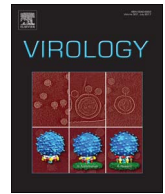




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Neutralization of genotype 2 porcine epidemic diarrhea virus strains by a novel monoclonal antibody

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) has two genotypes, G1 and G2. To research the immunogenicity differences of PEDV G1 and G2 genotype strains and obtain a neutralizing monoclonal antibody (mAb), we inoculated specific-pathogen-free BALB/c mice with a newly emerged strain, PEDV-LNCT2. After immunizations, cells from the spleen of the mice were fused with SP2/0 myeloma cells. Following culturing and subcloning, a strain, 1B9, secreting neutralizing antibody, was obtained. The 1B9 mAb neutralized new variant genotype 2 PEDV strains (LNCT2, LNSY, and Hjms), but it did not neutralize a genotype 1 PEDV strain (CV777), *in vitro*. Results showed that the epitope recognized by the 1B9 mAb lies in the spike protein, and that it is a conformational epitope. These findings confirm that allelic differences in the PEDV S gene between the G1 and G2 genotype strains led to changes in the S protein and, thus, differences in its immunogenicity.

1. Introduction

New variants of porcine epidemic diarrhea virus (PEDV) emerged in China in 2010 (Sun et al., 2012), and they spread to the United States in 2013 (Cima, 2013; Stevenson et al., 2013). These new PEDV variants have caused high morbidity and mortality rates in both newborn and suckling pigs (Wang et al., 2013). These animals present with diarrhea and vomiting, and PEDV infection has resulted in substantial economic losses to the swine industry, as well as increased pork prices in China.

Based on PEDV genome sequences, PEDV strains have been divided into two groups, the G1 genotype (including the prototype European PEDV strain CV777) and the G2 genotype (new variant strains that have emerged since 2011) (Chen et al., 2013; Lee and Lee, 2014). The pathogenesis of G1 and G2 strains has been reported since the 1980s and 2013, respectively (Coussement et al., 1982; Debouck et al., 1981; Stevenson et al., 2013). Both PEDV G1 and G2 strains lead to severe diarrhea and vomiting.

It is well known that the spike (S) protein of coronaviruses plays a crucial role in the induction of neutralizing antibodies, and it has been used to prepare effective vaccines (Gómez et al., 2000; Tuboly and

Nagy, 2001). The amino-terminal portion of the S protein of several coronaviruses has been shown to contain key antigenic sites that are responsible for eliciting humoral and cellular immune responses (Delmas et al., 1986; Gebauer et al., 1991; Zhang et al., 2016). The PEDV S gene is often used to evaluate the genetic diversity of coronaviruses (Li et al., 2012; Chen et al., 2013).

Based on research into the PEDV genome (Chen et al., 2013; Lee and Lee, 2014), the main differences between PEDV G1 and G2 strains occur in the S gene. The full length of S gene of G2 strains is 9 nt longer than that of the prototype PEDV strain CV777. In addition to its increased length, the S gene of G2 strains has many insertions and deletions, as well as other mutations, compared with the S gene of G1 strains (Lee and Lee, 2014).

Many pig herds that were vaccinated with inactivated or attenuated CV777 vaccines still experienced high mortality rates among newborn piglets (Li et al., 2012; Sun et al., 2012). A mutation in the S gene may lead to a change in the S protein and, thus, a difference in its immunogenicity, thereby resulting in a different neutralization profile. Therefore, in the present study, PEDV G2 virions were used to immunize BALB/c mice to produce a neutralizing monoclonal antibody (mAb) to test for immunogenicity differences that distinguish G1 and

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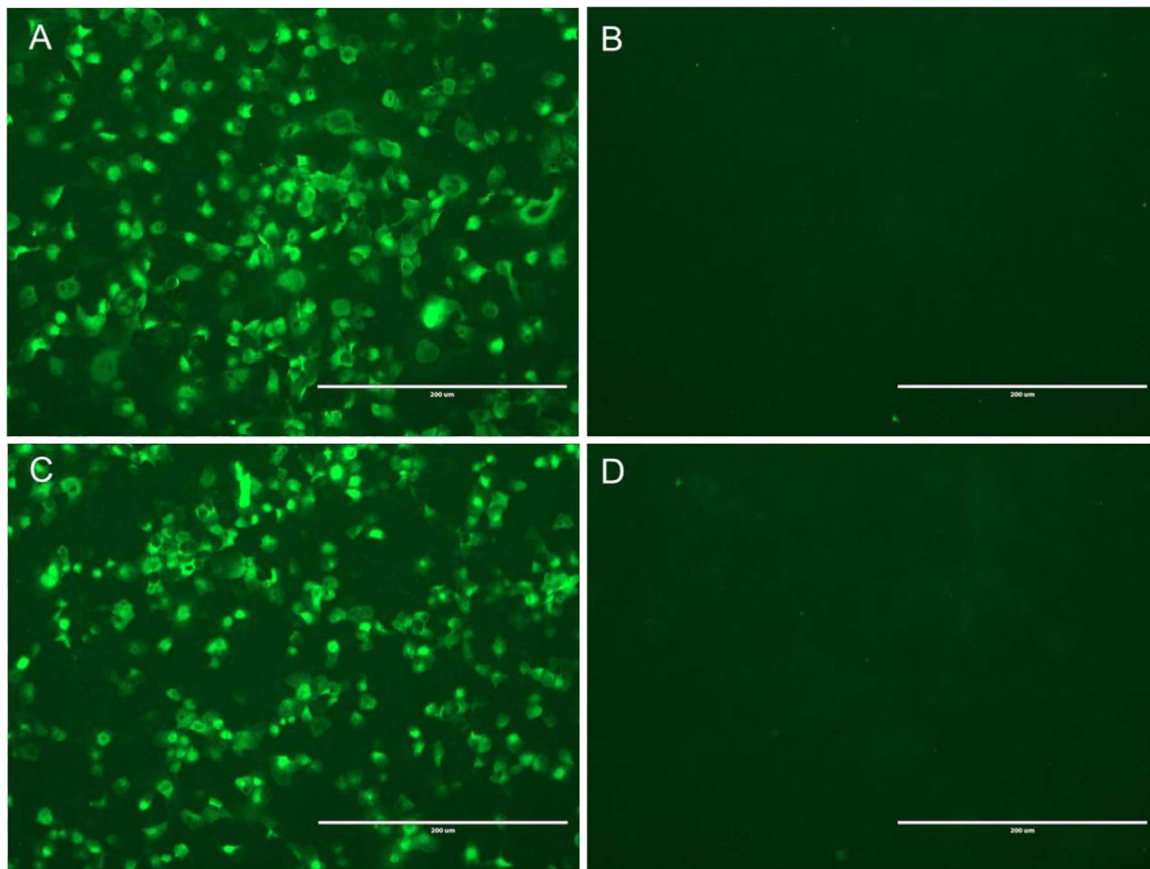


Fig. 1. Reaction of mAb 1B9 with PEDV-positive cells, as detected by an IFA. The cells in A) and C) were Vero E6 cells infected by PEDV strain LNCT2, which yielded positive signals; the cells in B) and D) were healthy Vero E6 cells used as a negative control. In A) and B), mAb 1B9 was used as the primary antibody, while in C) and D), a PEDV polyclonal antibody was used as the primary antibody.

G2 PEDV strains.

2. Results

2.1. Phylogenetic analysis based on PEDV genome and S gene sequences

Two phylogenetic trees of PEDV genomes and S genes were constructed, respectively (Suppl. Fig. S1(a, b)). Both the phylogenetic trees indicated that the LNCT2, LNSY, and Hjms strains belong to the G2 genotype, and the CV777 strain belongs to the G1 genotype. After translating the S genes, the S proteins were analyzed by MegAlign in DNASTar. As shown in Suppl. Fig. S2, we observed that the main differences lie in the S1 portion of the S protein.

2.2. A positive mAb clone against PEDV

After immunizing mice, their spleen cells fused with SP2/0 myeloma cells, and a positive clone of hybridoma cells was selected. A subclone, 1B9, reacted well with PEDV by enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence assay (IFA) (Fig. 1). The heavy chain was IgG2b, and the light chain was kappa, as detected by the SBA Clonotyping System-HRP (Southern Biotech, Birmingham, AL, USA). Western blotting was used to identify the epitope recognized by 1B9. However, mAb 1B9 did not react with PEDV strains LNCT2 and CV777, Vero-E6 cells, or the S protein of the LNCT2 strain (Suppl. Fig. S3a). Dot immunoblotting assay shows that mAb 1B9 binds LNCT2 virus stronger than that of CV777 (Suppl. Fig. S3b). These results suggested that the epitope of 1B9 was dependent on the protein conformation. The antibody titer in the culture supernatant

was higher than 1:1000, and the titer in the ascites fluid was higher than 1:100,000, as detected by an IFA.

2.3. Neutralization test

Purified mAb 1B9 (diluted to 1 mg per ml) was used in a neutralization test. As shown in Fig. 2, the LNCT2, LNSY, and Hjms strains were neutralized by mAb 1B9, while strain CV777 was not. Additionally, mAb 1B9 completely neutralized the LNCT2, LNSY, and Hjms strains at dilutions of 1:160, 1:80, and 1:160, respectively.

2.4. Identification of the protein recognized by mAb 1B9 using recombinant PEDV S, S1, and S2 proteins

Six plasmids, pcDNA3.1(-)-CV777-S, pcDNA3.1(-)-CV777-S1, pcDNA3.1(-)-CV777-S2, pcDNA3.1(-)-LNCT2-S, pcDNA3.1(-)-LNCT2-S1, and pcDNA3.1(-)-LNCT2-S2, were constructed. The plasmids were transfected into HEK293T cells to identify proteins that reacted with mAb 1B9. As shown in Fig. 3, cells transfected with plasmids pcDNA3.1(-)-CV777-S and pcDNA3.1(-)-LNCT2-S reacted well with mAb 1B9 and PEDV-positive pAb. Cells transfected with the other plasmids did not react with mAb 1B9, but reacted with the positive control antibodies. HEK293T cells transfected with these plasmids did not react with the negative serum (not shown in Fig. 3). These results suggested that mAb 1B9 recognized the S protein of the CV777 and LNCT2 strains. However, neither the S1 nor S2 proteins of CV777 were recognized by 1B9.

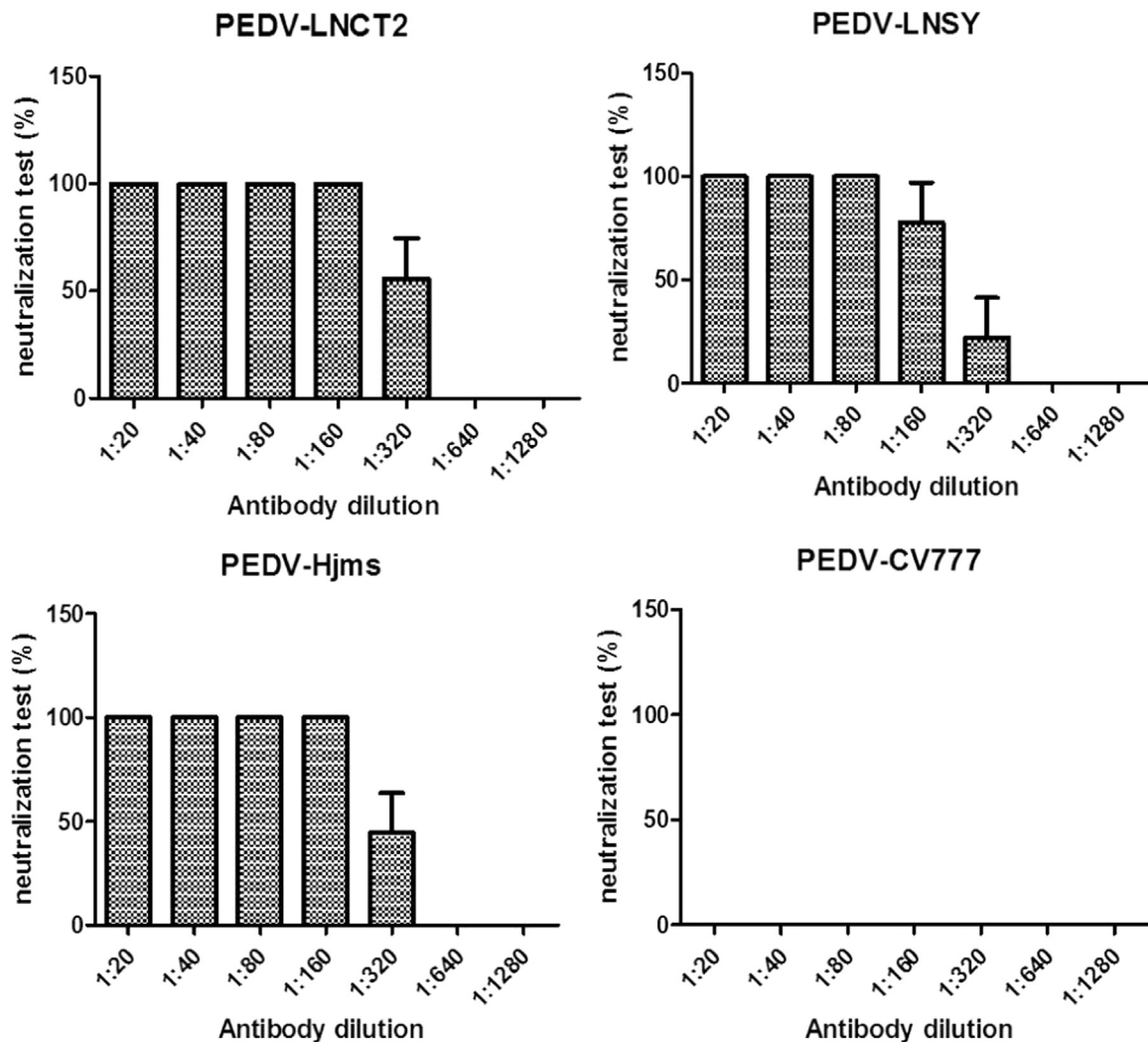


Fig. 2. The neutralization of antigenically distinct PEDV. PEDV strains LNCT2, LNSY, Hjms, and CV777 were used to test the neutralization features of mAb 1B9. The results show that PEDV strains LNCT2, LNSY, and Hjms were neutralized completely when mAb 1B9 was diluted to 1:160, 1:80, and 1:160, respectively. The CV777 strain was not neutralized by mAb 1B9.

3. Conclusions

The hybridoma cell line, 1B9, which secretes PEDV-specific neutralizing antibodies, was produced using the monoclonal antibody technique. The 1B9 mAb neutralized PEDV G2 strains (LNCT2, LNSY, and Hjms), but not a PEDV G1 strain (CV777). The epitope recognized by mAb 1B9 is a conformational epitope, and it lies within the S protein. The difference between mAb 1B9-mediated neutralization of PEDV G2 strains and the prototypical PEDV G1 strain suggests that they not only differ at the genomic level, but also in terms of their antigenic specificity. The 1B9 mAb could be used to distinguish the two genotypes of PEDV, and it could also be used to treat pigs infected with PEDV G2 strains at an early stage of infection.

4. Discussion

New variant strains of PEDV, belonging to the G2 genotype, first emerged in China in 2010 (Sun et al., 2012) and then spread to the USA in 2013 (Huang et al., 2013; Stevenson et al., 2013), and they have caused great losses to the swine industry. In China, inactivated and live attenuated vaccines based on the CV777 strain have been used to control the prevalence of PEDV. However, many pig herds that received those vaccines still experienced high mortality rates among newborn piglets (Li et al., 2012; Sun et al., 2012). The morbidity and mortality

rates of vaccinated herds were lower than those of nonvaccinated herds, which suggest that vaccines based on the CV777 strain could partially protect pigs against the new variant strains (Li et al., 2012).

Subsequently, the new variant strains were compared with the CV777 strain. PEDV strains were divided into two genotypes, the G1 genotype (CV777 strain) and the G2 genotype (new variant strains) based on their genome sequences (Lee and Lee, 2014; Wang et al., 2016). In the present study, three PEDV strains, LNCT2, LNSY, and Hjms, belonging to the G2 genotype were used in a phylogenetic analysis (Suppl. Fig. S1). To understand the differences in the PEDV G1 and G2 strains, apart from their genome sequences, we studied the immunogenicity of the newly emerged Chinese strains LNCT2, LNSY, and Hjms, and we compared these G2 strains with the prototypical G1 vaccine strain CV777. Purified PEDV LNCT2 particles were used to immunize BALB/c mice, and a mAb, 1B9, was obtained. Because mAb 1B9 completely neutralized the LNCT2, LNSY, and Hjms strains, while it did not neutralize the CV777 strain, whose S protein sequence differs from that of the G2 strains (Suppl. Fig. S2), we inferred that the epitope recognized by mAb 1B9 lies within the S protein or the S1 portion of the S protein. However, because PEDV particles consist of S, membrane, nucleocapsid, and envelope proteins, we were unsure which protein was recognized by mAb 1B9. It is known that the S protein of coronaviruses plays a crucial role in the induction of neutralizing antibodies, and it has been used to prepare effective vaccines (Tuboly

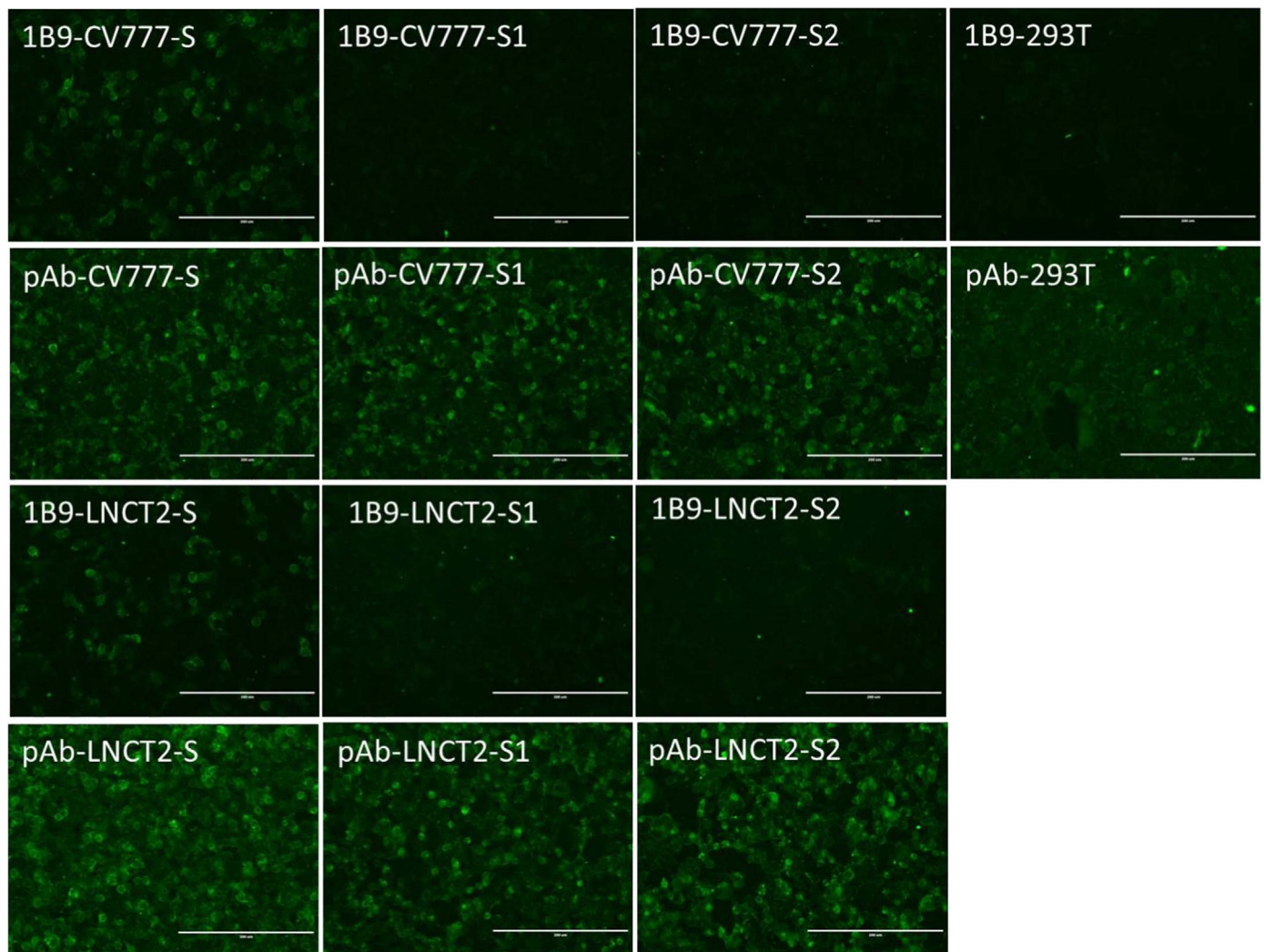


Fig. 3. Identification of the protein recognized by mAb 1B9 by IFA. Recombinant plasmids pcDNA3.1(-)-CV777-S, pcDNA3.1(-)-CV777-S1, pcDNA3.1(-)-CV777-S2, pcDNA3.1(-)-LNCT2-S, pcDNA3.1(-)-LNCT2-S1, and pcDNA3.1(-)-LNCT2-S2, were transfected into 293T cells. The results show that all the cells transfected with the six plasmids reacted well with the PEDV-positive pAb. Only the cells transfected with pcDNA3.1(-)-CV777-S and pcDNA3.1(-)-LNCT2-S plasmids reacted with mAb 1B9. The negative serum did not react with any cells, and were not shown in the results.

and Nagy, 2001).

To determine whether the S protein of PEDV was recognized by mAb 1B9, the S gene, as well as the S1 and S2 genes, of the CV777 and LNCT2 strains were cloned into plasmid pcDNA3.1(-). To improve the expression level of the S protein, the S gene sequences of the CV777 and LNCT2 strains were codon optimized using a ‘one amino acid-one codon’ strategy, without changing their amino acid sequences (Wang et al., 2016; Zyllicz-Stachula et al., 2014). The results showed that mAb 1B9 only reacted with cells that were transfected with pcDNA3.1(-)-CV777-S or pcDNA3.1(-)-LNCT2-S. These results suggested that the epitope recognized by mAb 1B9 lies within the S protein. To determine whether the epitope lies within the S1 or S2 regions of the S protein, the plasmids pcDNA3.1(-)-CV777-S1, pcDNA3.1(-)-CV777-S2, pcDNA3.1(-)-LNCT2-S1, and pcDNA3.1(-)-LNCT2-S2 were transfected into HEK293T cells, and then mAb 1B9 binding to cells. However, only a polyclonal antibody reacted with the S1 and S2 regions. To exclude the possibility that the epitope lies at the junction of the S1 and S2 regions, we designed 150-bp repetitive sequences in the S1 and S2 portions of the LNCT S gene, and 114-bp repetitive sequences in the S1 and S2 portions of the CV777 S gene (Table 1). Based on these results, we are sure that the S protein is the target of mAb 1B9. Furthermore, the epitope recognized by mAb 1B9 is a conformational epitope, because it did not react with viral particles by

Table 1

Oligonucleotide primers used for the construction of plasmids of PEDV S genes.

Primer	Sequence
LNCT-S1-F1	5'-GCAGAATTCATGAAGTCCCTGACCTACTTCTGGC-3'
LNCT-S1-R2460	5'-CTCGGATCCTCACTTGACACCGGCTGTGCCATTG-3'
LNCT-S2-F2311	5'-GCAGAATTCATGCAAGTGAAGATCGCCCTACCG-3'
LNCT-S2-R4161	5'-CTCGGATCCTCACTGCACATGCACTTCTCG-3'
CV777-S1-F1	5'-GCAGAATTCATGACCCCACTGATCTACTTCTGGC-3'
CV777-S1-R2430	5'-CTCGGATCCTCAGTTACACACGTATGTCACGCAATCG-3'
CV777-S2-F2317	5'-GCAGAATTCATGGTCACAGGAAATATCAGCATTTC-3'
CV777-S2-R4149	5'-CTCGGATCCTCACTGCACGTGGACCTTCTCAAAT-3'

Western blot or with the partial S proteins expressed by the transfected cells by IFA. And the partial S proteins expressed by the transfected cells might alter the conformation of the epitope. Although mAb 1B9 recognized the S protein of the CV777 and LNCT2 strains, it did not neutralize the CV777 virus, suggesting that differences in the S protein sequence between CV777 and LNCT2 might play an important role in epitope recognition or neutralization. Such differences in the S protein sequence likely alter the immunogenicity of S protein, thereby resulting in a different neutralization profile. Thus, the PEDV G1 and G2

genotypes not only differ in their genomes, but also in their antigenic specificity. Nevertheless, the conformational epitope of mAb 1B9 and the molecular mechanism by which 1B9 neutralized the LNCT2 strain, but not the CV777 strain, require further study.

5. Materials and methods

5.1. Virus, cells, and reagents

In November 2014, samples of small intestine obtained from piglets that had watery diarrhea were tested for enteric pathogens using the Antigen Rapid Porcine Epidemic Diarrhea Virus Ag Test Kit, the Antigen Rapid Rota Ag Test Kit, and the Antigen Rapid Transmissible Gastroenteritis Ag Test Kit (BioNote Inc., Suwon, South Korea). Only PEDV-positive samples were used to isolate viruses in this study. We obtained three PEDV strains that were Liaoning Changtu (LNCT2, GenBank accession no. KT323980), Shengyang (LNSY, KY007140), and Heilongjiang Jiamusi (Hjms, KY007139) strains. The CV777 vaccine strain (GenBank accession no. KT323979) was cultured in our laboratory. Vero E6 cells (no. CRL-1586, American Type Culture Collection, Manassas, VA, USA) were used for PEDV culture. The Vero E6 cell lines were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 5% fetal bovine serum (FBS) without an antibiotic-antimycotic solution. Trypsin-ethylenediaminetetraacetic acid and FBS were supplied by Gibco BRL Life Technologies. All other chemicals were of reagent grade.

5.2. Phylogenetic analysis of PEDV genomes and S genes

The full genome and S gene sequences of the LNCT2, LNSY, Hjms, and CV777 strains, along with other genomes that were submitted to GenBank, were used to construct phylogenetic trees by the neighbor-joining method using 1000 bootstrap replicates in MEGA 6.06 (<http://www.megasoftware.net/>). Additionally, DNAMAN and DNASTar were used to analyze the S gene sequences.

5.3. Virus propagation

Vero E6 cells were used to propagate PEDV according to the methods described by Oka et al. (2014). Briefly, confluent monolayers, from which growth medium (containing 5% FBS) had been removed, were washed with phosphate-buffered saline (PBS, pH 7.2). Then, 1×10^4 plaque-forming units (PFU) of PEDV, which were diluted in 10 ml of DMEM (containing 100 μ g of trypsin), were added to each culture bottle at 37 °C (the MOI was 0.01). The viruses were harvested after 90% of the cells exhibited a cytopathogenic effect, and then they were stored at -20 °C.

5.4. Virus purification by sucrose gradient ultracentrifugation

Cell cultures were harvested and then centrifuged at 8000 \times g for 5 min. The supernatant was collected, and the cell debris was discarded according to the method described by Hofmann and Wyler (1990), with modifications. Viruses in the suspensions were placed in 70-ml centrifuge tubes (Beckman Coulter, Pasadena, CA, USA) and pelleted by centrifugation at 120,000 \times g for 2 h in a 45Ti rotor. The pelleted viruses were suspended in PBS (1% of the original culture volume) and placed in 38-ml centrifuge tubes (Beckman Coulter), followed by under layering three sucrose solutions of different densities: 12 ml of 20% (w/w), 15 ml of 40% (w/w), and 8 ml of 60% (w/w) sucrose. The tubes were centrifuged at 100,000 \times g for 2 h in an SW32 rotor (Beckman Coulter). The resulting band between the 40% and 60% sucrose solutions were collected with a syringe, and, after removing the sucrose, used as antigen to immunize mice and in ELISA. The virus

was identified by electron microscopy. The sample was negatively stained with 3% phosphotungstic acid according to previously described procedures (Chen et al., 2014; Jung et al., 2014).

5.5. Preparation and characterization of mAbs against PEDV

Standard procedures created by Köhler and Milstein (1975) were used to generate hybridoma cells that secrete PEDV-specific antibodies, with some modifications. Briefly, 6-week-old female BALB/c mice were immunized subcutaneously with the purified PEDV particles in Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). The mice received two booster immunizations with the PEDV particles in Freund's incomplete adjuvant at 2-week intervals. Two weeks later, one additional intraperitoneal immunization without adjuvant was administered. Three days after the final dose, the mice were killed, and their spleen cells were harvested and fused with SP2/0 myeloma cells at a ratio of 5:1 using polyethylene glycol 1450 (Sigma-Aldrich). The culture and selection of hybridoma cells, as well as the culture supernatants of the surviving clones, were screened for reactivity and specificity with an indirect ELISA as described by Liu et al. (2013), with minor modifications. Briefly, the purified PEDV particles were used to coat Costar microplates (Corning, Corning, NY, USA). Positive clones were further detected by IFA to confirm the results. The positive clones were subcloned three times by limiting dilution. The antibody subtypes of the mAbs were detected by the SBA Clonotyping System-HRP according to the manufacturer's instructions. All the animal experiments in this study were performed with the approval of our institute, in accordance with animal ethics guidelines and approved protocols.

5.6. IFA

Monolayers of Vero E6 cells were grown to 100% confluency in 96-well plates and inoculated with 1×10^2 PFU per well of the PEDV LNCT2 strain that was suspended in DMEM supplemented with trypsin. The monolayers were fixed with 4% paraformaldehyde after 24 h, washed with PBS, and incubated with the supernatants of the hybridoma cells for 1 h. Then, they were incubated for 45 min with fluorescein isothiocyanate-labeled goat anti-mouse IgG (Sigma-Aldrich), and the cell layer was examined under a fluorescence microscope. Positive signals suggested that the supernatants of the hybridoma cells were PEDV-specific, IgG-positive mAbs.

5.7. Preparation of ascites fluid and antibody purification

The positive clone hybridoma cells were injected into the peritoneal cavities of Pristane (Sigma-Aldrich)-primed BALB/c mice to obtain their ascites fluid. The ascites fluid was purified by HiTrap™ Protein G HP column chromatography (GE Healthcare, Uppsala, Sweden) according to the manufacturer's protocol.

5.8. Neutralization test

To determine whether the antibody had neutralization activity, a neutralization test with mAb 1B9 was conducted according to a method described previously by Lefebvre et al. (2008), with modifications. Briefly, the purified ascites fluid of the mAb (diluted to 1 mg/ml) was filtered using a 0.22- μ m membrane. Then, 200 tissue culture infective dose of PEDV (in a volume of 200 μ l) was incubated for 1 h at 37 °C with an equal volume of 2-fold dilutions of the mAb. After incubation, this mixture, along with trypsin (each milliliter of solution contained 10 μ g of trypsin), was added to confluent (100%) monolayers of PEDV-negative Vero-E6 cells in four wells of a 96-well plate. Then, we observed the cytopathogenic effect in these cells for 5–7 days. Four PEDV strains (LNCT2, LNSY, Hjms, and CV777) were used to perform the neutralization test.

5.9. Dot immunoblot assay

Dot immunoblotting was performed as Ohashi et al. (1998) described. The PEDV-LNCT2 and CV777 strains (10 µl viruses containing 1000 pfu) and PEDV-LNCT2 S protein as well as CV777 S protein (100 ng) were adsorbed onto a nitrocellulose membrane. The antibody 1B9 was used as primary antibody. The horseradish peroxidase-labeled goat anti-mouse IgG (Zsbg-bio, Beijing, China) was used as secondary antibody. The color reaction was developed with 3, 3'-diaminobenzidine tetrahydrochloride (DAB) (Tiangen, Beijing, China) substrate.

5.10. Construction of expression vectors, and IFA detection

To identify the protein recognized by the mAb, the S gene of the CV777 and LNCT2 strains was amplified by polymerase chain reaction (PCR). Additionally, both the S1 and S2 genes were amplified by PCR. Primers (Table 1) containing *EcoR* I and *BamH* I sites were synthesized by Comate Bioscience Co., Ltd. (Changchun, China). Two plasmids, pAAV-opti CV777 S-flag and pAAV-opti-LNCT2 S-flag (Wang et al., 2016), were used as templates, respectively. Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) was used to amplify the genes according to the manufacturer's instructions. PCR products and pcDNA3.1(-) vectors were ligated by T4 DNA ligase (Toyobo, Osaka, Japan) after digestion by *EcoR* I and *BamH* I. Then, the ligation mixtures were transformed into *Escherichia coli* DH5α cells according to the manufacturer's instructions. The plasmids were verified by sequence analyses (Comate Bioscience Co., Ltd., Jilin, China). The verified plasmids containing the S, S1, and S2 genes were transfected into human embryonic kidney (HEK) 293T cells (80–90% confluency) in a 48-well plate, using the x-tremeGENE HP DNA Transfection Reagent (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. pcDNA3.1(-) vectors were also transfected into HEK293T cells as a negative control. After 24 h, the cells were fixed with 4% paraformaldehyde, and the reaction with mAb 1B9 was detected by an IFA. A PEDV-positive polyclonal antibody (pAb) (mouse) and negative serum of mice were used as controls.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2017.04.026.

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