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Cloning and further sequence analysis of the ORF3 gene of wild- and attenuated-type porcine epidemic diarrhea viruses

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Abstract The open reading frame (ORF3) genes of the parent DR13, attenuated DR13, KPED-9, P-5V, and 12 field samples were cloned and sequenced to further explore the functions of wild- and attenuated-type porcine epidemic diarrhea viruses (PEDVs). Sequencing revealed that wild-type PEDVs ORF3 genes 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. Attenuated-type PEDVs ORF3 genes had a single ORF of 624 nucleotides encoding a protein of 207 amino acids with a predicted M_r of 23.4 kDa. The coding region of the ORF3 gene of attenuated-type PEDVs including attenuated DR13, KPED-9, and P-5V had 51 nucleotide deletions that were not found in the ORF3 genes of wild-type PEDVs including CV777, Br1/87, LZC, parent DR13, and 12 field samples. In addition, attenuated-type PEDVs have previously been found to exhibit reduced pathogenicity in pigs. Therefore, 51 nucleotide deletions appear to be meaningful and may be significant for PEDV pathogenicity, because they lead to changes in the predicted amino acid sequences of attenuated-type PEDVs. Reverse transcriptase-polymerase chain reaction (RT-PCR) on the partial ORF3 gene including 51 nucleotide deletions revealed that all PEDVs fell into two types, wild- and attenuated-type PEDVs. Wild-type PEDVs containing parent DR13 and 12 field samples had RT-PCR products of 245 bp in size, while attenuated-type PEDVs containing PEDV vaccine strains (attenuated DR13, KPED-9, P-5V) had products of 194 bp. In addition, all PEDV vaccine strains were used as live virus vaccine, because they previously exhibited a reduced pathogenicity in pigs. Therefore, large deletion region, which is comprise 17 amino acid deletions caused by 51 nucleotide deletions and is seen in all PED live vaccine strains, may be important site for PEDV pathogenicity, and we can use it for differentiation of wild- and attenuated-type PEDVs.

Keywords Porcine epidemic diarrhea virus · ORF3 gene · Cloning · Pathogenicity · RT-PCR

Nucleotide sequence data reported is available in the GenBank database under the accession No. EU054929 and EU054930.

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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, 2]. Since then, outbreaks of the disease have been reported in many swine-producing countries, notably in Europe and Asia, including Japan, China, and Korea [3]. PEDV causes a devastating enteric disease with acute diarrhea, dehydration, and significant mortality in swine, resulting in heavy economic losses in Europe and Asia [4, 5].

Although serologically unrelated, PEDV and transmissible gastroenteritis virus (TGEV) cause digestive-tract infections that are extremely difficult to clinically differentiate [6–9]. Both viruses belong to the family Coronaviridae.

Genetic changes were reported in the open reading frame (ORF3) of a high cell cultured TGEV [10, 11]. The changes appear to have resulted from high passage of the virus through cell cultures. Virulence of TGEV in piglets was reduced through serial passage in cell cultures [11]. Similarly, many coronaviruses have insertions or deletions in the ORF3 gene. Some investigators have suggested this area of the genome might be involved in tropism and pathogenicity of TGEV [12–15].

Previous studies revealed that the ORF3 gene of PEDV has an unexpected genetic variability [16]. Especially, wild-type and cell culture adapted PEDVs almost have complete sequence identity with the exception of variations and truncations in the ORF3 gene observed, exclusively, in the cell culture adapted PEDV [16–19]. Similarly, the highly adapted PEDV, attenuated DR13, was differentiated from wild-type PEDVs by both reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP), which used sequence variations in the ORF3 gene of the highly adapted PEDV [20]. Moreover, the ORF3 gene has been suggested as an important determinant for PEDV biological properties.

The loss of ORF3 product demonstrated an unexpected feature caused by adaptation in cell culture [19, 21], which may explain why the adaptation of PEDV in cell culture may reduce the virulence of wild-type virus. Wild-type and cell culture adapted PEDVs differed in their abilities to cause diarrhea in neonate piglets [18, 22]. Piglets inoculated orally with CV777, wild-type PEDV, became sick and developed severe diarrhea [2]. However, Piglets inoculated with KPEDV-9, which is serially passaged in Vero cells, showed reduced disease and lesions [23]. Similarly, attenuated DR13, which is highly adapted to cell culture, exhibited reduced pathogenicity and induced immunogenicity in pigs [20]. These changes were supposed to result from adaptation and attenuation through serial passage in Vero cell cultures [20, 23].

In the present study, we constructed DNA clones of the parent DR13, attenuated DR13, KPED-9, P-5V, and 12 field samples ORF3 gene. In order to elucidate the genetic basis of the markedly different wild- and attenuated-type PEDV phenotypes, the nucleotide and deduced amino acid sequences of the ORF3 gene of parent DR13, attenuated DR13, KPED-9, P-5V, and 12 field samples were determined and were further analyzed and aligned with those of reference PEDV strains [16]. Furthermore, RT-PCR based on the ORF3 gene differences of wild- and attenuated-type PEDVs helped to differentiate wild- and attenuated-type PEDVs.

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).

Porcine epidemic diarrhea virus (PEDV) strain named DR13 was isolated from the intestinal tissues of piglets suspected with porcine epidemic diarrhea (PED), which had been submitted to the Department of Veterinary Medicine Virology Laboratory, College of Veterinary Medicine, Seoul National University, Seoul, Korea. Intestinal samples were made into 10% (v/v) suspensions through homogenization with phosphate buffered saline (PBS; 0.1 M, pH 7.2). The suspensions were vortexed and clarified by centrifugation for 10 min at $4{,}800 \times g$. 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) were removed and the cells were washed thrice with PBS (pH 7.4). Cells were inoculated with 1 ml per flask of the supernatants. After adsorption at 37°C for 1 h, the cells were incubated in α-MEM supplemented with 0.02% yeast extract, 0.3% tryptose phosphate broth, and 2 μg of trypsin, as described previously [20, 24]. The PEDV DR13 was continuously passaged in Vero cells. Sequential passage of virus was regularly conducted every 4–5 days postinfection in cells. The supernatant was harvested and used for next inoculation in Vero cells up to 100 passages. PEDV was identified through RT-PCR method [25]. Attenuated DR13 strain made through process described above was used for manufacturing commercial vaccine such as the Korean PED oral vaccine (Green Cross Veterinary Product Co., Ltd., Yong-In, Korea).

KPED-9 and P-5V used for the present study are two commercially available vaccine strains. KPED-9 strain was isolated from a neonatal pig in Korea, and was serially passaged in Vero cell cultures up to passage level 93 and became a candidate of live PEDV vaccine [23]. P-5V strain didn't have the paper about conducting serial passage or not, but is known to be originally isolated in Japan. KPED-9 was used for manufacturing the Korean PED live virus vaccine (Green Cross Veterinary Product Co., Ltd., Yong-In, Korea) and P-5V was used for the Japanese PED live virus vaccine (Nisseiken Co., Ltd., Tokyo, Japan). KPED-9 strain was kindly provided by the Green Cross Veterinary Products Co., Ltd. (Suwon, South Korea) and P-5V strain was provided by Nisseiken regional distributor in Korea.

A total of 12 porcine samples (from 12 farms) consisting of feces or intestinal contents, which had been taken from dead or sick young piglets showing watery diarrhea and dehydration, were submitted to the Department of Veterinary Medicine Virology Laboratory, College of Veterinary Medicine, Seoul National University, Seoul, Korea. Each commercial farm comprised at least 300 sows. All samples

(feces and intestinal contents) had been confirmed positive for PEDV by RT-PCR method [25].

Porcine epidemic diarrhea virus (PEDV) positive fecal samples were diluted with PBS (pH 7.2) to be 10% suspensions and PEDV positive intestinal contents were made into 10% suspensions through homogenization with PBS.

Infected cell cultures were prepared for the extraction of viral RNA. Infected cells were harvested when the cells reached 70–80% cytopathic effect (CPE). RNA was extracted from infected cells using TRIzol LS (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. The extracted 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.

Viral RNAs were extracted from the KPED-9, P-5V strains and the suspensions of the 12 field samples (PEDV positive fecal and intestinal samples) according to the method described above.

Published primers [20] designed based on the published sequences of spike (S) and small membrane (sM) genes were used for generating the full ORF3 gene of PEDV. Briefly, forward primer (ORF3–1), 5′-TCCTAGACTT CAACCTTACG-3′, and reverse primer (ORF3-2), 5′-GGTGACAAGTGAAGCACAGA-3′, were used for the amplification of the full ORF3 gene of PEDV. The size of expected product was 830 bp.

For reverse transcription, 10 μl of extracted RNA and 1 μl of random primer (hexa-deoxyribonucleotide mixture (TaKaRa BIO INC., Japan)) 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 5× 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 full ORF3 gene of PEDV. Exactly, 2 μl of cDNA was mixed with a reaction mixture containing 2.5 μl of 10× 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, CA). The 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 10 min, and then held at 4°C. The RT-PCR products were visualized by electrophoresis in 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.

Purified RT-PCR products corresponding to the full ORF3 gene were cloned using a QIAGEN PCR Cloning^{plus} Kit (QIAGEN), as described previously [26]. The cloned DNAs were extracted using the Wizard® Plus Minipreps DNA Purification System (Promega). Restriction enzyme digestion, with enzymes such as EcoRI, followed by electrophoresis through 1.5% agarose gels was employed for identification of recombinant DNA clones.

Purified RT-PCR products corresponding to the full ORF3 gene were cloned twice or thrice, as described above, and all ORF3 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 to verify the accuracy of the sequence data.

Nucleotide and deduced amino acid sequences were analyzed with the CLUSTALX v1.83 program and MegAlign software (DNAStar Inc., Madison, WI, USA). The ORF3 gene nucleotide and deduced amino acid sequences of the parent DR13 (GenBank accession No. EU054929), attenuated DR13 (GenBank accession No. EU054930), KPED-9, P-5V, and 12 field samples were compared with the PEDV CV777 (Br1/87) (EMBL accession No. Z24733) [16] and LZC (GenBank accession No. EF185992) strains.

A pair of sense and antisense primers was designed and aligned based on the nucleotide sequences of the ORF3 gene of CV777 (Br1/87) [16, 27], LZC, parent DR13, attenuated DR13 from the GenBank database (National Center for Biotechnology Information, USA) as well as those of KPED-9, P-5V and 12 field samples. These primers were used to generate cDNA for the partial ORF3 gene including large deletion region. The nucleotide sequences, locations and PCR product sizes of the primers are shown in Table 1.

Table 1 Primers for differentiation of wild- and attenuated-type PEDVs

Primer	Nucleotide sequence (5′ → 3′)	Position (on the ORF3 gene)	Mers	%GC	Strand	Wild-type product (bp)	Attenuated-type product (bp)
PEDO1	GATGCTGTCCAAGAGTTGGA	76–95	20	50.0	+	245	194
PEDO2	CAAAGCCTGCCAATAAGTGT	301–320	20	45.0	–		

Reverse Transcriptase (RT) was carried out as described above. In PCR, strategy such as amplification of the partial ORF3 gene including large deletion region was used to differentiate wild- and attenuated-type PEDVs through RT-PCR product sizes. PCR was also carried out as described above with simple modifications. The PCR was performed at 94°C for 5 min followed by 35 cycles of 94°C 20 s, 52°C 20 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 1.5% agarose gel containing ethidium bromide. The sizes of expected products of wild- and attenuated- type PEDVs were 245 and 194 bp, respectively.

In order to analyze the usefulness of this RT-PCR from the viewpoint of differentiation of wild- and attenuated-type PEDVs, the reaction was performed with the following agents: parent DR13, commercial vaccine strains (attenuated DR13 strain of the Korean PED oral vaccine, KPED-9 strain of the Korean PED live virus vaccine, P-5V strain of the Japanese PED live virus vaccine). And the 12 field samples, which were previously confirmed positive for PEDV, were analyzed by RT-PCR on the partial ORF3 gene including large deletion region.

In order to synthesize ds-cDNA of the parent DR13, attenuated DR13, KPED-9, P-5V and 12 field sample ORF3 gene, each DNA fragments were amplified by RT-PCR using a proper pair of sense (ORF3-1) and antisense (ORF3-2) primers. The DNAs of wild-type PEDVs including parent DR13 and 12 field samples, designated as Ofrag I (830 bp), Ofrag V–XVI (830 bp), and the DNAs of attenuated-type PEDVs including attenuated DR13, KPED-9 and P-5V, designated as Ofrag II–IV (779 bp) were each cloned into the pDrive Cloning Vector DNA (Fig. 1) and subjected to sequencing.

Nucleotide and deduced amino acid sequences of the parent DR13, attenuated DR13, KPED-9, P-5V, and 12 field sample ORF3 gene were determined and compared with the sequences of reference PEDV strains (Fig. 2). This revealed that the nucleotide sequences encoding the entire ORF3 gene of wild-type PEDVs including CV777, Br1/87, LZC, parent DR13, and 12 field samples contain a single 675-base ORF starting with an initiator, ATG, at position 1 nt and ending with a terminator, TGA, at position 673 nt. Prediction of molecular weight using the ExPASy (Expert Protein Analysis System) Proteomics Server of the Swiss Institute of Bioinformatics (SIB) revealed that wild-type PEDVs ORF3 genes encode a protein of 224 amino acids with a predicted M_r of 25.1–25.3 kDa.

Attenuated-type PEDVs including attenuated DR13, KPED-9, and P-5V contain a single 624-base ORF starting with an initiator, ATG, at position 1 nt and ending with a terminator, TGA, at position 622 nt. Attenuated-type PEDVs ORF3 genes encode a protein of 207 amino acids with a predicted M_r of 23.4 kDa.

Wild- and attenuated-type PEDVs had significant differences in their nucleotide and deduced amino acid sequences. Unlike wild-type PEDVs including CV777, Br1/87, LZC, parent DR13, and 12 field samples, attenuated-type PEDVs including attenuated DR13, KPED-9, and P-5V had 54 specific nucleotide and 17 specific deduced amino acid sequence changes.

More precisely, attenuated-type PEDVs had 17 specific deduced amino acid sequence deletions (at positions 82–98), which were produced by 51 nucleotide sequence deletions (at positions 245–295) and were not found in wild-type PEDVs.

Nucleotide and deduced amino acid sequence homology results are described in Table 2. We found that wild-type PEDVs ORF3 genes have 95.6–100% DNA sequence identities with each other and they have 89.3–91.1% DNA sequence identities with attenuated-type PEDVs. Likewise, they have 94.2–100% homologies with the deduced amino

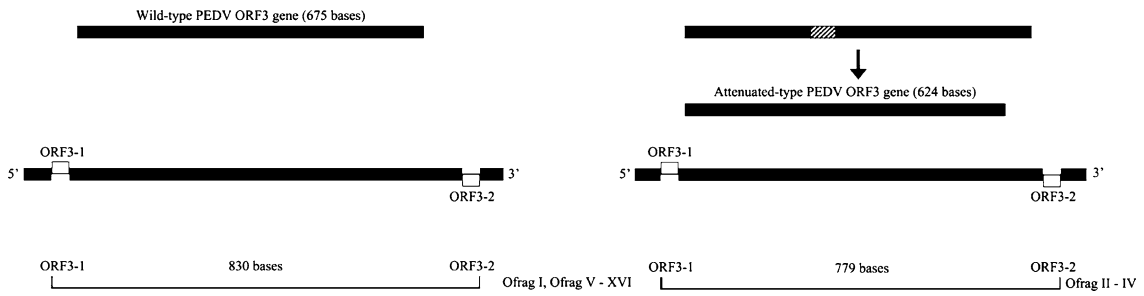

Fig. 1 Construction of cDNA clones for the full-length ORF3 gene of wild- and attenuated-type PEDVs by RT-PCR using a pair of sense (ORF3-1) and antisense (ORF3-2) primers: diagrammatic representation of the ORF3 gene of viral RNA (long solid rectangle) show primer-binding sites (small open rectangles) and large nucleotide deletion legion (stripe rectangles); 16 DNA fragments of wild- and attenuated-type PEDVs amplified by RT-PCR and cloned into the pDrive Cloning Vector are denoted as recombinant DNA clones Ofrag I–XVI. Wild-type PEDVs: Ofrag I (Parent DR13), Ofrag V (DBI825) Ofrag VI (BI976), Ofrag VII (BI1166), Ofrag VIII (M1763), Ofrag IX (M1764), Ofrag X (e1834), Ofrag XI (e2540), Ofrag XII (BI2804), Ofrag XIII (e3991), Ofrag XIV (PF4275), Ofrag XV (M4758), Ofrag XVI (e8066) Attenuated-type PEDVs: Ofrag II (Attenuated DR13), Ofrag III (KPED-9), Ofrag IV (P-5V)

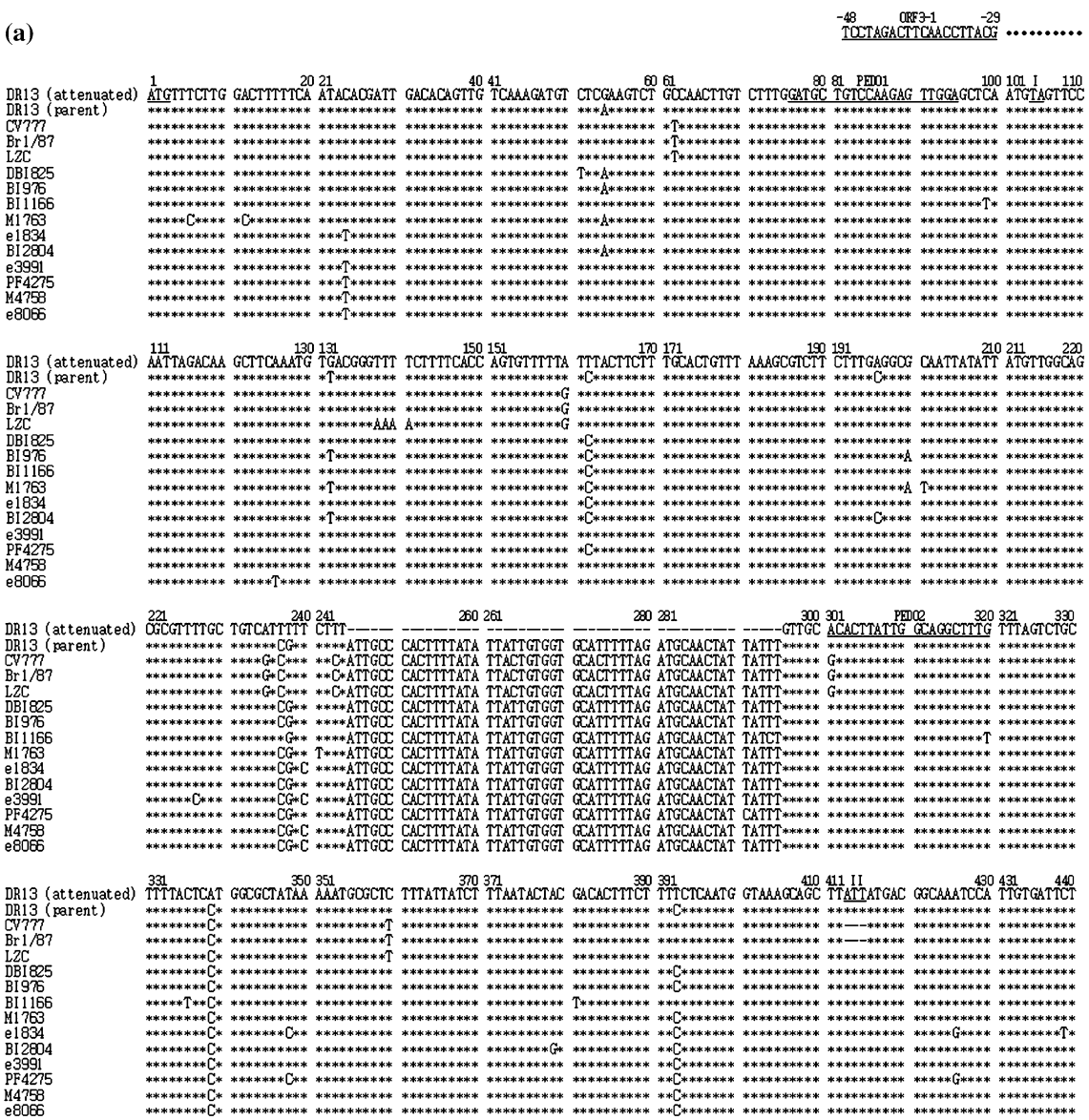

Fig. 2 Comparison of the (**a**) nucleotide and (**b**) deduced amino acid sequences of the ORF3 gene of wild- and attenuated-type PEDVs. Asterisks represent (**a**) nucleotides and (**b**) amino acids that are identical to those in the attenuated DR13 (GenBank accession No. EU054930). Dashed lines represent missing (**a**) nucleotides and (**b**) amino acids compared to the PEDV CV777 (Br1/87) (EMBL accession No. Z24733), LZC (GenBank accession No. EF185992), parent DR13 (GenBank accession No. EU054929) and 12 field samples. Start codon ATG and stop codon TGA are underlined. Regions corresponding to the primers used for cloning and differentiation of wild- and attenuated-type PEDVs are underlined and labeled above the sequence as ORF3-1-2 and PEDO1-2. Three variable regions previously reported are underlined and labeled above the sequence as Roman numbers, I–III. PEDVs in parentheses below had identical nucleotide and amino acid sequences with that in front of parentheses in the ORF3 genes and especially DBI825 and M4758 underlined below had different nucleotide but identical amino acid sequences with parent DR13. Wild-type PEDVs: CV777, Br1/87, LZC, Parent DR13, DBI825, BI976, BI1166, M1763 (M1764), e1834 (e2540), BI2804, e3991, PF4275, M4758, e8066 Attenuated-type PEDVs: Attenuated DR13 (KPED-9, P-5V)

acid sequences of each other and they have 88.4–92.0% homologies with the deduced amino acid sequences of attenuated-type PEDVs.

Attenuated-type PEDVs ORF3 genes have 100% DNA sequence identities with each other and they have 100% homologies with the deduced amino acid sequences of each other.

Wild- and attenuated-type PEDVs were rapidly identified through RT-PCR on the partial ORF3 gene including large deletion region. The primers amplified products of the expected sizes, 245 and 194 bp, from wild-type PEDV (parent DR13) and attenuated-type PEDV (attenuated DR13), respectively, which could be easily distinguished by agarose gel electrophoresis (Fig. 3).

Fig. 2 continued

In the analysis of usefulness of this RT-PCR method, signal was detected with the following PEDVs; parent DR13, attenuated DR13 strain of the Korean PED oral vaccine, KPED-9 strain of the Korean PED live virus vaccine, P-5V strain of the Japanese PED live virus vaccine. While wild-type PEDV, parent DR13, had 245 bp fragment, attenuated-type PEDVs, attenuated DR13, KPED-9, P-5V, had 194 bp fragments (Fig. 4). In addition, 12 field samples had 245 bp fragments as that of the wild-type PEDV (Fig. 5).

The full ORF3 genes of the parent DR13, attenuated DR13, KPED-9, P-5V, and 12 field samples were successfully cloned and sequenced. The sequencing results showed that wild-type PEDVs ORF3 genes 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. Attenuated-type PEDVs ORF3 genes had a single ORF of 624 nucleotides encoding a protein of 207 amino acids with a predicted M_r of 23.4 kDa. A single ORF of 675 nucleotides, with the potential to encode the coronavirus ORF3 protein, was identified [16, 28]. But, an ORF of 624 nucleotides, with the potential to encode the coronavirus ORF3 protein, was first identified. All PEDV ORF3 genes including wild- and attenuated-type PEDVs had a sequence (CTAGAC) of 46 nucleotides upstream of the initiator ATG, as previously recognized in Br1/87 [29]. This sequence is a hexameric motif common to coronaviruses and is similar to the hexameric motifs XUA(A/G)AC found adjacent to other PEDV ORFs. These hexameric motifs have been proposed as a starting site for the transcription of the subgenomic mRNAs [28].

Previous studies showed that wild-type and cell culture adapted PEDV exhibit remarkably different phenotypes in terms of pathogenicity in piglets [18, 20, 22]. Moreover, those two PEDV types have genetic differences that various deletions, which were observed in the ORF3 region (designated as variable region I, II, III) of the cell culture adapted PEDV, were not detected in the wild-type PEDV [16, 19]. However, these genetic differences may not be crucial for pathogenicity, because these various deletions observed in variable region I, II, III were not found in the attenuated-type PEDVs including attenuated DR13, KPED-9 and P-5V strains.

The coding region of the ORF3 genes of attenuated-type PEDVs including attenuated DR13, KPED-9, and P-5V had nucleotide and amino acid differences compared to wild-type PEDVs including CV777, Br1/87, LZC, parent DR13 and 12 field samples as described above. Out of all differences, only 51 nucleotide deletions, which were not found in the ORF3 genes of wild-type PEDVs, appear to be

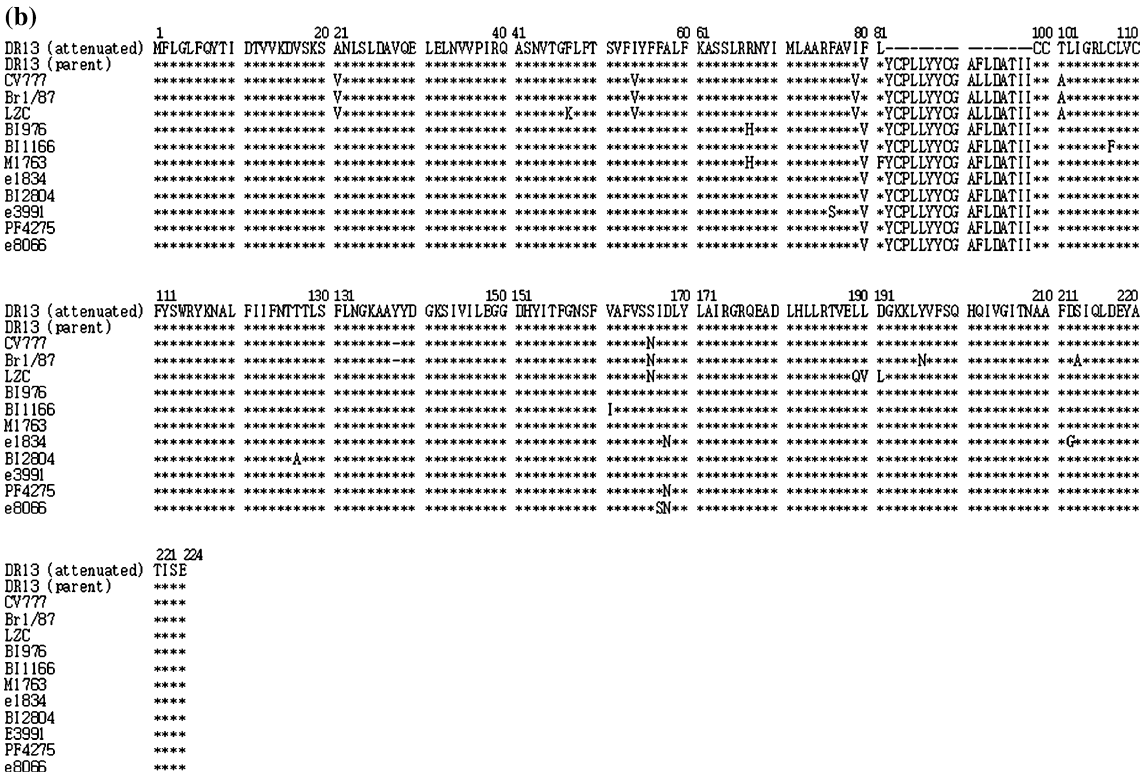

Fig. 2 continued

meaningful and may be significant for PEDV pathogenicity because they lead to changes in the predicted amino acid sequences of attenuated-type PEDVs. In addition, attenuated-type PEDVs (attenuated DR13, KPED-9) exhibited reduced pathogenicity in pigs when subjected to a high number of serial passages in Vero cell cultures [20, 23]. P-5V didn't have the papers on exhibiting reduced pathogenicity in pigs when subjected to a high number of serial passages in cell cultures but safety test is a mandatory clause for receiving the vaccine licence from Japanese and Korean governments.

Sequence homology analysis of the ORF3 genes indicated that wild-type PEDVs including CV777, Br1/87, LZC, parent DR13 and 12 field samples had homology differences at the nucleotide and deduced amino acid sequence levels, compared to attenuated-type PEDVs including attenuated DR13, KPED-9, and P-5V, as described above. Parent DR13 was highly homologous to wild-type PEDVs rather than to attenuated-type PEDVs including attenuated DR13, even though it is the origin of the attenuated DR13. In addition, these homology differences, which were caused by deletions and were shown in wild- and attenuated-type PEDVs, could well imply that the ORF3 product is not required for replication of PEDV in cell culture [19, 21]. Moreover, wild-type PEDVs including CV777, Br1/87, LZC, parent DR13 and 12 field samples had high pathogenicity and maximum possible length of the ORF3 genes, the only form which could be detected in wild-type PEDVs. Attenuated-type PEDVs including attenuated DR13, KPED-9, and P-5V had reduced pathogenicity and ORF3 genes, which contain 17 amino acid deletions produced by 51 nucleotide deletions and could be detected in attenuated-type PEDVs. Therefore, the facts described above could well imply that ORF3 may be of importance in vivo [21] and postulated 17 amino acid deletions, which were produced by 51 nucleotide deletions observed in the ORF3 genes of attenuated-type PEDVs and were caused in process of adaptation to serial propagation in cell culture conditions, may influence the pathogenicity of PEDV.

Reverse transcriptase-polymerase chain reaction (RT-PCR) method on the partial ORF3 gene including 51 nucleotide deletions revealed that all PEDVs fell into two types, wild- and attenuated-type PEDVs. According to those results described above, wild-type PEDVs containing parent DR13 and 12 field samples had RT-PCR products of 245 bp in size, while attenuated-type PEDVs containing PEDV vaccine strains (attenuated DR13, KPED-9, P-5V) had products of 194 bp. In addition, all PEDV vaccine strains were used as live virus vaccine because they previously exhibited reduced pathogenicity in pigs. Moreover, this RT-PCR require less labor, less money, and less

Table 2 Nucleotide and deduced amino acid sequence homology of the ORF3 gene of wild- and attenuated-type PEDVs

	PEDV		Percentage identity (%)[a]																	
			Wild-type PEDVs															Attenuated-type PEDVs		
		No[c]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Percentage identity (%)[b]	Wild-type PEDVs	1	***	99.7	98.7	96.9	97.9	97.0	96.6	96.4	96.4	96.4	96.4	96.7	97.2	96.7	97.3	97.0	90.2	90.2	90.2
		2	99.1	***	98.4	96.6	96.7	96.7	96.3	96.1	96.1	96.1	96.1	96.4	96.9	96.4	97.0	96.7	89.9	89.9	89.9
		3	98.2	97.3	***	96.0	96.1	96.1	95.7	95.6	95.6	95.6	95.6	95.9	96.3	95.9	96.4	96.1	89.3	89.3	89.3
		4	96.4	95.5	95.1	***	99.3	99.6	97.9	99.0	99.0	98.1	98.1	99.9	98.8	98.4	99.0	98.4	90.7	90.7	90.7
		5	96.4	95.5	95.1	100	***	99.4	98.1	98.8	98.8	98.2	98.2	99.1	99.0	98.5	99.1	98.5	90.8	90.8	90.8
		6	96.0	95.1	94.6	99.6	99.6	***	98.1	99.4	99.4	98.2	98.2	99.4	99.0	98.5	99.1	98.5	90.8	90.8	90.8
		7	95.5	94.6	94.2	99.1	99.1	98.7	***	97.5	97.5	97.5	97.5	97.8	97.9	97.8	98.1	97.8	90.8	90.8	90.8
		8	95.5	94.6	94.2	99.1	99.1	99.6	98.2	***	100	97.6	97.6	98.8	98.4	97.9	98.5	97.9	90.2	90.2	90.2
		9	95.5	94.6	94.2	99.1	99.1	99.6	98.2	100	***	97.6	97.6	98.8	98.4	97.9	98.5	97.9	90.2	90.2	90.2
		10	95.5	94.6	94.2	99.1	99.1	98.7	98.2	98.2	98.2	***	100	97.9	98.7	99.4	98.8	98.8	90.2	90.2	90.2
		11	95.5	94.6	94.2	99.1	99.1	98.7	98.2	98.2	98.2	100	***	97.9	98.7	99.4	98.8	98.8	90.2	90.2	90.2
		12	96.0	95.1	94.6	99.6	99.6	99.1	98.7	98.7	98.7	98.7	98.7	***	98.7	98.2	98.8	98.2	90.5	90.5	90.5
		13	96.0	95.1	94.6	99.6	99.6	99.1	98.7	98.7	98.7	98.7	98.7	99.1	***	98.7	99.9	99.3	91.0	91.0	91.0
		14	96.0	95.1	94.6	99.6	99.6	99.1	98.7	98.7	98.7	99.6	99.6	99.1	99.1	***	98.8	98.8	90.7	90.7	90.7
		15	96.4	95.5	94.6	100	100	99.6	99.1	99.1	99.1	99.1	99.1	99.6	99.6	99.6	***	99.4	91.1	91.1	91.1
		16	95.5	94.6	95.1	99.1	99.1	98.7	98.2	98.2	98.2	99.1	99.1	98.7	98.7	99.6	99.1	***	90.8	90.8	90.8
	Attenuated-type PEDVs	17	89.7	88.8	88.4	92.0	92.0	91.5	91.1	91.1	91.1	91.1	91.1	91.5	91.5	91.5	92.0	91.1	***	100	100
		18	89.7	88.8	88.4	92.0	92.0	91.5	91.1	91.1	91.1	91.1	91.1	91.5	91.5	91.5	92.0	91.1	100	***	100
		19	89.7	88.8	88.4	92.0	92.0	91.5	91.1	91.1	91.1	91.1	91.1	91.5	91.5	91.5	92.0	91.1	100	100	***

[a] Percentage of nucleotide identity (upper triangle)

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

[c] Number of virus: 1. CV777, 2. Br1/87, 3. LZC, 4. Parent DR13, 5. DBI825, 6. BI976, 7. BI1166, 8. M1763, 9. M1764, 10. e1834, 11. e2540,12. BI2804, 13. e3991, 14. PF4275, 15. M4758, 16. e8066, 17. Attenuated DR13, 18. KPED-9, 19. P-5V

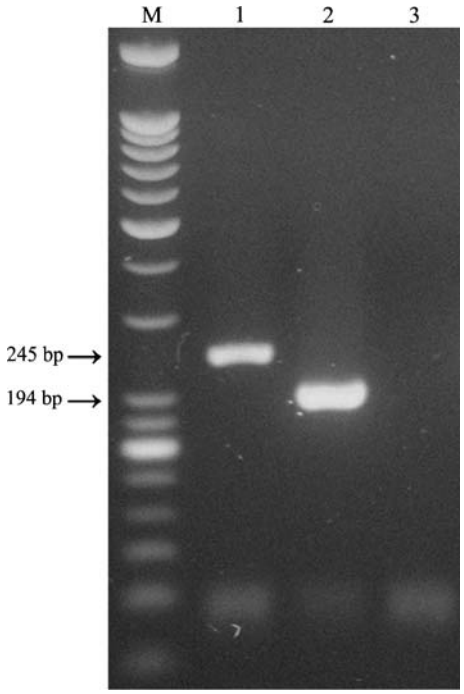

Fig. 3 RT-PCR on the partial ORF3 gene including large deletion region. From left to right: Lane M, 25/100 bp DNA ladder; Lane 1, parent DR13; Lane 2, attenuated DR13; Lane 3, negative control

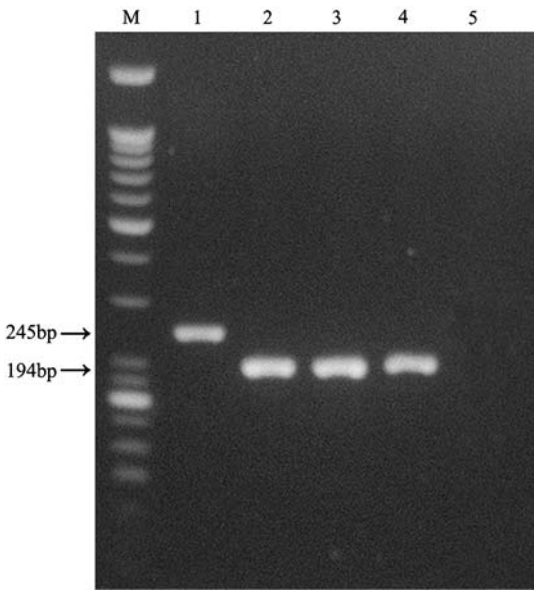

Fig. 4 RT-PCR on the commercial vaccine strains used in Korea. From left to right: Lane M, 25/100 bp DNA ladder; Lane 1, parent DR13; Lane 2, attenuated DR13 strain of the Korean PED oral vaccine; Lane 3, KPED-9 strain of the Korean PED live virus vaccine; Lane 4, P-5V strain of the Japanese PED live virus vaccine; Lane 5, negative control

analysis time compared with the previously established method, which differentiates the highly adapted PEDV from wild-type PEDVs by sequential use of both RT-PCR

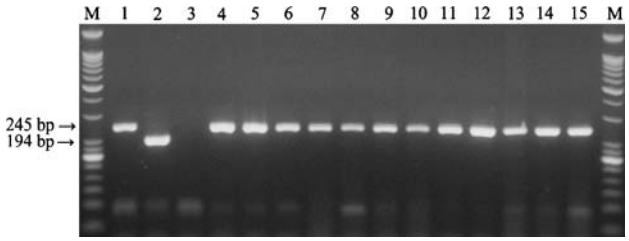

Fig. 5 RT-PCR on the 12 field samples. From left to right; Lane M, 25/100 bp DNA ladder; Lane 1, parent DR13; Lane 2, attenuated DR13; Lane 3, negative control, Lane 4, DBI825; Lane 5, BI976; Lane 6, BI1166; Lane 7, M1763; Lane 8, M1764; Lane 9, e1834; Lane 10, e2540; Lane 11, BI2804, Lane 12, e3991; Lane 13, PF4275; Lane 14, M4758; Lane 15 e8066

and RFLP [20]. Therefore, large deletion region, which is comprised of 17 amino acid deletions caused by 51 nucleotide deletions and is seen in all PED live vaccine strains, may be crucial for PEDV pathogenicity and we can use it for differentiation of wild- and attenuated-type PEDVs.

In the present study, the complete nucleotide and deduced amino acid sequences of the ORF3 gene of the parent DR13, attenuated DR13, KPED-9, P-5V and 12 field samples were determined and compared to reference PEDV strains, to find determinants of PEDV pathogenicity. We first found an ORF3 of 624 nucleotides having 51 nucleotide deletions compared to wild-type PEDVs and this deletion region might be important site of PEDV pathogenicity. In addition, RT-PCR using that deletion region is very useful to differentiate wild- and attenuated-type PEDVs. Moreover, the complete nucleotide and deduced amino acid sequences of the ORF3 gene of the parent DR13, attenuated DR13, KPED-9, P-5V and 12 field samples will now form the basis for further functional exploration of both wild- and attenuated-type PEDVs. However, a clear correlation between specific genomic differences and pathogenicity is only possible with manipulation of an infectious clone of the virus. Therefore, further large scale experiments through construction of an infectious clone of virus will be needed for the functional studies of PEDV.

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