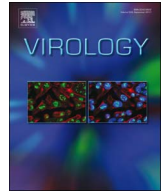




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The S2 glycoprotein subunit of porcine epidemic diarrhea virus contains immunodominant neutralizing epitopes

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ABSTRACT

The porcine epidemic diarrhea virus (PEDV) spike (S) protein is the major target of neutralizing antibodies against PEDV. Here immunodominant neutralizing epitopes of PEDV were identified using a panel of S-specific monoclonal antibodies (mAbs). Ten of eleven S-specific mAbs successfully neutralized PEDV infectivity *in vitro*. Notably, epitope mapping by peptide ELISAs revealed that nine of these mAbs recognized linear neutralizing epitopes located in the N-terminus of the S2 glycoprotein subunit (amino acids [aa] 744–759, 747–774 and/or 756–771). Additionally, one mAb recognized a neutralizing epitope located in the C-terminus of S2 (aa 1371–1377), while only one neutralizing mAb reacted against a region of the S1 glycoprotein subunit (aa 499–600). Notably, mAbs that recognized epitopes within the S2 subunit presented the highest neutralizing activity against PEDV. Together these results indicate that the S2 glycoprotein subunit contains major antigenic determinants and, perhaps, the immunodominant neutralizing epitopes of PEDV.

1. Introduction

Porcine epidemic diarrhea virus (PEDV) is a member of the genus *Alphacoronavirus* of the family *Coronaviridae*. The virus replicates primarily in enterocytes of the small intestine causing villous atrophy and malabsorptive diarrhea which lead to electrolyte imbalance, metabolic acidosis and death (Alvarez et al., 2015; Annamalai et al., 2015). The characteristic clinical signs of PEDV infection are watery diarrhea, vomiting, anorexia, and dehydration which are followed by high mortality rates (50–100%) in suckling piglets or weight loss due to diarrhea in older pigs (Pensaert and Martelli, 2016).

The PEDV genome consists of a large (~28 Kb) single-stranded, positive sense RNA molecule which contains seven open reading frames (ORF1ab, and ORFs2–6) (Huang et al., 2013; Lawrence et al., 2014). It is organized in a central coding region that is flanked by 5'- and 3'-untranslated regions (UTRs). The first gene ORF1ab encompasses approximately 2/3 of the viral genome and encodes large polyproteins (pp1a and pp1b), that are cleaved by viral encoded proteases into 16 non-structural proteins (nsp1–16) (Huang et al., 2013). In addition, four structural proteins are encoded by ORFs2, –4, –5 and –6, including the S glycoprotein (180–220 kDa), membrane

(M; 27–32 kDa), envelope (E; 7 kDa), and the nucleocapsid proteins (N; 55–58 kDa), respectively. ORF3 encodes for a non-structural accessory protein (Huang et al., 2013; Lee, 2015; Song and Park, 2012).

The S protein is the major envelope glycoprotein responsible for virus attachment, receptor binding, cell membrane fusion and entry (Cruz et al., 2008; Sun et al., 2008; Wicht et al., 2014). The S protein is expressed as a 1386 amino acid (aa) precursor protein (180–200 kDa) that is cleaved by host proteases into two major subunits: the S1 subunit (residues 20–729) that mediates virus attachment to the cell surface receptor; and the S2 subunit (residues 730–1386) involved in virus and host cell membrane fusion. Like other coronavirus' S proteins, the PEDV S is a type I membrane glycoprotein that forms homotrimeric projections (spikes) on the virion surface and contains an N-terminal signal peptide (residues 1–18), a large extracellular region, a single transmembrane domain (residues 1328–1350) and a short cytoplasmic tail (residues 1351–1386) (Li et al., 2016). The S1 subunit has been shown to have a modular architecture with four discrete domains, including an N-terminal domain (NTD; residues 19–233) that exhibits sialic acid binding activity and a C-terminal domain (CTD; residues 477–629) that can interact with protein receptor(s) (Li

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et al., 2016). The S2 subunit presents the typical structural features found in class I fusion proteins, including a hydrophobic fusion peptide (FP, residues 891–908), two heptad repeat regions (HR1, residues 978–1117 and HR2, residues 1274–1313), and a C-terminal transmembrane domain (residues 1328–1350) (aa positions based on PEDV strain CO13 S sequence, GenBank accession no. KF272920) (Li et al., 2016). The homotrimeric S proteins on the virion surface are uncleaved and undergo proteolytic processing required for fusion during entry (Shirato et al., 2011; Wicht et al., 2014).

Given its critical functions during cell entry, the PEDV S protein is the main target of host neutralizing antibodies against the virus (Song et al., 2016). Indeed, several studies have recently shown that immunization of pigs with full-length or truncated versions of the S protein elicits antibody responses and protection against PEDV (Hain et al., 2016; Makadiya et al., 2016; Oh et al., 2014). Most importantly, at least four neutralizing domains have been identified in the S protein, including: 1) a domain recently mapped to the NTD/S0 region (Li et al., 2017), 2) a domain that is homologous to the collagenase resistant fragment (CO-26K) of TGEV S (Godet et al., 1994), thus being named “collagenase equivalent” (COE) in PEDV (residues 499–638) (Chang et al., 2002), 3) an epitope that has been mapped to the S1D region (residues 636–789) and spans the S1-S2 junction region (Sun et al., 2008), and 4) an epitope mapped to C-terminus of the S protein (residues 1371–1377) (Cruz et al., 2008).

Here we have shown that immunization of BALB/c mice with purified and inactivated whole virus preparations of PEDV followed by somatic cell fusion resulted in hybridoma cell lines secreting mAbs specific for the S protein that exhibited potent neutralizing activity against PEDV *in vitro*. Epitope mapping of the resultant neutralizing mAbs led to the identification of important antigenic determinants within the S protein. Notably, most mAbs obtained here reacted against linear epitopes within the S2 subunit, indicating that this region contains immunodominant neutralizing epitopes of PEDV.

2. Results

2.1. Selection and characterization of PEDV S-specific mAbs

PEDV S-specific mAbs were generated and selected based on the reactivity of the mAbs with full length PEDV S expressed by a recombinant viral vector (ORFV-PEDV-S) (Hain et al., 2016) (Fig. 1). Approximately 25 primary hybridomas secreting S-specific mAbs were obtained and their reactivity was confirmed by immunofluorescence (IFA) and Western blot assays (data not shown). The reactivity of select (n=11) mAbs was assessed by IFA against the prototype PEDV strain USA/Colorado/2013 (Marthaler et al., 2013) and the S-INDEL strain USA/Iowa106/2013 (Oka et al., 2014) (Fig. 1). Additionally, their neutralizing activity was assessed by fluorescent focus neutralization (FFN) assay, with supernatants from nine hybridomas presenting neutralizing titers between 4 and 16 (data not shown). These hybridoma lines were subcloned by limiting dilution (10^{-1} to 10^{-12}) and further characterized in our study. One additional hybridoma cell line (SD33-1; Fig. 1) secreting S-specific mAbs that did not neutralize PEDV and another line previously established at the SD Animal Disease Research and Diagnostic Laboratory (SD37-11) (unpublished data) were also included in our study. Following subcloning one-to-two clones of each primary hybridoma line was selected and subjected to ascites production (Envigo, Inc.). A summary of the properties and characteristics of the mAbs generated here is presented in Table 1.

All mAbs specifically recognized cells infected with the recombinant ORFV-PEDV-S, expressing the full length PEDV S and cells infected with the PEDV strain CO13 (Fig. 1; Table 1). Notably, ten out of eleven mAbs recognized cells infected with the S-INDEL variant PEDV strain USA/IOWA/106/2013, with mAb SD125-2 not recognizing S-INDEL infected cells (Fig. 1; Table 1).

The reactivity of the PEDV-S mAbs was also evaluated by Western

blots and ELISAs using whole virus preparations, and recombinant S- (amino acid [aa] 630–800 or aa 499–600) and N (full length) proteins expressed in *E. coli*. On Western blots, ten of eleven mAbs recognized the full-length spike protein in whole PEDV lysates (Fig. 2) and the truncated S protein spanning the S1-S2 junction region (aa 630–800) (Fig. 2). Notably while mAb SD37-11 did not recognize the full-length spike in whole virus lysates, mAb SD125-2 did not react with the S1-S2 recombinant protein (aa 630–800) (Fig. 2). None of the mAbs reacted with the recombinant N protein (Fig. 2). The reactivity of the mAbs with the S protein under denaturing Western blot conditions indicate that they recognize linear epitopes, with most of them being specific for epitopes within the S1-S2 junction region (Fig. 2). When tested by ELISA, all mAbs reacted against PEDV strains CO13 (Fig. 3A) and ten of eleven (except for SD125-2) reacted with S-INDEL strain Iowa106 (Fig. 3A and B). These results corroborate the findings of the IFA assays, confirming the reactivity of the mAbs with PEDV.

Next, we assessed the reactivity of the mAbs against truncated versions of the S protein (aa 499–600 and aa 630–800). Notably, nine of eleven mAbs reacted against the S1-S2 region (aa 630–800), with mAb SD131-3 also reacting with the product corresponding to aa 499–600, which contains the putative receptor binding domain of the S1 subunit (Fig. 3C and D). These results confirmed the Western blots findings, indicating that most mAbs developed here specifically recognize epitopes located in the S1-S2 junction of the S protein. It is important to note that, while mAb SD125-2 reacted with whole PEDV strain CO13 (Figs. 1, 2 and 3A), it did not recognize the S1- nor the S1-S2 truncated proteins (Fig. 3C and D), suggesting that this mAb may recognize another region of the S protein. Additionally, mAb SD131-3 reacted with S1-S2 protein on Western blot and with the S1 and S1-S2 truncated proteins on ELISAs, suggesting a mixed hybridoma population.

2.2. Neutralizing activity of PEDV S-specific mAbs

The neutralizing activity of S-specific mAbs was assessed by FFN and plaque reduction neutralization (PRN) assays. For this, all mAbs were purified from ascites fluid and diluted to a working concentration of 1.5 mg/mL (in PBS). Two-fold serial dilutions of the mAbs' working stocks (1:20 – 1:2560) were tested in triplicate by FFN or PRN assays. Endpoint titers were considered the reciprocal of the highest mAb dilution capable of reducing PEDV infectivity by 90 (FFN) or 80% (PRN) *in vitro*. Ten out of eleven mAbs presented neutralizing titers ranging from 1:40 to 1:640 against PEDV strain CO13 (Table 2, Fig. 4) and from 1:40 to 1:160 against PEDV S-INDEL variant strain (Table 2). Similarly, PRN titers against PEDV strain CO13 ranged between 1:40 to 1:640 (Table 2, Fig. 4). Consistent with the results of our preliminary screening, no neutralizing activity was observed for PEDV S-specific mAb SD33-1 (Fig. 4). These results demonstrate potent neutralizing activity of ten S-specific mAbs against PEDV *in vitro*.

2.3. Epitope mapping of S-specific mAbs

To identify neutralizing domains in the PEDV S, the epitope specificity of the neutralizing mAbs developed here was assessed by peptide ELISAs. Four regions of PEDV S protein have been shown to be the targets of neutralizing antibodies, including the NTD/S0 region, the RBD (aa 499–600), the S1-S2 junction (aa 639–789) and a region in the carboxi terminus of the S protein (Chang et al., 2002; Li et al., 2017; Ostrowski et al., 2002; Sun et al., 2007). A few linear epitopes within these regions have been shown to induce neutralizing antibodies against PEDV, including epitopes at aa positions 747–774 and 1371–1377 (Cruz et al., 2006, 2008; Sun et al., 2007). Since most mAbs developed here recognized the S1-S2 (aa 630–800) recombinant protein in Western blot and/or ELISAs, they were initially screened against epitope 747–774 located in the N-terminus of S2 (Fig. 5A).

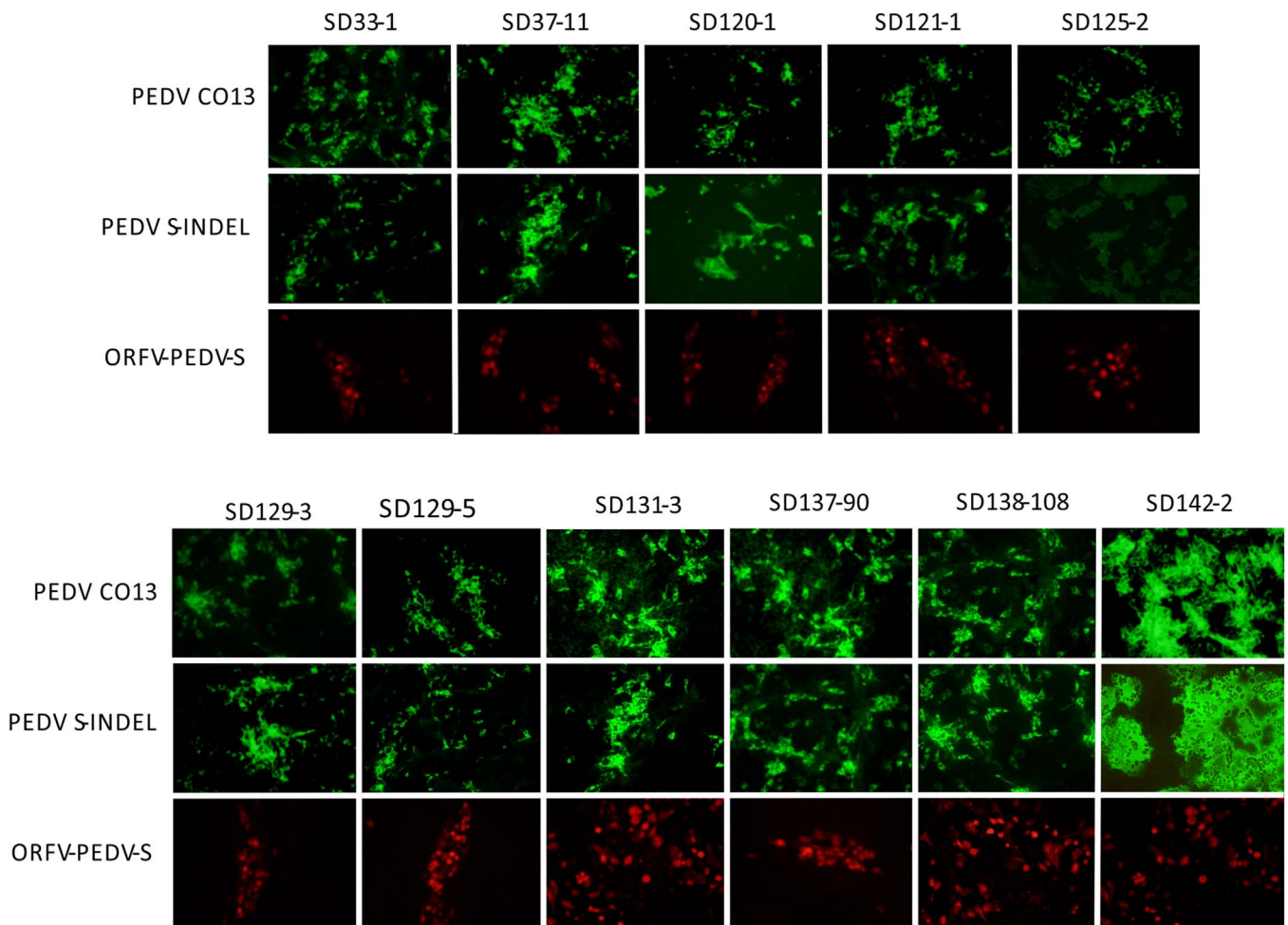


Fig. 1. Immunoreactivity of PEDV specific monoclonal antibodies with PEDV and ORFV-PEDV-S infected cells. Vero cells were infected with PEDV strain CO13 or S-INDEL variant strain USA/IOWA/106/2013. Primary ovine fetal turbinate (OFTu) cells were infected with the recombinant ORFV-PEDV-S. Cells were fixed with 80% acetone (Vero) or 3.7% formaldehyde (OFTu) and incubated with cell culture supernatants of hybridoma cultures secreting PEDV S-specific mAbs. Goat anti-mouse IgG+IgM+IgA secondary antibodies (FITC or DyLight594) were added and cells evaluated under a fluorescence microscope (200 X).

Next, mAbs were screened against epitope aa 1371–1377 located at the C-terminus of S (Fig. 5B). Notably, six mAbs (SD120-1, SD129-5, SD131-3, SD137-90, SD138-108, and SD142-2) recognized epitope

747–774 (Fig. 5A, Fig. 6), while mAb SD125-2 specifically reacted against epitope 1371–1377 (Fig. 5B, Fig. 6, Table 2).

To confirm the specificity of the mAbs and further dissect neutraliz-

Table 1

Characterization of mouse mAbs against PEDV spike protein.

		Reactivity of mAbs against PEDV antigens							
		Immunofluorescence				Western blot			
		ORFV-PEDV-S	PEDV N	CO13 ^a	S-INDEL ^b	S1-S2 630–800	PEDV N	CO13	S-INDEL
SD33-1	IgM	+ ^c	- ^d	+	+	+	-	+	+
SD37-11	IgG2a	+	-	+	+	+	-	-	+
SD120-1	IgG	+	-	+	+	+	-	+	+
SD121-1	IgG	+	-	+	+	+	-	+	+
SD125-2	IgG	+	-	+	-	-	+	+	-
SD129-3	IgG	+	-	+	+	+	-	+	+
SD129-5	IgG	+	-	+	+	+	-	+	+
SD131-3	IgG2b	+	-	+	+	+	-	+	+
SD137-90	IgG1	+	-	+	+	+	-	+	+
SD138-108	IgG3	+	-	+	+	+	-	+	+
SD142-2	IgG2a	+	-	+	+	+	-	+	+

^a PEDV strain Colorado 13.

^b PEDV S-INDEL strain USA/IOWA/106/2013.

^c Positive: intense fluorescence staining, indicating reactivity of antibody.

^d Negative: no visible fluorescence staining, indicating lack of reactivity of mAb.

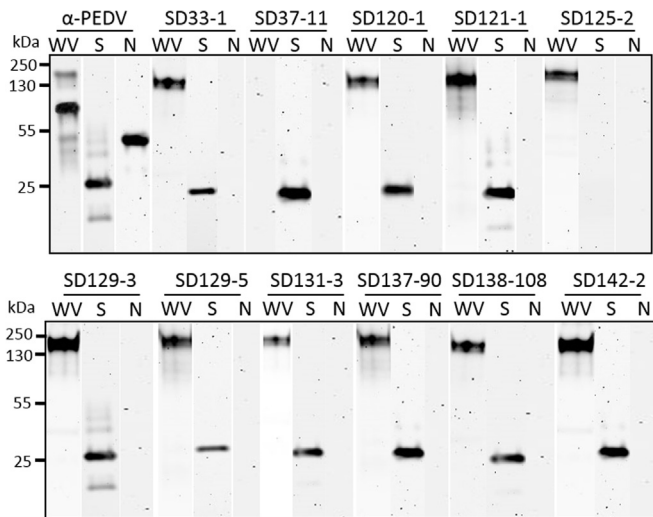


Fig. 2. Immunoreactivity of PEDV specific monoclonal antibodies for linear epitopes of the S protein. Purified whole PEDV preparations (WV, full length S: ~150 kDa, cleaved S1/S2: ~80 kDa), recombinant S (S1-S2; aa 630–800, S: ~24 kDa) and recombinant nucleocapsid (N, ~49 kDa) proteins were subjected to SDS-PAGE in 4–20% gradient acrylamide gels. Proteins were blotted into nitrocellulose membranes and probed with each PEDV S-specific mAb. Swine convalescent serum (α -PEDV) was used as positive control. Blots were developed with a chemiluminescent method.

ing epitopes within the S1-S2 junction region, all mAbs were screened against two smaller epitopes contained almost entirely within the 747–774 epitope (744–759 and 756–771). As shown in Fig. 5C eight mAbs

recognized epitope 744–759 (SD37-11, SD121-1, SD129-3, SD129-5, SD-131-3, SD137-90, SD138-108, and SD142-2), while only two MABs recognized epitope 756–771 (SD120-5 and SD129-5). A summary of the epitope specificity of individual mAbs is presented in Table 2 and Fig. 6. Notably, two mAbs specific to the S1-S2 region (SD37-11 and SD121-1) recognized only epitope 744–759, four mAbs (SD131-3, SD137-90, SD138-108 and SD142-2) recognized both epitopes 744–759 and 747–774, while two mAbs (SD120-1 and SD129-5) recognized epitopes 756–771 and 747–774 (Fig. 6; Table 2). These results indicate that the amino terminus of the S2 subunit protein contains highly antigenic, perhaps, immunodominant neutralizing epitopes of PEDV, and further suggest that epitope 744–759 may represent a core neutralizing epitope within S1-S2 junction region (Fig. 6).

2.4. Neutralizing activity of S-specific mAbs correlates with their reactivity with the truncated S1-S2 (aa 630–800) protein

The S1-S2 junction region of the S protein has been shown to contain major neutralizing epitopes (Sun et al., 2007). Notably, most neutralizing mAbs developed here specifically recognized a truncated form of the S protein (aa 630–800) spanning the S1-S2 junction region (Fig. 2). To assess whether the neutralizing activity of the mAbs correspond to their ability to recognize this region of the S, the correlation between endpoint FFN and ELISA titers was determined. As shown in Fig. 7A, endpoint neutralizing and S1-S2 ELISA titers were similar for all mAbs, resulting in a strong positive correlation between FFN and ELISA ($R^2 = 0.704$; Fig. 7B) test results. Notably, neutralizing titers detected in convalescent polyclonal swine serum also presented a high correlation with antibody titers detected by the S1-S2

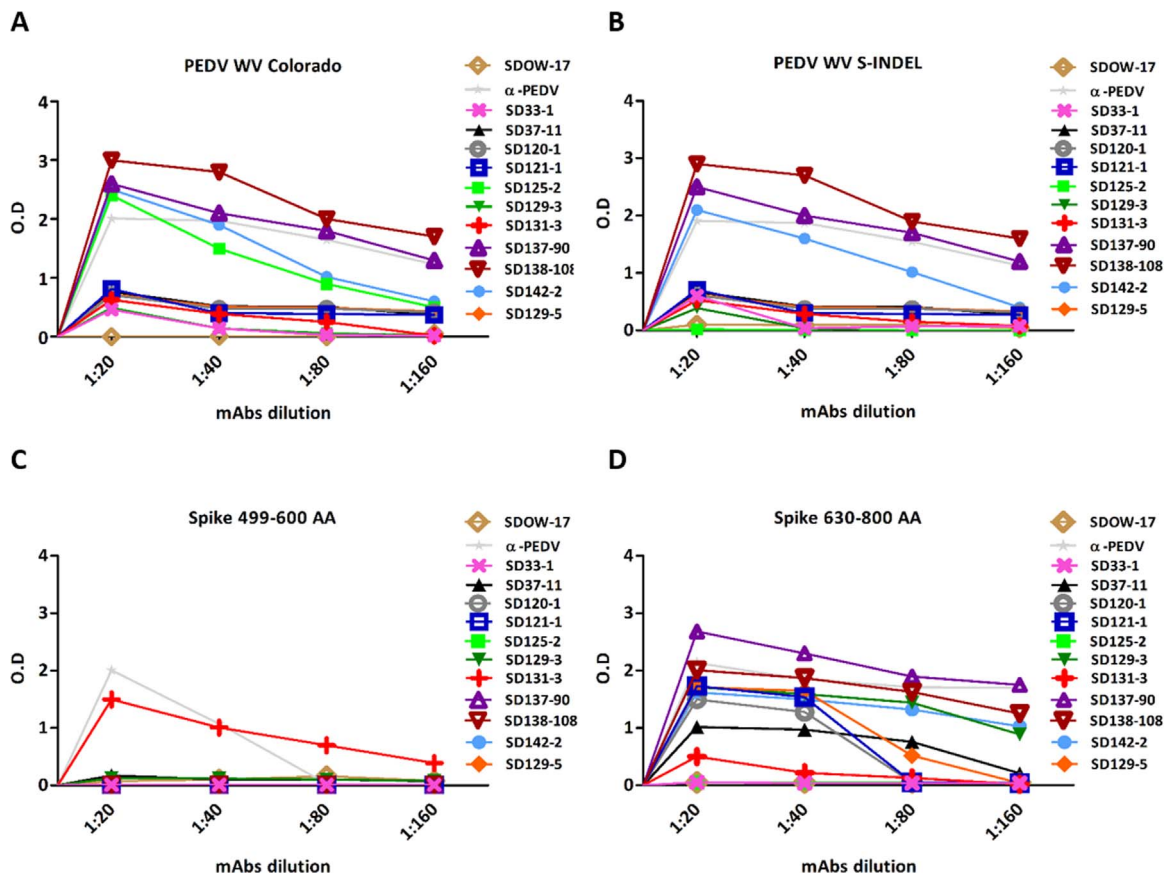


Fig. 3. Reactivity of PEDV specific monoclonal antibodies to prototype and S-INDEL PEDV strains and truncated regions of S protein. The reactivity of each PEDV-S mAb with whole PEDV preparations (WV; strains CO13 [A] and S-INDEL [B]) and truncated recombinant S proteins (COE; aa 499–600 [C]; and aa 630–800 [D]) were evaluated by indirect ELISAs. Swine convalescent serum (α -PEDV) was used as positive control and anti-PRRSV mAb SDOW-17 was used as a negative control. Each mAb was subjected to 2-fold dilutions (1:20–1:160) and tested in three independent experiments. All antibodies were initially diluted to a working solution of 3 mg/mL. Optical densities (OD) shown represent the average of three independent experiments.

Table 2
Summary of neutralizing activity and epitope specificity of mouse mAbs against PEDV S.

mAb	Reactivity of mAbs against PEDV antigens										
	ELISA				Peptide ELISA ^a				FFN		PRNT
	CO13 ^a	S-INDEL ^b	499-600 ^c	630-800 ^d	747-774	744-759	756-771	1371-1377	CO13	S-INDEL	CO13
SD33-1	+ ^f	+	- ^g	-	-	-	-	-	< 20	< 20	< 20
SD37-11	+	+	-	+	-	+	-	-	80	80	80
SD120-1	+	+	-	+	+	-	+	-	80	40	40
SD121-1	+	+	-	+	-	+	-	-	40	40	80
SD125-2	+	-	-	-	-	-	-	+	80	< 20	160
SD129-3	+	+	-	+	-	+	-	-	80	40	80
SD129-5	+	+	-	+	+	+	-	-	80	40	40
SD131-3	+	+	+	+	+	-	-	-	40	40	20
SD137-90	+	+	-	+	+	-	-	-	640	160	640
SD138-108	+	+	-	+	+	-	-	-	640	160	640
SD142-2	+	+	-	+	+	-	-	-	80	80	160

^a PEDV strain Colorado 13.

^b PEDV S-INDEL strain USA/IOWA/106/2013.

^c Recombinant S protein aa 499–600.

^d Recombinant S protein aa 630–800.

^e Peptides corresponding to aa 747–774, 744–759, 756–771, 1371–1377.

^f Positive: high OD, indicating reactivity of antibody.

^g Negative: low OD, indicating lack of reactivity of mAb.

indirect ELISA (Fig. 7A and B).

3. Discussion

Given its critical functions in cell entry, the coronavirus S protein is the main target of host humoral responses. Neutralizing antibodies directed against the S protein likely play a key role on protection against PEDV infection. A recent study demonstrated that neutralizing activity against PEDV in colostrum and milk correlated with levels of IgA antibodies to the S protein (S1 and S2 subunits) (Song et al., 2016). Notably, at least four neutralizing regions have been identified in the S protein (Chang et al., 2002; Cruz et al., 2006; Cruz et al., 2008; Li et al., 2017; Sun et al., 2007). Recently, a neutralizing domain was identified in the NTD/S0 region of the S1 subunit (Li et al., 2017), with other epitope(s) mapping to a domain in S1 that is homologous to the collagenase resistant fragment (CO-26 K) of TGEV S (Godet et al., 1994) (aa 499–638; named “collagenase equivalent” in PEDV, COE) (Chang et al., 2002; Li et al., 2017). A region spanning the S1-S2 junction (aa 638–789) in PEDV S contains other neutralizing epitope(s) (Sun et al., 2007), with a well defined neutralizing epitope mapping to the carboxy-terminus of the S protein (aa 1371–1377) (Cruz et al., 2006, 2008). Results here showing the specificity of ten PEDV neutralizing mAbs for these domains in the S protein confirm that epitopes located in these regions are indeed responsible for eliciting neutralizing antibodies against PEDV. The fact that these mAbs were generated by immunization of mice with whole virus preparations indicates that these regions are highly antigenic, further suggesting that epitopes within these regions may represent immunodominant neutralizing epitopes of the PEDV S protein.

While the amino-terminal region of S (S1, aa 1–729) is involved in virus attachment to cellular receptors, the carboxy-terminal region (S2, aa 730–1383) undergoes conformational changes after the attachment of S1 to facilitate fusion of the virus envelope with the host cell membrane, thus allowing entry of the viral genome into the host cell (Li et al., 2016). Notably, antibodies directed against both S subunits have been correlated with neutralization of PEDV infectivity (Song et al., 2016). Immunization of mice with inactivated whole PEDV virion preparations followed by somatic cell fusion here resulted in production of hybridoma cell lines secreting mAbs specific for the PEDV S protein and capable of neutralizing the virus infectivity *in vitro* (titers ranging from 1:40 to 1:640). Interestingly, epitope mapping ELISAs

revealed that most mAbs developed here (ten of eleven) specifically recognized epitopes located within the S2 subunit (SD37-11, SD120-1, SD121-1, SD125-2, SD129-3, SD129-5, SD131-3 SD137-90, SD138-108 and SD142-2), while only one mAb (SD131-3) reacted with the S1 subunit (Fig. 3C and D). While UV- or formalin inactivated PEDV virions likely do not retain the exact antigen conformation and architecture of infective PEDV particles, the data here suggest that the S2 subunit contains immunodominant antigenic determinants of PEDV. Results from a recent study showing higher levels of S2-specific IgG antibodies when compared to S1-specific antibodies in serum and colostrum of sows naturally infected with PEDV, further support this hypothesis (Song et al., 2016). Nevertheless, antibodies against the S1 subunit also play a critical role on immune responses against PEDV (Song et al., 2016), and immunization with the recombinant S1 protein subunit have induced neutralizing antibodies and passive protection against infection (Oh et al., 2014). Similar experiments, however, have to be performed with the S2 subunit to assess its immunogenicity and to determine the contribution of S2-specific antibodies on protection against PEDV.

Infection with PEDV induces neutralizing antibody responses within 14–21 days post-infection, with NA titers in convalescent polyclonal serum usually ranging between 1:20 to 1:320 (Chen et al., 2016; Clement et al., 2016). As shown in Fig. 3, most S-specific mAbs produced here (exception SD33-1) were capable of completely blocking PEDV infectivity (titers of 1:40 to 1:640), with a few of these mAbs (SD137-90, SD138-108 and SD142-2) presenting markedly higher neutralizing activity (titers up to 1:640) (Table 2). Considering the highly stringent endpoint criteria used in the *in vitro* neutralization assays (90% reduction in fluorescent focus-forming units or 80% reduction in plaque forming-units), and the fact that these are monospecific antibodies these titers represent potent neutralizing activities. It is important to note, that while mAbs SD137-90, SD138-108 and SD142-2 specifically recognized two overlapping epitopes located in the amino-terminus of the S2 subunit (aa 744–759 and 747–774), they did not react against epitope 756–771, which is also contained within the neutralizing epitope aa 747–774 in S2. Together these results indicate that these mAbs either recognize epitope 744–759 or, perhaps, a smaller epitope within this region. These observations suggest that epitope 744–759 might represent a core immunodominant neutralizing epitope of PEDV S. However, the possibility that these results reflect a higher avidity and/or affinity of

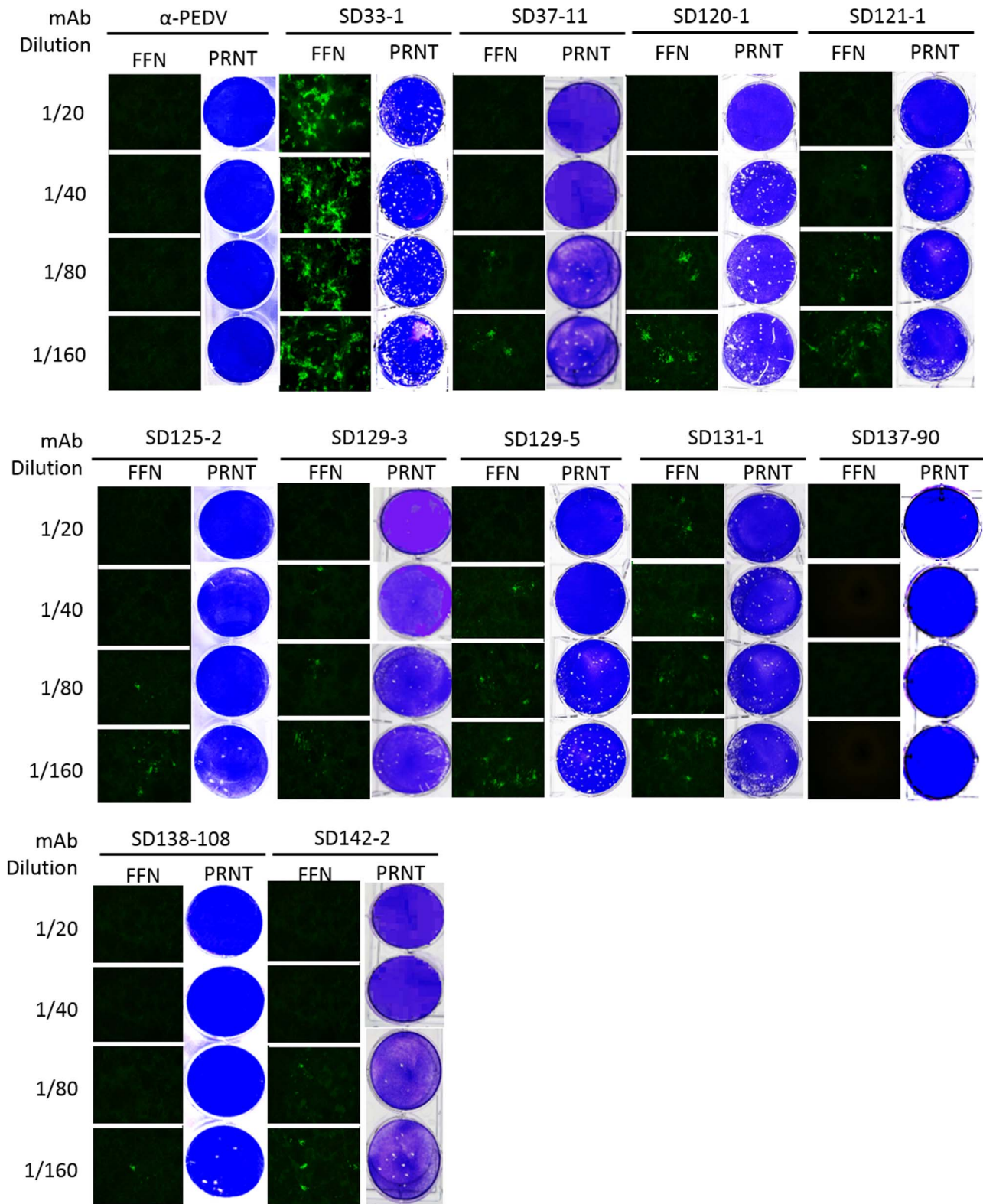


Fig. 4. Neutralizing activity of PEDV S-specific mAbs. The neutralizing activity of each PEDV-S mAb was determined by fluorescent focus neutralization assay (FFN; 90% reduction cutoff) and plaque reduction neutralization test (PRNT, 80% reduction cutoff). Swine convalescent serum (α-PEDV) was used as positive control. Each mAb was tested in three independent experiments.

these specific mAbs for their target epitope cannot be formally excluded.

At least two genetically distinct strains of PEDV (prototype and S-INDEL variant strains) have been described in the US and in other parts of the world (Chen et al., 2016; Oka et al., 2014). The S-INDEL

variant strains contain aa insertions, deletions and/or substitutions mainly in the N-terminus of the S1 protein subunit (first 350 aa). Although a recent study has shown that there is significant serological and neutralizing cross-reactivity between two representative strains obtained in the US (Chen et al., 2016), significant antigenic differences

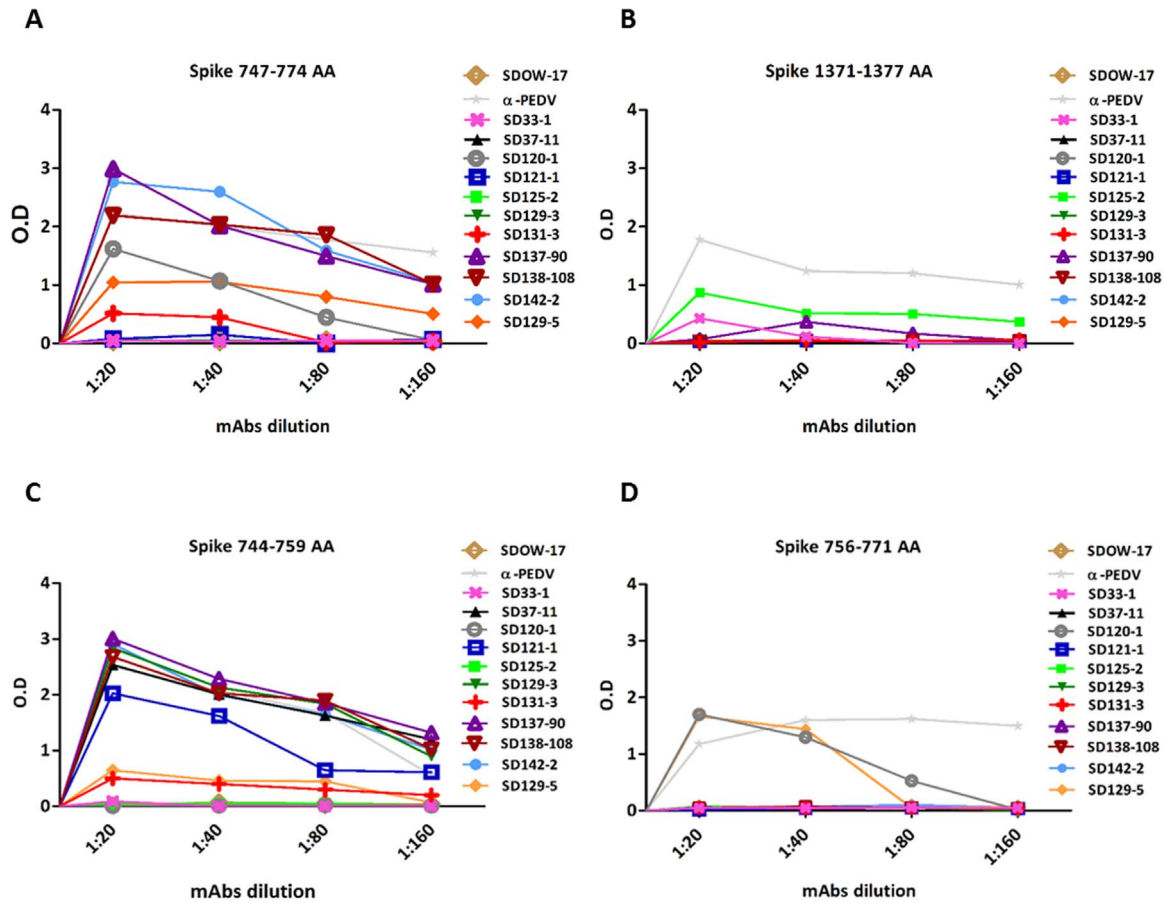


Fig. 5. Epitope mapping ELISA of PEDV S-specific monoclonal antibodies. The epitope specificity of each neutralizing mAb was assessed by pepscan ELISAs (A–D). Swine convalescent serum (α -PEDV) was used as positive control and anti-PRRSV mAb SDOW17 was used as a negative control. Each mAb was subjected to 2-fold dilutions (1:20–1:160) and tested in three independent experiments. All antibodies were initially diluted to a working solution of 3 mg/mL. Optical densities (OD) shown represent the average of three independent experiments.

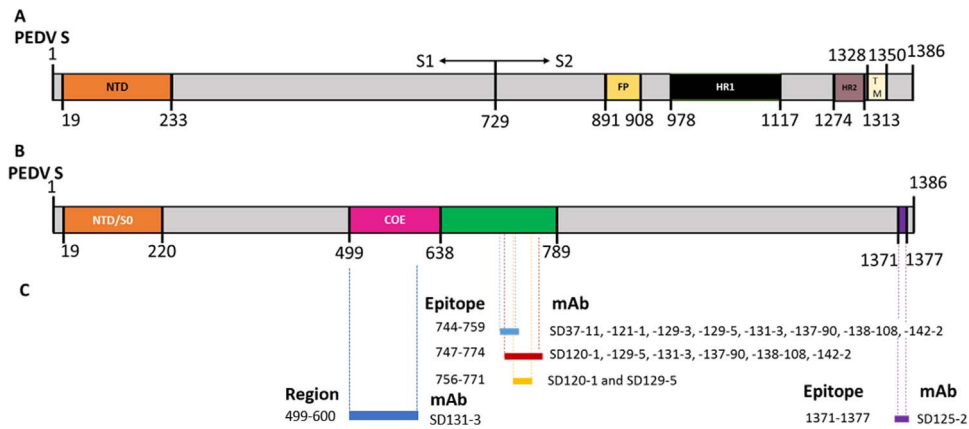


Fig. 6. Schematic representation of PEDV S and its immunodominant neutralizing epitopes (PEDV strain CO13, GenBank accession no. KF272920). (A) Diagram depicting the main features of the PEDV S protein, including: putative cleavage site between S1 and S2 subunits at aa 729, signal peptide (aa 1–18), N-terminal domain (aa 19–233), fusion peptide (aa 891–908), heptad repeat region 1 (HR1, aa 978–1117), heptad repeat region 2 (HR2, aa 1274–1313), and the transmembrane domain (aa 1328–1350). (B) Diagram depicting the regions containing B-cell epitopes (N-terminal domain [NTD]/S0; aa 19–220; collagenase equivalent [COE] aa 499–638; and aa 638–789) and targeted by neutralizing antibodies (NTD/S0, aa 19–220; COE, aa 499–638, and aa 1371–1377). (C) Spike immunodominant neutralizing regions (aa 499–600, aa 744–759, aa 747–774, aa 756–771, and aa 1371–1377) and mAb reactivity with neutralizing epitopes.

have been demonstrated between PEDV strains by using mAbs against the S1 protein subunit (Li et al., 2017). Here we showed that ten of eleven mAbs recognized both prototype (CO13) and S-INDEL (Iowa106) strains in IFA and/or whole virus ELISAs (Figs. 1 and 3A and B), while only one mAb (SD125-2) did not react with S-INDEL infected cells. Most importantly, all mAbs that recognized prototype or US-INDEL infected cells also presented neutralizing activity against

both PEDV strains. These results are consistent with the fact that the target neutralizing epitopes of these mAbs (744–759, 747–774 or 756–771) are highly conserved (100% aa id; data not shown) between the two PEDV strains used in our study. The reactivity and neutralizing activity of these mAbs with other more diverse S-INDEL strains, however, may vary and awaits further experimentation.

Recently, Song and collaborators have demonstrated a high corre-

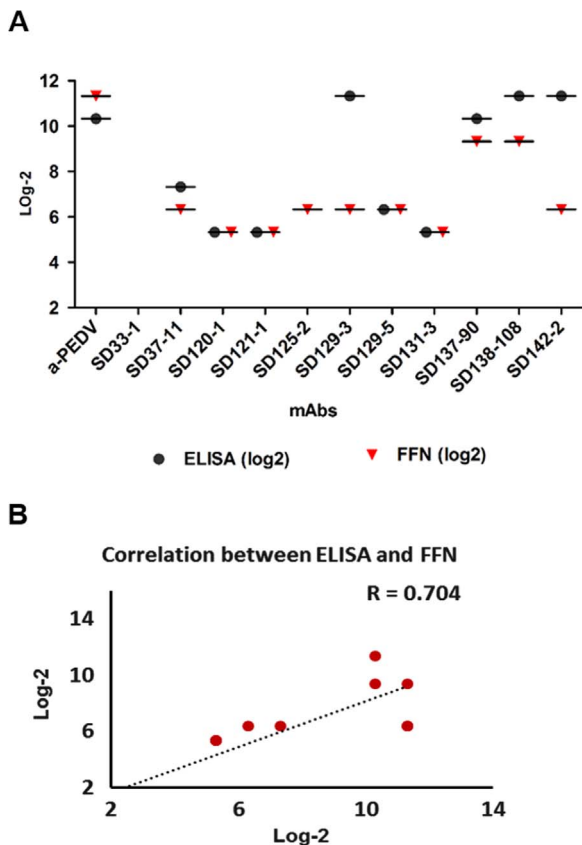


Fig. 7. Neutralizing activity of PEDV S-specific mAbs correlates with endpoint ELISA titers against recombinant S1-S2 recombinant protein. (A) Endpoint neutralizing antibody titers as determined by FFN assay were transformed to log₂ and compared to endpoint S ELISA (aa 630–800) titers. End point neutralizing titers were considered the reciprocal of the highest dilution capable of inhibiting virus infectivity by 90%, whereas endpoint ELISA titers were considered as the highest mAb dilution above the assay cutoff OD value of 0.16. Each antibody was tested in three independent experiment and the average endpoint titer is presented. (B) Correlation plot between neutralizing and S ELISA endpoint titers for PEDV S mAbs.

lation between the levels of neutralizing antibodies in serum, colostrum and milk of naturally infected sows with the levels of S1- and S2-specific antibodies in these samples (Song et al., 2016). Given that most neutralizing mAbs developed here are specific for epitopes located within the S1-S2 junction region, we performed endpoint ELISA titrations with these mAbs using the recombinant S1-S2 protein (aa 630–800) and assessed the correlation between ELISA and neutralizing antibody titers. Notably, a strong positive correlation between the ELISA titers and FFN titers was observed for all mAbs developed here. Similarly, neutralizing antibody titers detected on a convalescent swine serum sample used as positive control also matched the endpoint titers detected in the S1-S2 ELISA. These observations indicate the importance of the S protein as the major target of neutralizing antibody responses against PEDV, and highlight the S1-S2 junction as an immunodominant region with strong neutralizing activity within the S protein.

In summary, by using a panel of PEDV S-specific neutralizing mAbs we have shown that immunodominant neutralizing epitopes of PEDV S map to the amino-terminus of the S2 subunit. While seven neutralizing mAbs generated after immunization of mice with whole virion preparations recognized a domain in S2, only one mAb reacted against S1. These results suggest that S2 subunit contains immunodominant neutralizing determinants of PEDV. This study provides important information on PEDV infection biology and immunity and shed light into major determinants of neutralizing protective immunity within the S protein. Such information may lead to the design and development of

improved vaccines and diagnostic assays for this important pathogen of swine.

4. Materials and methods

4.1. Cells and viruses

Vero-76 (ATCC[®] CRL-1587[™]) and primary ovine fetal turbinate cells (OFTu) were cultured at 37 °C with 5% CO₂ in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), and 2 mM L-glutamine, and containing penicillin (100 U/mL), streptomycin (100 µg/mL) and gentamicin (50 µg/mL). PEDV strain USA/CO/2013 (CO13; GenBank accession no: KF272920.1) was obtained from the National Veterinary Services Laboratory (NVSL). PEDV strains BIVI-1 and BIVI-2 were provided by Boehringer Ingelheim Vetmedica and PEDV S-INDEL-variant strain USA/IOWA/106/2013 (GenBank accession no: KJ645695.1) was kindly provided by Dr. QiuHong Wang, The Ohio State University. All PEDV strains were propagated in Vero-76 cells in the presence of 1.5 µg tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin (Sigma Aldrich, St. Louis, MO). A recombinant Orf virus expressing the full length PEDV S (ORFV-PEDV-S) was recently developed in our laboratory (Hain et al., 2016) and was used here for selection of S-specific mAbs.

4.2. Production of S-specific monoclonal antibodies

Four-week-old female BALB/c mice were randomly allocated into three groups as follows: Group 1: PEDV CO13-immunized (n=3), Group 2: PEDV BIVI-1-immunized (n=3), and Group 3: PEDV BIVI-2 immunized (n=3). After a week of acclimation, mice were immunized with sucrose purified UV-(PEDV CO13) or formalin (BIVI-1 and BIVI-2) inactivated whole PEDV virions in a water-in-oil (W/O) adjuvant emulsion (Montanide ISA 50 V2; Seppic, France). After a series of three intraperitoneal immunizations (2-week intervals), splenic cells were fused with myeloma NS-1 cell lines and cultured in the presence of hypoxanthine-aminopterin-thymidine (HAT) medium for selection of hybridoma cells following standard protocols (Okda et al., 2015). After 10–14 days of culture, hybridoma cell culture supernatants were screened for the presence of S-specific mAbs by indirect immunofluorescence assays. All animal experiments were reviewed and approved by the SDSU Institutional Animal Care and Use Committee (IACUC; protocol approval no. 14-087A).

4.3. Indirect immunofluorescence assay (IFA)

The supernatant of hybridoma cell cultures was screened for the presence of S-specific mAbs by using IFA. For this, primary OFTu cells infected with the recombinant ORFV-PEDV-S, expressing the full-length PEDV S (Hain et al., 2016), were incubated with the hybridoma cell culture supernatants for 1 h at 37 °C. Unbound antibodies were removed by washing with phosphate buffered saline (PBS) and S-specific mAbs detected with a goat anti-Mouse IgG+IgM+IgA DyLight[®]594-conjugated antibody (Bethyl Laboratories, Inc., Montgomery, TX). Positive clones were also screened in PEDV CO13-infected cell cultures using a goat anti-Mouse IgG+IgM+IgA FITC-conjugated antibody (MP Biomedicals, Santa Ana, CA) as above. Positive primary clones were subcloned, expanded and re-screened in ORFV-PEDV-S- and PEDV (CO13 and S-INDEL) infected cells.

4.4. Neutralization assays

The neutralizing activity of S-specific mAbs was initially assessed by FFN assays as previously described (Okda et al., 2015). Hybridoma culture supernatants were first screened and clones secreting neutralizing antibodies at titers greater than 4 were used for ascites production (Envigo, Inc., Indianapolis, IN). Immunoglobulin isotyping was per-

formed using a lateral flow assay kit (Bio-Rad, Hercules, CA) and mAbs were purified using NAb™ protein G spin columns (IgG; Thermo Fisher Scientific, Waltham, MA) or the ammonium sulfate precipitation method (IgM). Following purification, all MAb were diluted in PBS to final concentration of 1.5 mg/mL. Normalized mAbs were re-screened by FFN assays to confirm their neutralizing activity against PEDV. Neutralizing antibody titers were expressed as the reciprocal of the highest mAb dilution capable of reducing PEDV infectivity by 90%.

To confirm the neutralizing activity the mAbs were also screened by plaque reduction neutralization (PRN) assay (Hofmann and Wyler, 1989). For this, Vero cells were seeded in six-well plates at a density of 4×10^5 cells/well and incubated at 37 °C with 5% CO₂ until the cells were approximately 90% confluent. The mAbs were subjected to serial 2-fold dilutions (1:20–1:2560) and each dilution incubated with 50 PFU of PEDV strain CO13 for 1 h at 37 °C with 5% CO₂. After incubation the antibody-virus mixture was transferred to Vero cells and incubated for 1.5 h with gentle rocking of the plates (every 15 min) to distribute the inoculum homogeneously throughout the well. Cells were overlaid with 2% agar medium (2X MEM, and 3 µg/mL TPCK-treated trypsin) and incubated at 37 °C with 5% CO₂ for 72–96 h. Next, 1 mL of 0.2% crystal violet (Sigma-Aldrich, St Louis, MO) in 0.01% formaldehyde and 20% acetone solution was added to each well and the cells were fixed and stained for 15 min at RT. Viral plaques were counted and used to determine the PRN for each mAb. Plaque counts for each antibody were used to calculate percent reduction in plaques as follows: % reduction = $100 \times [1 - (\text{average number of plaques for each dilution} / \text{average number of plaques in the virus control well})]$. An 80% reduction in the number of plaques was used as a cutoff to determine neutralizing antibody titers. Each mAb was tested in duplicate and four control wells used on each assay, including, a virus control (no antibody) and a negative control (MEM only). Additionally, convalescence PEDV polyclonal serum and unrelated mAbs controls (anti-PRRS, and anti-PEDV N protein antibody), were used on every PRN assay.

4.5. Western blots

The reactivity of PEDV mAbs developed here were evaluated by Western blots. Each antibody was tested against sucrose purified whole virus preparations, a recombinant/truncated version of the S protein (aa 630–800; Hain et al., 2016), and a recombinant N protein (Okda et al., 2015). Approximately, 20 µg of each antigen were resolved by SDS-PAGE in 4–20% acrylamide gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes. Blots were incubated with 5% non-fat-dry-milk tris buffered saline (TBS)–0.1% Tween 20 (TBS-T) solution for 1 h at RT and probed with the MABs and control antibodies overnight at 4 °C. Blots were washed three times with TBS-T for 10 min at RT and incubated with a goat anti-mouse IgG-HRP- or goat anti-mouse IgM-HRP-conjugated antibody for 2 h at RT. Blots were washed three times with TBS-T for 10 min and developed by using a chemiluminescent substrate (Clarity, ECL; Bio-Rad, Hercules, CA).

4.6. ELISAs

The specificity and epitope mapping of the neutralizing mAbs was investigated by ELISAs. Initially, the mAbs were screened against whole virus preparations (prototype or I-INDEL strains) or truncated versions of the S protein known to contain neutralizing epitopes (aa 499–630 or 630–800) (Chang et al., 2002; Sun et al., 2007). Optimal assay conditions, including the amount of antigen, and secondary antibody dilution were determined by a checkerboard titration and conditions that resulted in the highest signal to noise ratio were used. Polystyrene microtiter plates (Immunolon 1B, Thermo Scientific, Waltham, MA) were coated with the appropriate antigen (50 ng/well of UV inactivated sucrose purified whole virus antigen; 100 ng/well of S/630–800, or 25 ng/well of S/499–630) as previously described (Okda

et al., 2015). Serial 2-fold dilutions of the MABs and appropriate controls were diluted in PBS-T 5% non-fat dry milk, and 100 µl of diluted samples were added to paired coated and uncoated control wells and incubated at room temperature for 1 h. Unbound antibodies were washed with PBS-T (three times) followed by incubation with streptavidin-HRP specific IgG and IgM conjugate (Pierce, Rockford, IL) according to each MAB isotype. Reactions were developed with 3,3',5'5' – tetramethylbenzidine substrate (TMB) (KPL Inc., Gaithersburg, MA) and OD values determined at 450 nm using a microplate reader (BioTek Instruments Inc., Winooski, VT). OD values for each test and control samples were normalized to the OD value of uncoated wells and results expressed as OD values. An internal cutoff value of 0.16 for WV and recombinant protein ELISAs was calculated using MedCalc version 11.1.1.0 (MedCalc software, Mariakerke, Belgium). Each antibody dilution (tested in triplicate) was treated as a sample resulting in a total of 225 positive and 75 negative samples used in the cutoff calculation. Endpoint S1-S2 (aa 630–800) ELISA titers were determined for each mAb based on the cutoff value of 0.16.

The epitope specificity of neutralizing mAbs was assessed by peptide ELISAs. Previously described epitopes (744–759, 747–774 and/or 756–771) located near the S1-S2 junction or the neutralizing antibody located in the C-terminus of S2 (1371–1377) were chemically synthesized (Genscripts, NJ) and used in pepscan ELISAs. Optimal assay conditions, including the amount of peptide, and secondary antibody dilution were determined by a checkerboard titration and conditions that resulted in the highest signal to noise ratio were used. Nunc™ Immobilizer® amino plates (ThermoFisher Scientific, Waltham, MA) were coated with individual peptides (744–759, 80 ng/well; 756–771, 133 ng/well; 747–771, 200 ng/well and 1371–1377, 400 ng/well) in bicarbonate/carbonate coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, pH 9.6) in alternate wells. After incubation at 37 °C for 1 h, plates were incubated overnight at 4 °C. Next, plate were washed four times with PBS tween 20 (PBS-T, 0.05%) and blocked 2 h at 37 °C with PBS-T 2% non-fat dry milk. Blocking reagent was removed and plates washed three times with PBS-T (300 µl). mAb incubations and reaction development were performed as above. OD values for each test and control samples were normalized to the OD value of uncoated wells and results expressed as OD values.

4.7. Statistical analysis

The linear correlation between S1-S2 ELISA and FFN endpoint titers was assessed by using the Pearson's correlation method using the Medcalc software.

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