, and E), and the whole virus (WV) antigen in pigs (n = 12 per group) inoculated with PEDV (USA/IN/2013/19338E), TGEV Miller (ATCC VR-1740), TGEV Purdue (ATCC VR-763), PRCV (ATCC VR-2384), PDCoV (USA/IL/2014), and a negative control (sham inoculated). Different letters denoted statistical differences ($p \leq 0.05$). Samples above the estimated S/P cut-off (dashed line) were considered positive.

Figure 3. Distribution of cumulative FMIA IgG sample-to-positive (S/P) ratios obtained for each recombinant polypeptide (S1 non S-INDEL, S1 S-INDEL, N, M, E) on serum samples (n = 792 total; n = 132 per group) collected at DPI -7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42 from pigs (n = 72 total; n = 12 per group) inoculated with PEDV (USA/IN/2013/19338E), TGEV Miller (ATCC VR-1740), TGEV Purdue (ATCC VR-763), PRCV (ATCC VR-2384), PDCoV (USA/IL/2014), and a negative control (sham inoculated). The FMIA S/P cut-off values estimated for each individual antigen are presented in the graph (dashed line).

559 **Figure 4.** Performance of PEDV whole virus (WV) indirect ELISA on experimental samples of
560 precisely known porcine coronavirus infectious status, i.e., pigs inoculated with PEDV
561 (USA/IN/2013/19338E; n = 12), TGEV Miller (ATCC VR-1740; n = 12), TGEV Purdue (ATCC
562 VR-763; n = 12), PRCV (ATCC VR-2384; n = 12), PDCoV (USA/IL/2014; n = 12), and a
563 negative control (sham inoculated; n = 12). A) Sample-to-positive (S/P) ratios of serum antibody
564 (IgG) response (mean, SE) over time in each inoculation group. B) Distribution of cumulative
565 ELISA WV IgG sample-to-positive (S/P) on serum samples (n = 792 total; n = 132 per group)
566 collected at DPI -7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42. Different letters denoted statistical
567 differences ($p=\leq 0.05$). Samples above the estimated S/P cut-off (dashed line) were considered
568 positive.
569

Figure 1

Figure 2

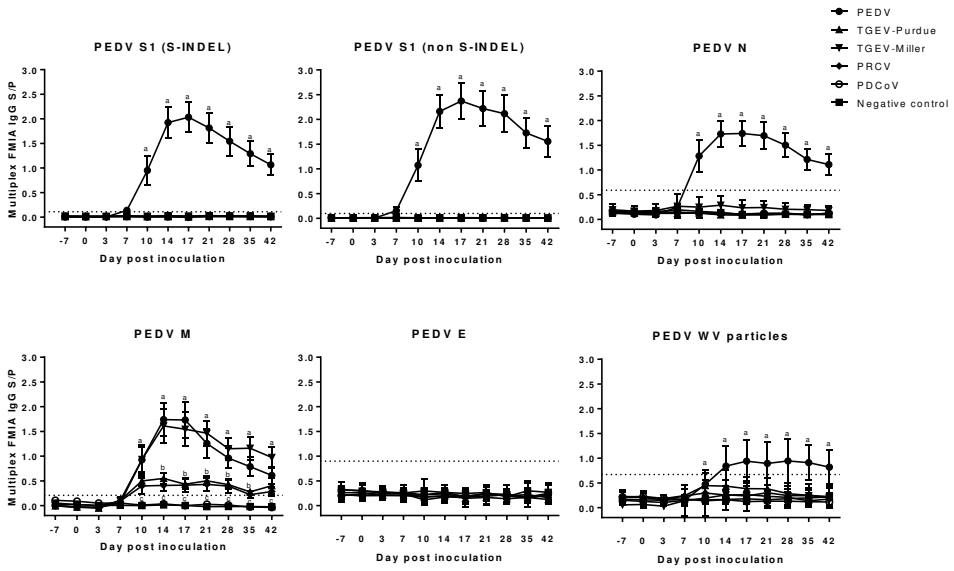

Figure 3

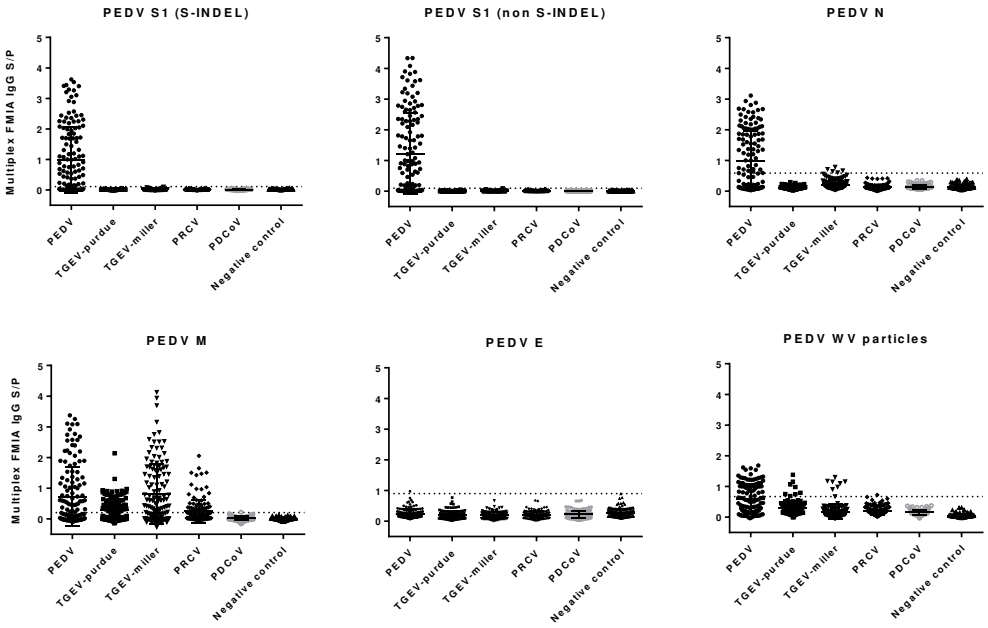

Figure 4