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# Differentiation of a Vero cell adapted porcine epidemic diarrhea virus from Korean field strains by restriction fragment length polymorphism analysis of ORF 3

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## Abstract

A porcine epidemic diarrhea virus (PEDV) designated DR13 was isolated in Vero cells and serially passaged by level 100. The virus was titrated at regular intervals of the passage level. Open reading frame (ORF) 3 sequences of the virus at passage levels 20, 40, 60, 80, and 100 were aligned and compared using a computer software program. Suitability of the restriction fragment length polymorphism (RFLP) analysis for differentiating the virus from other Korean field strains was investigated. The DR13 field isolate was successively adapted in Vero cells as observed through polymerase chain reaction (PCR) and titration of the virus. RFLP analysis identified change in cleavage sites of *Hind*III and *Xho* II from passage levels 75 and 90, respectively; these RFLP patterns of ORF 3 differentiated the Vero cell-adapted virus from its parent strain, DR13, and 12 other strains of PEDV studied. The cell adapted DR13 was tested for its pathogenicity and immunogenicity in piglets and pregnant sows. The results indicated that cell adapted DR13 revealed reduced pathogenicity and induced protective immune response in pigs. Differentiation between highly Vero cell-adapted virus and wild-type virus could be the marker of adaptation to cell culture and a valuable tool for epidemiologic studies of PEDV infections. The results of this study supported that the cell attenuated virus could be applied as a marker vaccine candidate against PEDV infection.

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**Keywords:** Porcine epidemic diarrhea virus; Attenuation marker; RFLP pattern

## 1. Introduction

Porcine epidemic diarrhea virus (PEDV), a member of the family *Coronaviridae*, is an enveloped, single-stranded RNA virus [5,9,16], causing severe entero-pathogenic diarrhea in swine and incurring heavy economic losses in Asia [6,18]. PEDV and transmissible gastroenteritis virus (TGEV), although serologically not related [17,20,23], are known as causative agents in digestive tract infections. Clinically, differentiation of these two virus infections is extremely difficult. Both viruses belong to the family *Coronaviridae*.

Genetic changes were reported in the open reading frame (ORF) 3 of highly cell-cultured TGEV [4,25]. 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 [25]. And ORF 3 has been suggested as an important determinant for the

virulence of the virus. PEDV consists of spike (S), membrane (M), small membrane (sM), ORF 3, and nucleocapsid (N) genes, and all have been sequenced [2,7]. The loss of ORF 3 product demonstrated an unexpected feature caused by the adaptation in cell culture [3,21], which may explain why the adaptation of PEDV in cell culture may reduce the virulence of wild-type virus. Piglets inoculated with a PEDV serially passaged in Vero cells showed reduced disease and lesions [13]. Therefore, unexpected features observed through cell culture is of much interest.

In this study, a PEDV was isolated from a field specimen, designated DR13, and serially passaged in Vero cell until level 100. Nucleotide sequence of ORF 3 was aligned and compared as passage level for the identification of markers. In addition, to differentiate the virus from other field isolates, restriction fragment length polymorphism (RFLP) analysis of ORF 3 was performed. Further we discussed the derivation of DR13 of PEDV, as a vaccine candidate that could be differentiated from wild-type viruses and a vaccine strain.

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## 2. Materials and methods

### 2.1. Cells and viruses

Continuous Vero cell line (ATCC, CCL-81) was regularly maintained in  $\alpha$ -minimum essential medium ( $\alpha$ -MEM) supplemented with 5% fetal bovine serum, penicillin (100 units/ml), streptomycin (100  $\mu$ g/ml), and amphotericin B (0.25  $\mu$ g/ml). The cell passaged PEDV, KPEDV-9 strain [13] kindly provided by the Green Cross Veterinary Product Co. Ltd. (Suwon, South Korea) for manufacturing live PEDV vaccine by the Korean government, was used.

### 2.2. PEDV isolation and serial passage

Intestines were collected from suckling pigs 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. They were made into 10% (v/v) suspension through homogenization with phosphate buffered saline (PBS; 0.1 M, pH 7.2). Suspensions were vortexed and clarified by centrifugation for 10 min at  $4800 \times g$ . Supernatants passed through a 0.2  $\mu$ m syringe filter (Acrodisk, Gelman) were used for virus isolation in Vero cells. Prior to inoculation, growth medium of the confluent cells grown in 25 cm<sup>2</sup> flask (Falcon, USA) was removed, and the cells were washed three times with PBS. One milliliter of the supernatant per flask was then inoculated into the cells. After adsorption at 37 °C for 1 h, the cells were incubated in  $\alpha$ -MEM supplemented with 0.02% yeast extract, 0.3% tryptose phosphate broth, and 2  $\mu$ g of trypsin as described previously [10]. Serial passages of the DR13 isolate of PEDV, were continued in a 25 cm<sup>2</sup> flask by level 100 according to the method described above. PEDV was identified through the reverse transcription-polymerase chain reaction (RT-PCR) [11].

### 2.3. Virus titration

DR13 was titrated at regular intervals of the passage level. Virus titration was carried out using a 96-well microplate with Vero cells as described previously [12]. Virus cultures were 10-fold serially diluted with the virus replication medium containing trypsin. Confluent Vero cells of the microplate were washed three times with PBS and inoculated at 0.1 ml per well into five wells. Following adsorption for 1 h at 37 °C, the inocula were removed, and the cells were washed three times with PBS. Subsequently, 0.1 ml of fresh virus replication medium containing trypsin was transferred into each well, and the cells were further incubated for 5 days at 37 °C. Fifty percent tissue culture infective doses (TCID<sub>50</sub>) were expressed as the reciprocal of the highest virus dilution showing cytopathic effect (CPE).

### 2.4. Extraction of genomic PEDV RNA

Infected cell cultures and intestines were prepared for the extraction of genomic RNA. Infected cells were harvested when the cells reached 70–80% CPE. RNA was extracted from infected cells using TRIzol reagent (Gibco BRL, Grand Island, NY) according to the manufacturer's instructions. PEDV-infected cells were lysed directly in a cell culture flask by adding 1 ml of TRIzol reagent per 10 cm<sup>2</sup> of the cell monolayer area. For intestines, 500  $\mu$ l of TRIzol reagent was mixed with 200  $\mu$ l of 10% (v/v) homogenized intestine. Subsequently, 300  $\mu$ l of chloroform was added to the mixture, and the suspension was centrifuged for 10 min at  $12,000 \times g$ . RNA containing aqueous phase was precipitated with isopropanol of the same volume, maintained at –70 °C for 2 h, and centrifuged for 10 min at  $12,000 \times g$ . RNA pellet was washed with 1 ml of 75% ethanol, centrifuged for 10 min at  $12,000 \times g$ , and dried, followed by resuspension in 30  $\mu$ l of diethyl-pyrocabonate (DEPC; Sigma)-treated deionized water.

### 2.5. RT-PCR of PEDV on the S gene

PEDV was routinely identified on the S gene through RT-PCR as described previously [11]. Briefly, forward primer, 5'-TTCTGAGTCACGAACAGCCA-3', and reverse primer, 5'-CATATGCAGCCTGCTCTGAA-3', were used for the amplification of PEDV. The size of the amplified product was 651 bp. Reverse primer was used for the synthesis of complementary DNA. Three-step procedures were performed in a thermal cycler (Perkin-Elmer, Applied Biosystems Inc., Foster City, CA). Samples were amplified using a program that consisted of: incubation at 94 °C for 5 min; followed by five cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s, adding 1 s every cycle. At the completion of cycling, samples were kept at 72 °C for 7 min and then cooled. S genes of serially passaged PEDV were amplified through RT-PCR at levels 0, 1, 30, 60, 90, and 100.

### 2.6. RT-PCR of PEDV on the ORF 3 gene

Primers were designed based on the published sequences of S and sM genes to cover the ORF 3 gene of PEDV. The primers were ORF 3-1 (forward), 5'-TCCTAGACTTCAA-CCTTACG-3'; and ORF 3-2 (reverse), 5'-GGTGACAAGT-GAAGCACAGA-3'. The size of amplified product was predicted to be 833 bp. Reverse transcription was carried out using ORF 3-2. The reverse primer and 6  $\mu$ l of resuspended RNA in DEPC-treated water were denatured by heating at 95 °C and immediately placed on ice. The remaining reagents, which consisted of 5  $\mu$ l of 5 $\times$  first strand buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl<sub>2</sub>), 10 mM DTT, 25 pmol of reverse primer, 0.3 mM of each dNTP, and

100 units of M-MLV reverse transcriptase at a final volume of 25  $\mu$ l, were added. The mixture was incubated at 37 °C for 60 min, and the reaction was stopped by heating at 95 °C for 2–3 min. The cDNA was either stored at –20 °C or amplified immediately. DNA amplification was performed in 25  $\mu$ l of distilled water using 25 pmol oligonucleotide primers, 0.2 mM each of dNTP, 10 mM Tris–HCl (pH 8.8), 1.25 mM MgCl<sub>2</sub>, 50 mM KCl, and 1 unit of *Taq* polymerase. The PCR profile used included a denaturing step at 94 °C for 30 s. Three-step procedures were performed according to the protocol described above, followed by a final extension at 72 °C for 10 min. Specificity of PCR was evaluated using several causative agents of diarrhea, TGEV, porcine rotavirus, and bovine viral diarrhea virus.

### 2.7. ORF 3 sequencing and RFLP analysis

Amplified fragments were separated in 1.5% agarose gels containing 0.2  $\mu$ g/ml ethidium bromide prior to visualization by UV transillumination. Each specific PCR were performed in triplicate, and the amplicons were pooled and then purified using a QIAquick Gel Extraction Kit according to the manufacturer's recommendation (Qiagen, Germany). PCR primers (ORF 3-1, ORF 3-2) were used to sequence the appropriate amplicons in both directions using a FS Dye Primer Kit (Perkin-Elmer, Applied Biosystems Inc.). ORF 3 genes of the virus at passage levels 20, 40, 60, 80, and 100 were sequenced. Sequencing reactions were visualized on an ABI 373 automated sequencer (Perkin-Elmer, Applied Biosystems Inc.) through the use of a commercial service (Takara, Japan). Each fragment was sequenced twice in its entirety in both directions to avoid misincorporation of nucleotide during PCR. The raw data obtained was subsequently edited and aligned to the published sequence using the BCM Search Launcher Multiple Sequence Alignment Program (Human Genome Sequencing Center, Houston, TX, USA). And the 12 field isolates of PEDV which were identified by RT-PCR previously were analyzed by RT-PCR RFLP using *Hind*III

and *Xho* II. These 12 field isolates of PEDV were detected by RT-PCR [11].

### 2.8. S gene and M gene sequencing

The PCR products of DR13 field isolate and passage level 100 were sequenced using S gene specific primer [11]. In addition, M gene of DR13 field isolate and passage level 100 was amplified by RT-PCR as described previously [13]. For the sequencing of M gene, specific primers designated as P1(M), 5'-CCCCAGTACTGTTATTGACGTATAAAC-3'; and P2(M), 5'-GTTTAGACTAAATGAAGCACTTTC-3', were used [13]. Each fragment was sequenced twice in its entirety in both directions and analyzed.

The purified DNA was then digested with restriction enzymes, *Hind*III and *Xho* II (Takara, Japan), for 1 h at 37 °C. The digested DNA fragments were analyzed by electrophoresis on 1.5% agarose gel containing 0.2  $\mu$ g/ml ethidium bromide prior to visualization by UV transillumination. The size of digested DNA fragments were estimated based on migration distances of molecular weight standards.

### 2.9. Pathogenicity and immunogenicity of cell adapted DR13 in 14-day-old piglets and pregnant sows

For the identification of attenuation, viral stock was tested in 14-day-old piglets and pregnant sows. The experiment was designed as Table 1. To compare the pathogenicity, field isolate DR13 before cell adaptation was prepared from small intestines of piglets. The small intestine was homogenated in PBS. The 10% small intestine suspension was then filtered using 0.8 and 0.2  $\mu$ m microfilter (Sartorius, Germany). A total of 2520 pregnant sows from 4 commercial farms (300, 500, 1200, 1300 sow herd each) were inoculated orally with 1 ml of the virus (passage level 100) containing 10<sup>5.8</sup> TCID<sub>50</sub>/0.1 ml. The animals were observed for clinical signs of diarrhea and mortality in the inoculated animals for 10 days in piglets and 5 days in pregnant sows. And, the

Table 1  
Pathogenicity and immunogenicity of cell adapted DR13 in 14-day-old piglets and pregnant sows

Group	Virus		Number of pigs	Inoculation	
	Name	Titer <sup>a</sup>		Route	Volume (ml)
Fourteen-day-old piglets	P90 <sup>b</sup>	10 <sup>6.0</sup>	4	Oral	5
	P75 <sup>c</sup>	10 <sup>6.0</sup>	4	Oral	5
	Parent <sup>d</sup>	10% suspension <sup>e</sup>	4	Oral	5
	Control	$\alpha$ -MEM <sup>f</sup>	4	Oral	5
Pregnant sows	P100 <sup>g</sup>	10 <sup>5.8</sup>	2520	Oral	1

<sup>a</sup> TCID<sub>50</sub>/0.1 ml.

<sup>b</sup> Cell adapted DR13 (passage level 90).

<sup>c</sup> Cell adapted DR13 (passage level 75).

<sup>d</sup> Parent DR13 (passage level 0).

<sup>e</sup> Ten percent suspension of small intestine (virus titer was not determined).

<sup>f</sup>  $\alpha$ -Minimum essential media.

<sup>g</sup> Cell adapted DR13 (passage level 100).

average litter sizes were compared with the data of control pregnant sows at the corresponding farm during the same period of time.

For immune response, the collected sera in 14-day-old piglets and colostrums at delivery were tested for the presence of antibodies by ELISA as previously described [13]. The total number of 230 pregnant sows were received twice at 2-week interval. The 12 colostrums were collected at delivery from farrowing sows inoculated with the virus. Also, 10 colostrums were collected from control sows.

### 3. Results

#### 3.1. Virus isolation and serial passage

The isolate of PEDV designated as DR13 was successfully passaged in Vero cells. Serial passages of DR13 virus were conducted at a 5-day interval post-infection in cells. After 72 h of inoculation, CPE characterized by cell-fusion and syncytial formation, followed by cell destruction was observed. Virus titration was performed at 5 each passage level (Fig. 1).  $TCID_{50}/0.1$  ml of the isolate, DR13, was 2.2 in Vero cells. Titer of the virus gradually increased, reaching a plateau at passage level 55, and maintained  $10^{5.0}$  to  $10^{6.0}$   $TCID_{50}/0.1$  ml. At passage levels 0, 1, 30, 60, 90, and 100, PEDVs were identified through RT-PCR on the S gene. The PCR primers amplified a DNA fragment of the expected size of 651 bp at each passage level (Fig. 2) as well as KPEDV-9. No signal was detected with the negative control of mock-infected Vero cell. In addition, the cell culture-adapted DR13 and its parent strain were identified through RT-PCR on the ORF 3 gene. The PCR reaction yielded an expected 833 bp fragment similar in size to that observed from KPEDV-9 strain extracted RNA as a positive

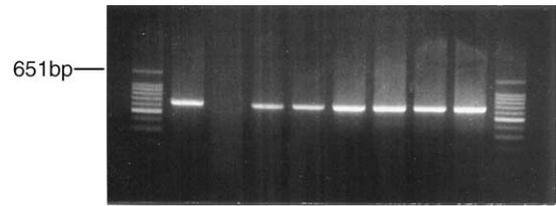


Fig. 2. Agarose gel electrophoresis of PCR products on the S gene of porcine epidemic diarrhea virus. From left to right: lane M, 100 bp DNA ladder; lane 1, KPEDV-9; lane 2, negative control; lanes 3–8, passage levels 0, 1, 30, 60, 90, and 100, respectively.

control (Fig. 3). In addition no signal was detected with a negative control.

#### 3.2. ORF 3, S and M gene sequencing

Sequence analysis of ORF 3 gene of PEDV was aligned and compared using a computer software program. Those of the prototype PEDV, CV777, cell-culture adapted DR13 strain at passage levels 20, 40, 60, 80, and 100, and its parent strain are shown in Fig. 4. As a reference, ORF 3 sequence of CV777 was obtained from GenBank (accession number Z24733). Among the 652 nucleotides of ORF 3, only one nucleotide change (nucleotide 245, from A to C) in the parent DR13 strain and its cell cultured virus (passage levels 20–100) observed compared with the prototype PEDV, CV777. A change found in the parent virus and its cultured virus at passage level 20, compared to passage level 40 and higher was one deletion in nucleotide 526. Further, one nucleotide change (nucleotide 160, from G to A) was found from passage level 60. Nucleotide changes observed in the cell cultured virus between passage level 60, and passage level 80 consisted of one deletion (nucleotide 19) and 3 nucleotides changes (nucleotide 301 from G to A, nucleotide

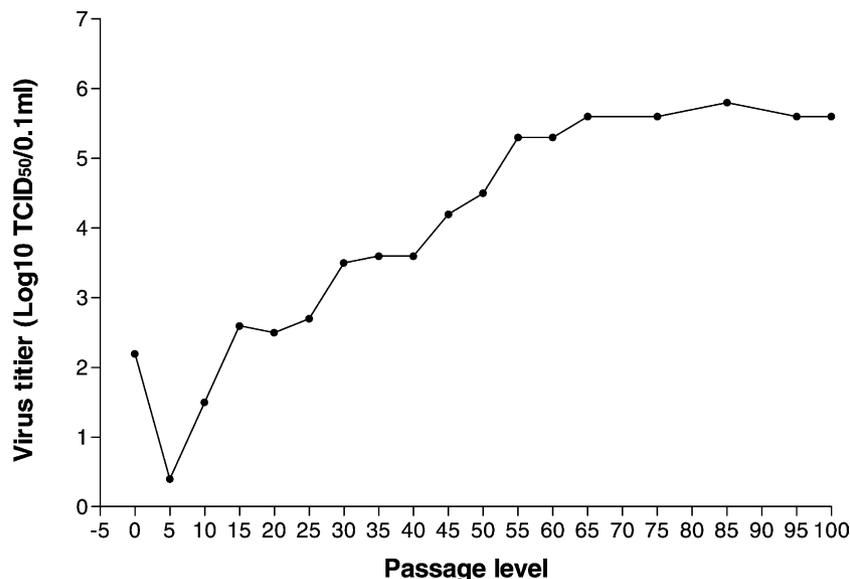


Fig. 1. Virus titers of porcine epidemic diarrhea virus, DR13, on the course of Vero cell passage up to passage level 100.

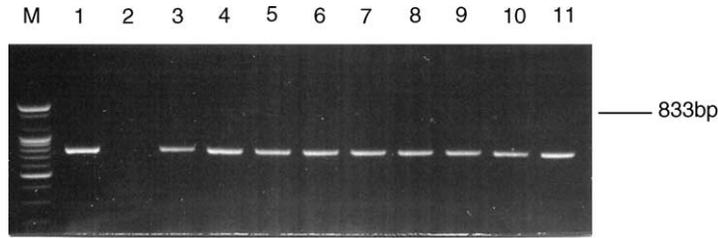


Fig. 3. Agarose gel electrophoresis of PCR products on the ORF 3 gene of porcine epidemic diarrhea virus. From left to right: lane M, 100 bp DNA ladder; lane 1, KPEDV-9; lane 2, negative control; lanes 3–11, passage levels 0, 1, 30, 60, 70, 75, 85, 90, and 100, respectively.

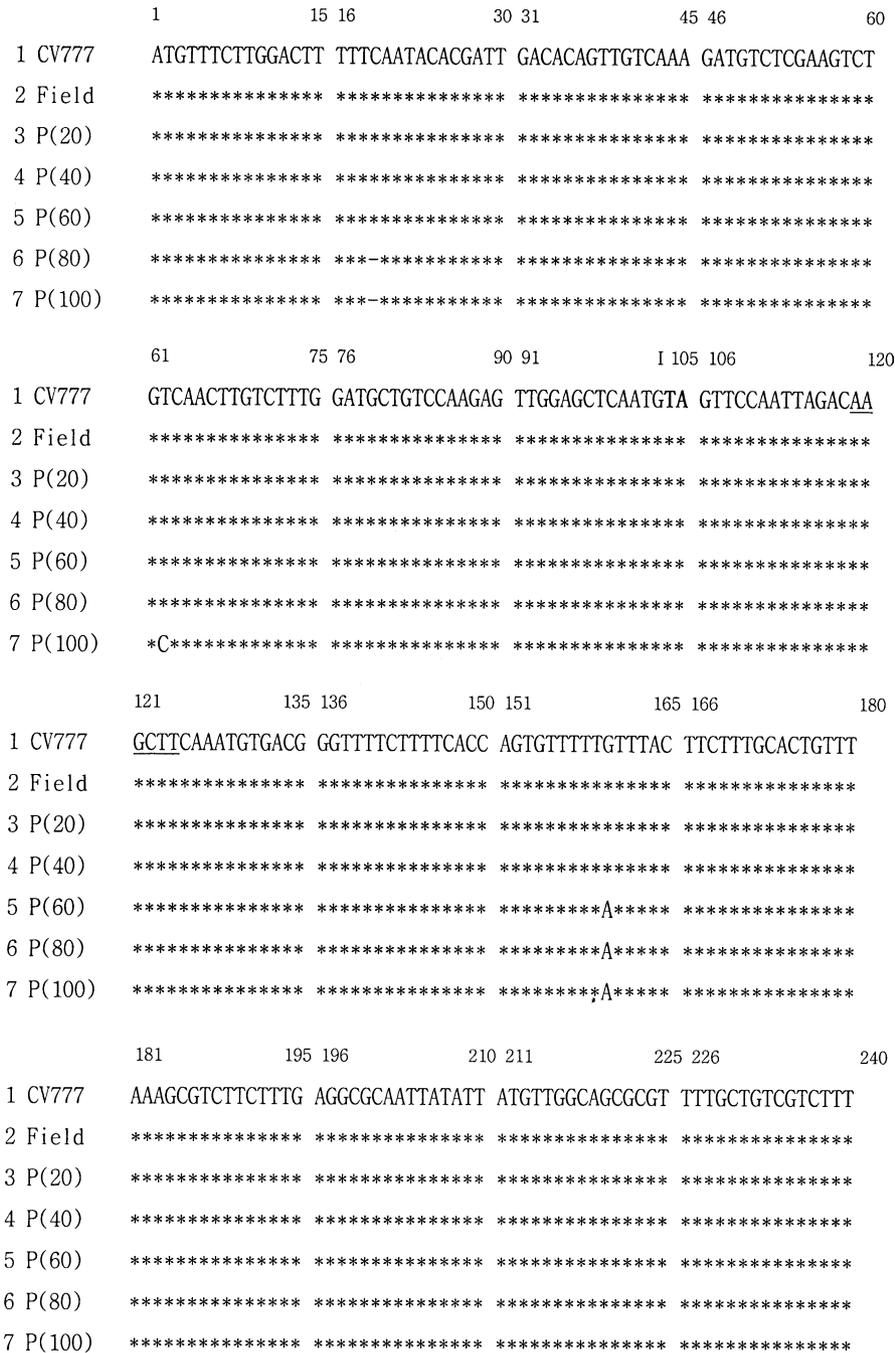


Fig. 4. Sequence alignments of ORF 3 gene of porcine epidemic diarrhea virus, CV777, parent DR13, and cell-passaged DR13 at passage levels 20, 40, 60, 80, and 100.

	241	255 256	270 271	285 286	300
1 CV777	CTCTATTGCCACTT	TTATATTACTGTGGT	GCACTTTTAGATGCA	ACTATTATTGTTCG	
2 Field	***C*****	*****	*****	*****	
3 P(20)	***C*****	*****	*****	*****	
4 P(40)	***C*****	*****	*****	*****	
5 P(60)	***C*****	*****	*****	*****	
6 P(80)	***C*****	*****	*****	*****	
7 P(100)	***C*****	*****	*****	*****	
	301	315 316	330 331	345 346	360
1 CV777	GCACTTATTGGCAGG	CTTGTTTAGTCTGC	TTTACTCCTGGCGC	TATAAAAATGCGCTT	
2 Field	*****	*****	*****	*****	
3 P(20)	*****	*****	*****	*****	
4 P(40)	*****	*****	*****	*****	
5 P(60)	*****	*****	*****	*****	
6 P(80)	A*****	*****	*****	*****	
7 P(100)	A*****	*****	*****	*****	
	361	375 376	390 391	405 406 II	420
1 CV777	TTTATTATCTTAAAT	ACTACGACACTTCT	TTTCTCAATGGTAAA	GCAGCTTATGACGGC	
2 Field	*****	*****	*****	*****	
3 P(20)	*****	*****	*****	*****	
4 P(40)	*****	*****	*****	*****	
5 P(60)	*****	*****	*****	*****	
6 P(80)	*****	*****	*****	*****	
7 P(100)	*****	*****	*****	*****	
	421	435 436	450 451	465 466	480
1 CV777	AAATCCATTGTGATT	CTAGAAGGTGGCGAC	CATTACATCACTTTT	GGCAACTCTTTTGT	
2 Field	*****	*****	*****	*****	
3 P(20)	*****	*****	*****	*****	
4 P(40)	*****	*****	*****	*****	
5 P(60)	*****	*****	*****	*****	
6 P(80)	*****	*****T***	*****	*****	
7 P(100)	*****	*****T***	*****	*****	

Fig. 4. (Continued).

447 from C to T, nucleotide 577 from A to C). Furthermore, three nucleotides changes (nucleotide 62 from T to C, nucleotide 505 from T to G, nucleotide 536 from A to C) were found in passage level 100 compared to passage level 80. Two deletions and seven changes were found between the parent and the cell culture-adapted DR13 strain (passage level 100). In addition, two cleavage sites of the

restriction enzyme, *Hind*III, were recognized both in CV777 as in the parent DR13 strain, while only one was found in the cell culture-adapted DR13 strain from passage level 80. No cleavage site of the restriction enzyme, *Xho* II, was found in CV777 and the parent DR13 strain. However, the cell culture-adapted DR13 strain from passage level 100 revealed one cleavage site of *Xho* II.

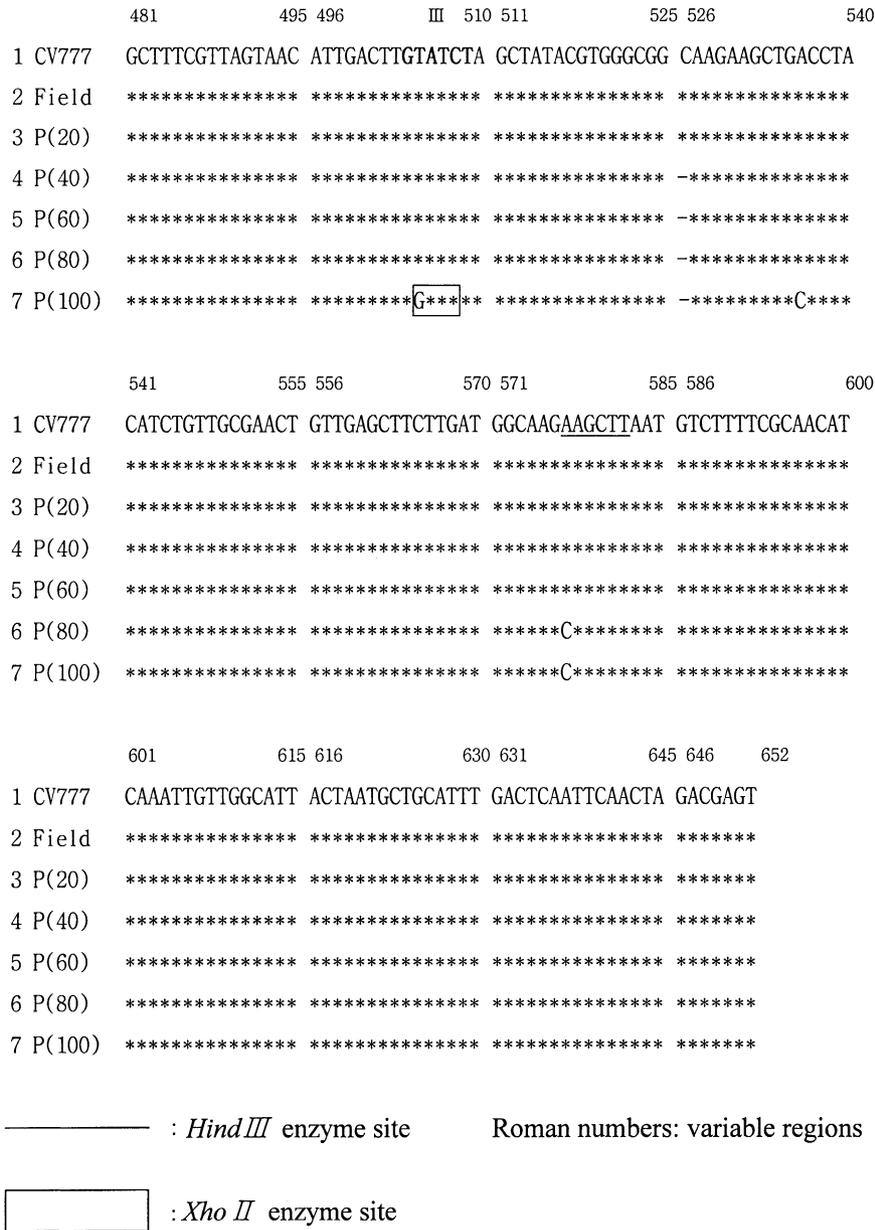


Fig. 4. (Continued).

The comparison of M gene of cell adapted DR13 (passage level 100) showed the 99.11% (675/681) and 98.67% (672/681) in nucleotides identity with previously reported Br 1/87 and KPEDV-9 strains. And the comparison of M gene between cell adapted DR13 (passage level 100) and parent DR13 showed 98.53% (671/681) in nucleotides identity. In addition, the comparison of partial S gene among cell adapted DR13 (passage level 100), parent DR13, CV777 strain was performed. The sequences of cell adapted DR13 (passage level 100) showed 98.77% (643/651), 99.07% (645/651) nucleotides identity with CV777 and parent DR13 strain. And the parent DR13 showed 97.85% (637/651) homologies with CV777 strain.

### 3.3. RFLP analysis

RT-PCR reaction on the ORF 3 gene yielding a fragment of the expected 833 bp was further analyzed using restriction enzymes, *Hind* III and *Xho* II. The PCR products digested by *Hind* III generated three fragments of 471, 183, and 179 bp at the low passage of the virus, while two of 650 and 183 bp from passage level 75 (Fig. 5). The 183 and 179 bp fragments in low passage level are overlapped in agarose gel electrophoresis. The PCR products digested by *Xho* II generated two fragments of 563 and 270 bp from passage level 90 (Fig. 6). In addition, 12 other field strains of PEDV used in this study revealed the same RFLP pattern as that of the

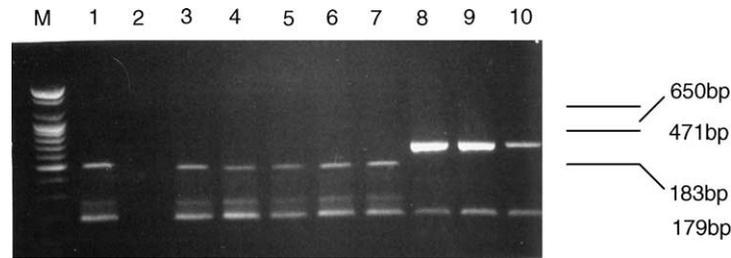


Fig. 5. RFLP patterns of porcine epidemic diarrhea virus, DR13, on the ORF 3 gene PCR products digested with restriction enzyme *Hind*III. From left to right: lane M, 100 bp DNA ladder; lane 1, KPEDV-9; lane 2, negative control; lanes 3–10, passage levels 0, 1, 30, 60, 70, 75, 85, and 100, respectively.

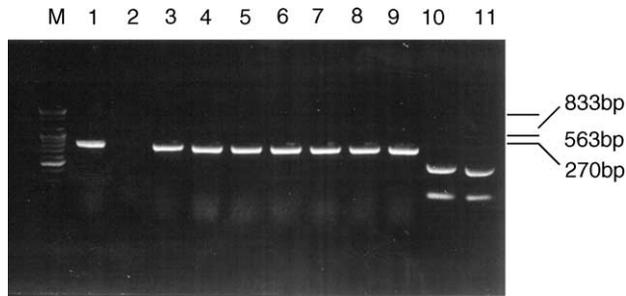


Fig. 6. RFLP patterns of porcine epidemic diarrhea virus, DR13, on the ORF 3 gene PCR products digested with restriction enzyme *Xho* II. From left to right: lane M, 100 bp DNA ladder; lane 1, KPEDV-9; lane 2, negative control; lanes 3–11, passage levels 0, 1, 30, 60, 70, 75, 85, 90, and 100, respectively.

low passage of the virus (Figs. 7 and 8 for *Hind*III and *Xho* II, respectively).

#### 3.4. Pathogenicity and immunogenicity of cell adapted DR13 in 14-day-old piglets and pregnant sows

In this experiment all piglets and pregnant sows inoculated cell culture adapted DR13 failed to show signs of diarrhea and symptoms like anorexia, pyremia related to PEDV infection. Although one piglet in 14-day-old piglets showed signs of mild diarrhea after inoculation, it seemed to be transient. A piglet inoculated cell adapted DR13 with mild diarrhea recovered in 3 days. However, in the group of piglets fed with parent DR13, all piglets developed symptoms of

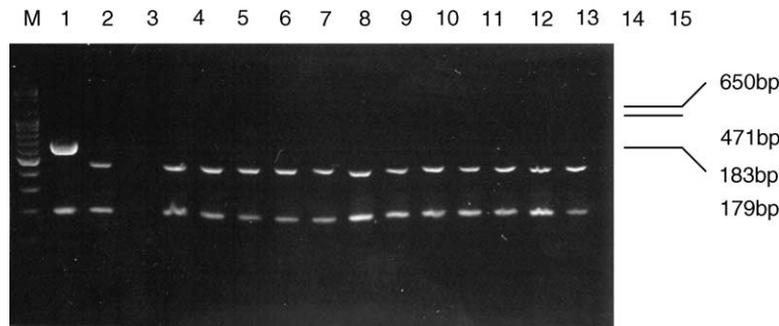


Fig. 7. RFLP patterns of 12 isolates of porcine epidemic diarrhea virus on the ORF 3 gene PCR products digested with restriction enzyme *Hind*III. From left to right: lane M, 100 bp DNA ladder; lane 1, DR13 at passage level 100; lane 2, parent DR13; lane 3, negative control; lanes 4–15, PEDV isolates DR11, B409, DR183, B640, DR195, GC2, B719, 1101, KJY1, KJY2, B720, and DH1, respectively.

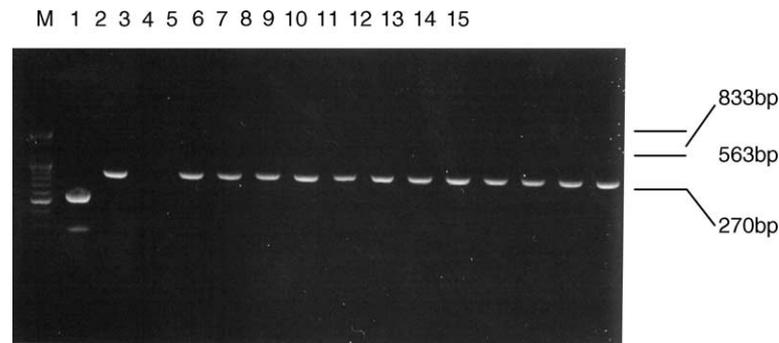


Fig. 8. RFLP patterns of 12 isolates of porcine epidemic diarrhea virus on the ORF 3 gene PCR products digested with restriction enzyme *Xho* II. From left to right: lane M, 100 bp DNA ladder; lane 1, DR13 at passage level 100; lane 2, parent DR13; lane 3, negative control; lanes 4–15, PEDV isolates DR11, B409, DR183, B640, DR195, GC2, B719, 1101, KJY1, KJY2, B720, and DH1, respectively.

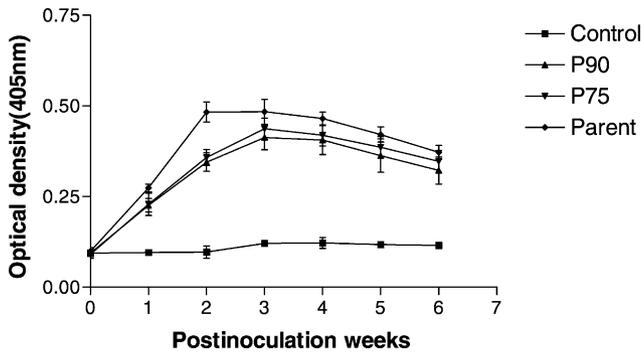


Fig. 9. Immune response of cell adapted DR13 and parent DR13 in 14-day-old piglets.

severe watery diarrhea and dehydration for 3 days. Virus shedding lasted by 7 days in a group inoculated cell adapted DR13 and by 8 days in a group inoculated parent DR13. Pregnant sows inoculated cell adapted DR13 (passage level 100) did not show any clinical signs like diarrhea, anorexia, and pyremia. There was no significant difference of average litter size (average 10.4 pigs born) between the inoculated pregnant sows and control sows.

All inoculated piglets and sows produced protective ELISA titer (Fig. 9). Antibody against PEDV in control piglets and a sow did not detect during the experiment. In addition, colostrums of corresponding sows at delivery showed high level of antibodies against PEDV. In ELISA, optical density (OD) values of colostrum from pregnant sows inoculated with cell adapted DR13 ranged from 0.479 to 0.718, while those of control group did from 0.168 to 0.231.

#### 4. Discussion

The DR13 strain, which was serially passaged in Vero cells up to passage level 100, was distinguishable from the wild-type PEDV at low passage level. ORF 3 A–B gene of several coronaviruses, including TGEV, was recognized with insertions and deletions, which suggested that this area of the genome may be involved in cell tropism and pathogenicity of the virus [14,15,22,24]. In other studies, highly cell-passaged PEDV showed sequence variations and truncations in ORF 3 gene, which were not detected in the wild-type PEDV [3,8,19]. Comparison of the nucleotide sequence available from different PEDV isolates revealed almost complete sequence identity with the exception of changes in ORF 3 observed exclusively in the cell culture-adapted PEDV [19].

In the present study, ORF 3 sequences of the virus DR13 at passage levels 20, 40, 60, 80, and 100 were aligned, and the nucleotide sequences of the cell culture-adapted PEDV and its parent were compared. The authors found nine nucleotide changes over the course of attenuation in Vero cells; however, in the case of its parent PEDV, only one nucleotide

change was identified from the ORF 3 of CV777. And the M gene and partial S gene sequences were compared between cell adapted DR13 and parent DR13. Cell adapted DR13 showed 98.53 and 99.07% sequence homologies, respectively. In addition, cleavage sites of the restriction enzymes, *Hind*III and *Xho* II, were identified for RFLP analysis. Serial passage of DR13 strain conducted up to passage level 100 showed distinct RFLP patterns. In sequence data, different *Hind*III and *Xho* II sites were found from passage level 80 and passage level 100, respectively. But the change in RFLP patterns of *Hind*III and *Xho* II could be identified from passage levels 75 and 90, suggesting that the different *Hind*III and *Xho* II recognition sites changed from passage levels 75 and 90, respectively. The cleavage site of *Xho* II located at one of the three variable regions observed in PEDV ORF 3. In a previous study, three variable regions in ORF 3 gene were reported [8]. In this study, the sequence variation of cell culture-adapted PEDV (passage level 100) occurred in only one region among the three variable regions.

This modification may have resulted from adaptation and attenuation through serial passage in Vero cells. Highly cell culture-adapted PEDV appeared to have lost the ability to synthesize the ORF 3 product in Vero cells [21]. Change in ORF 3 demonstrated an unexpected feature as a consequence of the adaptation to the cell culture. However, RFLP patterns of KPEDV-9 and P-5V of Japanese PED live virus vaccine strain (Nisseiken Co. Ltd., Tokyo, Japan) were the same as those of wild-type PEDVs by two restriction enzymes. Highly cell culture-adapted PEDV could be differentiated from KPEDV-9 and P-5V through the RFLP method (data not presented here).

A fragment of ORF 3 gene of virulent TGEV was smaller than the corresponding fragment of serially passaged virus, and its virulence through cell culture was reduced in piglets [25]. In previous reports, wild-type and cell culture-adapted PEDVs differed in their abilities to cause diarrhea in neonate piglets [1]. Piglets inoculated orally with CV777, a wild-type PEDV, became sick and developed severe diarrhea [5]. When compared with wild PEDVs, animals inoculated with a highly cell-passaged virus did not show any severe signs of diarrhea [13]. The KPEDV-9 strain was serially passaged in Vero cell cultures up to passage level 93 and became a candidate of live PEDV vaccine, so we continued to serial passage up to passage level 100.

In this study, we investigated the attenuation of DR13 strain through serial passages in Vero cell cultures and its immune response in 14-day-old piglets and pregnant sows. When compared with the parent DR13, the piglets inoculated with the high passage level of DR13 did not show any severe clinical signs such as diarrhea, supporting attenuation. And also all the inoculated sows had no clinical signs like abortion, diarrhea, anorexia, and pyrexia. Antibody was detected through serum from piglets and colostrums from pregnant sows. Because the protection of the piglets against PEDV is based on the specific antibodies which have been demonstrated in the colostrums of the immune sows, we evaluated

the antibody titer of colostrums from pregnant sows. However there would be variable in piglets' immunity according to the degree of colostrums uptake of a piglet, litter size, the concentration of antibodies and the quality of colostrums.

Distinct nucleotide changes and RFLP profiles could be meaningful markers for the highly cell-passaged viruses. The results of this study supported that the cell adapted DR13 could be applied as a vaccine candidate of PEDV that could be differentiated from other PEDVs.

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