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Identification of a novel linear B-cell epitope within the collagenase equivalent domain of porcine epidemic diarrhea virus spike glycoprotein

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ABSTRACT

The porcine epidemic diarrhea virus (PEDV) collagenase equivalent domain (COE, residues 499–638), a crucial antigenic region within the viral spike (S) glycoprotein, has been widely utilized for the development of subunit vaccines to prevent viral infection. In the current study, we immunized BALB/c mice with recombinant truncated PEDV COE protein and obtained 14 COE-specific monoclonal antibodies (mAbs). Based on the reactivity analysis of the mAbs with two prevalent PEDV strains in G2 type and the attenuated CV777 strain in G1 type, 6 mAbs were selected for subsequent identification of COE mAb-binding epitopes. Dot-blot hybridization and enzyme-linked immunosorbent assays (ELISAs) identified the peptide ^{592}TSLLASACTIDLFGYP^{607} as a novel linear B-cell epitope involved in binding of mAbs 4D8F10 and 6F3E3. Subsequently, alanine (A)-scanning mutagenesis demonstrated that residues 606Y, 605G and 604F were core residues involved in recognition. Importantly, this novel COE epitope, including core residues, is conserved among G1 and G2 type PEDV strains. Further experiment indicates that the mAbs 4D8F10 and 6F3E3 were suitable for PEDV detection via mAb binding to the conserved epitope. The current work actually provides potential uses for the development of diagnostic methods to detect PEDV.

1. Introduction

Porcine epidemic diarrhea (PED) is a serious swine viral disease (Lee, 2015) characterized by vomiting, diarrhea and dehydration. The causative agent of this disease is PED virus (PEDV), a member of the *Alphacoronavirus* genus of the *Coronaviridae* family, *Nidovirales* order. Since its re-emergence in 2010 in China, PED has become prevalent in swine herds (Sun et al., 2012). PED was detected in the USA in April 2013 and spread rapidly across the country (Huang et al., 2013). The mortality of PED approaches 100% for newborn piglets, incurring huge economic losses to the global pork industry during recent years. Due to the lack of effective vaccines, PED still circulates worldwide.

PEDV is an enveloped, positive-sense, single stranded RNA virus with a genome size of approximately 28 kb. The PEDV genome encodes spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins, as well as two large polyproteins ORF1a and ORF1b (Park et al., 2012). PEDV S protein, a glycoprotein found on the PEDV surface, can be divided into S1 and S2 subunits; the S1 subunit participates in receptor recognition and the S2 subunit mediates cell membrane fusion (Belouzard et al., 2012). Notably, the S protein contains multiple neutralizing epitopes and has been frequently utilized for PEDV vaccine development (Chang et al., 2002; Cruz et al., 2008; Li et al., 2017). Based on S gene variations, PEDV strains can be classified into genogroup 1 (G1) and genogroup 2 (G2). The G1 type encompasses classical strains including prototype strains (virulent CV777 and DR13) and their respective cell culture-adapted vaccine strains (attenuated CV777 and DR13), while the G2 type includes other variant strains that are prevalent around the world (Huang et al., 2013; Wang et al., 2016; Yu et al., 2018).

Previous studies have shown that a fragment of PEDV S1 protein (residues 499–638) efficiently induces neutralizing antibodies. This fragment, which corresponds to the collagenase-digested fragment

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(namely CO-26K) of transmissible gastroenteritis virus (TGEV), is designated CO-26K equivalent (COE) (Chang et al., 2002). COE has been considered an ideal antigenic candidate for creation of subunit vaccines (Bae et al., 2003; Ge et al., 2012; Ma et al., 2018). However, as few studies have focused on identification of epitopes within the COE fragment (Chang et al., 2019), such studies would be of great importance for vaccine development.

In this study, we utilized the recombinant truncated PEDV COE construct (COE-t, residues 505–629) to immunize BALB/c mice and subsequently prepared 14 mAbs with binding specificities for prevalent PEDV strains in China. Based on antibody binding analysis, we chose 6 mAbs for more precise epitope mapping and identified a novel linear B-cell epitope within the COE-t region. In addition, mAbs 4D8F10 and 6F3E3 were shown to strongly bind to other prevalent PEDV strains as a first step toward development of diagnostic PEDV detection methods.

2. Materials and methods

2.1. Cells and viruses

The African green monkey kidney epithelial Vero cell line and human embryonic kidney HEK-293T cell line were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco) and antibiotics (100 U/ml penicillin, 100 μg/mL streptomycin; Gibco) at 37 °C with 5% CO_2. The PEDV strains CH/hubei/2016 (GenBank accession number: KY496315.1), CH/HNHB/2016 (GenBank accession number: MK111632.1), CH/HNAY/2016 (GenBank accession number: MK111633.1), CH/HNZK/2017 (GenBank accession number: MK726310.1) and attenuated CV777 (GenBank accession number: KT323979.1) were maintained and stored in our laboratory.

2.2. Virus propagation

PEDV strains were propagated in Vero cells as previously described (Hofmann and Wyler, 1988). Briefly, when cell confluence reached 80%, Vero cells were washed three times with PBS and inoculated with a 200 $TCID_{50}$ (50% tissue culture infective dose) of PEDV and incubated at 37 °C for 1 h. Next, the supernatant was removed and cells were maintained in growth medium containing 0.3% tryptose phosphate broth (TPB), 0.02% yeast extract and 3 μg/ml tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma Aldrich, St. Louis, MO, USA). When obvious cytopathic effect (CPE) appeared, cells were frozen and thawed three times followed by centrifugation at 2000 rpm at 4 °C for 10 min. The supernatant containing virus was stored at −80 °C until needed.

2.3. Production of mAbs targeting PEDV

The antigen COE-t of the PEDV CH/HNXC strain (GenBank accession number: MK093253.1, a prevalent strain of G2 type) was prepared as previously described (Sun et al., 2018b). Each six-week-old female BALB/c mouse was immunized with 50 μg COE-t in Freund's complete adjuvant (Sigma-Aldrich). Next, a total of six mice received three intramuscular injections each of PEDV COE-t in Freund's incomplete adjuvant at 4-week intervals. Two mice with the highest antibody titers against COE-t were further boosted by intraperitoneal injection with 100 μg COE-t in 200 μl of PBS. Three days after the final injection, the mice were sacrificed and their spleens were collected for preparation of hybridoma cells using PEG 1500. All experiments were performed according to the *Chinese Regulations of Laboratory Animals-The Guidelines for the Care of Laboratory Animals* (Ministry of Science and Technology of People's Republic of China). Supernatants of hybridoma cell cultures were screened for the presence of PEDV-specific mAbs via immunoperoxidase monolayer assay (IPMA).

2.4. IPMA

The PEDV CH/hubei/2016 strain was propagated in Vero cells in 96-well plates until obvious CPE was observed. Cells were washed three times with PBS and fixed with methanol containing 3% H_2O_2 at room temperature (RT) for 10 min. Next, cells were washed with PBST (PBS containing 0.05% Tween-20) and incubated with blocking buffer containing 5% skim milk at 4 °C overnight. Then, cells were incubated with supernatants of hybridoma cell cultures and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (H + L) (Jackson ImmunoResearch, West Grove, PA, USA). Finally, plated cells were stained with 3-amino-9-ethylcarbazole (AEC) solution (ZSGB-BIO, Beijing, China) at RT and examined by light microscopy. Cells incubated with PEDV COE-t antiserum and PEDV-negative serum served as positive and negative controls, respectively. Positive hybridoma cells for mAbs specific for PEDV were subcloned and neutralizing activities of PEDV-specific mAbs were determined as previously described (Li et al., 2017). In addition, IMPA was also used to evaluate binding of MAbs to PEDV CH/hubei/2016, CH/HNHB/2016 and attenuated CV777 strains.

2.5. Expression of PEDV COE-t truncated constructs

In order to identify the epitope, we first designed the PEDV COE-t truncated constructs according to predicted COE secondary structures based on the alignment between PEDV COE-t and TGEV S RBD (Reguera et al., 2012). Truncated COE-t constructs retained integrity of α-helices and β-strands and were designed as a series of overlapping fragments such that each fragment overlapped with the next fragment in a 6- or 9-amino-acid overlap zone (Fig. 2A and B). Three mini-fragments spanning residues 592–629, with a 4-amino-acid overlapping zone between each, were synthesized as previously described (Hua et al., 2004). Primers used to clone each truncated construct are listed in Tables 1 and 2. Next, cDNAs encoding truncated PEDV COE-t constructs were inserted into the pFuse-hIgG1-FC2 vector (Invivogen, San Diego, USA) using restriction enzyme sites EcoRI and NcoI. After transient expression in HEK-293T cells for 48 h, truncated PEDV COE-t proteins were enriched using nProtein A Sepharose 4 Fast Flow beads (GE Healthcare, Piscataway, NJ, USA). Target proteins were eluted with glycine/HCl buffer (pH 3.0) and neutralized using 1 M Tris/HCl (pH 9.0).

2.6. Dot-blot hybridization assay

Human IgG1-FC-tagged PEDV COE-t truncated constructs were spotted onto nitrocellulose (NC) membranes (Pierce, Rockford, IL, USA). Membranes were dried and blocked with 5% skim milk at 4 °C overnight. Next, membranes were incubated with mAbs for 1 h at 37 °C. After washing three times with PBST, membranes were incubated with HRP-conjugated goat anti-mouse IgG antibody and detected by

Table 1
Primers used in this work.

Name	Sequence (5'-3')	Residues sites
N F	CCGGAATTCGCCCTCCTTCAACGACCAT	505–569
N R	CATGCCATGGATCCTGGGACTTGCTCAC	
C F	CCGGAATTCGGTGAGCAAGTCCCAGGAT	564–629
C R	CATGCCATGGTGTAATGAGCTCCCCCTTT	
N1 F	CCGGAATTCGCCCTCCTTCAACGACCAT	505–544
N1 R	CATGCCATGGCCACGCAAAAGGAAGAGAA	
N2 F	CCGGAATTCGATCAACGGATTCTCTTCC	536–569
N2 R	CATGCCATGGCATCCTGGGACTTGCTCAC	
C1 F	CCGGAATTCGGTGAGCAAGTCCCAGGAT	564–600
C1 R	CATGCCATGGCTGTGCAGGCGCTGGCCAG	
C2 F	CCGGAATTCGACTAGCCTGCTGGCCAGC	592–629
C2 R	CATGCCATGGCTGTAATGAGCTCCCCCTTT	

The underlined letters represent restriction enzyme sites.

Table 2
The synthesized mini-fragments and their amino acid sequences.

Name	Sequence (5'-3')	Amino acid sequences
C2-1 F	AATTC**G**ACTAGCCTGCTGGCCAGCGCCTGCACAATTGACCTGTTCGGCTACCCT**G**C	^{592}TSLLASACTIDLFGYP^{607}
C2-1 R	CATGG**C**AGGGTAGCCGAACAGGTCAATTGTGCAGGCGCTGGCCAGCAGGCTAGT**C**G	
C2-2 F	AATTC**G**TTCGGCTACCCTGAGTTCGGGTCCGGAGTGAAGTTTACCAGCCTCTAC**G**C	^{604}FGYPEFGSGVKFTSLY^{619}
C2-2 R	CATGG**C**GTAGAGGCTGGTAAACTTCACTCCGGACCCGAACTCAGGGTAGCCGAA**C**G	
C2-3 F	AATTC**G**ACCAGCCTCTACTTCCAGTTTACAAAGGGGGAGCTCATTACA**G**C	^{616}TSLYFQFTKGELIT^{629}
C2-3 R	CATGG**C**TGTAATGAGCTCCCCCTTTGTAAACTGGAAGTAGAGGCTGGT**C**G	

The underlined letters represent restriction enzyme sites. The boldface letters were added to avoid frame shift mutation.

enhanced chemiluminescence (ECL) plus reagent (Solarbio, Beijing, China). Rabbit anti-human IgG antibody (Abcam, Camb, UK) was used to test adsorption of proteins to NC membranes.

2.7. ELISA

For analysis of the core residues recognized by the mAbs 6F3E3 and 4D8F10, the truncated COE-t construct C2-1 was further truncated from its N- and C-termini through synthesis of various peptides (GL Biochem, Shanghai, China) to perform pepscan analysis. Sequences of peptides are shown in Fig. 4. First, the peptides were coupled with bovine serum albumin (BSA) using EDC/NHS (N1-((ethylimino) methylene)-N3,N3-dimethylpropane-1,3-diamine/N-hydroxysuccinimide;1-hydroxypyrrolidine-2,5-dione; Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. ELISA plates were coated with coupled peptides (5 μg/ml) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After three washes with PBST, plates were blocked with 5% skim milk at 37 °C for 1 h. Next, hybridoma supernatants containing 4D8F10 or 6F3E3 mAbs and HRP-labeled goat anti-mouse IgG (H + L) were added to peptide-coated wells of ELISA plates. A substrate solution of TMB (3,3′,5,5′-tetramethylbenzidine, Sigma-Aldrich) was added and incubed at RT for 10 min before the reaction was stopped via addition of 2 M H_2SO_4. Absorbance readings at 450 nm were measured using a microplate reader (Omega, Ortenberg, Germany). BSA was used as negative control.

2.8. Amino-acid sequence analysis

The conservation of the novel epitope (^{592}TSLLASACTIDLFGYP^{607}) was determined via alignment of COE protein sequences of PEDV strains of both G1 and G2 types (Wang et al., 2016). Alignments were carried out using the Clustal W method provided by the MEGA 5.0 software package.

2.9. Indirect immunofluorescence assay (IFA)

After infection with PEDV strains, Vero cells exhibiting obvious CPE were fixed with ice-cold methanol/acetone (1:1 v/v) and incubated with blocking buffer containing 5% skim milk at 4 °C overnight. Next, cells were incubated with mAbs 4D8F10 or 6F3E3 and goat anti-mouse IgG (H + L)-Alexa Fluor 488 (Invitrogen, Rockford, IL, USA). Finally, cell nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI) and visualized by fluorescence microscopy (ZEISS, Jena, Germany).

3. Results

3.1. Generation of mAbs against PEDV

To generate mAbs specific for PEDV, we immunized female BALB/c mice with PEDV COE-t followed by hybridomascreening via IPMA. Positive hybridoma cell lines were subcloned and 14 mAbs were chosen for further characterization. Fig. 1 shows strong positive binding of mAbs to Vero cells infected with the native prevalent PEDV CH/hubei/2016 strain. Unfortunately, in spite of these binding results, none of the mAbs neutralized PEDV CH/hubei/2016 strain (data not shown).

3.2. Reactivity of the mAbs with three PEDV strains

Subsequently, we tested the reactivity of mAbs with PEDV strains CH/hubei/2016, CH/HNHB/2016 and attenuated CV777 via IPMA. PEDV CH/hubei/2016 and CH/HNHB/2016 strains are epidemic strains in China that are classified as G2a and G2b subtypes, respectively. The attenuated CV777 is a cell culture-adapted vaccine strain of G1 type. As Table 3 shows, mAbs 11F2C3B9, 7G4A9, 6F3E3, 1E5C2, 4D8F10 and 9D6D6 exhibited strong binding to all three PEDV strains. However, mAbs 9D7C7G7, 2D3A7C5, 1D3F7D2, 2D3G11 and 2F2B3 reacted strongly to PEDV CH/hubei/2016 and attenuated CV777 strains, but did not bind to the PEDV CH/HNHB/2016 strain. Meanwhile, mAbs 3H3A8, 9D6B10 and 4H5D2 bound strongly to PEDV strains CH/hubei/2016 and CH/HNHB/2016, with poor binding observed to the attenuated CV777 strain. Based on patterns of positive binding to the three PEDV strains, the 14 mAbs were next sorted into three categories: mAbs that strongly bound to all three PEDV strains (11F2C3B9, 7G4A9, 6F3E3, 1E5C2, 4D8F10 and 9D6D6); mAbs that did not recognize the PEDV CH/HNHB/2016 strain (9D7C7G7, 2D3A7C5, 1D3F7D2 and 2D3G11); mAbs that weakly bound to the attenuated CV777 strain (3H3A8, 9D6B10 and 4H5D2). From binding specificity pattern results, mAbs 9D7C7G7, 2D3A7C5, 1D3F7D, 6F3E3, 4D8F10 and 7G4A9 were selected for more precise epitope characterization.

3.3. Epitope mapping of PEDV specific mAbs

First, COE-t was divided into overlapping N (residues 505–569) and C (residues 564–629) regions. Next, N and C regions were further subdivided to create truncated constructs designated N1 (residues 505–544), N2 (residues 536–569) and C1 (residues 564–600), C2 (residues 592–629), respectively (Fig. 2B). All truncated constructs were expressed in eukaryotic HEK-293T cells as the S protein is glycosylated. Fig. 2C shows mAb binding to proteins spotted onto NC membranes whereby 4D8F10 and 6F3E3 bound to fragments C and C2, thus localizing the binding epitope to residues 592-629. However, the other mAbs did not specifically react to truncated COE-t fragments (data not shown) and thus were not assayed in subsequent experiments. The C2 fragment was further truncated into C2-1 (residues 592–607), C2-2 (residues 604–619) and C2-3 (residues 616–629) (Fig. 2B) that were each expressed in HEK-293T cells. The results of dot-blot hybridization assays suggest that mAbs 4D8F10 and 6F3E3 specifically bound to the C2-1 protein (residues 592–607) via the epitope ^{592}TSLLASACTIDLFGYP^{607} (Fig. 2C).

3.4. Epitope mapping of the C2-1 protein through pepscan

In order to define critical recognition sites of mAbs 4D8F10 and 6F3E3, N- and C-terminally truncated C2-1 proteins were synthesized via solid-phase peptide synthesis and evaluated by ELISA. Fig. 3 shows that mAbs 4D8F10 and 6F3E3 strongly bound to the peptide T16 P, demonstrating that they share the epitope ^{592}TSLLASACTIDLFGYP^{607}, consistent with results of the dot-blot hybridization assay. In addition,

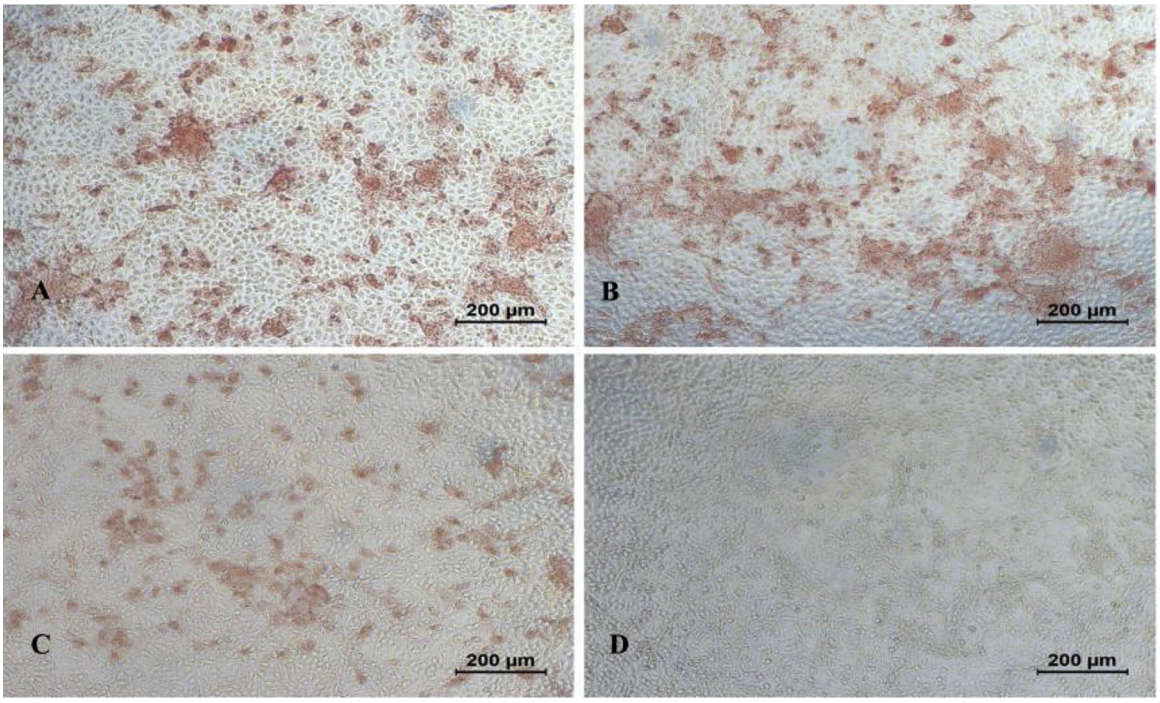


Fig. 1. Screening of mAbs against PEDV by IPMA. CPE regions shown in red are associated with positive binding of mAbs to the prevalent PEDV CH/hubei/2016 strain. Representative binding is shown for mAbs (A) 6F3E3, (B) 9D7C7G7, (C) 4D8F10 and (D) PEDV-negative mouse serum.

mAbs 4D8F10 and 6F3E3 bound to all N-terminally truncated proteins including peptide T8 P (^{600}TIDLFGYP^{607}), which only contains 8 amino acids (OD_{450} > 1.0) (Fig. 3A). These results suggest that the epitope sequence for mAb binding was located within the C-terminus of peptide T16 P. Although removal of residue P from the C-terminus did not reduce binding of mAbs 4D8F10 and 6F3E3, a complete loss of mAb binding was observed after removing both Y and P residues (Fig. 3B). This result indicates that the 606Y residue is essential for recognition by these mAbs. To further identify residues important for binding by these mAbs, the peptide T8 P (^{600}TIDLFGYP^{607}) was N-terminally truncated and mAb binding was detected by ELISA. Fig. 3C shows that mAbs 4D8F10 and 6F3E3 effectively bound to all truncated T8 P peptides, even to peptide F4P. Since peptides containing fewer than four amino acids are difficult to prepare, peptide F4P was not further truncated.

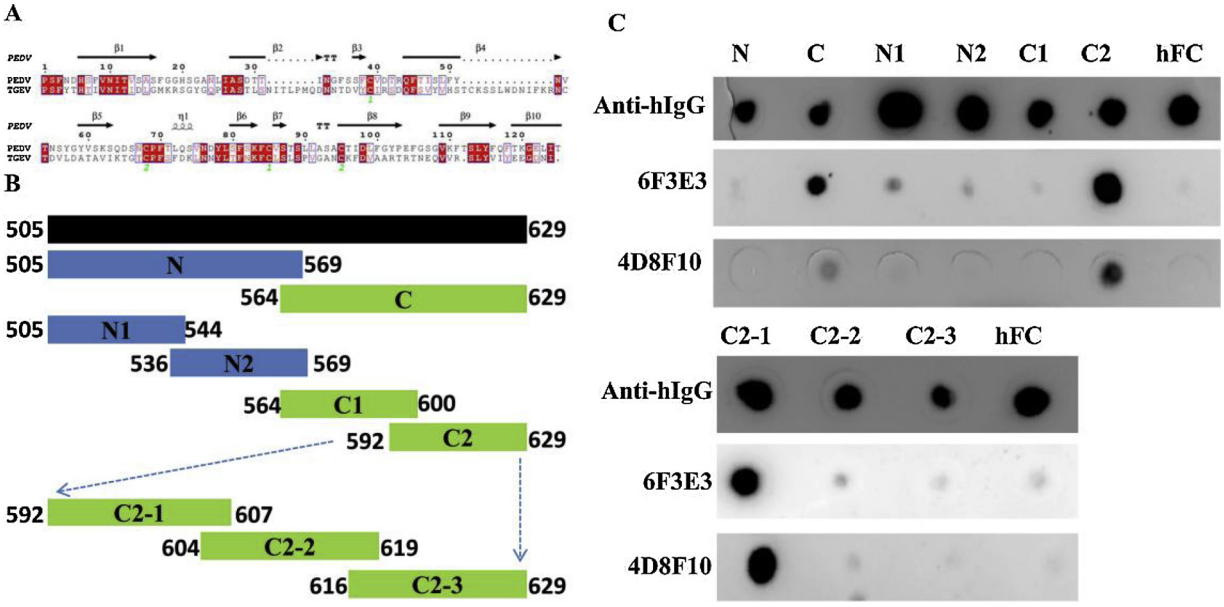


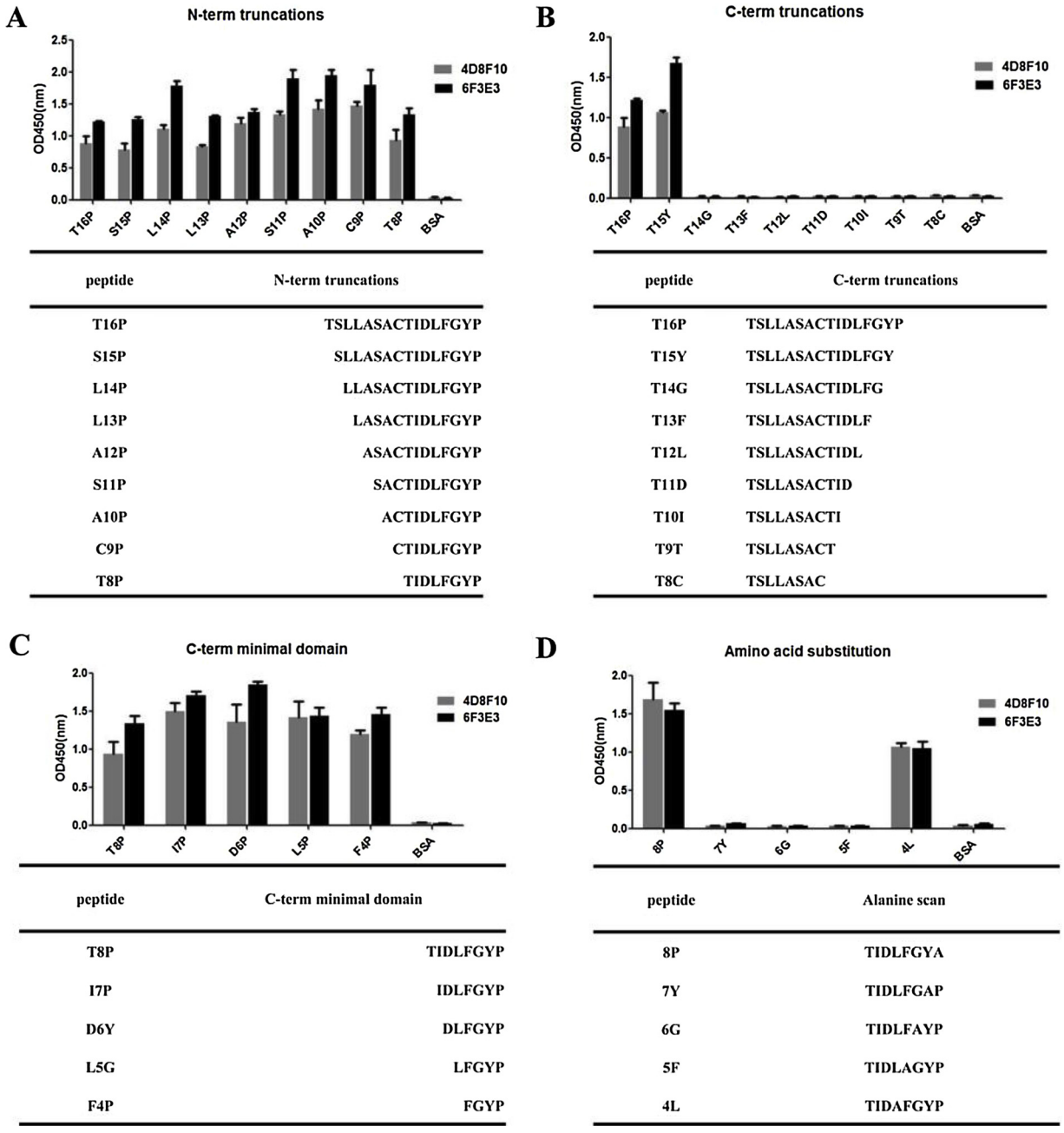
Fig. 2. Truncated COE-t constructs and epitope mapping. (A) Amino acid sequence alignment between PEDV COE-t and TGEV RBD proteins. The three-dimensional structure of PEDV COE-t was predicted using SWISS-MODEL based on the published structure of TGEV RBD (PDB: 4F2M). (B) A schematic showing truncated COE-t constructs. COE-t was first divided into two fragments (N and C). Next, N and C fragments were further subdivided to form N1, N2, C1 and C2. Finally, the positive fragment C2 was subdivided into C2-1, C2-2 and C2-3 fragment regions as shown in the schematic. (C) Binding results of mAbs with truncated COE-t constructs were obtained using a dot-blot hybridization assay. Positive binding appears as black dots that represent binding of hFC (human IgG FC tag) to mAb bound to epitope on COE-t fragment.

Table 3
Reactivity of mAbs with different PEDV strains determined by IPMA.

Strains	9D7C7G7	2D3A7C5	1D3F7D2	11F2C3B9	7G4A9	6F3E3	2D3G11
CH/hubei/2016	√	√	√	√	√	√	√
CH/HNHB/2016	×	×	×	√	√	√	×
attenuated CV777	√	√	√	√	√	√	√

Strains	1E5C2	4D8F10	3H3A8	2F2B3	9D6B10	4H5D2	9D6D6
CH/hubei/2016	√	√	√	√	√	√	√
CH/HNHB/2016	√	√	√	×	√	√	√
attenuated CV777	√	√	weak	√	weak	weak	√

"√" indicates that the mAb strongly reacts to the PEDV strain and the color of stained cells is markedly red; "×" indicates that the mAb does not react to the PEDV strain; "weak" indicates that the mAb weakly binds to PEDV strain and the color of stained cells is light red.



peptide	N-term truncations
T16P	TSLLASACTIDLFGYP
S15P	SLLASACTIDLFGYP
L14P	LLASACTIDLFGYP
L13P	LASACTIDLFGYP
A12P	ASACTIDLFGYP
S11P	SACTIDLFGYP
A10P	ACTIDLFGYP
C9P	CTIDLFGYP
T8P	TIDLFGYP

peptide	C-term truncations
T16P	TSLLASACTIDLFGYP
T15Y	TSLLASACTIDLFGY
T14G	TSLLASACTIDLFG
T13F	TSLLASACTIDLF
T12L	TSLLASACTIDL
T11D	TSLLASACTID
T10I	TSLLASACTI
T9T	TSLLASACT
T8C	TSLLASAC

peptide	C-term minimal domain
T8P	TIDLFGYP
I7P	IDLFGYP
D6Y	DLFGYP
L5G	LFGYP
F4P	FGYP

peptide	Alanine scan
8P	TIDLFGYA
7Y	TIDLFGAP
6G	TIDLFAYP
5F	TIDLAGYP
4L	TIDAFGYP

Fig. 3. Mapping of critical residues of mAbs 4D8F10 and 6F3E3 via pepscan. (A, B and C) N- and C-terminally truncated peptides of C2-1 fragment were used to identify key recognition sites of mAbs 4D8F10 and 6F3E3 by ELISA. (D) Identification of core residues of peptide T8 P for binding of mAbs 4D8F10 and 6F3E3 through alanine scanning.

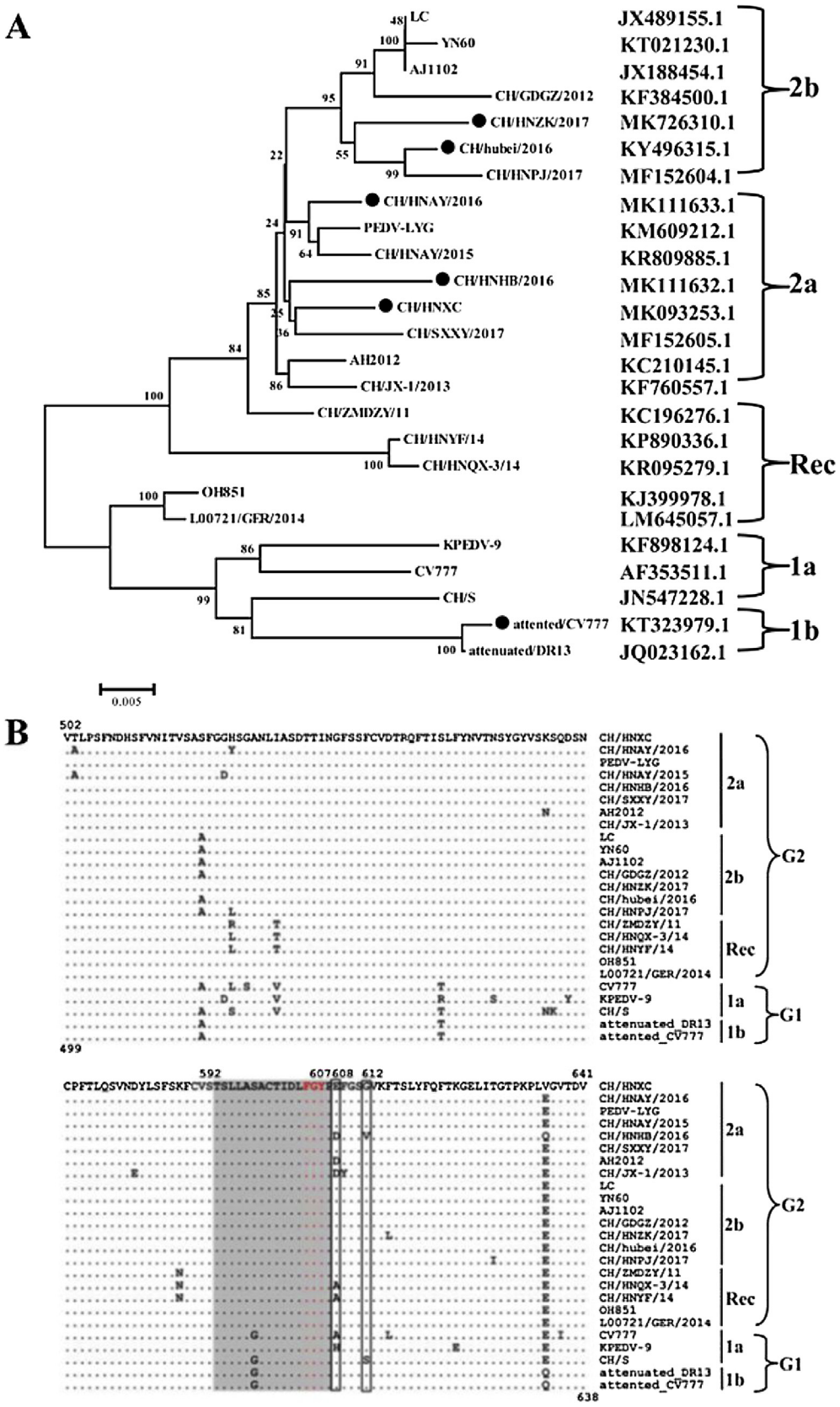


Fig. 4. Conservation analysis of the epitope ^{592}TSLLASACTIDLFGYP^{607}. (A) Genotyping of 25 PEDV strains based on S protein sequences. The phylogenetic tree was constructed using the neighbor-joining method in MEGA 5.0 with bootstrap values from 1000 resamplings shown for each node. Strain names and GenBank accession numbers are shown. Strains labeled with black dots were used in this study. (B) Alignment of COE protein sequences of different PEDV strains of both G1 and G2 types is shown. The epitope ^{592}TSLLASACTIDLFGYP^{607} is marked in gray and core residues for mAb binding are shown in red. Boxes represent unique amino acid mutations at sites 608 and 612. Rec: Recombinant PEDV (INDEL-like PEDV).

3.5. Core residues of the peptide T8P for the mAbs 4D8F10 and 6F3E3 binding

In case peptide F4P may lose additional binding residues, peptide T8P was utilized for subsequent experiments. To define crucial T8P residues involved in peptide binding by mAbs 4D8F10 and 6F3E3, alanine-scanning mutagenesis was performed to create a panel of peptides with individual substitutions with alanine (A) residues. After

testing peptides for mAb binding by ELISA, the results demonstrated that both mAbs bound to peptide 8 P (^{600}TIDLFGYA^{607}), but no binding of mAbs was observed to peptides with alanine substitutions of 606Y, 605G and 604F. However, both mAbs still bound to peptide 4 L (Fig. 3D), suggesting that residues 606Y, 605G and 604F are core residues involved in binding of mAbs 4D8F10 and 6F3E3.

3.6. Conservation analysis of the identified epitope

Here, COE protein sequences of 6 PEDV strains used in this work and 19 representative PEDV strains of both G1 and G2 types were selected for phylogenetic analysis through amino acid alignments by MEGA 5.0. As shown in Fig. 4A, the G1 type included 1a (CV777, KPEDV-9 and CH/S) and 1b (attenuated DR13 and attenuated CV777) subtypes, while the G2 type included field epidemic strains of 2a (CH/HNXC, CH/HNAY/2016, PEDV-LYG, CH/HNAY/2015, CH/HNHB/2016, CH/SXXY/2017, AH2012 and CH/JX-1/2013), 2b (LC, YN60, AJ1102, CH/GDGZ/2012, CH/HNZK/2017, CH/hubei/2016 and CH/HNPJ/2017) and recombinant (INDEL-like; CH/ZMDZY/11, CH/HNQX-3/14, CH/HNYF/14, OH851 and L00721/GER/2014) subtypes. All of these strains represent PEDV strains existing worldwide. Although amino acid sequence alignments of these strains demonstrate mutations within COE region, the epitope ^{592}TSLLASACTIDLFGYP^{607} was conserved among diverse PEDV stains, with only one exception of an amino acid substitution (S→G) at site 597 in G1 type strains. Notably, core amino acid residues 606Y, 605G and 604F were highly conserved among all PEDV strains (Fig. 4B). These data confirm that COE epitopes bound by mAbs 4D8F10 and 6F3E3 are highly conserved in PEDV S proteins of diverse PEDV strains circulating globally.

In order to verify that mAbs 4D8F10 and 6F3E3 were applicable for recognition of the conserved epitope, another two prevalent PEDV strains CH/HNAY/2016 (G2a) and CH/HNZK/2017 (G2b) having the conserved epitope (Fig. 4B) were further tested using IFA. Fig. 5 shows that mAbs 4D8F10 and 6F3E3 specifically bound to both PEDV strains.

4. Discussion

The PEDV S protein is the main target for induction of host antibodies, such as neutralizing antibodies and colostrum immunoglobulin A (IgA) (Song et al., 2016). Previous studies have identified several domains containing neutralizing epitopes within the S protein, such as residues 19–220 (Li et al., 2017), residues 499–638 (Chang et al., 2002), residues 636–789 (Sun et al., 2007), residues 744–759, 747–774 and/or 756–771 (Okda et al., 2017), residues 1268–1322 (Zhao et al., 2018) and residues 1371–1377 (Cruz et al., 2006). Notably, the COE fragment, which consists of residues 499–638, has been evaluated for development of PEDV subunit vaccines (Bae et al., 2003; Ge et al., 2012; Ma et al., 2018). A recent study (Chang et al., 2019) identified an neutralizing epitope (residues 575–639) within the COE. However, many studies have reported amino acid changes within this domain that may lessen its effectiveness as a vaccine target epitope (Kim et al., 2015; Sun et al., 2018a; Yu et al., 2018). Therefore, determination of COE epitopes would enhance understanding of the relevance of antigenic changes to the development of effective vaccines.

In this study, we generated mAbs against COE-t that were shown to recognize native PEDV via IPMA. PEDV mAb epitopes were then mapped more precisely using dot-blot hybridization assay and pepscan analyses. The results demonstrated that mAbs 4D8F10 and 6F3E3 specifically bound to the 16-amino acid peptide ^{592}TSLLASACTIDLFGYP^{607}, which had not been previously identified as an epitope and thus is a novel linear B-cell epitope. Further N-terminal truncation of this peptide determined that ^{604}FGYP^{607} comprises the minimal crucial epitope for binding recognition by non-neutralizing mAbs. Furthermore, alanine substitution analysis suggests that 604F, 605G and 606Y are core binding residues (Fig. 3D). Meanwhile, amino acid sequence alignment analysis demonstrated that the epitope ^{592}TSLLASACTIDLFGYP^{607} is highly conserved among COE proteins of PEDV strains of G1 and G2 types, especially with regard to the core residues (604F, 605G and 606Y) (Fig. 4B). Moreover, further experimentation determined that mAbs 4D8F10 and 6F3E3 specifically recognized prevalent G2 type PEDV strains, which all share the novel epitope identified here. Therefore, mAbs 4D8F10 and 6F3E3 should be further evaluated for use in ELISA or rapid immunochromatographic strip testing to detect PEDV infection.

PED has re-emerged and increased in prevalence in China since 2010 (Sun et al., 2012), while highly virulent PEDV has spread rapidly throughout the USA since 2013 (Huang et al., 2013). Importantly, the S protein of prevalent PEDV strains shows mutations, insertions and/or deletions compared to S proteins of classical PEDV strains, thus aligning with previous speculations that amino acid changes in S protein might be associated with antigenic changes of prevalent PEDV strains (Huang et al., 2013; Li et al., 2014). Here, binding of mAbs was tested by IPMA and the result suggests that mAb binding to PEDV differed among strains of both G1 and G2 types. Indeed, mAbs 9D7C7G7, 2D3A7C5, 1D3F7D2, 2D3G11 and 2F2B3 did not recognize PEDV CH/HNHB/2016 strain (Table 3). We hypothesize that the absence of binding of mAbs to this virus strain was due to amino acid substitutions within mAb-binding epitopes. Upon further amino acid sequence alignment analysis, two unique substitutions (608D and 612 V) were observed in COE domains of the PEDV CH/HNHB/2016 strain compared with PEDV strains CH/HNXC, CH/hubei/2016 and attenuated CV777 (Fig. 4B). Therefore, mutations E608D and G612 V may be responsible for antigenic change, although confirmation of this hypothesis awaits future studies. Intriguingly, both 608 and 612 sites are highly variable among different PEDV strains. As a consequence, mAbs 9D7C7G7, 2D3A7C5, 1D3F7D2, 2D3G11 and 2F2B3 might be useful for identifying PEDV strains with these unique substitutions.

Virus neutralization assays showed that mAbs did not prevent PEDV infection of Vero cells (data not shown). Therefore, the sequence ^{592}TSLLASACTIDLFGYP^{607} is a non-neutralizing epitope. However, a previous study had demonstrated that a non-neutralizing epitope peptide could significantly inhibit adsorption of PEDV to Vero cell surfaces (Cao et al., 2015). In addition, non-neutralizing antibodies have also been shown to play important roles in antiviral responses via antibody-dependent cellular cytotoxicity or Fc gamma receptor- and complement-dependent effector mechanisms *in vivo* (Abreu-Mota et al., 2018; Jegaskanda et al., 2013; Vogt et al., 2011). Previously, only two non-neutralizing epitopes (residues 748–755 and 764–771) had been identified within the PEDV S protein (Sun et al., 2008). Here, by screening 14 mAbs for binding to the PEDV S protein COE region, a novel non-neutralizing epitope ^{592}TSLLASACTIDLFGYP^{607} was identified. This peptide will be the focus of future studies that evaluate its relevance to host antiviral responses.

In summary, we prepared mAbs that bind specifically to PEDV and demonstrated different mAb binding to various PEDV strains, suggesting antigenic differences among S spike glycoprotein COE regions of different strains. Notably, one novel linear B cell epitope was identified within the COE region. This epitope was highly conserved among PEDV strains of both G1 and G2 types and may be useful for the future development of diagnostic methods to detect PEDV.

Conflicts of interest

The authors declare that they have no competing interest.

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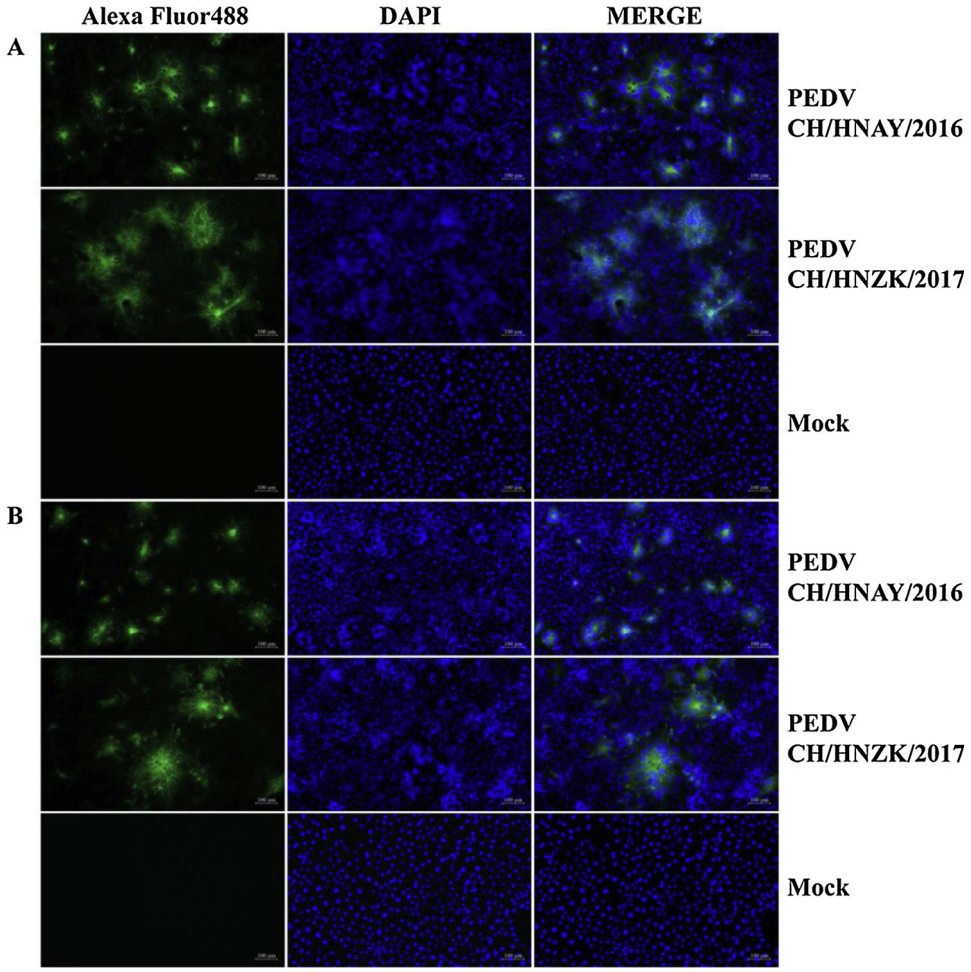

Fig. 5. Binding of mAbs 4D8F10 and 6F3E3 with PEDV-infected Vero cells as determined by IFA. Prevalent PEDV strains CH/HNAY/2016 and CH/HNZK/2017 of G2a and G2b subtypes were detected by binding of mAbs 4D8F10 (A) and 6F3E3 (B). "Mock" represents uninfected Vero cells as a negative control. Scale bars, 100 μm.

decision to submit the work for publication.

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