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Reactivity of Porcine Epidemic Diarrhea Virus Structural Proteins to Antibodies against Porcine Enteric Coronaviruses: Diagnostic Implications

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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 to 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 cutoff values were selected to provide 100% diagnostic specificity for each target. The earliest IgG antibody response, mainly directed against S1 polypeptides, was observed at days 7 to 10 postinfection. With the exception of nonreactive protein E, we observed similar antibody ontogenies and patterns of seroconversion for S1, N, M, and WV antigens. Recombinant S1 provided the best diagnostic sensitivity, regardless of the 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 to TGEV Miller and TGEV Purdue antisera, while N protein presented some cross-reactivity to TGEV Miller. The M protein was highly cross-reactive to TGEV and PRCV antisera. Differences in the antibody responses to specific PEDV structural proteins have important implications in the development and performance of antibody assays for the diagnosis of PEDV enteric disease.

KEYWORDS PEDV, recombinant structural proteins, whole virus, multiplex FMIA, ELISA, antibody response, cross-reactivity

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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 of the genome contains 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 as the causative agent of PED in the early 1970s (4). PEDV classical CV777-like strains were subsequently reported in Europe and Asia (5),

but PEDV was absent from the Americas, Africa, and Oceania prior to 2013 (6). The emergence of high-virulence PEDV strains was first recognized in late 2010 in China, with outbreaks reported in April 2013 in the United States (7). Since then, 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 (7–9). The emergent PEDV strains are genetically distinct from the classical CV777-like strains that continue to circulate in the field (7, 10, 11). On the basis of 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 (6, 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 (CoV) 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 (7, 8, 18). In general, PEDV and TGEV are considered more virulent than PDCoV, but the three pathogens are clinically and histopathologically indistinguishable (7, 14, 19). Porcine respiratory coronavirus (PRCV) has a predilection for residence in 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., 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) enzyme-linked immunosorbent assay (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.

RESULTS

(i) Dynamics of antibody responses to different PEDV antigens after experimental inoculation. The IgG serum antibody responses to individual PEDV antigens (recombinant spike 1 [rS1] S-INDEL and rS1 non-S-INDEL], rN, rM, rE, and WV) were evaluated over time (days postinfection [DPI] −7 to 42) in pigs inoculated with PEDV, TGEV Miller, TGEV Purdue, PRCV, PDCoV, or a negative control by 6-plex FMIA (Fig. 1) or PEDV WV ELISA (Fig. 2A). In the PEDV-inoculated group, similar antibody dynamics against rS1, rN, rM, and WV antigens were observed, with significantly ($P < 0.05$) higher antibody levels than were seen with the negative-control group at DPI ≥10. Likewise, antibody responses to the rS1's (FMIA), rN (FMIA), and WV (ELISA) in the PEDV-inoculated group were significantly higher ($P < 0.05$) at DPI ≥10 than to those 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 responses were found in pairwise comparisons between the following treatment groups: PEDV and TGEV Miller (over time), PEDV and TGEV Purdue (DPI −7 to 10 and DPI 28 to 42), and PEDV and PRCV (DPI −7 to 10 and DPI 42). No differences in the FMIA sample/positive result (S/P) ratios with respect to the IgG 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.

(ii) Diagnostic sensitivity and specificity of PEDV antigens. The optimal S/P cutoff points were selected to ensure 100% diagnostic specificity. Selected cutoff values

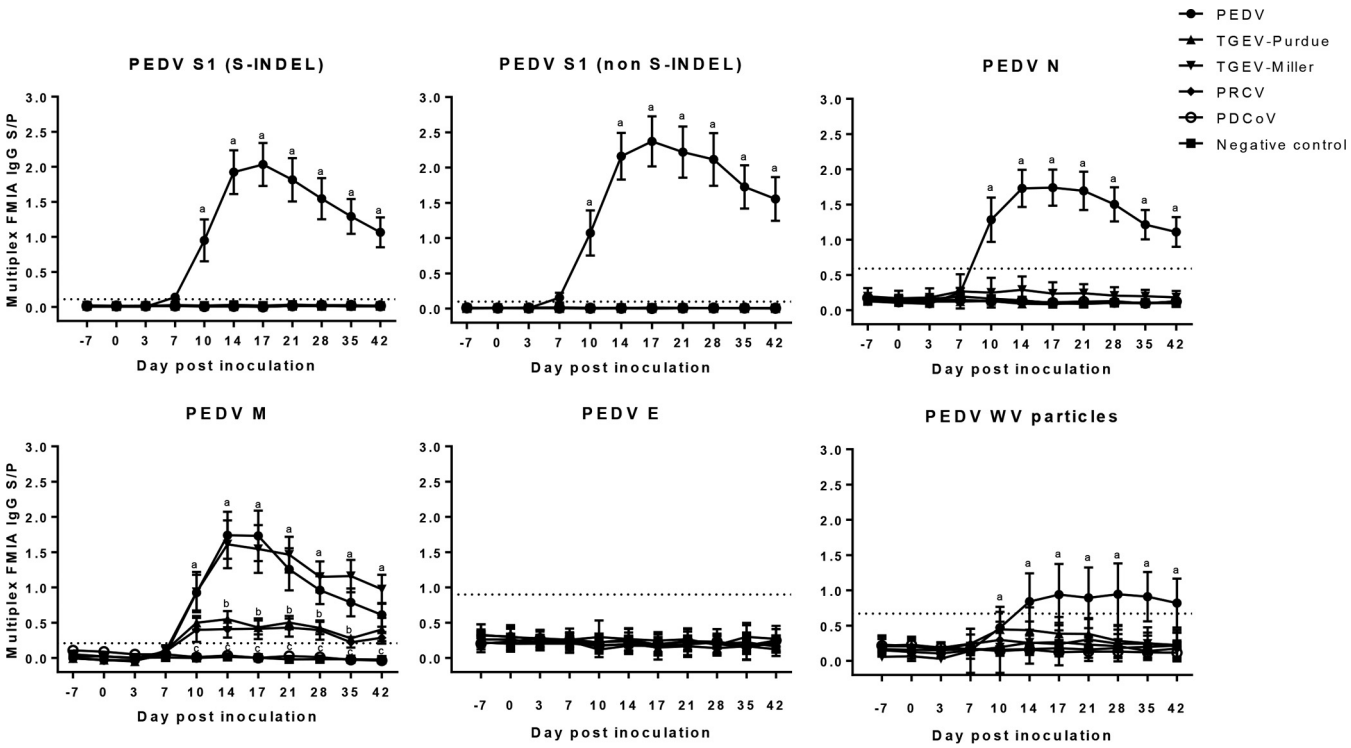

FIG 1 PEDV multiplex (6-plex) fluorescent microbead-based immunoassay (FMIA) sample/positive-result (S/P) ratios of serum antibody (IgG) responses (means and standard errors [SE]) over time to the 5 recombinant polypeptides (S1 non-S-INDEL, S1 S-INDEL, N, M, and E) and to the whole-virus (WV) antigen 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), or PDCoV (USA/IL/2014) or with a negative control (sham inoculation). Different letters denoted statistical differences ($P = \leq 0.05$). Samples above the estimated S/P cutoff (dashed line) were considered positive.

and the associated diagnostic sensitivity for each target tested by FMIA (rS1 non-S-INDEL, rS1 S-INDEL, rN, rM, rE, and WV) or ELISA (WV) are presented in Table 1.

The time of detection and the over[time detection of PEDV antibodies to different PEDV antigens are presented in Table 2. The first PEDV antibody-positive pigs were observed on DPI 7 for all PEDV antigens, except for rE protein. Recombinant E protein showed poor antigenicity, inducing seroconversion in only one animal infected with PEDV (1/12) at DPI 35. With the exception of the nonreactive rE protein, no significant differences ($P > 0.05$) in the numbers of seropositive animals were observed over time among the PEDV antigens. Likewise, no differences were observed over time in the proportions of seropositive animals detected by both rS1's derived from the PEDV non-S-INDEL and S-INDEL strains. Interestingly, the rate of seropositivity using the WV antigen was higher when it was used on the ELISA platform than when it was used on the FMIA platform.

(iii) Cross-reactivity of PEDV antigens against PEDV-related porcine enteric coronaviruses. The cumulative distribution of serum antibody responses (S/P) against specific PEDV markers is given in Fig. 3 (6-plex FMIA) and Fig. 2B (WV ELISA) for each inoculation group. On the basis of the selected cutoff points, the overall analytical specificities of individual PEDV antigens were 100% for both rS1 polypeptides and rE protein, 98.7% for rN protein, 51.6% for rM polypeptide, 95.6% for WV in the 6-plex FMIA, and 96.3% for WV ELISA. The cross-reactivity of individual antigens to antiserum obtained over time from pigs inoculated with PEDV-related porcine coronaviruses (TGEV Miller, TGEV Purdue, PRCV, and PDCoV) tested by the 6-plex FMIA and/or WV ELISA is presented in Table 2. PEDV rN protein cross-reacted to TGEV Miller antisera (2/12 pigs) at DPI 7 and 14. Similarly, WV FMIA detected one positive pig in both the TGEV Miller-infected and PRCV-infected groups, and WV ELISA detected one positive animal in the TGEV Purdue-infected group. The WV antibody response to PEDV was higher and discriminated better between groups with ELISA (Fig. 2A) than with FMIA

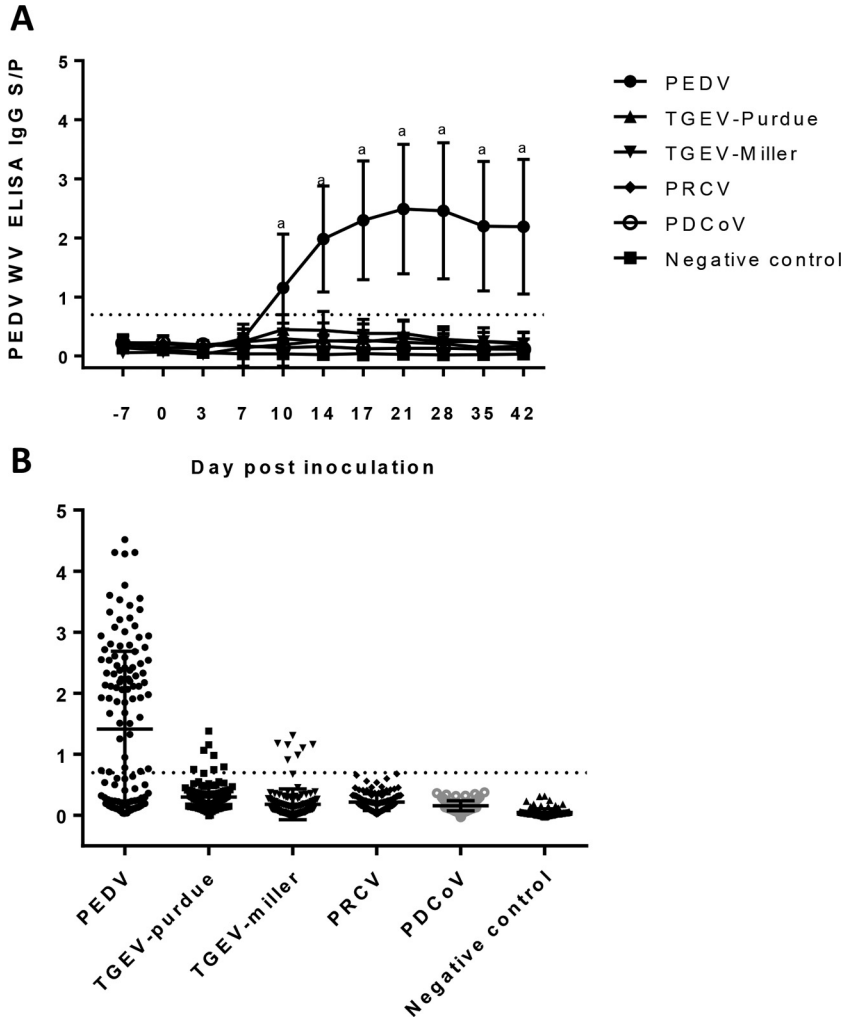

FIG 2 Performance of PEDV whole-virus (WV) indirect ELISA on experimental samples of precisely known porcine coronavirus infectious status, i.e., pigs inoculated with PEDV (USA/IN/2013/19338E; $n = 12$), TGEV Miller (ATCC VR-1740; $n = 12$), TGEV Purdue (ATCC VR-763; $n = 12$), PRCV (ATCC VR-2384; $n = 12$), or PDCoV (USA/IL/2014; $n = 12$) or with a negative control (sham inoculation; $n = 12$). (A) Sample/positive result (S/P) ratios of serum antibody (IgG) responses (mean, SE) over time in each inoculation group. (B) Distribution of cumulative ELISA WV IgG sample/positive result (S/P) ratios in serum samples ($n = 792$ total; $n = 132$ per group) collected at DPI −7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42. Different letters denoted statistical differences ($P = \leq 0.05$). Samples above the estimated S/P cutoff (dashed line) were considered positive.

(Fig. 3). The rM protein was highly reactive to pigs experimentally inoculated with TGEV Miller (12/12), TGEV Purdue (9/12), and PRCV (7/12) between DPI 7 and DPI 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 homologies 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 corresponding to porcine coronaviruses PEDV, TGEV Miller, TGEV Purdue, PRCV, and PDCoV used during experimental inoculation is presented in Table 3.

DISCUSSION

The pig immune system has the ability to recognize specific PEDV proteins and to respond by producing specific antibodies (21, 24–27), 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 to

TABLE 1 Optimal S/P cutoff points and diagnostic sensitivity of serum IgG antibody response to each PEDV antigen by 6-plex FMIA or WV ELISA

Assay	PEDV marker(s)	Cutoff (S/P)[a]	No. of samples that tested positive/ total no. (%) of positive samples; 95% CI[b]
6-plex FMIA	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	0.59	59/72 (81.9); 71.5–89.1
	M	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
ELISA	WV particles	0.70	63/72 (87.5); 77.9–93.3

[a]The optimal S/P cutoff points were selected to ensure 100% diagnostic specificity.
[b]CI, confidence interval.

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 on the basis of gene size, hydrophobicity, and antigenic features to facilitate overexpression and efficient purification. A C-terminal intravirion topological

TABLE 2 Detection of serum IgG antibody responses among inoculation groups by day postinoculation[a]

Inoculation group	Total no. of pigs	PEDV marker(s)	Assay	No. of pigs positive by day postinoculation:										
				−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

[a]Data are presented as numbers of samples (with values above selected S/P cutoff values) that tested positive with respect to each PEDV antigen by 6-plex FMIA or WV ELISA. Data in bold indicate true-positive results, and data in italics indicate false-positive results (cross-reactivity).

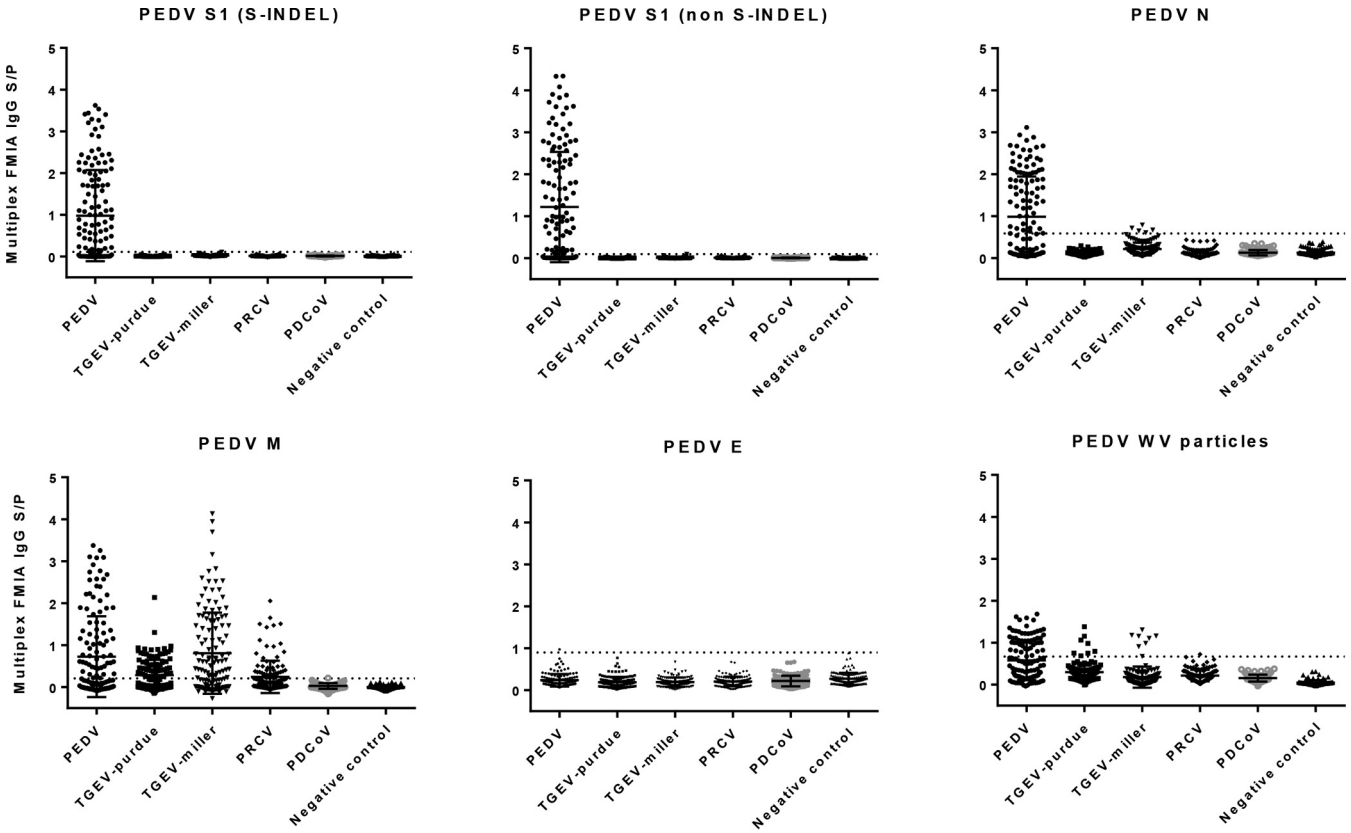

FIG 3 Distribution of cumulative FMIA IgG sample/positive result (S/P) ratios obtained for each recombinant polypeptide (S1 non-S-INDEL, S1 S-INDEL, N, M, or E) on serum samples ($n = 792$ total; $n = 132$ per group) collected at DPI −7, 0, 3, 7, 10, 14, 17, 21, 28, 35, and 42 from pigs ($n = 72$ total;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

domain of the M protein, previously identified as the major immunodominant region in the M protein, was selected (28). 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 (29, 30). The S1 subunit was selected because it contains major antigenic sites and antiviral neutralizing determinants in many animal coronaviruses, including TGEV (31, 32). However, the S1 subunits diverge 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 conserved region of the protein (33, 34). Therefore, two recombinant S1 proteins derived from PEDV non-S-INDEL and S-INDEL strains, respectively, were generated and comparatively evaluated.

The analysis showed that serum antibody from PEDV-inoculated pigs reacted to all

TABLE 3 Amino acid sequence homology of PEDV recombinant polypeptides S1, N, M, and E compared to homologous regions of PEDV-related porcine coronaviruses TGEV Miller, TGEV Purdue, PRCV, and PDCoV used during experimental inoculation[a]

Strain used for exptl inoculation	% homology for amino acid sequence of PEDV recombinant polypeptide				
	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

[a]Sequence analysis was performed using UGene multiplatform software (version 1.25; Unipro, Novosibirsk, Russia). S1, spike (globular head); N, nucleocapsid (full length); M, membrane (C-terminal intravirion topological domain); E, envelope (full length).

proteins evaluated, except rE. These data are in agreement with previous results of studies examining the utility of N antigen (25), S antigen (27), M antigen (26), and WV antigen (24) for PEDV antibody detection. In general, the magnitude of an antibody response is dependent upon the amount of antigen presented to the immune system. The M protein is the most abundant protein in the virion envelope (35), the N protein is the most abundant coronavirus antigen produced throughout infection (36), and the S protein forms the crown-like projection of the viral surface (37). In contrast, E protein is present in only small amounts in infected cells and the viral envelope (38), which may explain its poor antigenicity.

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

PEDV IgG antibodies against S1 (S-INDEL and non-S-INDEL), N, M, and WV were first detected between 7 to 10 DPI and thereafter for the duration of the study. However, our data confirmed that the antibody response to S1 proteins was the most sensitive marker of early PEDV infection and the best choice for reliable detection of weak seroresponders (Table 2). Notably, antibodies produced against the PEDV non-S-INDEL (USA/IN/2013/19338E) strain used for experimental inoculation reacted similarly to 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* (39). Our results confirmed that the high percentage of homology in the amino acid sequences of S1 protein among PEDV non-S INDEL and S-INDEL strains ($>$90%) ensures 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 (40) 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 candidates for antibody-based differential testing of porcine coronaviruses on the basis of a complete absence of detectable cross-reactivity (100% analytical specificity). Protein N showed some cross-reactivity (2/12 pigs) to TGEV Miller, but not to TGEV Purdue antisera, and only at the early postinfection stages (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 those of other porcine coronaviruses (Table 3) and should be ruled out as a marker for differential diagnosis, as it was highly cross-reactive to 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 (41). 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 (41, 42). Alternatively, cross-reactivity could be corrected by increasing the S/P ratio cutoff value from $\geq$0.59 to $\geq$0.80, but this would reduce the diagnostic sensitivity from 83.3% to 76.3%. Similarly, the cross-reactivity of the WV antigen to TGEV Miller (1/12 pigs) and TGEV Purdue (1/12) antisera

FIG 4 Amino acid sequences of 5 recombinant polypeptides derived from the following PEDV structural proteins: (i) envelope (E) full-length protein (CV777 PEDV strain); (ii) full-length nucleocapsid (N) protein from a consensus among PEDV strains (CV777, non-S-INDEL, and S-INDEL); (iii) truncated (C-terminal intravirion topological domain) membrane (M) protein from a consensus among PEDV strains (CV777, S-INDEL, and non-S-INDEL); (iv) truncated spike protein (globular head; S1) from a consensus among PEDV non-S-INDEL strains; (v) S1 from a consensus among PEDV S-INDEL strains.

revealed by ELISA (Table 2) could be corrected using a more conservative cutoff (an S/P ratio cutoff value of ≥1.3 instead of ≥0.7) without severely impacting diagnostic sensitivity (which would change from 88.8% to 83.3%).

This study demonstrated that variations in the antibody responses to 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.

MATERIALS AND METHODS

(i) Experimental design. The study was conducted with 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 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 reverse transcription-PCR (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 a 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 S1 in the supplemental material. 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 days postinfection (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) were 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 the use of a penetrating captive bolt (Accles and Shelvoke, Ltd., Sutton Coldfield, United Kingdom) 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 a sham control. The diagnostic sensitivity and specificity and the analytical specificity were evaluated for each individual antigen.

(ii) Generation of PEDV recombinant spike (rS)-derived proteins. The coding regions of the amino-terminal receptor-binding (S1) domains derived from a consensus sequence (Fig. 4) based on 10 PEDV non-S-INDEL strains (2,151 nucleotides [nt]) or 5 S-INDEL strains (2,142 nt) (see Table S2 in the supplemental material) 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 MKSLTYFWLFLPVLSTLSLP) and a 3′ terminal tobacco etch virus (TEV) cysteine protease site

(encoding ENLYFQS), followed by the Fc portion of human IgG1 (GenBank accession number JX292764.2). The genes were amplified or manipulated using 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 293 (HEK293) cells (Invitrogen, Thermo Fisher Scientific, Grand Island, NY, USA) (1×10^6 cells/ml) using polyethylenimine (PEI) (Thermo Fisher Scientific) at an optimal 1:4 (wt/wt) plasmid/PEI ratio. 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 posttransfection, culture supernatants were harvested by centrifugation at 3,500 $\times$ *g* for 20 min and were subjected to filter sterilization using a 0.45-μm-pore-size filter. The soluble expression of Fc tag-fused S1 proteins (S1-Fc) (107.5 kDa) was confirmed by 12% dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). PEDV S1-Fc proteins were enzymatically cleaved by incubation with TEV (20 IU/mg sample) for 3 h at 25°C under conditions of endotoxin control and purified from culture supernatant by protein A chromatography (GE Healthcare, Pittsburgh, PA, USA) and nickel (Ni)-chelating Sepharose Fast Flow (SFF) affinity chromatography (GE Healthcare), according to the manufacturer's instructions. Purified rS1 non-S-INDEL (717-amino-acid [aa]) and rS1 S-INDEL (714-aa) proteins were dialyzed against phosphate-buffered saline (PBS) (10 mM phosphate and 150 mM NaCl, pH 7.4) and analyzed by SDS-PAGE and Western blotting.

(iii) Generation of PEDV recombinant nucleocapsid (rN) protein. An *Escherichia coli* codon-optimized consensus version (Fig. 4) obtained from a multiple-sequence alignment (non-S-INDEL, S-INDEL, and CV777 strains) of the full-length PEDV N (1,356-nt) gene (see Table S2 in the supplemental material) was synthesized *in vitro* (Shanghai Genery Biotech Co., Ltd., Shanghai, China). The gene was amplified using forward primer 5′-CAT CAT CAT CAT CAT CAT ATG GCA TCT GTT AGC TTT CAG GAT CG-3′ and reverse primer 5′-AGA CTG CAG GTC GAC AAG CTT TTA ATT GCC GGT ATC GAA GAT C-3′. The amplicon was cloned into pCold II expression plasmid (Novoprotein Scientific Inc., Shanghai, China), confirmed by sequencing (Genewiz Inc., Suzhou, China), and then transformed into an *E. coli* BL21(DE3) host strain (Invitrogen, Carlsbad, CA, USA). The transformants were resuspended and grown in 1 liter of Luria-Bertani (LB) medium (Invitrogen) containing 100 μg/ml ampicillin at 16°C with shaking at 250 rpm. When an A_{600} value of 0.9 was reached, 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added, and cultures were grown for an additional 16 h at 16°C. Cells were chilled at 4°C and harvested by centrifugation at 3,500 $\times$ *g* for 15 min, resuspended in 150 ml of lysis buffer (20 mM Tris-HCl and 500 mM NaCl, pH 8.0), and disrupted by ultrasonication (Vibra-Cell sonicator; Sonics & Materials, Newtown, CT, USA). The crude extracts were centrifuged at 15,000 $\times$ *g* for 30 min at 4°C, and the soluble expression of a 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 and 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 blotting.

(iv) Generation of PEDV recombinant membrane (rM) protein. An *E. coli* codon-optimized consensus version (Fig. 4) 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 intravirion topological domain of the M protein (see Table S2 in the supplemental material) was synthetically generated (Shanghai Genery Biotech Co., Ltd.). The gene was amplified using 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–6 M urea (pH 7.4) at 4°C, and analyzed by SDS-PAGE and Western blotting.

(v) Generation of recombinant envelope (rE) small membrane protein. A commercial *E. coli*-expressed PEDV (strain CV777) recombinant His-tagged E protein (76 aa) was purchased from Cusabio (CSB-EP771125PPW; Cusabio Biotech Co., MD, USA), and its sequence is presented in Fig. 4.

(vi) PEDV multiplex fluorescent microbead-based immunoassay (FMIA). The covalent coupling of purified PEDV WV antigen and recombinant polypeptides to microbead sets was performed as previously described (43). A total of 25 μg of each protein, i.e., rS1 non-S-INDEL (0.6 mg/ml; bead region 53), rS1 S-INDEL (0.3 mg/ml; bead region 45), rN (0.44 mg/ml; bead region 64), rM (1 mg/ml; bead region 12), and rE (1 mg/ml; bead region 54), and 60 μl of purified WV antigen were coupled to 5×10^6 carboxylated magnetic microspheres (MagPlex-C Microspheres; Luminex Corp., Austin, TX, USA). Each set of coupled beads was individually evaluated using different assay buffers to exclude matrix inhibitory effects and to select the optimum buffer combination for all bead sets. After it was verified that each of the bead sets worked properly in a single-analyte assay, they were sequentially added into progressively larger multiplex assays to exclude interference between bead regions. Serum samples were diluted to 1/50 in assay buffer (Sea Block Blocking Buffer; Thermo Fisher Scientific) and mixed with 50 μl of the bead

suspension (~2,500 beads per well for each target) in each well (Bio-Plex Pro flat-bottom plates; Bio-Rad Laboratories Inc., Hercules, CA, USA). The plates were incubated for 30 min on a microplate shaker (VWR, Radnor, PA, USA) at 400 rpm and were washed three times with 200 μl of 0.1 M PBS (pH 7.4) containing 0.1% Tween 20 (PBST). All incubations were performed at ~22°C in a dark environment. Next, 50 μl of biotin-labeled goat anti-pig IgG (Fc) (Bethyl Laboratories Inc., Montgomery, TX, USA) at a 1/3,000 dilution in assay buffer was added to each well, followed by a 30-min incubation. After a washing step, 50 μl streptavidin phycoerythrin (SAPE; Moss Inc., Pasadena, MD, USA) at a 1/100 dilution 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.). The antibody response, reported as median fluorescent intensity (MFI), was expressed as the sample/positive result (S/P) ratio as follows:

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

(vii) Data analysis. The cutoff value for and diagnostic performance of each individual PEDV antigen were 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 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 and 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 numbers 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 analysis of variance (ANOVA) with Tukey's correction was used for multiple comparisons with an alpha value of 0.05 (GraphPad Prism 6). Specifically, we compared differences in the antibody responses of the inoculation groups by day postinoculation for each antigen target.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.02507-16.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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