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Sequence analysis of the partial spike glycoprotein gene of porcine epidemic diarrhea viruses isolated in Korea

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Abstract Porcine epidemic diarrhea virus (PEDV) causes a devastating enteric disease with acute diarrhea, dehydration and significant mortality in swine, thereby incurring heavy economic losses in Korea. Spike (S) glycoprotein has been suggested as an important determinant for PEDV biological properties. In this study, the nucleotide and deduced amino acid sequences of the partial S glycoprotein genes of Korean PEDV isolates, including epitope region that is capable of inducing PEDV-neutralizing antibodies, were determined. The partial S glycoprotein genes were amplified by RT-PCR, cloned, sequenced, and compared with each other as well as with reference PEDV strains. By phylogenetic analysis, the Korean PEDV isolates were divided into three groups (G1, G2, G3), which had three subgroups (G1-1, G1-2, G1-3). Group1 (G1) Korean PEDV isolates were highly homologous to CV777, Br1/87, JS-2004-2, KPED-9, P-5V, SM98-1, parent DR13, and attenuated DR13, group2 (G2) Korean PEDV isolates were highly homologous to Spk1, and group3 (G3) was Chinju99 at the nucleotide and deduced amino acid sequence levels. In addition, the G1 Korean PEDV isolates didn't had several specific nucleotides and amino acids which were found in the G2 and G3 Korean PEDV isolates, and especially the G1-1 Korean PEDV isolates had specific nucleotides and amino acids which were not found in the G1-2, G1-3, G2, and G3 Korean PEDV isolates. It was suggested that many Korean PEDV isolates are closely related to the G1 including CV777, Br1/87, JS-2004-2, KPED-9, P-5 V, SM98-1, parent DR13, and attenuated DR13 rather than to the G2 and G3 including Spk1 and Chinju99, and notably more prevalent PEDVs isolated in Korea are especially close to the Chinese PEDV strain JS-2004-2 rather than Korean PEDV strains Spk1, Chinju99, KPED-9, SM98-1, parent DR13, and attenuated DR13.

Keywords Porcine epidemic diarrhea virus · Spike glycoprotein gene · Epitope region · Cloning · Phylogenetic analysis

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Introduction

Porcine epidemic diarrhea virus (PEDV), a member of the family Coronaviridae, is an enveloped, single-stranded RNA virus. PEDV was first reported in Belgium and the United Kingdom in 1978 [1]. Since then, outbreaks of the disease have been reported in many swine-raising countries, notably in Europe and Asia, including Japan, China and Korea [2]. PEDV causes a devastating enteric disease with acute diarrhea, dehydration and significant mortality in swine, thereby incurring heavy economic losses in Europe and Asia [3].

Reading in the 5′ to 3′ direction, the PEDV genome contains genes for pol1 (P1) protein, spike (S) protein, an open reading frame (ORF3), envelope (E) protein,

membrane (M) protein, and nucleocapsid (N) protein [4–6]. Among the proteins encoded by these genes, S protein, a glycoprotein peplomer (surface antigen) on the viral surface, plays an important role in binding to specific host cell receptor glycoproteins with subsequent penetration into the cells occurring via membrane fusion. The S protein also stimulates induction of neutralizing antibodies in the host [4].

Although serologically unrelated, PEDV and transmissible gastroenteritis virus (TGEV), cause digestive tract infections which are extremely difficult to differentiate clinically [7, 8]. Both viruses belong to the family Coronaviridae. The S gene of TGEV is an important site of virus neutralization. In addition, the S glycoprotein forms the peplomers on the virion envelope and contains receptor binding regions and four major antigenic sites [9–11]. The sites A and D are known to be regions inducing major neutralizing antibodies [9, 11]. The sequence variation in the S glycoprotein gene and antigenic diversity of TGEV isolates has been reported [12–15]. Based on partial sequence analysis of S glycoprotein genes, TGEV strains isolated in Korea are different from foreign TGEV isolates [16]. In addition, diversity among the TGEV isolated in Korea has been reported [17].

PEDV occurs frequently in Korea since the virus was first isolated [2]. In spite of using the vaccine strategy at present, damage caused by PEDV infection is continuous and serious in Korea. Accomplished an accurate analysis on genome of prevalent PEDVs in Korea, we prevent outbreaks of the PEDV-induced diarrhea more effectively, according to choosing the correct PEDV vaccine strain.

Therefore, the purpose of the present study was to investigate the diversity among the Korean PEDV isolates, according to sequence analysis of the S glycoprotein genes including epitope region with Korean and non-Korean reference PEDV strains and was to find out more prevalent PEDVs in Korea through sequence and phylogenetic analyses. Present study focused on especially epitope region of S glycoprotein because S glycoprotein is considered a primary target antigen for developing an effective vaccine against PEDV, since it has been suggested as an important determinant for PEDV biological properties [18], and moreover epitope region is capable of inducing PEDV-neutralizing antibodies [19, 20].

Materials and methods

Cells

The continuous Vero cell line (ATCC, CCL-81) was regularly maintained in α-minimum essential medium (α-MEM) supplemented with 5% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml).

Source of specimens and PEDV isolation

A total 737 porcine samples (from 472 farms) consisting of feces or intestinal contents, which had been taken from young piglets showing watery diarrhea, dehydration and high mortality, were submitted to the Department of Veterinary Medicine Virology Laboratory, College of Veterinary Medicine, Seoul National University from 8 provinces in Korea between January 2002 and August 2005. The farms each had 300 or more sows. One to 20 fecal samples or intestinal contents were obtained from each outbreak of diarrhea. A total 319 cases of all samples (feces and intestinal contents) had been confirmed positive for PEDV by reverse transcription polymerase chain reaction (RT-PCR) method [21] and especially PEDV positive intestinal samples once more had been confirmed positive by histopathological assessment.

PEDV positive fecal samples were diluted with phosphate-buffered saline (PBS; 0.1 M, pH 7.2) to be 10% (v/v) suspensions and PEDV positive intestinal contents were made into 10% suspensions through homogenization with PBS. The suspensions were vortexed and clarified by centrifugation for 10 min at 4800*g*. Supernatants passed through a 0.2 μm syringe filter (Acrodisk, Gelman) were used for virus isolation in Vero cells. Prior to inoculation, the growth media of confluent cells grown in 25 cm^2 flasks (Falcon, USA) was removed, and the cells were washed three times with PBS (pH 7.4). One milliliter of the supernatant per flask was then inoculated into the cells. After adsorption at 37°C for 1 h, the cells were washed again and incubated in α-MEM supplemented with 0.02% yeast extract, 0.3% tryptose phosphate broth, and 2 μg of trypsin as described previously [21–23]. Serial passages of the PEDVs were continued in 25 cm^2 flasks by level 7 according to the method described above. Forty-five PEDVs were isolated from fecal samples or intestinal contents taken from 213 each farm and were identified through RT-PCR [21]. The isolates, used for nucleotide sequence analysis, amino acid sequence analysis and phylogenetic analysis in this study, were described in Table 1.

Viral RNA extraction

Viral RNA was extracted from infected cells using TRIzol LS (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. For PEDV-infected cells, 250 μl suspensions were lysed directly in 1.7 ml microtubes by adding 750 μl TRIzol LS reagent. Then 200 μl of chloroform was added to the mixture, and the suspensions

Table 1 45 Korean PEDV isolates taken from feces or intestinal contents

Name of Isolates	Sample origin	Date of isolation	Geographic origin	Status of PED vaccine
V803	Intestine	May 2002	Chungnam	Use (Injectable vaccine)
DBI825	Intestine	August 2002	Gyeonggi	Use (Injectable vaccine)
DBI865	Intestine	October 2002	Gyeonggi	Use (Injectable vaccine)
e942	Intestine	January 2003	Gyeonggi	Use (Injectable vaccine)
BI960	Feces	January 2003	Gyeonggi	Use (Injectable vaccine)
BI961	Feces	January 2003	Gyeonggi	Use (Injectable vaccine)
BI976	Intestine	February 2003	Gyeonggi	Use (Injectable vaccine)
BI981	Intestine	February 2003	Chungnam	Use (Injectable vaccine)
BI1108	Intestine	March 2003	Gyeonggi	Use (Injectable vaccine)
BI1166	Intestine	March 2003	Gyeonggi	Use (Injectable vaccine)
BI1401	Intestine	July 2003	Chungnam	Use (Injectable vaccine)
BI1482	Intestine	September 2003	Gyeonggi	Use (Injectable vaccine)
M1595	Intestine	November 2003	Gyeonggi	Use (Injectable vaccine)
e1642	Intestine	November 2003	Chungnam	Use (Oral vaccine)
e1693	Intestine	December 2003	Chungnam	Use (Oral vaccine)
e1695	Intestine	December 2003	Chungnam	Use (Oral vaccine)
e1696	Intestine	December 2003	Chungnam	Use (Oral vaccine)
e1697	Intestine	December 2003	Chungnam	Use (Oral vaccine)
M1763	Intestine	December 2003	Chungnam	Use (Injectable vaccine)
M1764	Intestine	December 2003	Chungnam	Use (Injectable vaccine)
DBI1784	Intestine	January 2004	Gyeongbuk	Use (Oral vaccine)
e1833	Feces	February 2004	Chungnam	Use (Oral vaccine)
e1834	Intestine	February 2004	Gyeongbuk	Use (Oral vaccine)
DBI2169	Intestine	July 2004	Gyeongbuk	Use (Oral vaccine)
M2227	Intestine	August 2004	Gyeonggi	Use (Injectable vaccine)
BI2357	Intestine	November 2004	Gyeonggi	Not use
M2366	Intestine	November 2004	Gyeonggi	Use (Injectable vaccine)
BI2439	Intestine	December 2004	Jeonbuk	Use (Injectable vaccine)
M2466	Intestine	January 2005	Chungbuk	Use (Injectable vaccine)
V2501	Intestine	January 2005	Gyeonggi	Not known
M2503	Intestine	January 2005	Gyeonggi	Use (Oral vaccine)
M2537	Intestine	February 2005	Gyeonggi	Use (Oral vaccine)
e2539	Intestine	February 2005	Gyeonggi	Use (Oral vaccine)
e2540	Intestine	February 2005	Chungnam	Use (Oral vaccine)
BI2804	Feces	March 2005	Gyeongbuk	Use (Injectable vaccine)
BI2944	Intestine	March 2005	Chungnam	Use (Injectable vaccine)
e3975	Intestine	May 2005	Gyeonbuk	Use (Oral vaccine)
e3981	Intestine	May 2005	Gyeonbuk	Use (Oral vaccine)
e3984	Intestine	May 2005	Gyeonbuk	Use (Oral vaccine)
e3988	Intestine	May 2005	Chungnam	Use (Oral vaccine)
e3991	Intestine	June 2005	Chungnam	Use (Oral vaccine)
e3997	Intestine	June 2005	Chungnam	Use (Oral vaccine)
PF4275	Intestine	July 2005	Chungnam	Use (Oral vaccine)
M4758	Feces	August 2005	Chungnam	Use (Oral vaccine)
M4759	Feces	August 2005	Chungnam	Use (Oral vaccine)

were centrifuged for 10 min at 12,000*g*. The RNA-containing aqueous phase was precipitated with an isopropanol of the same volume, maintained at –70°C for 2 h, and centrifuged for 10 min at 12,000*g*. The RNA pellet was washed with 1 ml of 75% ethanol, centrifuged for 10 min at 12,000*g*, and dried, following which it was resuspended

in 30 μl of diethyl-pyrocarbonate (DEPC)-treated deionized water.

Primers used for RT-PCR

Published primers [24] were used for generating partial S glycoprotein genes including the epitope region of PEDV (nucleotides 1495–1914 of the coding sequence of the S glycoprotein gene of PEDV strain Br1/87; EMBL accession No. Z25483) is an important site of virus neutralization [20]. Briefly, forward primer, 5′-TTCTGAGTCACG AACAGCCA-3′, and reverse primer, 5′-CATATGCAGCC-TGCTCTGAA-3′, were used for the amplification of PEDV S glycoprotein gene. The size of amplified product was 651 bp.

RT-PCR

For reverse transcription, 10 μl of extracted RNA and 1 μl of reverse primer were mixed. And the mixture was denaturated by heating 95°C and was immediately placed on ice. The remaining reagents, which were 10 μl of 5X first strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, 0.3 mM each of dNTP, and 100 units of M-MLV reverse transcriptase in a final volume of 50 μl, were added. The mixture was incubated at 37°C for 60 min and the reaction was stopped by heating to 95°C for 2–3 min. The cDNA was either stored at –20°C or amplified immediately.

In PCR, a pair of specific primers was used to amplify the partial S glycoprotein genes of PEDV. Exactly, 2 μl of cDNA was mixed with a reaction mixture containing 2.5 μl of 10X Taq DNA polymerase buffer (Promega, Madison, WI), 3 mM of $MgCl_2$, 2.0 μl of dNTPs (2.5 mM/μl), 0.5 μl of each specific primer (10 pmol), 1 μl of Taq DNA polymerase (Promega, Madison, WI) and brought to 25 μl with autoclaved, filtered (0.2 μm) distilled water. The amplification was carried out with a commercial amplification system (Perkin-Elmer, Applied Biosystems, Foster City, Calif). The RT-PCR was performed at 94°C for 5 min, followed by 30 cycles of 94°C 30 s, 53°C 30 s, 72°C 30 s, and a final extension at 72°C for 7 min, and then held at 4°C. The RT-PCR products were visualized by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Bands of the correct size were excised and purified using a QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions.

Cloning of cDNA

Purified RT-PCR products corresponding to the partial S glycoprotein gene were cloned using a QIAGEN PCR Cloning^{plus} Kit (QIAGEN) according to the manufacturer's instructions with simple modifications.

For cloning of cDNA, 4 μl of purified RT-PCR product, 1 μl of pDrive Cloning Vector (50 ng/μl), and 5 μl of 2X ligation Master Mix were mixed gently and incubated for 4 h at 16°C. The ligation-reaction mixture was then subjected to the transformation protocol, which renders cells competent through heat-shock. For transformation, a number of tubes of QIAGEN EZ Competent Cells were thawed on ice and SOC medium was warmed to room temperature following which 5 μl of ligation-reaction mixture was added per tube of cells, mixed gently for 3 s and incubated on ice for 30 min. The tubes were heated in a 42°C water bath for 90 s and incubated on ice immediately. Room temperature SOC medium (250 μl) was added to each tube and 100 μl of each transformation mixture was immediately plated onto LB agar plates containing ampicillin. The plates were incubated at room temperature until the transformation mixture had absorbed into the agar, following which they were inverted and then incubated at 37°C overnight. Colonies grown in LB agar plates were cultured in LB broth with shaking at 37°C overnight, and DNA was extracted using the Wizard® Plus Minipreps DNA Purification System (Promega). Restriction enzyme digestion, with enzymes such as EcoRI, followed by electrophoresis through a 1.5% agarose gels was employed for identification of recombinant DNA clones.

Sequencing

All partial S glycoprotein gene recombinant DNA clones were sequenced by Genotech Co. Ltd (Korea). All sequencing reactions were performed in duplicate and all sequences were confirmed by sequencing both strands.

Sequence analysis

Nucleotide and deduced amino acid sequences were aligned, edited, and analyzed with the CLUSTALX v1.83 program, Bioedit v7.0.5.2 program, and MegAlign software (DNAStar Inc., Madison, WI, USA). A phylogenetic tree was then generated using an alignment of partial S glycoprotein gene nucleotide and deduced amino acid sequences with reference PEDV strains by applying the neighbor-joining method in the MEGA 3.1 program. To assess the relative support for each clade, bootstrap values were calculated from 1000 replicate analyses. The reference strains used for sequence alignment, sequence analysis, and phylogenetic analysis with Korean PEDV isolates were described in Table 2.

Results

Nucleotide and deduced amino acid sequence analyses

Nucleotide and deduced amino acid sequences of the partial S glycoprotein genes including the epitope region of 45 PEDVs isolated in Korea were determined and compared with the sequences of reference PEDV strains (Fig. 1).

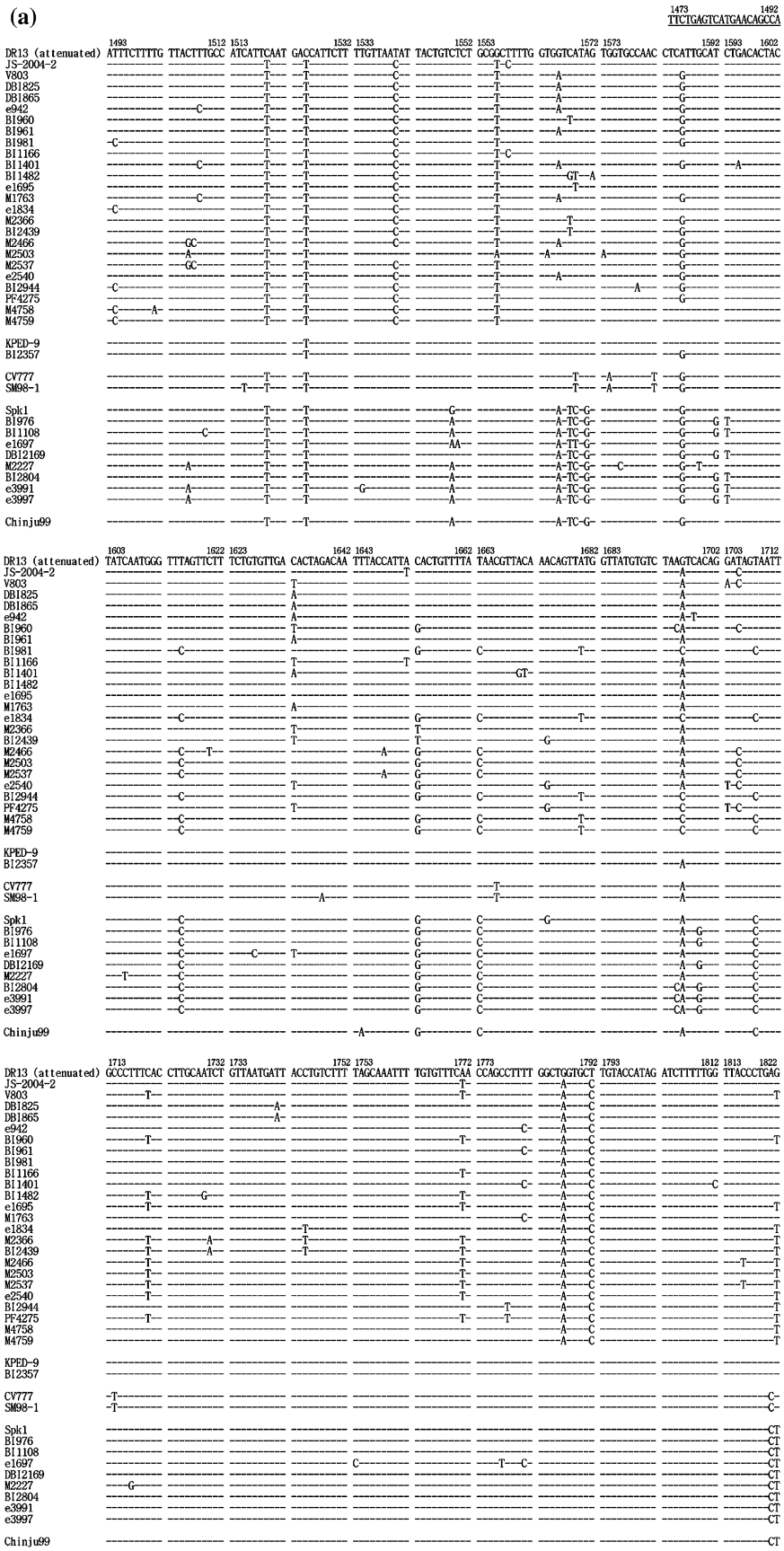
Group1 (G1), group2 (G2), and group3 (G3) had significant differences in their nucleotide and deduced amino acid sequences. The G1 Korean PEDV isolates including CV777, Br1/87, JS-2004-2, KPED-9, P-5 V, SM98-1, parent DR13, and attenuated DR13 strains didn't have 2 specific deduced amino acid sequence changes (from H to S or F at 520, from S to G at 521), which were produced by 3 nucleotide sequence changes (from CA to TC or TT at 1568, from A to G at 1571) and were found in other Korean PEDV isolates (G2, G3) including Spk1 and Chinju99 strains. The G3, Korean PEDV Chinju99 strain, had 13 specific deduced amino acid sequence changes (from F to Y at 545, from K to N at 610, from F to Y at 611, from T to R at 612, from S to W at 623, from G to W at 626, from T to A at 627, from Q to N at 632, from V to L at 634, from T to L at 635, from D to K at 636, from V to G at 637, from S to G at 638), which were produced by 18 nucleotide sequence changes (from T to A at 1644, from G to T at 1840, from T to A at 1842, from C to G at 1845, from C to G at 1878, from G to T at 1886, from C to G at 1888, from A to G at 1889, from C to A at 1904, from A to C at 1906, from G to C at 1910, from AC to TT at 1913, from G to A at 1916, from C to G at 1918, from T to G at 1920, from TC to GG at 1922) and were not found in other Korean PEDV isolates (G1, G2). Especially, the G1-1 Korean PEDV isolates including JS-2004-2 strain only had 2 specific deduced amino acid sequence differences (from A to S or T at 516, from G to S at 593) compared to other Korean PEDV isolates (G1-2, G1-3, G2, G3) including CV777, Br1/87, Spk1, Chinju99, KPED-9, P-5 V, SM98-1, parent DR13 and attenuated DR13 strains because 2 nucleotide sequence changes (from G to T or A at 1556, from G to A at 1787) led to changes in the deduced amino acid sequences of the G1-1.

Fig. 1 Comparison of the (**a**) nucleotide and (**b**) deduced amino acid sequences of the partial S glycoprotein genes including epitope region of Korean PEDV isolates and following reference PEDV strains: CV777 (GenBank accession No. AF353511), Br1/87 (EMBL accession No. Z25483), JS-2004-2 (GenBank accession No. AY653204), Spk1 (GenBank accession No. AF500215), Chinju99 (GenBank accession No. AY167585), KPED-9 of the Korean PED live virus vaccine strain (Green Cross Veterinary Product Co., Ltd., Yong-In, Korea), P-5 V of the Japanese PED live virus vaccine strain (Nisseiken Co. Ltd., Tokyo, Japan), SM98-1 of the inactivated vaccine strain (Green Cross Veterinary Product Co., Ltd., Yong-In, Korea), parent DR13 (DQ862099) and attenuated DR13 of the Korean PED oral vaccine strain (Green Cross Veterinary Product Co., Ltd., Yong-In, Korea) (GenBank accession No. DQ462404). The numbering of the alignment of the (**a**) nucleotide and (**b**) deduced amino acid sequences start at position 1,493 nt and 495 aa, as compared with those of attenuated DR13. Dashes represent nucleotides and amino acids that are identical to those in attenuated DR13. Regions corresponding to the primers used for cloning are underlined. We changed the sentences. Korean PEDV isolates in parentheses below had identical nucleotide and amino acid sequences with that in front of parentheses in the partial S glycoprotein genes and especially Korean PEDV isolates DBI865, M1763, M4759, and DBI2169 underlined below had different nucleotide but identical amino acid sequences with isolates DBI825, BI961, e1834, and BI976, respectively, G1: G1-1: JS-2004-2, V803, DBI825, DBI865, e942, BI960 (M1595, e1642, e1693, DBI1784), BI961, BI981, BI1166, BI1401, BI1482, e1695 (e1696), M1763 (M1764, e1833), e1834 (e2539), M2366, BI2439, M2466 (V2501), M2503, M2537, e2540 (e3975, e3981, e3984, e3988), BI2944, PF4275, M4758, M4759, G1-2: KPED-9 (P-5 V), attenuated DR13, BI2357, G1-3: CV777 (Br1/87, parent DR13), SM98-1, G2: Spk1, BI976, BI1108, e1697, DBI2169, M2227, BI2804, e3991, e3997. G3: Chinju99

Table 2 The reference strains used for sequence alignment, sequence analysis, and phylogenetic analysis with Korean PEDV isolates

Viruses	Strains	Countries	Database Accession Numbers	References
PEDV	CV777	Belgium	AF353511	29
	Br1/87	Britain	Z25483	4
	Js-2004-2	China	AY653204	Unpublished
	Spk1	South Korea	AF500215	20
	Chinju99	South Korea	AY167585	30
	KPED-9 (The Korean PED live virus vaccine strain)	South Korea	In this study	Unpublished
	P-5V (The Japanese PED live virus vaccine strain)	Japan	In this study	Unpublished
	SM98-1 (The inactivated vaccine strain)	South Korea	In this study	Unpublished
	Parent DR13	South Korea	DQ862099	34
	Attenuated DR13 (The Korean PED oral vaccine strain)	South Korea	DQ462404	34
TGEV	PUR46-MAD	USA	M94101	14
	TS	China	AY335548	Unpublished

(a)


1473 1492
TTCTGAGTCATGAACAGCCA
1493 1512 1513 1532 1533 1552 1553 1572 1573 1592 1593 1602
DR13 (attenuated) ATTTCTTTTG TTACTTTGCC ATCATTCAAT GACCATTCTT TTGTTAATAT TACTGTCTCT GCGGCTTTTG GTGGTCATAG TGGTGCCAAC CTCATTGCAT CTGACACTAC
JS-2004-2
V803
DB1825
DB1865
e942
BI960
BI961
BI981
BI1166
BI1401
BI1482
e1695
M1763
e1834
M2366
BI2439
M2466
M2503
M2537
e2540
BI2944
PF4275
M4758
M4759
KPED-9
BI2357
CV777
SM98-1
Spk1
BI976
BI1108
e1697
DB12169
M2227
BI2804
e3991
e3997
Chinju99
1603 1622 1623 1642 1643 1662 1663 1682 1683 1702 1703 1712
DR13 (attenuated) TATCAATGGG TTTAGTTCTT TCTGTGTTGA CACTAGACAA TTTACCATTA CACTGTTTTA TAACGTTACA AACAGTTATG GTTATGTGTC TAAGTCACAG GATAGTAATT
JS-2004-2
V803
DB1825
DB1865
e942
BI960
BI961
BI981
BI1166
BI1401
BI1482
e1695
M1763
e1834
M2366
BI2439
M2466
M2503
M2537
e2540
BI2944
PF4275
M4758
M4759
KPED-9
BI2357
CV777
SM98-1
Spk1
BI976
BI1108
e1697
DB12169
M2227
BI2804
e3991
e3997
Chinju99
1713 1732 1733 1752 1753 1772 1773 1792 1793 1812 1813 1822
DR13 (attenuated) GCCCTTTCAC CTTGCAATCT GTTAATGATT ACCTGTCTTT TAGCAAATTT TGTGTTTCAA CCAGCCTTTT GGCTGGTGCT TGTACCATAG ATCTTTTTGG TTACCCTGAG
JS-2004-2
V803
DB1825
DB1865
e942
BI960
BI961
BI981
BI1166
BI1401
BI1482
e1695
M1763
e1834
M2366
BI2439
M2466
M2503
M2537
e2540
BI2944
PF4275
M4758
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KPED-9
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CV777
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M2227
BI2804
e3991
e3997
Chinju99

Fig. 1 continued

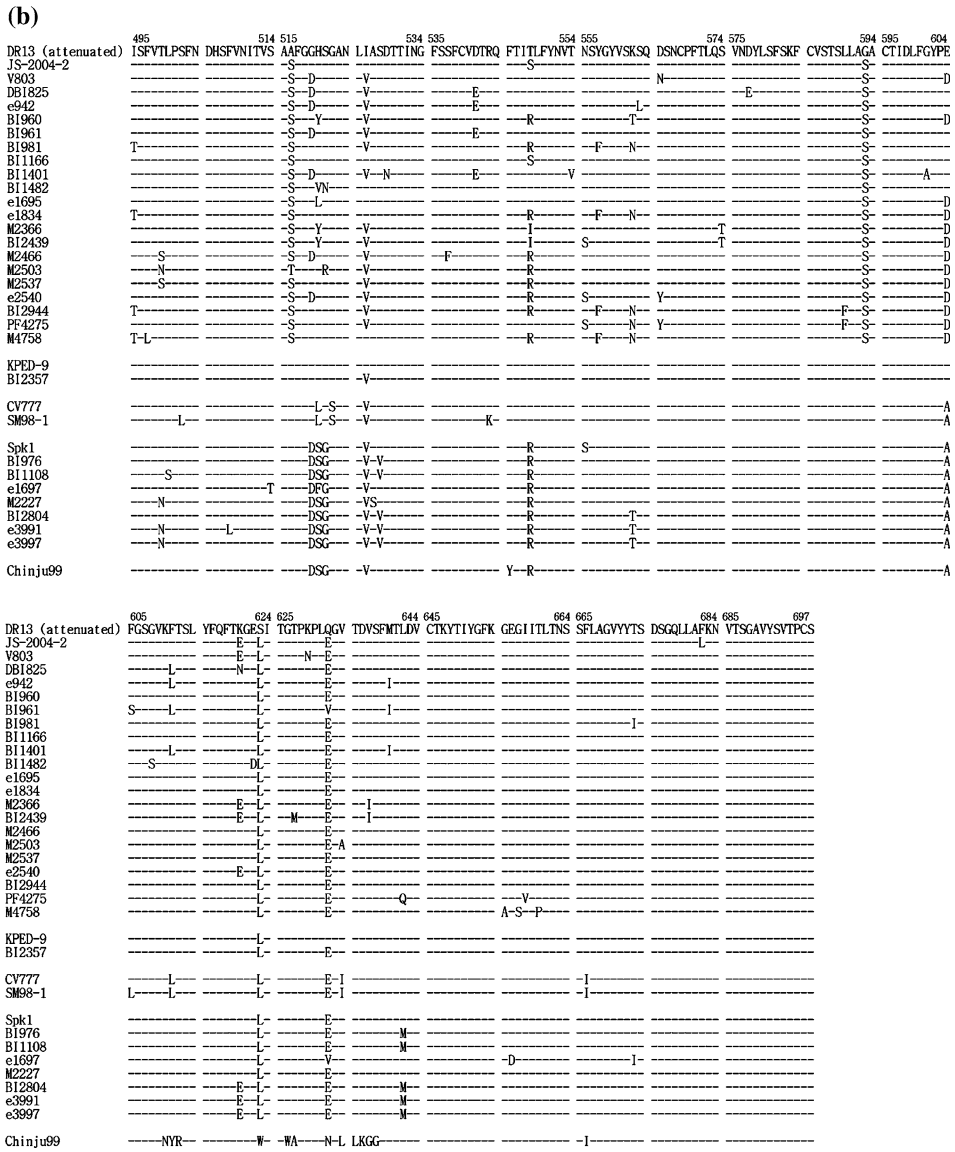

Fig. 1 continued

Sequence homology analysis

Nucleotide and deduced amino acid sequence homology results are described in Table 3. We found that the G1 Korean PEDV partial S glycoprotein genes have 95.1–100% DNA sequence identities with each other and they have 93.5–96.7% and 88.7–91.5% DNA sequence identities with the G2 and G3. Likewise, they have 91.6–100% homologies with the deduced amino acid sequences of each other and they have 90.6–97.0% and 86.2–91.1% homologies with the deduced amino acid sequences of the G2 and G3.

The G2 Korean PEDV partial S glycoprotein genes have 96.7–99.8% DNA sequence identities with each other and they have 91.8–93.0% DNA sequence identities with the G3. Likewise, they have 94.6–100% homologies with the deduced amino acid sequences of each other and they have 90.1–92.6% homologies with the deduced amino acid sequences of the G3.

More precisely, the G1-1 Korean PEDV partial S glycoprotein genes have 95.1–100% DNA sequence identities with each other and they have 95.3–97.9%, 93.6–96.6%, 93.5–96.6%, and 88.7–90.7% DNA sequence identities with the G1-2, G1-3, G2, and G3. Likewise, they have 91.6–100% homologies with the deduced amino acid sequences of each other and they have 93.6–98.0%, 90.1–96.1%, 90.6–96.6%, and 86.2–89.7% homologies with the deduced amino acid sequences of the G1-2, G1-3, G2, and G3. The G1-2 Korean PEDV partial S glycoprotein genes have 98.7–100% DNA sequence identities with each other and they have 96.4–97.4%, 95.1–96.7%, and 90.8–91.0% DNA sequence identities with the G1-1, G2, and G3.

Table 3 Nucleotide and deduced amino acid sequence homology of the partial S glycoprotein genes of Korean PEDV isolates and reference PEDV strains

Group			Percentage identity (%)[a]				
			G1			G2	G3
			G1-1	G1-2	G1-3		
Percentage identity (%)[b]	G1	G1-1	***	95.3–97.9	93.6–96.6	93.5–96.6	88.7–90.7
		G1-2	93.6–98.0	***	94.4–97.4	95.1–96.7	90.8–91.0
		G1-3	90.1–96.1	94.1–97.0	***	94.1–95.9	91.0–91.5
	G2		90.6–96.6	93.1–97.0	91.6–95.6	***	91.8–93.0
	G3		86.2–89.7	90.1–90.6	89.7–91.1	90.1–92.6	***

Bold line in the table means the border among the G1 (G 1-1, G 1-2, G1-3), G 2 and G3.

[a] Percentage of nucleotide identity (upper triangle).

[b] Percentage of deduced amino acid identity (lower triangle).

[c] Group including Korean PEDV isolates and reference PEDV strains: G1: G1-1: JS-2004-2, V803, DBI825, DBI865, e942, BI960, BI961, BI981, BI1166, BI1401, BI1482, M1595, e1642, e1693, e1695, e1696, M1763, M1764, DBI1784, e1833, e1834, M2366, BI2439, M2466, V2501, M2503, M2537, e2539, e2540, BI2944, e3975, e3981, e3984, e3988, PF4275, M4758, M4759, G1-2: KPED-9, P-5 V, attenuated DR13, BI2357, G1-3: CV777, Br1/87, SM98-1, parent DR13, G2: Spk1, BI976, BI1108, e1697, DBI2169, M2227, BI2804, e3991, e3997, G3: Chinju99.

Likewise, they have 98.5–100% homologies with the deduced amino acid sequences of each other and they have 94.1–97.0%, 93.1–97.0%, and 90.1–90.6% homologies with the deduced amino acid sequences of the G1-3, G2, and G3. The G1-3 Korean PEDV partial S glycoprotein genes have 99.5–100% DNA sequence identities with each other and they have 94.1–95.9% and 91.0–91.5% DNA sequence identities with the G2 and G3. Likewise, they have 98.5–100% homologies with the deduced amino acid sequences of each other and they have 91.6–95.6% and 89.7–91.1% homologies with the deduced amino acid sequences of the G2 and G3.

Phylogenetic analysis

Phylogenetic trees were generated on the basis of nucleotide and deduced amino acid sequences (Fig. 2). The left hand phylogenetic tree (Fig. 2a) was generated based on nucleotide sequences and the right hand tree (Fig. 2b) was based on deduced amino acid sequences. While these phylogenetic trees did differ slightly, overall they showed high similarity. In brief, all fifty-five PEDVs, which were used for comparison, including Korean PEDV isolates and reference PEDVs, fell into three groups (G1, G2, G3).

One group (G1) comprised CV777, Br1/87, JS-2004-2, KPED-9, P-5 V, SM98-1, parent DR13, attenuated DR13, and 37 Korean PEDV isolates. The second group (G2) consisted of Spk1 and 8 Korean PEDV isolates. The third group (G3) is Chinju99. The G1 containing CV777, Br1/87, JS-2004-2, KPED-9, P-5 V, SM98-1, parent DR13, attenuated DR13, and 37 Korean PEDV isolates had three subgroups (G1-1, G1-2, G1-3). PEDV JS-2004-2 and 36 Korean PEDV isolates formed one subgroup (G1-1) and KPED-9, P-5 V, and attenuated DR13 formed second subgroup (G1-2) with Korean PEDV isolate BI2357. CV777, Br1/87, SM98-1, and parent DR13 formed third subgroup (G1-3).

Discussion

The partial S glycoprotein genes including epitope region of Korean PEDV isolates were amplified by RT-PCR, cloned, and sequenced to determine the genetic diversity among the Korean PEDV isolates and to find out more prevalent PEDVs in Korea through sequence and phylogenetic analyses with reference PEDV strains.

In case of TGEV, belong to the family Coronaviridae with PEDV, only one serotype of TGEV is described. Recently, however, antigenic, genomic, and pathogenic diversity among TGEV strains has been described by using monoclonal antibodies and various molecular genetic techniques [12, 14, 15, 25, 26]. The sequence variation in the TGEV S glycoprotein genes and antigenic diversity of TGEV isolates has been reported [12–15]. In addition, based on partial sequence analysis of S glycoprotein genes, TGEV strains isolated in Korea have genetic diversity compared with foreign TGEV isolates as well as with each other [17, 27].

Similarly, although PEDV is known to have only one serotype at present, genome of PEDV is widely known to show genetic diversity, according to analysis results using molecular biological methods [3, 28–30]. Based on sequence analyses of M glycoprotein gene and N phosphoprotein gene, Korean PEDV isolates are different from foreign PEDV strains. In addition, diversity among the

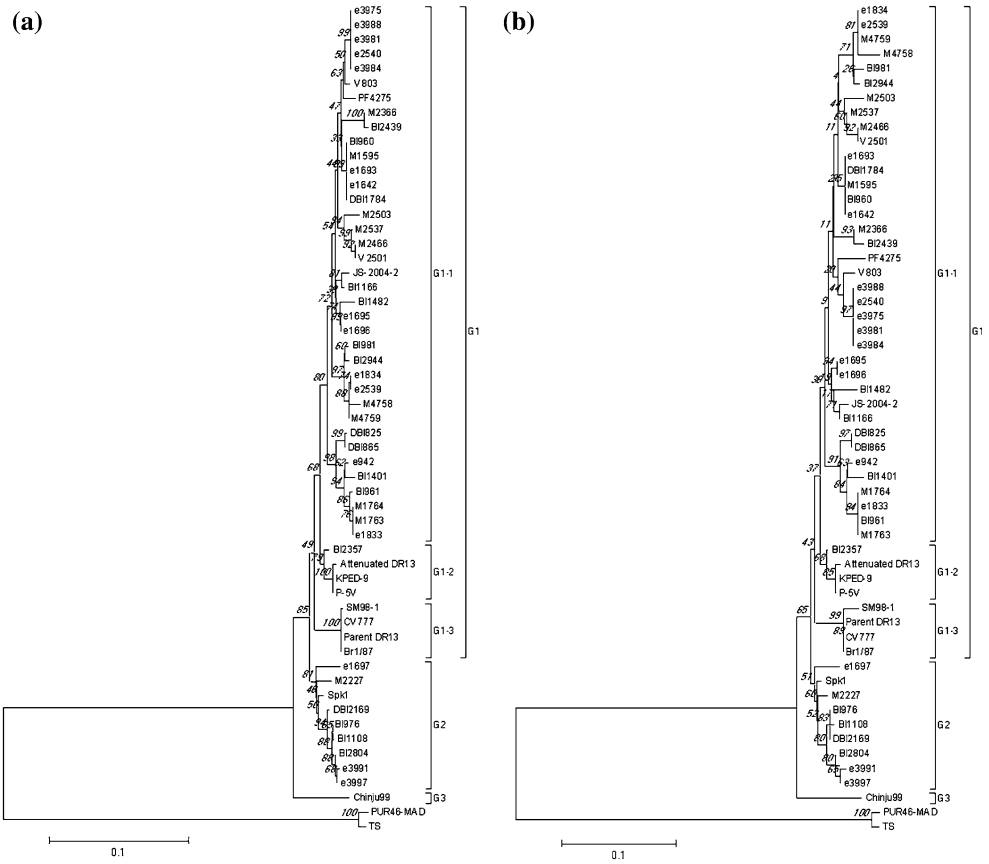

Fig. 2 Phylogenetic trees generated on the basis of (**a**) nucleotide and (**b**) deduced amino acid sequences of the partial S glycoprotein genes of Korean PEDV isolates with reference PEDV strains. Trees constructed with neighbor-joining method using MEGA 3.1 program. Horizontal branch lengths are proportional to genetic distances among the Korean PEDV isolates including reference PEDV strains. Bootstrap figures are shown in italics for the major nodes

Korean PEDV isolates has been reported [31]. However, this is the first report of genetic diversity in the S glycoprotein genes from Korean PEDV isolates and reference PEDV strains. Our findings show that, as with TGEV strains, Korea PEDV isolates are genetically diverse in their S glycoprotein genes, both among themselves and as compared with reference strains.

Phylogenetic analysis indicated that recent Korean PEDV isolates were divided into quite different three groups (G1, G2, G3) because they appear to originate from different ancestors and result from accumulation of mutations, although in case of TGEV, some isolates remained genetically stable over year [13]. It has been suggested that the reemergence of similar viruses into field, laboratory cross-contamination, point mutations, or recombination could affect the appearance of various PEDV isolates such as that of various TGEV strains [13, 32, 33].

There are several unique characteristics among the Korean PEDV isolates. The G1 Korean PEDV isolates didn't have 3 unique nucleotides between positions 1568 and 1571 and didn't have 2 unique amino acids between 521 and 522, as compared with G2 and G3 Korean PEDV isolates. The G3 Korean PEDV isolates had 18 unique nucleotides between positions 1644 and 1922 and 13 unique amino acids between 546 and 639, as compared with G1 and G2 Korean PEDV isolates. Especially, the G1-1 Korean PEDV isolates including JS-2004-2 strain had 2 unique nucleotides between positions 1556 and 1787 and 2 unique amino acids between 517 and 594, as compared with G1-2, G1-3, G2 and G3 Korean PEDV isolates. Although more PEDV isolates need to be analyzed, these nucleotide or amino acid differences may be used to differentiate specific PEDV group from Korean PEDV isolates

Sequence homology and phylogenetic analyses of partial S glycoprotein genes indicated that the G1 Korean PEDV isolates were highly homologous to CV777, Br1/87, JS-2004-2, KPED-9, P-5 V, SM98-1, parent DR13, and attenuated DR13, the G2 Korean PEDV isolates were highly homologous to Spk1, and the G3 was Chinju99 at the nucleotide and deduced amino acid sequence levels. In addition, the G1 Korean PEDV isolates were found to belong to a group that includes CV777, Br1/87, JS-2004-2, KPED-9, P-5 V, SM98-1, parent DR13, and attenuated DR13 and the G2 Korean PEDV isolates were found to belong to a group that includes Spk1. The G3 was found to belong to a group that includes Chinju99. More precisely, the G1 Korean PEDV isolates had 3 subgroups, that is, JS-2004-2 and 36 Korean PEDV isolates constituted the G1-1, KPED-9, P-5 V, attenuated DR13, and Korean PEDV

isolate BI2357 constituted the G1-2, and others formed the G1-3 based on nucleotide and deduced amino acid sequences. Taken together, it appears that many Korean PEDV isolates are closely related to the G1 including CV777, Br1/87, JS-2004-2, KPED-9, P-5 V, SM98-1, parent DR13, and attenuated DR13 rather than to the G2 and G3 including Spk1 and Chinju99. It is notable that prevalent PEDVs in Korea is especially close to the Chinese PEDV strain JS-2004-2 rather than to the Korean PEDV strains Spk1, Chinju99, KPED-9, SM98-1, parent DR13, and attenuated DR13, even though it is isolated in Korea.

PED vaccines are used in many Korean swine farms as damage by PEDV infection increases. All individual farms (except 2 farms) used in the present study also use the PED vaccines as described in Table 1. The relation between vaccination status and PEDV isolates in each farms indicated that all PEDVs isolated in each farms, which use either injectable or oral PED vaccine, belong to G1-1 and G2 including JS-2004-2 and Spk1, whereas only BI2357 isolated in a farm, which doesn't use any PED vaccines, belongs to G1-2 including commercial PED vaccine strains, KPED-9, P-5 V, and attenuated DR13. These results reflected existence of genetic diversity among the Korean PEDV isolates and suggested the possibility that PED vaccine use or not in each farms could affect the appearance of various PEDV isolates.

In the present study, the partial S glycoprotein genes including epitope region of Korean PEDV isolates were determined and compared to each other as well as reference PEDV strains, to find genetic diversity of PEDV in S glycoprotein gene. Epitope region used for sequence analysis herein, that is responsible for inducing the virus neutralizing antibodies, is known to be highly conserved in PEDV strains [19], but nevertheless, sequence changes in this conserved region are observed and are supposed to be remarkably meaningful. Sequence and phylogenetic analyses results using the partial S glycoprotein genes that include the epitope region show high similarity with the results using the full S glycoprotein genes [34] and require less labor, less money, and less analysis time. Phylogenetic trees were constructed and analyzed, according to partial S glycoprotein gene nucleotide and deduced amino acid sequences. Similarities and differences among the Korean PEDV isolates, including reference PEDV strains, were demonstrated, and these helped elucidate the phylogenetic relationship among the Korean PEDV isolates as well as reference PEDV strains. By phylogenetic analysis, the Korean PEDV isolates were divided into three groups (G1, G2, G3), which had three subgroups, and it was recognized that prevalent PEDVs in Korea are closely related to Chinese PEDV strain JS-2004-2. We expect to prevent outbreaks of the PEDV-induced diarrhea more effectively, according to choosing the more efficient PEDV vaccine strain by accurate analysis on genome of prevalent PEDVs in Korea. Moreover, genetic variation existence in S glycoprotein genes of Korean PEDV isolates and more prevalent PEDVs discovery in Korea will now form the basis for further functional exploration of PEDV.

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