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# Molecular characterization and phylogenetic analysis of porcine epidemic diarrhea virus (PEDV) field isolates in Korea

Seong-Jun Park · Hye-Kwon Kim · Dae-Sub Song · Hyoung-Joon Moon · Bong-Kyun Park

**Abstract** Porcine epidemic diarrhea virus (PEDV) has caused enteric disease with devastating impact since the first identification of PEDV in 1992 in Korea. In this study, we investigated molecular epidemiology, showed genetic diversity, and analyzed phylogenetic relationships of Korean PEDV field isolates with other PEDV reference strains. Genetic analysis of the complete M and ORF3 genes showed that each PEDV group had several unique characteristics, and this indicated that specific groups of PEDVs may be differentiated from the other PEDVs by specific nucleotide differences. Especially, ORF3 gene analysis can be used for discrimination between vaccine and wild-type PEDVs. Sequence and phylogenetic analysis showed that recent, prevalent Korean PEDV field isolates have close relationships to Chinese field strains and differ genetically from European strains and vaccine strains used in Korea. These results raise questions as to whether a new type of PEDV vaccine may be necessary for preventing PEDV infection more effectively in Korea.

S.-J. Park · H.-K. Kim · B.-K. Park (✉)
Department of Veterinary Medicine Virology Lab,
College of Veterinary Medicine and BK21 Program
for Veterinary Science, Seoul National University,
Gwanak-gu, Seoul 151-742, Korea
e-mail: parkx026@snu.ac.kr

D.-S. Song
Viral Infectious Disease Research Center,
Korea Research Institute of Bioscience and Biotechnology,
Daejeon 305-806, Korea

H.-J. Moon
Research Unit, Green Cross Veterinary Products,
Yongin 449-903, Korea

## Introduction

Porcine epidemic diarrhea virus (PEDV), a member of the family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus*, is an enveloped, single-stranded RNA virus. PEDV was first reported in Belgium and the United Kingdom in 1978 [21]. Since the first identification of PEDV, outbreaks of PEDV infections have been reported in many swine-producing countries, notably in Europe and Asia [22]. Porcine epidemic diarrhea (PED), caused by PEDV, is an acute, highly contagious, and devastating enteric disease that is characterized by severe diarrhea, dehydration and significant mortality in swine, resulting in severe economic losses in the European and Asian swine industry [22].

Coronaviruses have a genome organization with a common set of five genes arranged in a conserved order [7]. The polymerase gene, occupying 70% of the genome, encodes the replicase polyproteins. The genes for structural proteins S, E, M, and N are located downstream of the polymerase gene [7]. In addition, a variety of genes encoding accessory proteins whose number and sequence vary among different coronaviruses are studded between the structural genes, and are called accessory genes [16].

One of the four structural proteins, the M protein, a structural membrane glycoprotein and the most abundant envelope component, is a triple-spanning membrane protein with a short amino-terminal domain on the exterior of the virion and a long carboxy-terminal domain on the inside [29]. In addition to playing an important role in the viral assembly process [5, 17], the M protein induces antibodies that neutralize virus in the presence of complement [23, 24]. The M protein has also been proposed to play a role in α-interferon (α-IFN) induction [14]. It has been demonstrated that coexpression of M and E proteins

allows the formation of pseudoparticles, which exhibit an interferogenic activity similar to that of complete virions [1].

Unlike the structural proteins, in most cases, little is known about the functions of the accessory proteins, and in general they are not required for virus replication in cultured cells [4, 25, 31, 32]. Quite the opposite, their expression might lead to a decrease in viral fitness in vitro, and mutants with inactivated accessory genes are easily selected during serial passage in cell cultures [11, 15, 26, 30]. In field strains, however, accessory genes are generally maintained [8, 11], and their loss mainly results in attenuation in the natural host [6, 10, 18]. Especially, in the case of PEDV, the ORF3 gene is the only accessory gene, and it has been suggested to be an important determinant for virulence of this virus. Virulence of PEDV can be reduced by altering the ORF3 gene through cell culture adaptation [26] in a manner similar to TGEV [30], and differentiation of ORF3 genes between the highly cell-adapted viruses and field viruses could be a marker of adaptation to cell culture and attenuation of virus [19, 26]. In addition, differentiation of the ORF3 gene could be a valuable tool for molecular epidemiology studies of PEDV [2, 19, 26].

In Korea, PEDV was first isolated in 1992 [12]. It has been detected frequently in many provinces and has become one of the most important viral enteric diseases. In spite of using the vaccine strategy at present, damage caused by PEDV infection is continuous and serious in Korea. To better control and prevent PEDV infection, it is necessary for us to investigate the molecular epidemiology of PEDV field isolates in Korea. In this study, therefore, we investigate the molecular epidemiology and genetic diversity of Korean PEDV field isolates and analyze phylogenetic relationships of the Korean PEDV field isolates to other PEDV reference strains reported previously. The present study focused on the M and ORF3 genes because of their characteristics described above.

## Materials and methods

### PEDV field isolates

Porcine intestinal and fecal samples were collected between October 2002 and June 2007 from 26 swine farms in six provinces of Korea. All pigs from the 26 farms showed signs of watery diarrhea and dehydration at the time of sample collection. Fresh samples were collected from individual pigs, placed into a sterile specimen container, and submitted to the Department of Veterinary Medicine Virology Laboratory, College of Veterinary Medicine, Seoul National University. These intestinal and fecal samples were confirmed to be positive for PEDV by reverse transcription-polymerase chain reaction (RT-PCR) [20].

### PEDV vaccine strains

The attenuated DR13 strain and its parent strain were obtained from our laboratory [26]. The attenuated DR13 strain was derived from the parent strain by 100 serial passages in Vero cells and was used for manufacture of the Korean PED oral vaccine by the Green Cross Veterinary Product Co., Ltd. (Yongin, Korea). The KPED-9 strain, used for manufacture of the Korean live PEDV vaccine, was kindly provided by the Green Cross Veterinary Product Co., Ltd. (Yongin, Korea), and the P-5 V strain for manufacture of the Japanese live PEDV vaccine was provided by the Nisseiken regional distributor in Korea.

### RNA extraction and reverse transcription (RT)

PEDV-positive fecal samples were prepared as 10% (v/v) fecal suspensions in phosphate-buffered saline (PBS; 0.1 M, pH 7.2), and positive intestinal contents were also prepared as 10% (v/v) intestinal suspensions in PBS by homogenization. The sample suspensions were vortexed and centrifuged for 10 min at 4800 x g. RNA was extracted from a 250-μl starting volume of the centrifuged 10% sample suspensions using TRIzol LS (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Viral RNAs were extracted from attenuated DR13, KPED-9 and P-5V strains as described above. Reverse transcription (RT) was carried out using random hexamer primers (TaKaRa Bio Inc., Otsu, Japan), and the cDNA was immediately used for amplification or stored at −20°C.

### PCR amplification of the M gene

Primers were designed based on the published sequences of the E and N genes to cover the complete M gene of PEDV. The primers were PEDM1 (forward), 5'-GTCTTACATG CGAATTGACC-3', and PEDM2 (reverse), 5'-GGCATA GAGAGATAATGGCA-3'. The size of the amplified product was predicted to be 808 bp. PCR was carried out using a commercial amplification system (Perkin-Elmer, Applied Biosystems, Foster City, CA) as described previously [20] with simple modifications. The PCR was performed at 94°C for 5 min, followed by 35 cycles of 94°C 30 s, 50°C 30 s, 72°C 30 s, and a final extension at 72°C for 7 min, and the sample was then held at 4°C.

### PCR amplification of the ORF3 gene

The primer pair ORF3-1/ORF3-2 [26], targeting the S/E regions (corresponding to nt 24,741-25,570 of CV777;

830 bp) of PEDV, was used for generating the complete ORF3 gene of PEDV, and PCR was performed using the protocol described previously [19].

## Cloning of cDNA and sequencing

The RT-PCR products for each M and ORF3 gene were analyzed by 1.5% agarose gel electrophoresis and visualized by ultraviolet illumination after ethidium bromide staining. Bands of the correct size were excised and purified using a QIAquick Gel Extraction Kit (QIAGEN GmbH, Hiden, Germany) according to the manufacturer's instructions. The purified RT-PCR products corresponding to the full-length M and ORF3 genes of PEDV were cloned into pDrive cloning vector (QIAGEN GmbH, Hiden, Germany) as described previously [20], and the cloned plasmids were purified using a QIAprep® Spin Miniprep Kit (QIAGEN GmbH, Hiden, Germany) before sequencing. Sequencing of plasmid DNA was carried out at least twice in both directions at the Genotech Institute (Genotech Co., Ltd., Korea) using T7 and SP6 primers and an automated DNA sequencer (ABI system 3700, Applied Biosystems Inc., Foster City, USA).

## Molecular analysis

The nucleotide sequences of the full-length M and ORF3 genes of Korean PEDV field isolates were aligned using ClustalX version 1.83 [28], edited using Bioedit version 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and compared with those of PEDV reference strains in the GenBank database as well as in previous papers. Sequence similarity analysis was performed for the aligned nucleotide and amino acid sequences using MegAlign software (DNASTar Inc., Madison, WI, USA). Phylogenetic analysis of the Korean PEDV field isolates with other PEDV reference strains based on the nucleotide alignments was done by the neighbor-joining method and the minimum-evolution method of Molecular Evolutionary Genetics Analysis (MEGA version 4.1) using pairwise distances [27]. The Korean PEDV field isolates and the other PEDV reference strains used for sequence alignment, sequence analysis and phylogenetic analysis are indicated in the figure legends.

## Results

### Phylogenetic analysis of the M gene

Phylogenetic analysis based on a complete M gene fragment of Korean PEDV field isolates, together with other PEDV reference strains, confirmed that all PEDVs, including Korean field isolates, fell into three groups, and

all PEDVs isolated from Korea belonged to group 3 (Fig. 1). More precisely, as shown in Fig. 1, group 3 had four subgroups (3-1, 3-2, 3-3, and 3-4). Seven Korean strains in 1993-2001 made up subgroup 3-1, together with the Japanese JMe2 strain; and three Korean field isolates from 2003-2004 formed subgroup 3-2, together with three vaccine strains and nine Chinese strains. Subgroup 3-3 comprised nine Korean field isolates from 2007, Chinese strain JS-2004-2, and four Thai strains in 2007-2008; and subgroup 3-4 consisted of 13 Korean field isolates from 2003-2007, the parent DR13 strain, and two Chinese strains.
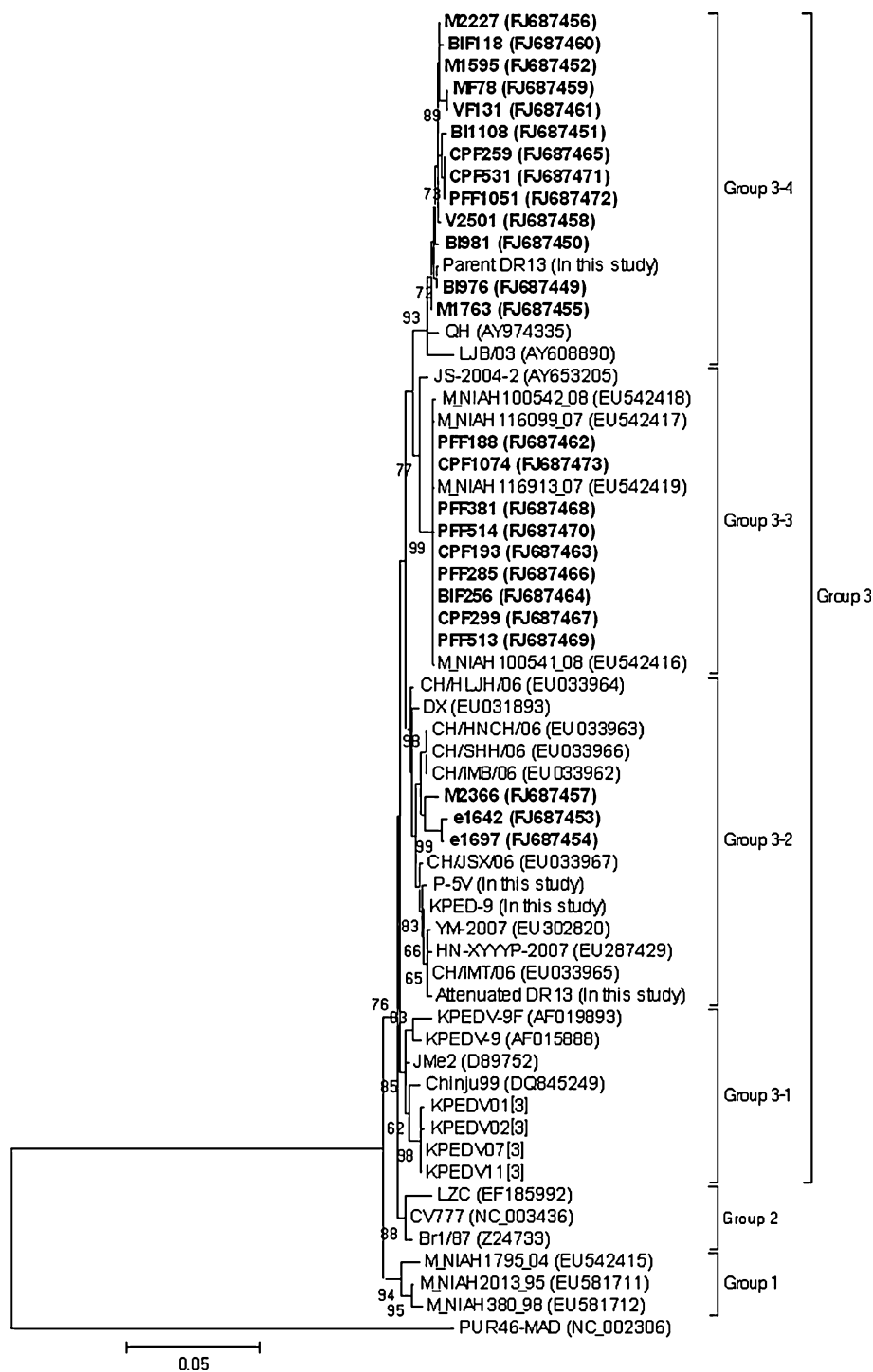
### Genetic analysis of the M gene

Sequences of complete M genes of Korean PEDV field isolates were determined and compared to those of other PEDV reference strains. All Korean PEDV field isolates had a single ORF of 681 nucleotides encoding a protein of 226 amino acids with a predicted $Mr$ of 25.3-25.4 kDa. They did not have nucleotide deletions or insertions but did have point mutations. These isolates had a conserved ATAAAC sequence at 11 nucleotides upstream of the initiator ATG, as previously recognized in Br1/87 [9].

Sequence analysis of the complete M genes showed that all PEDVs, including the Korean field isolates, fell into three groups, and group 3 had four subgroups (3-1, 3-2, 3-3, and 3-4), as can be seen in the phylogenetic tree (Fig. 1). Each group had unique differences in its sequences. Group 1 had seven specific nucleotide changes (G→C at 139, T→C at 255, C→T at 387, A→G at 444, A→C at 570, G→A at 574, and A→C at 621) that were not found in the other groups, and two of the seven changes lead to amino acid changes (V→L at 47 and G→S at 192). Group 2 had one specific nucleotide change (C→T at 618). The specific differences between group 3 and other groups are as follows: Subgroup 3-1 exhibited one specific nucleotide change (T→A at 588). In particular, in the case of subgroups 3-2, 3-3 and 3-4, which include all of the Korean field isolates, subgroups 3-2 and 3-3 shared one specific nucleotide change (C→T at 198), and subgroups 3-3 and 3-4 shared two specific nucleotide changes (T→C at 285, and T→A at 348). Subgroup 3-4 had one specific nucleotide change (C→A at 597). These results indicated that specific groups of PEDVs may be differentiated from all other PEDVs, including Korean field isolates, by specific nucleotide differences, although more PEDVs need to be analyzed for more accurate analysis.

### Sequence homology analysis of the M gene

Sequence homology results based on the complete M gene of all PEDVs, including the Korean PEDV field isolates,

M2227 (FJ687456)
BIFI18 (FJ687460)
M1595 (FJ687452)
MF78 (FJ687459)
89 VFI31 (FJ687461)
BI1108 (FJ687451)
CPF259 (FJ687465)
CPF531 (FJ687471)
78 PFF1051 (FJ687472)
V2501 (FJ687458)
BI981 (FJ687450)
Parent DR13 (In this study)
73 BI976 (FJ687449)
93 M1763 (FJ687455)
QH (AY974335)
LJB/03 (AY608890)
JS-2004-2 (AY653205)
M_NIAH100542_08 (EU542418)
M_NIAH116099_07 (EU542417)
PFF188 (FJ687462)
77 CPF1074 (FJ687473)
M_NIAH116913_07 (EU542419)
PFF381 (FJ687468)
PFF514 (FJ687470)
99 CPF193 (FJ687463)
PFF285 (FJ687466)
BIF256 (FJ687464)
CPF299 (FJ687467)
PFF513 (FJ687469)
M_NIAH100541_08 (EU542416)
CH/HLJH/06 (EU033964)
DX (EU031893)
CH/HNCH/06 (EU033963)
98 CH/SHH/06 (EU033966)
CH/IMB/06 (EU033962)
M2366 (FJ687457)
e1642 (FJ687453)
99 e1697 (FJ687454)
CH/JSX/06 (EU033967)
P-5V (In this study)
KPED-9 (In this study)
83 YM-2007 (EU302820)
66 HN-XYYYP-2007 (EU287429)
65 CH/IMT/06 (EU033965)
Attenuated DR13 (In this study)
76
83 KPEDV-9F (AF019893)
KPEDV-9 (AF015888)
JMe2 (D89752)
85 Chinju99 (DQ845249)
KPEDV01[3]
62 KPEDV02[3]
98 KPEDV07[3]
KPEDV11[3]
LZC (EF185992)
CV777 (NC_003436)
88 Br1/87 (Z24733)
M_NIAH1795_04 (EU542415)
M_NIAH2013_95 (EU581711)
94 M_NIAH380_98 (EU581712)
95
PUR46-MAD (NC_002306)

Group 3-4

Group 3-3

Group 3

Group 3-2

Group 3-1

Group 2

Group 1

0.05

are shown in Table 1, and it was found that group 1 PEDVs have 98.3-99.4% (97.7-99.1%) nucleotide (deduced amino acid) sequence identity with each other, and they have 96.7-98.0% (95.5-98.6%) and 96.0-97.7% (94.6-98.6%) sequence identity with members of group 2 and 3, respectively. Group 2 PEDVs have 98.6-99.7% (97.3-99.1%) sequence identity with each other, and they have 96.9-99.4% (95.5-100%) sequence identity with members

of group 3. Group 3 PEDVs have 96.7-100% (96.0-100%) sequence identity with each other.

More precisely, comparison of each subgroup in group 3 with each other as well as other groups revealed that subgroup 3-1 PEDVs have nucleotide (deduced amino acid) identities of 98.0-100% (96.9-100%) with each other and 96.4-97.6% (94.6-97.7%), 97.3-99.2% (95.5-99.1%), 97.0-98.9% (96.0-99.5%), 97.3-98.3% (97.3-99.5%) and

◄ **Fig. 1** Relationships between Korean PEDV field isolates (accession no. FJ687449-FJ687473 in **boldface**) and other PEDV reference strains based on the full-length M gene. The phylogenetic trees were constructed by using the neighbor-joining method in MEGA version 4.1 with pairwise distances [27]. Bootstrap values (based on 1000 replicates) for each node are given if >60%. The *scale bar* indicates nucleotide substitutions per site. Accession numbers (or references) for the Korean PEDV field isolates and the other PEDV reference strains used in the analysis are as follows: CV777 (NC_ 003436), Br1/87 (Z24733), M_NIAH2013_95 (EU581711), M_NIAH380_98 (EU581712), M_NIAH1795_04 (EU542415), M_NIAH100541_08 (EU542416), M_ NIAH116099_07 (EU542417), M_NIAH100542_08 (EU542418), M_NIAH116913_07 (EU542419), KPEDV-9F (AF019893), KPEDV-9 (AF015888), Chinju99 (DQ845249), KPEDV01 [3], KPEDV02 [3], KPEDV07 [3], KPEDV11 [3], parent DR13 (this study), JMe2 (D89752), attenuated DR13 (this study), KPED-9 (this study), P-5V (this study), LZC (EF185992), DX (EU031893), CH/IMB/06 (EU033962), CH/ HNCH/06 (EU033963), CH/HLJH/06 (EU033964), CH/IMT/06 (EU033965), CH/ SHH/06 (EU033966), CH/JSX/ 06 (EU033967), HN-XYYYP-2007 (EU287429), YM-2007 (EU302820), JS-2004-2 (AY653205), LJB/03 (AY608890), QH (AY974335), **BI976 (FJ687449)**, **BI981 (FJ687450)**, **BI1108 (FJ687451)**, **M1595 (FJ687452)**, **e1642 (FJ687453)**, **e1697 (FJ687454)**, **M1763 (FJ687455)**, **M2227 (FJ687456)**, **M2366 (FJ687457)**, **V2501 (FJ687458)**, **MF78 (FJ687459)**, **BIF118 (FJ687460)**, **VF131 (FJ687461)**, **PFF188 (FJ687462)**, **CPF193 (FJ687463)**, **BIF256 (FJ687464)**, **CPF259 (FJ687465)**, **PFF285 (FJ687466)**, **CPF299 (FJ687467)**, **PFF381 (FJ687468)**, **PFF513 (FJ687469)**, **PFF514 (FJ687470)**, **CPF531 (FJ687471)**, **PFF1051 (FJ687472)**, **CPF1074 (FJ687473)**

96.9-98.6% (96.4-99.5%) with members of group 1, 2, 3-2, 3-3 and 3-4, respectively. Subgroup 3-2 PEDVs have identities of 98.0-100% (96.9-100%) with each other and 96.4-97.7% (95.5-98.6%), 97.3-99.4% (96.4-100%), 97.0-98.9% (96.4-100%) and 96.7-98.5% (96.0-100%) with members of group 1, 2, 3-3 and 3-4, respectively. Subgroup 3-3 PEDVs have identities of 98.9-100% (99.1-100%) with each other and 96.4-97.0% (95.5-97.7%), 97.0-98.5% (96.4-99.1%) and 97.5-98.5% (96.9-99.1%) with members of groups 1, 2, and 3-4. Subgroup 3-4 PEDVs have identities of 98.2-100% (97.3-100%) with each other and 96.0-97.3% (95.1-98.2%) and 96.9-98.8% (96.0-99.5%) with members of groups 1 and 2.

Phylogenetic analysis of the ORF3 gene

Phylogenetic analysis based on a complete ORF3 gene fragment of Korean PEDV field isolates, together with other PEDV reference strains, confirmed that all PEDVs, including Korean field isolates, fell into three groups (Fig. 2). One group comprised the CV777, Br1/87, and LZC strains. The second group consisted of vaccine strains (the attenuated strains DR13, KPED-9, P-5V and CV777 vs), the CH/GSJIII/07 strain, and the DBI865 Korean field isolate. The third group was made up of 25 Korean field isolates, Chinju99, the parent strain DR13, and 11 Chinese strains. The third group had two subgroups (3-1 and 3-2). Nine Korean field isolates from 2007 and ten Chinese

strains formed one subgroup (3-1), and 15 Korean field isolates from 2003-2007 formed a second subgroup (3-2) together with Chinese strain CH/S, Korean strain Chinju99, and the parent strain DR13.

Genetic analysis of the ORF3 gene

Sequences of complete ORF3 genes of Korean PEDV field isolates were determined and compared to those of other PEDV reference strains. All Korean PEDV field isolates except DBI865 had a single ORF of 675 nucleotides encoding a protein of 224 amino acids, with a predicted $M$r of 25.1-25.3 kDa. On the other hand, DBI865 had a single ORF of 276 nucleotides encoding a protein of 91 amino acids, with predicted $M$r of 10.2 kDa because of a 49-nucleotide deletion at position 245-293. All Korean PEDV field isolates, including DBI865, had a conserved sequence (CTAGAC) at 46 nucleotides upstream of the initiator ATG, similar to that described above for the M gene.

Sequence analysis of the complete ORF3 genes showed that all PEDVs, including the Korean field isolates, fell into three groups, and group 3 had two subgroups (3-1 and 3-2), as can be seen in the phylogenetic tree (Fig. 2). Each group had differences in its sequences. Group 1 had eight specific nucleotide changes (C→T at 62, A→G at 160, A→G at 235, T→C at 243, A→G at 301, C→T at 360, T→C at 450 and G→A at 497) that were not found in the other groups, and four of the eight changes led to amino acid changes (A→V at 21, I→V at 54, T→A at 101 and S→N at 166). Group 2 showed one specific nucleotide change (C→A at 339) and had two classes of large nucleotide deletions at position 245-295, which are predicted to produce truncated proteins. The specific differences between group 3 (containing all Korean field isolates except DBI865) and the other groups are as follows: group 3 had three specific nucleotide changes (T→G at 238, C→T at 264 and C→T at 274), and two of three changes led to amino acid changes (F→V at 80 and L→F at 92). In particular, subgroup 3-1, which includes nine Korean field isolates from 2007 and ten Chinese strains, was differentiated from subgroup 3-2 by one specific nucleotide change (C→T at 237). These results showed that these specific nucleotide differences may be used to differentiated specific groups of PEDVs from other PEDVs, including Korean field isolates. However, more PEDVs need to be analyzed. In addition, complete ORF3 gene analysis can be used for differentiation between vaccine- and wild-type PEDVs because all vaccine-type PEDVs had a large nucleotide deletion in the ORF3 gene.

Sequence homology analysis of the ORF3 gene

Sequence homology results based on the complete ORF3 gene of all PEDVs, including Korean PEDV field isolates,

**Table 1** Nucleotide and deduced amino acid sequence similarity based on the full-length M genes of the Korean PEDV field isolates and those of the other PEDV reference strains

| Group | | Group 1[a] | Group 2[b] | Group 3 | | | |
|---|---|---|---|---|---|---|---|
| | | | | Subgroup 3-1[c] | Subgroup 3-2[d] | Subgroup 3-3[e] | Subgroup 3-4[f] |
| Group 1 | | | 96.7–98.0 | 96.4–97.6 | 96.4–97.7 | 96.4–97.0 | 96.0–97.3 |
| Group 2 | | 95.5–98.6 | | 97.3–99.2 | 97.3–99.4 | 97.0–98.5 | 96.9–98.8 |
| Group 3 | Subgroup 3-1 | 94.6–97.7 | 95.5–99.1 | | 97.0–98.9 | 97.3–98.3 | 96.9–98.6 |
| | Subgroup 3-2 | 95.5–98.6 | 96.4–100 | 96.0–99.5 | | 97.0–98.9 | 96.7–98.5 |
| | Subgroup 3-3 | 95.5–97.7 | 96.4–99.1 | 97.3–99.5 | 96.4–100 | | 97.5–98.5 |
| | Subgroup 3-4 | 95.1–98.2 | 96.0–99.5 | 96.4–99.5 | 96.0–100 | 96.9–99.1 | |

Upper and lower triangles indicate percent nucleotide (nt) and amino acid (aa) sequence similarity, respectively

[a] Three Thai strains (M_NIAH2013_95, M_NIAH380_98 and M_NIAH1795_04)

[b] European strains (CV777 and Br1/87) and Chinese LZC strain

[c] Seven Korean strains (KPEDV-9F, KPEDV-9, Chinju99, KPEDV01, KPEDV02, KPEDV07 and KPEDV11) from 1993–2001 and Japanese JMe2 strain

[d] Vaccine strains (attenuated DR13, KPED-9 and P-5V), nine Chinese strains (DX, CH/ HLJH/06, CH/HNCH/06, CH/IMB/06, CH/IMT/06, CH/JSX/06, CH/SHH/06, HN-XYYYP-2007 and YM-2007) and three Korean field isolates (e1642, e1697 and M2366) from 2003–2004

[e] Chinese JS-2004-2 strain, four Thai strains (M_NIAH100541_08, M_NIAH116099_07, M_NIAH100542_08 and M_NIAH116913_07) and nine Korean field isolates (PFF188, CPF193, BIF256, PFF285, CPF299, PFF381, PFF513, PFF514 and CPF1074) from 2007

[f] Two Chinese strains (LJB/03 and QH), the Korean parent DR13 strain and 13 Korean field isolates (BI976, BI981, BI1108, M1595, M1763, M2227, V2501, MF78, BIF118, VF131, CPF259, CPF531 and PFF1051) from 2003–2007

are shown in Table 2, and these revealed that group 1 PEDVs have 98.3-99.7% (97.3-99.1%) nucleotide (deduced amino acid) sequence identity with each other, and they have 95.8-98.0% (90.1-97.5%) and 95.4-98.0% (93.7-96.8%) sequence identity with members of group 2 and 3, respectively. Group 2 PEDVs have 99.8-100% (94.5-100%) sequence identity with each other, and they have 97.2-98.8% (91.2-99.5%) sequence identity with members of group 3. Group 3 PEDVs have 97.3-100% (97.3-100%) sequence identity with each other.

More precisely, comparison of each subgroup in group 3 with each other as well as other groups revealed that subgroup 3-1 PEDVs have nucleotide (deduced amino acid) identities of 97.9-100% (97.3-100%) with each other and 95.4-97.4% (93.7-96.4%), 97.4-98.8% (92.3-99.0%), and 97-3-99.1% (97.3-100%) with members of group 1, 2, and 3-2, respectively. Subgroup 3-2 PEDVs have identities of 97.6-100% (97.7-100%) with each other and 95.5-98.0% (93.7-96.8%) and 97.2-98.5% (91.2-99.5%) with groups 1 and 2.

## Discussion

In this study, the complete M and ORF3 genes of Korean PEDV field isolates were amplified by RT-PCR, cloned and sequenced to investigate their molecular and epidemiological characteristics and their genetic diversity. In addition, phylogenetic relationships between Korean PEDV field isolates and other PEDV previously reported reference strains were also analyzed.

All Korean PEDV field isolates (excluding DBI865) have only point mutations in the M and ORF3 genes, and all of them, including DBI865, have ATAAAC and CTAGAC sequences at 11 and 46 nucleotides upstream of the initiator ATG of the M and ORF3 genes, as recognized previously [9]. These sequences are hexameric motifs that are common to coronaviruses and are similar to the hexameric motifs XUA(A/G)AC found adjacent to other PEDV ORFs. These hexameric motifs have been proposed to be a start site for the transcription of subgenomic mRNAs [13]. The DBI865 isolate has a 49-nucleotide deletion at position 245-293 of the ORF3 gene, resulting in a truncated protein of 91 amino acids in size. This size of this deletion is exactly the same as the one present in the vaccine strain CV777 and the Chinese field strain CH/GSJIII/07 [2].

Genetic analysis based on complete M and ORF3 genes showed that each PEDV group had several unique characteristics, and these results indicated that specific groups of PEDVs may be differentiated from other PEDVs, including Korean field isolates, by specific nucleotide differences, but more PEDVs need to be analyzed for more accurate analysis. Especially, complete ORF3 gene analysis can be used for discrimination between vaccine and wild-type PEDVs because only vaccine-type PEDVs had large deletions in the ORF3 gene.

According to sequence and phylogenetic analysis using the complete M gene, all PEDVs isolated in Korea are
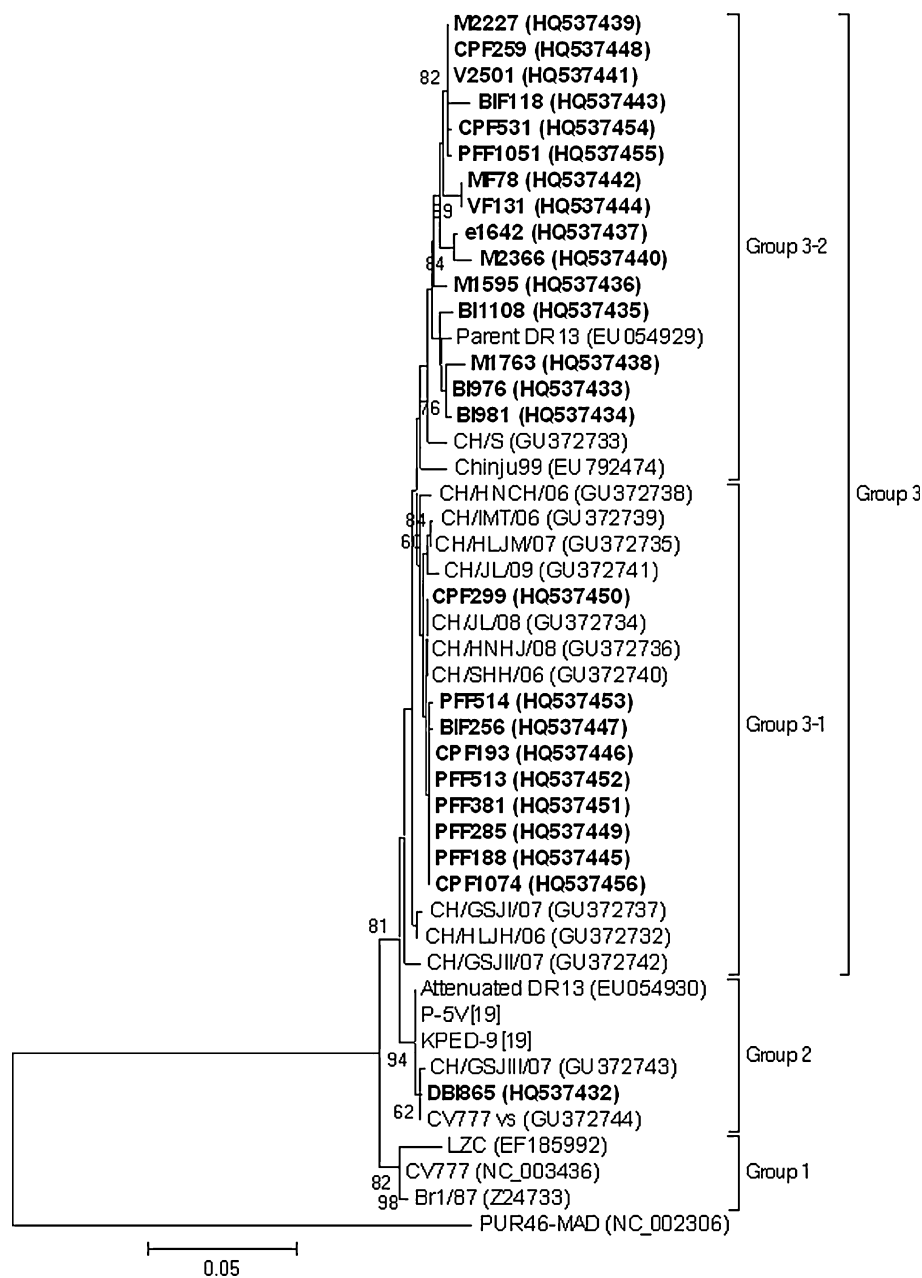
**Fig. 2** Relationships between Korean PEDV field isolates (accession no. HQ537432- HQ537456 in **boldface**) and other PEDV reference strains based on the full-length ORF3 gene. The phylogenetic trees were constructed by using the neighbor-joining method in MEGA version 4.1 with pairwise distances [27]. Bootstrap values (based on 1000 replicates) for each node are given if >60%. The *scale bar* indicates nucleotide substitutions per site. Accession numbers (or references) for the Korean PEDV field isolates and the other PEDV reference strains used in the analysis are as follows: CV777 (NC_003436), Br1/87 (Z24733), Chinju99 (EU792474), parent DR13 (EU054929), attenuated DR13 (EU054930), KPED-9 [19], P-5V [19], CV777 vs (GU372744), LZC (EF185992), CH/HLJH/06 (GU372732), CH/S (GU372733), CH/JL/08 (GU372734), CH/ HLJM/07 (GU372735), CH/HNHJ/08 (GU372736), CH/GSJI/07 (GU372737), CH/ HNCH/06 (GU372738), CH/IMT/06 (GU372739), CH/SHH/06 (GU372740), CH/JL/09 (GU372741), CH/GSJII/07 (GU372742), CH/GSJIII/07 (GU372743), **DBI865** (**HQ537432**), **BI976** (**HQ537433**), **BI981** (**HQ537434**), **BI1108** (**HQ537435**), **M1595** (**HQ537436**), **e1642** (**HQ537437**), **M1763** (**HQ537438**), **M2227** (**HQ537439**), **M2366** (**HQ537440**), **V2501** (**HQ537441**), **MF78** (**HQ537442**), **BIF118** (**HQ537443**), **VF131** (**HQ537444**), **PFF188** (**HQ537445**), **CPF193** (**HQ537446**), **BIF256** (**HQ537447**), **CPF259** (**HQ537448**), **PFF285** (**HQ537449**), **CPF299** (**HQ537450**), **PFF381** (**HQ537451**), **PFF513** (**HQ537452**), **PFF514** (**HQ537453**), **CPF531** (**HQ537454**), **PFF1051** (**HQ537455**), **CPF1074** (**HQ537456**)

closely related to those of group 3, and recent prevalent Korean PEDV field isolates are especially closely related to members of subgroup 3-3 and 3-4, including parent strain DR13, three Chinese field strains, and four Thai strains from 2007-2008, and they are less closely related to members of subgroups 3-1 and 3-2, which include seven Korean strains

**Table 2** Nucleotide and deduced amino acid sequence similarity based on the full-length ORF3 genes of the Korean PEDV field isolates and those of the other PEDV reference strains

| Group | | Group 1[a] | Group 2[b] | Group 3 | |
|---|---|---|---|---|---|
| | | | | Subgroup 3-1[c] | Subgroup 3-2[d] |
| Group 1 | | | 95.8–98.0 | 95.4–97.4 | 95.5–98.0 |
| Group 2 | | 90.1–97.5 | | 97.4–98.8 | 97.2–98.5 |
| Group 3 | Subgroup 3-1 | 93.7–96.4 | 92.3–99.0 | | 97.3–99.1 |
| | Subgroup 3-2 | 93.7–96.8 | 91.2–99.5 | 97.3–100 | |

Upper and lower triangles indicate percent nucleotide (nt) and amino acid (aa) sequence similarity, respectively

[a] European strains (CV777 and Br1/87) and Chinese LZC strain

[b] Vaccine strains (attenuated strains DR13, KPED-9, P-5V and CV777 vs), Chinese CH/GSJIII/ 07 strain and Korean DBI865 field isolate

[c] Ten Chinese strains (CH/HLJH/06, CH/JL/08, CH/HLJM/07, CH/HNHJ/08, CH/GSJI/ 07, CH/HNCH/06, CH/IMT/06, CH/SHH/06, CH/JL/09 and CH/GSJII/07) and nine Korea field isolates (PFF188, CPF193, BIF256, PFF285, CPF299, PFF381, PFF513, PFF514 and CPF1074) from 2007

[d] Chinese strains CH/S, Korean strains (Chinju99 and parent DR13) and 15 Korean field isolates (BI976, BI981, BI1108, M1595, e1642, M1763, M2227, M2366, V2501, MF78, BIF118, VF131, CPF259, CPF531 and PFF1051) from 2003–2007

from 1993-2001, the Japanese JMe2 strain, three vaccine strains and nine Chinese strains. The complete ORF3 gene sequence and phylogenetic analysis showed that all Korean PEDV field isolates (except DBI865) have a close relationship to Chinese field strains and differ genetically from European PEDV strains. The Korean PEDV field isolates (except DBI865) are also genetically different from the vaccine strains (attenuated DR13, KPED-9 and P-5V), which have been used for prevention of PEDV infection in Korea. In other words, recent prevalent Korean PEDV field isolates represent a new genotype that differs from the genotype including the vaccine strains.

PED vaccines are commonly used in Korea as losses caused by PEDV infection increase. However, most of the Korean PEDV field isolates analyzed in this study differ from members of the group that includes the vaccine strains, and only a few isolates belonged to that group. These results may be attributable to immune pressure due to widespread vaccine use in Korea. In addition, this reflects the existence of genetic diversity among the Korean PEDV field isolates and raises questions as to whether a new type of PEDV vaccine may be necessary for more effective prevention of PEDV infection.

The DBI865 isolate has high sequence identity and a close phylogenetic relationship to the vaccine strains according to genetic and phylogenetic analysis of the complete ORF3 gene, and this result implies that it might be derived from a vaccine strain. Although the mechanism by which the DBI865 isolate emerged is unclear, two possibilities are suggested: (1) the DBI865 isolate might have evolved from a vaccine strain; (2) DBI865 isolate might have been produced in nature through recombination between a PEDV field isolate and a vaccine strain. Of these two possibilities, the latter is more likely because the partial S and ORF3 genes of the DBI865 isolate are closely

related to those of G1-1 (which is genetically different from the vaccine strains) [20] and group 2 (which are genetically similar to the vaccine strains). This is similar to what was shown for other Korean field isolates used in this study. In the case of the e1642 and M2366 isolates, partial S [20] and ORF3 genes were different from those of the vaccine strains, and the M genes were similar to those of the vaccine strains. The partial S gene of the e1697 isolate differs from that of the vaccine strains [20], and the M gene is closely related to those of the vaccine strains. However, despite trying several times to amplify the complete M gene of the DBI865 isolate, we did not obtain any amplicons from this isolate, and for that reason, further studies, such as full-length sequencing as well as complete M gene sequencing of the DBI865 isolate, are needed to determine how the DBI865 isolate originated.

The present study allows a better understanding of the molecular epidemiology, genetic diversity, and phylogenetic relationships of Korean PEDV field isolates with other PEDV reference strains. We expect that the results of this study will help to prevent and control PEDV infection more effectively.

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