SPIKE PROTEIN REGION (aa 636–789) OF PORCINE EPIDEMIC DIARRHEA VIRUS IS ESSENTIAL FOR INDUCTION OF NEUTRALIZING ANTIBODIES

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Summary. – To analyze antigenic structure of the spike (S) protein of Porcine epidemic diarrhea virus (PEDV), the gene encoding its major immunodominant region S1 was amplified by PCR. We prepared four truncated S1 proteins spanning the entire S1 domain fused to GST protein. To identify the most important antigenic region of S1, the truncated S1-GST fusion proteins were examined for their ability to react with immune serum against PEDV and to elicit the formation of neutralization antibodies in immunized animals. We found that the region of S1 signed as S1D (aa 636–789) was able to react with PEDV antiserum and to elicit formation of neutralization antibodies in mice. Moreover, the immune serum against S1D showed the binding ability to the native S protein of PEDV.

Key words: epitope; Porcine epidemic diarrhea virus; spike protein; virus neutralization

Introduction

Porcine epidemic diarrhea (PED) is a highly contagious enteric disease of swine caused by Porcine epidemic diarrhea virus (PEDV), a member of the genus *Coronavirus,* the family *Coronaviridae*, the order *Nidovirales*. The disease is characterized by the severe diarrhea, vomiting, and dehydration leading to death with a mortality rate of up to 90% (Pensaert *et al*., 1978). The PED was reported in Belgium and the UK in 1978 (Pensaert *et al*., 1978; Chasey *et al*., 1978). Since then, the disease has frequently broken out in many European countries as well as China, Japan and Korea and led to a severe economic loss (Debouck *et al*., 1982; Jimenez *et al*., 1986; Chae *et al*., 2000).

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Abbreviations: GST = glutathione S-transferase; IFA = immunofluorescence assay; PED = porcine epidemic diarrhea; PEDV = Porcine epidemic diarrhea virus; S = spike glycoprotein; TGEV = Transmissible gastroenteritis virus; VN = virus neutralization

The genome of PEDV is a positive single-stranded RNA approximately 28 kb in length. Subgenomic mRNAs are transcribed from the genome and are translated to viral proteins such as the spike protein S of 180–220 K, membrane protein M 27–32 K, and nucleocapsid protein N, 55–58 K (Egberink *et al*., 1988). The S protein is a glycoprotein localized on the virion surface that plays a key role in the induction of the neutralizing antibodies against PEDV (Duarte *et al*., 1994). Thus, S protein is essential for the development of an effective vaccine against PEDV. The S protein is not cleaved into the S1 and S2 subunits during infection, but it can be divided into the S1 domain (aa 1–789) and S2 domain (aa 790–1383). This division is predicted according to the presence of the conserved nonamer and GxCx motifs at the proteolytic cleavage site of S protein in other members of coronavirus, group II (Follis *et al*., 2006). The S1 domain contains major neutralization epitopes of S protein (Posthumus *et al*., 1990; Kida *et al*., 1999; Yoo *et al*., 2001; He *et al*., 2004). Chang *et al*. (2002) reported the neutralization epitope region COE (aa 499–638) on the S1 domain of S protein according to the nucleotide sequence for the neutralization epitope of the Transmissible gastroenteritis virus (TGEV).

In this study, we identified a new epitope region signed S1D (aa 636–789) on the S1 domain of PEDV S protein that was highly conserved across PEDV isolates. This region had the capacity to induce a production of virus neutralization (VN) antibodies. This characteristic was very important especially in the development of diagnostic reagents and effective vaccine against PEDV.

Materials and Methods

Cloning of PEDV S1 gene. PEDV strain CV777, a gift from Dr. Pensaert, Ghent University, was propagated according to Hofmann *et al*. (1988). The viral RNA was extracted according to Yeo *et al*. (2002). The primers specific for S1 gene: PS1 (5'-ATTT GTGGCTTTTCTAATCA-3') and PS2 (5'-TACTCATACTAA AGTTGGTGGG-3') were based on the nucleotide sequence of the S gene of PEDV strain CV777 (GenBank Acc. No. AF353511). The first strand of cDNA of S1 gene was prepared by the M-MLV reverse transcriptase reagent kit (Promega). Then, the S1 sequence was amplified by PCR using the cDNA as a template. The PCR products were cloned into the vector pMD-18T (TaKaRa). The recombinant plasmid of the S1 gene was designated as pMD-18TS1.

Cloning of the truncated forms of S1 gene fused to GST gene, their expression, and purification. Four pairs of primers containing *Bam*HI and *Sal*I sites at 5' and 3' termini were designed for amplification of four truncated S1 genes with signal peptides removed (Table 1). Nucleotides of the four truncated S1 genes were prepared from the plasmid pMD-18TS1 by PCR. The PCR products were digested with *Bam*HI and *Sal*I restriction enzymes (Fermentas) and cloned into the vector pGEX-6p-1 with GST tag (Invitrogen). The recombinant plasmids were cloned into *Escherichia coli* Rosetta™ (DE3) plysS cells (Novagen). The truncated S1-GST fusion proteins expressed in *E. coli* were purified by B-PER® GST Spin Purification Kit (Pierce). Additionally, GST protein alone as a control was prepared and purified by the same procedure.

Preparation of antisera against PEDV, truncated PEDV S1-GST proteins and GST. PEDV was purified by sucrose density gradient centrifugation (Miyamoto *et al*., 1992). The rabbits were immunized with 200 μg of purified PEDV emulsified in complete Freund's adjuvant (Sigma). Two boosters of 200 μg of purified PEDV emulsified in incomplete Freund's adjuvant (Sigma) were administered in 2-week intervals. The animals were bled one week after the last booster. The immunoglobulin fraction was prepared from immune sera by ammonium persulfate precipitation.

Similarly, five groups of 6-week-old BALB/c mice were immunized with 50 μg of purified fusion proteins or GST protein emulsified in complete Freund's adjuvant. At 2-week intervals two boosters of 50 μg of purified fusion proteins or GST protein emulsified in incomplete Freund's adjuvant were administered. After each inoculation, blood samples were collected from the immunized animals and tested for the presence of specific antibodies by ELISA. Before immunization, the pre-immunization rabbit or mouse sera were collected and used as a negative control.

ELISA. The ELISA plate was coated with 1 μg/ml of purified fusion proteins in 50 mmol/l carbonate buffer (pH 9.6) at 4°C for 12 hrs and blocked with 5% skimmed milk in PBS with 0.05% Tween-20 (PBST, pH 7.4) for 1 hr at 37°C. 100 μl of PEDV antisera diluted 1:100 in PBST was added to the wells and incubated at 37°C for 1 hr. 100 μl of HRP-conjugated goat anti-rabbit IgG diluted 1:8,000 in PBST was added and incubated at 37°C for 45 mins. After addition of 100 μl of 200 μg/ml 3,3'-5,5'-tetramethyl benzidine solution and incubation at room temperature for 10 mins, the reaction was stopped, and A_{450} was taken with a microplate reader (Model 550, BioRad).

Western blot analysis of truncated PEDV S1-GST fusion proteins and native S protein. The truncated PEDV S1-GST fusion proteins, native S protein, and GST protein were analyzed on the SDS-PAGE using the 12% gel. The separated proteins were transferred to the nitrocellulose membrane (Amersham, Pharmacia). After blocking, the blots were incubated with PEDV or PEDV S1-GST antisera diluted 1:100 in PBST. HRP-conjugated goat anti-rabbit or anti-mouse IgG diluted 1:2,000 in PBST was used as secondary antibody and the staining was carried out with 3,3'-diaminobenzidine solution.

Immunofluorescence assay (IFA). Vero cells cultivated in 96-well plates were infected with PEDV strain CV777 at MOI 1 PFU/cell. As soon as the 70–80% CPE was observed, the plates were fixed with alcohol at 4°C for 10 mins. Pre-immune and immune sera against fusion proteins, PEDV, and GST were diluted to 1:100 in PBS. 100 μl of each diluted serum was added to 4 wells containing infected or non-infected cells. The plates were incubated at

Table 1. Primers for amplification of truncated forms of PEDV S1 gene by PCR

Fragment	Primer sequence	Position of the amplified fragments*
S1A	F: 5'-CTAGGATCCGATGTCACTAGGTGCCAGT-3'	
	R: 5'-CAAGTCGACTTATGTTCCTAAAGCAGTATGA-3'	nt 64–1020
S1B	F: 5'-ACTGGATCCATTCTTGCTGAAGGCTCA-3'	
	R: 5'-ATTGTCGACTTACAAAGTAACAAAAGAAAT-3'	nt 976–1515
S1C	F: 5'-GTTGGATCCGTTACTTTGCCATCATTCA-3'	
	R: 5'-AGTGTCGACTTAAACGTCTGTGATACCTTC-3'	nt 1495–1914
S1D	F: 5'-GGTGGATCCGACGTTTCTTTTATGACTCTG-3'	
	R: 5'-ATAGTCGACTTAAATACTCATACTAAAGTTG-3'	nt 1909–2367

*The position was calculated from the initial codon ATG of PEDV S gene (GenBank Acc. No. AF353511). Sequences "GGATCC" and "GTCGAC" represented *Bam*HI and *Sal*I restriction sites, respectively.

37°C for 1 hr and washed three times with PBS. 100 µl of FITC-conjugated goat anti-mouse IgG (Sigma) diluted 1:200 in PBS was added and incubated at 37°C for 1 hr. The plates were washed with PBS as described above, air dried for 10 mins and examined under a fluorescence microscope.

Virus neutralization (VN) test. Immune sera used for VN assay were filtered through 0.45 µm membrane filter (Acrodisk, Gelman) and inactivated at 56°C for 30 mins. The S1A, S1B, S1C and S1D antisera were diluted 8-, 16-, 32-, 64-, and 128-fold with MEM containing streptomycin (100 µg/ml), penicillin (100 U/ml) and trypsin (10 µg/ml). The diluted PEDV stocks (MOI = 0.1) were mixed with the serial 2-fold dilutions of serum samples in a sterile centrifuge tube and then incubated at 37°C for 1 hr. After incubation, 100 µl of the mixture was added to 4 wells of 96-well microtiter culture plate containing Vero cells. The 96-well culture plate was incubated at 37°C until the infected cells showed 70–80% CPE. VN titer of serum samples was calculated with Spearman-Karber method. The MOI from 0.1 to 0.0001 of PEDV for confirming viral infectivity and all serum samples for detecting their toxicity were included with each test run.

Results

Cloning of S1 gene

PCR products of 2.4 kb approx. were detected using the primers of PS1 and PS2 specific for the S1 gene of PEDV. The negative control e.g. H_2O as PCR template did not show any bands in the ethidium bromide-stained 0.8 % agarose gel (Fig. 1).

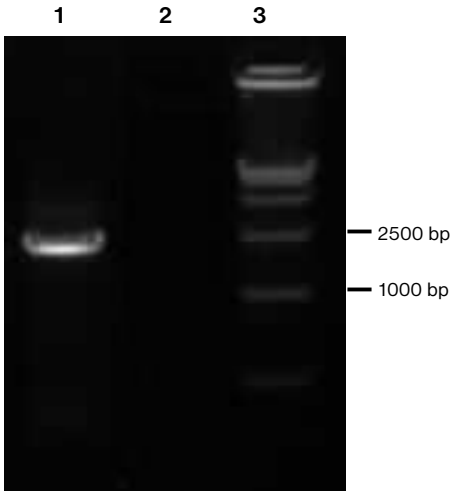

Fig. 1

Agarose gel electrophoresis of PCR product of PEDV S1 gene

PCR products of PEDV S1 gene (1), H_2O as negative control (2), DNA Mark DL 15000 (3).

Identification of the epitope region within S1 domain of S protein

To identify epitope regions within the S1 domain of S protein, four truncated forms of S1 gene covering entire S1 domain were expressed as GST fusion proteins in *E. coli* (Fig. 2).

The fusion proteins were purified by a B-PER® GST Spin purification kit. Western blotting and ELISA assays demonstrated that the S1D fusion protein showed a strong reaction with immune serum against PEDV. On the other hand, the S1A, S1B, and S1C fusion proteins showed only negative or weak reaction with PEDV antisera (Fig. 3 and 4). These results revealed that the S1D region contained the most important epitopes of S1 domain.

Immunogenicity of S1D region

The Western blot analysis with native S protein and IFA on PEDV infected Vero cells were carried out to find out whether antisera to fusion proteins were able to recognize the native S protein of PEDV. In Western blotting test S1D antisera formed a specific band at about 180 K (Fig. 5) that corresponded to the S protein of PEDV. However, S1A, S1B, S1C antisera, GST antiserum, and pre-immune mouse serum did not show any visible bands.

The intensive fluorescence signal was observed in the PEDV-infected cells treated with S1D antiserum in IFA (Fig. 6H). In contrast, only weak fluorescence signal was observed in the PEDV-infected cells using S1A, S1B and S1C antisera (Fig. 6E, F, G, respectively). As expected, the fluorescence signal was not observed in the non-infected cells stained with PEDV antiserum (Fig. 6B) and the PEDV-infected cells stained with pre-immune mouse serum and GST antiserum (Fig. 6C, and D, respectively). However, intensive fluorescence signal appeared in the PEDV-infected cells stained with PEDV antiserum used as positive control (Fig. 6A). These data indicated that the S1D fusion protein had a strong immunogenicity and was able to induce S1-specific antibodies with binding abilities to the native S protein of PEDV.

VN activity of the S1D antisera

In the VN assay, the S1D antisera diluted 1:210 could inhibit 50% CPE caused by the PEDV strain CV777 and its VN titer was equal to 210 and presented about 65% of the VN titer of PEDV antiserum that equaled to 380. However, the immune sera against S1A, S1B, S1C, GST and pre-immune mouse serum showed lower VN titers equaled to 75, 90, 45, 40 and 40, respectively (Fig. 7). Those results suggested that the S1D antisera could inhibit multiplication of PEDV in Vero cells. It is noteworthy that the GST

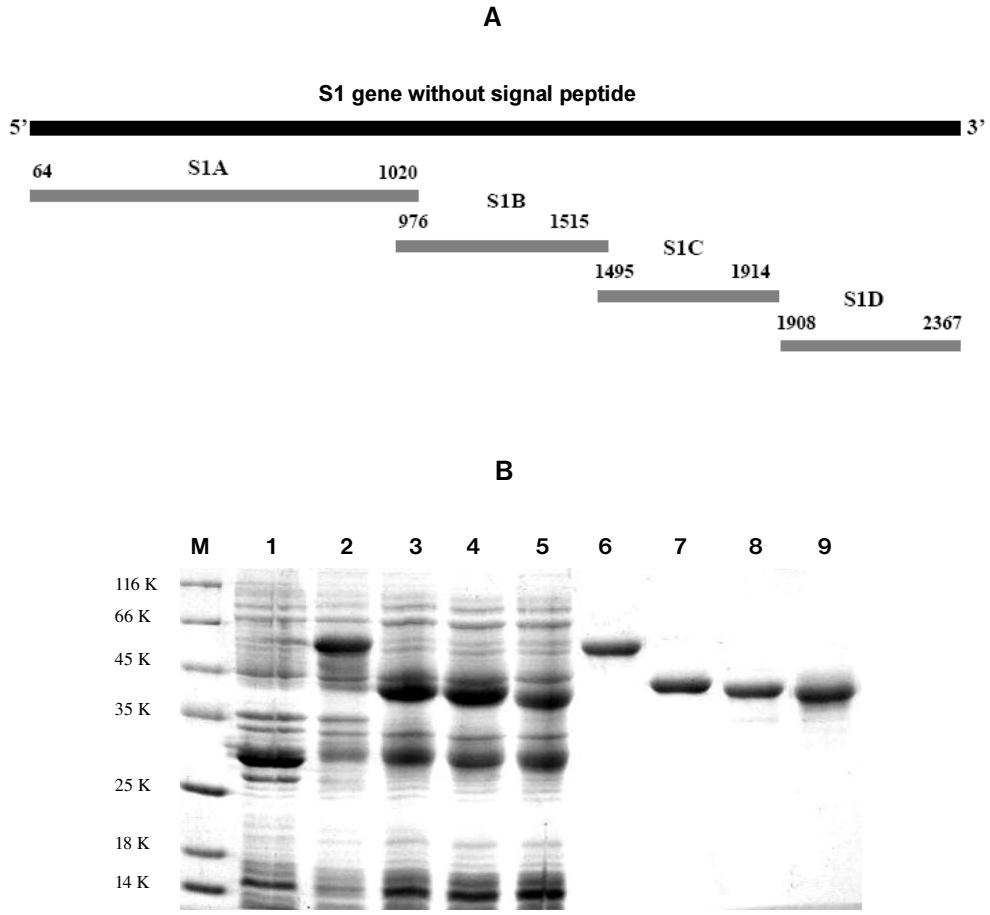

Fig. 2

The map of truncated forms of the S1 gene (A) and PAGE of S1 truncated forms as fusion proteins expressed in *E. coli* (B)

Cell lysate of pGEX-6p-1-transformed *E. coli* (1), cell lysates of S1A-GST, S1B-GST, S1C-GST and S1D-GST (2, 3, 4, and 5, respectively), purified fusion proteins S1A-GST, S1B-GST, S1C-GST and S1D-GST (6, 7, 8, and 9, respectively), molecular size markers (M).

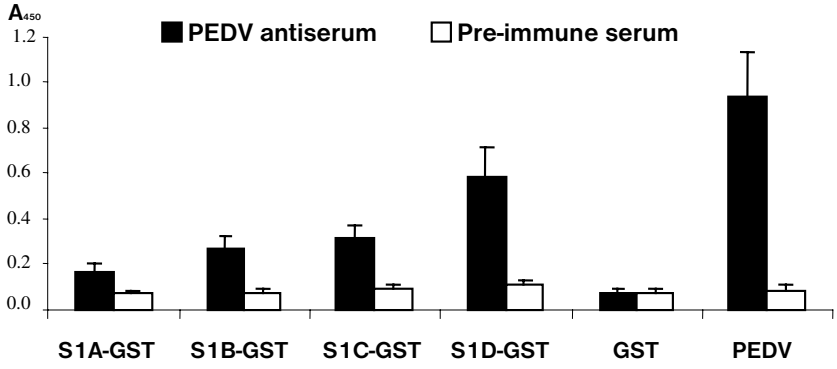

Fig. 3

Reactivity of purified S1A, S1B, S1C, and S1D fusion proteins to antibodies against PEDV in ELISA

Purified PEDV and GST antigen are included as positive, negative control, respectively.

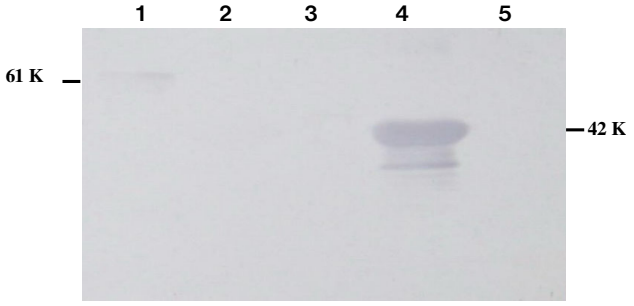

Fig. 4

Western blot analysis of purified S1A, S1B, S1C and S1D fusion proteins (1, 2, 3, and 4, respectively) with antibodies against PEDV

GST as negative control (5).

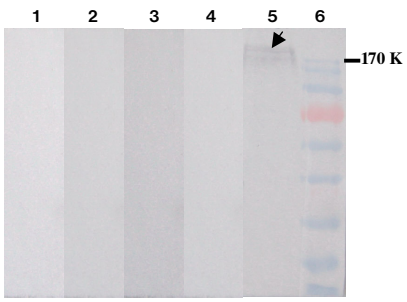

Fig. 5

Western blot analysis of native PEDV S protein with immune sera to S1A, S1B, S1C and S1D fusion proteins (2, 3, 4, and 5, respectively)

GST antiserum as negative control (1), molecular size markers (6).

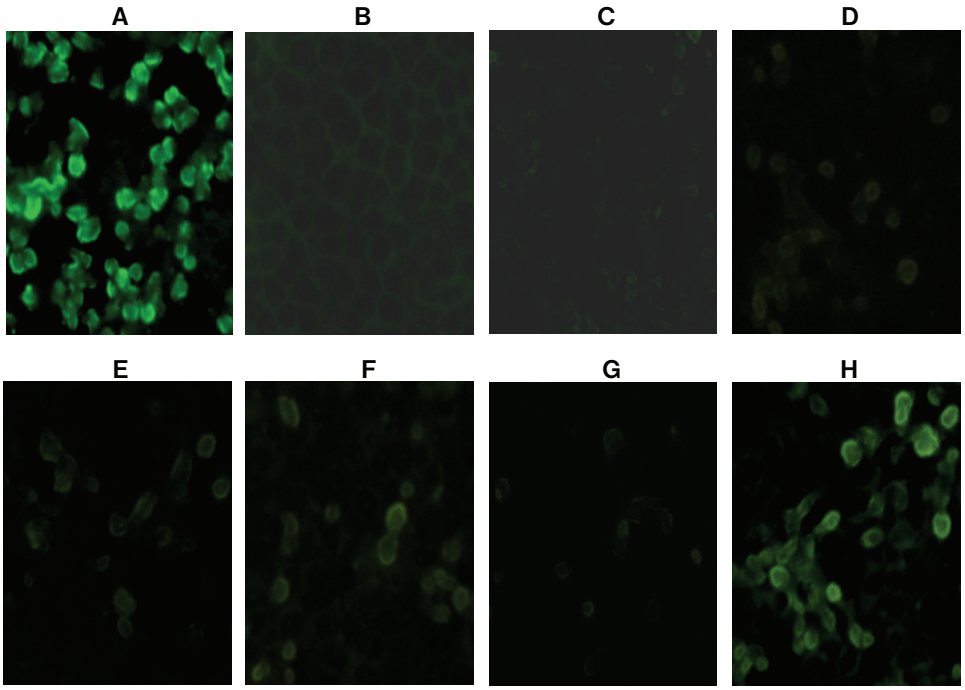

Fig. 6

Immunofluorescence assay of PEDV-infected Vero cells with immune sera against S1A, S1B, S1C and S1D fusion proteins (E, F, G, and H, respectively)

Immune serum against PEDV as positive control (A), non-infected Vero cells treated with immune serum against PEDV as negative control (B), pre-immune mouse serum (C), immune serum against GST (D).

antiserum and pre-immune mouse serum also showed VN activity to PEDV to a certain degree. We believed that this phenomenon was due to the low dilution of tested serum that was able to degrade trypsin present in the cultivation medium. The enzymatic activity of trypsin was required to activate receptor-ligand interaction between Vero cells and PEDV and the lowered activity of trypsin could delay the appearance of CPE in the Vero cells infected with PEDV.

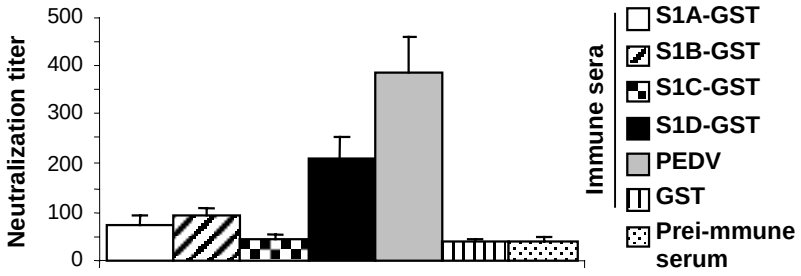

Fig. 7

Virus neutralization titers of immune sera to S1A, S1B, S1C and S1D fusion proteins

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S1D         TDVSFMTLDVCTKYTIYGFKGEGIITLTNSSILAGVYYTSDSGQLLAFKNVT  52
Brl/87      TDVSFMTLDVCTKYTIYGFKGEGIITLTNSSILAGVYYTSDSGQLLAFKNVT  52
JS          TDVSFMTLDVCTKYTIYGFKGEGIITLTNSSFLAGVYYTSDSGQLLALKNVT  52
KPEDV       TDVSFMTLDVCTKYTIYGFKGEGIITLTNSSFLAGVYYTSDSGQLLAFKNVT  52
Chinju99    LKGGFMTLDVCTKYTIYGFKGEGIITLTNSSILAGVYYTSDSGQLLAFKNVT  52

S1D         SGAVYSVTPCSFSEQAAYVNDDIVGVISSLSNSTFNNTRELPGFFYHSNDGS 104
Brl/87      SGAVYSVTPCSFSEQAAYVNDDIVGVISSLSNSTFNNTRELPGFFYHSNDGS 104
JS          SGAVYSVTPCSFSEQAAYVDDDIVGVISSLSSSTFNSTRELPGFFYHSNDGS 104
KPEDV       SGAVYSVTPCSFSEQAAYVDDDIVGGISSLSNSTFNNXRDFPGFFYHSNDGS 104
Chinju99    SGAVYSVTPCSFSEQAAYVNDDIVGVISSLSNSTFNNTRELPGFFYHSNDGS 104

S1D         NCTEPVLVYSNIGVCKSGSIGYVPSQYGQVKIAPTVTGNISIPTNFSMSI   154
Brl/87      NCTEPVLVYSNIGVCKSGSIGYVPSQYGQVKIAPTVTGNISIPTNFSMSI   154
JS          NCTEPVLVYSNIGVCKSGSIGYVPSQSGQVKIAPTVTGNISIPTNFSMSI   154
KPEDV       NCTEPVLVYSNIGVCKSGSIGYVPLQDGQVKIAPTVIGNISIPTNFSMSI   154
Chinju99    NCTEPVLVYSNIGVCKSGSIGYVPSQYGQVKIAPTVTGNISIPTNFSMSI   154
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Fig. 8

Multiple alignment of the S1D region amino acid sequences of various PEDV strains

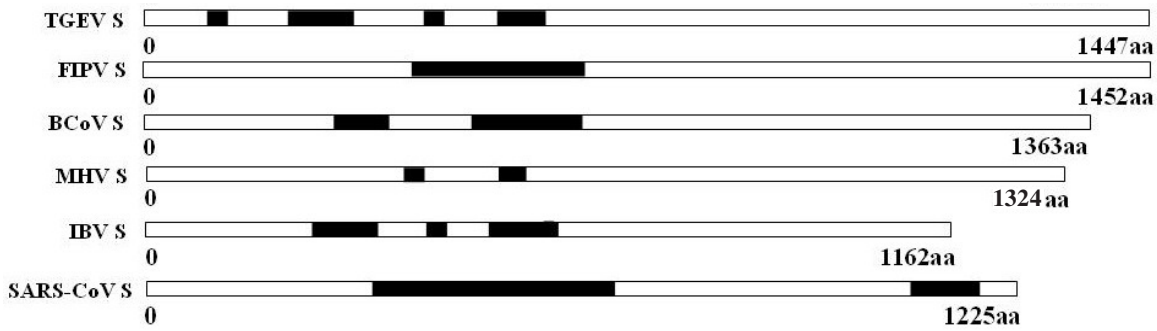

Fig. 9

S protein epitope regions of various coronaviruses

Aligning of S1D amino acid sequence among known PEDV strains

A multiple alignment of aa sequences of the S1D region and the S1 domain on known PEDV strains was carried out by DNAMAN software. The result indicated that in aa sequences of the S1 domain displayed 94.84% identity in strains CV777 (GenBank, Acc. No. AF353511), Brl/87 (Accession No. Z25483), JS (Acc. No. AY653204), Chinju99 (Acc. No. AY167585), and KPEDV (Acc. No. AF500215). The aa sequence of S1D region of S protein had 97.55% identity among examined strains as determined by the same method (Fig. 8). These data demonstrated the region S1D was highly conserved among PEDV strains.

Discussion

The S1 domain of S protein constitutes the globular ectodomain that is exposed on the surface of PEDV particles. When PEDV infects permissible animal, the host immune system is activated by the S1 domain and the differentiated B-lymphocytes produce specific antibodies against S protein. Within the genus *Coronavirus*, the epitopes of the S protein have been identified and mapped in TGEV, Bovine coronavirus (BCoV), Feline infectious peritonitis virus (FIPV), Infectious bronchitis virus (IBV), Murine hepatitis virus (MHV), and Severe acute respiratory syndrome coronavirus (SARS-CoV). Major epitopes of coronavirus members localize in S1 domain of the S protein and their characteristic and location are very similar (Fig. 9). For PEDV, Chang *et al*. (2002) reported the neutralization epitope region COE (aa 499–638) on the S protein according to the nucleotide sequence for the neutralization epitope of TGEV and the antisera to COE recombinant protein could reduce the plaque formation by 66.2%, when 1000-fold diluted virus stock was used to infect the host cells (Chang *et al*., 2002). Cruz *et al*. (2006) recently identified a mocking epitope (^{1368}GPRLQPY^{1374}) on the C-terminal endodomain of PEDV S protein by phage display. The result of the neutralization inhibition assays showed that the mocking epitope had antigenic similarities with PEDV neutralizing epitopes. The epitope region S1D reported in this study localizes in the different regions in comparison with the epitopes identified earlier and therefore this region is a novel B-cell antigenic epitope region of S protein. The S1D fusion protein could elicit S1-specific antibodies. S1D antisera could recognize the native S protein of PEDV and showed VN activity in vitro. By aligning the S protein sequences of five PEDV strains, which have been published in GenBank, we discovered that the S1 domain of S glycoprotein has 94.84% identity in aa sequence at these strains CV777, Brl/87, JS, Chinju99 and KPEDV of PEDV. Furthermore, the epitope region S1D among these strains has 97.55% identity in aa sequence. Thus, the identified epitope region, S1D is highly conserved among PEDV strains. Epitopes and the epitope regions of SARS S protein were analyzed and localized close to the N-teminus of the protein (He *et al*., 2004). The epitope region S1D of PEDV reported here is similar to that of SARS S protein. According to this study, better strategies of immune prevention should be devised for inducing neutralizing antibodies against PEDV. Further studies are needed to identify other conserved VN epitopes on the S protein and to use these data to develop effective vaccine against PEDV.

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