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Derivation of attenuated porcine epidemic diarrhea virus (PEDV) as vaccine candidate

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Abstract

The field isolate of porcine epidemic diarrhea virus (PEDV) was serially passaged in Vero cells. The cell passaged PEDV, designated KPEDV-9, was tested for its pathogenicity in the neonatal pigs, immunogenicity and safety in the pregnant sows. The result indicated that KPEDV-9 at the 93rd passage revealed reduced pathogenicity in the neonatal pigs. Pregnant sows inoculated with the attenuated virus showed increased immune responses by ELISA. In addition, delivered piglets were protected from challenge of wild type PEDV. The safety test in pregnant sows indicated that all inoculated animals farrowed the average numbers of litters of piglets. The results of this study supported that the attenuated virus derived from serial passage could be applied as vaccine for protecting suckling piglets against PEDV infection. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Porcine epidemic diarrhea virus; Attenuation; Vaccine candidate

1. Introduction

Porcine epidemic diarrhea virus (PEDV), a member of Coronaviridae, is the etiological agent of enteropathogenic diarrhea in swine [1–3]. Although the clinical symptoms of PEDV infection are similar to transmissible gastroenteritis virus (TGEV) infection, PEDV has a wider variety of clinical signs in pigs [4]. Propagation of PEDV *in vitro* was rather limited until Vero cells were found to support the growth of virus in the presence of trypsin [5]. In this study, we described the derivation of an attenuated strain of PEDV, as a potential vaccine candidate, through cell adaptation.

2. Materials and methods

2.1. Cell and virus

Vero cells obtained from ATCC (Vero C1008) were regularly maintained in alpha-MEM supplemented with 5% fetal bovine serum, penicillin (100 unit/ml), streptomycin (100 unit/ml) and amphotericin (0.25 g/ml). The isolate of PEDV was originated from a neonatal pig and plaque purified in Vero cells. The isolate, designated KPEDV-9, was passaged in 80–90% monolayers of Vero cells in alpha-MEM with 0.02% yeast extract, 0.3% tryptose phosphate broth (TPB) and 1–2 µg of trypsin as described [5,6]. Sequential passages of the virus were normally conducted in roller culture. Each passage level of virus was stored at –70°C or freeze-dried with equal volume of stabilizer (0.217 M

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Lactose, 0.0038 M KHPO₄, 0.0072 M K₂HPO₄, 0.0049 M monosodium glutamate, 1% Gelatin). The final stock of the PEDV was characterized in two ways. Firstly, the culture supernatant was subjected to direct centrifugation at 60,000 × *g* for 2 h and the pellet was resuspended to 1/200 of initial volume for morphological identification by transmission electron microscopy. Secondly, the virus infected cells were subjected to reverse transcription polymerase chain reaction (RT-PCR) to detect specific PEDV sequences. Three primers for RT-PCR were selected from the sequences information of membrane protein (M) gene of Duarte et al. [7]. P1 (27mer); 5′-CCCCAGTACTGTTATGACGTATAAAC-3′ (position 974–1000), P2 (24mer); 5′-GTTTAGACTAAATGAAGCACTTTC-3′ (position 1665–1688) for PCR and P3 (25mer); 5′-GCCATAAAGTTTCTGTTTAGACTAA-3′ (1702–1678) as the primer for synthesis of complementary DNA, respectively. The extraction of RNA and RT-PCR were conducted according to the instructions of commercially available kit (Stratagene). PCR reactions were performed in the conditions as described previously [8]. After amplification, the PCR products were cloned into pUC19 vector for sequencing using Sequenase version 2.0 (USB, USA). In addition, the presence of adventitious virus such as porcine parvovirus (PPV), Japanese encephalitis virus (JEV), Hog cholera virus (HCV) and other cythopathogenic viruses were examined as described previously [9–11].

2.2. Examination on attenuation of cell passaged PEDV

For the detection of attenuation, viral stocks at 90 passages were tested in 4 days old piglets. From 10^{6.0} TCID₅₀/ml to 10^{8.0} TCID₅₀/ml of cell adapted PEDV was inoculated into suckling piglets intramuscularly or through the oral route. In order to compare the pathogenicity, PEDV isolate before cell adaptation was prepared from the small intestines of neonatal piglet. The intestine was ground in phosphate buffered saline (PBS, pH 7.4). The 10% suspension was then filtered using a 0.2-μm membrane filter (Acrodisk, Gelman) and further diluted to 5-, 10- and 20-fold in PBS. Four groups of five piglets were orally fed with the suspensions of diluted stock of intestine. The animals were observed for clinical symptoms of diarrhea and mortality in the inoculated animals was observed for 10 days.

2.3. Immunogenicity of attenuated virus

Three pregnant sows 4–5 weeks prior to farrowing were tested for the detection of immune responses. Pigs were inoculated intramuscularly with the attenuated viruses at the titer of at 10^{7.0} TCID₅₀/ml. Two pregnant sows remained as uninoculated control. After

two weeks, second inoculations of same titer were followed. Each paired serum before and after inoculation was collected at two-week interval. The collected sera and colostrum at delivery were tested for the presence of antibodies by ELISA. After delivery, suckling piglets of 2 days old were orally challenged with 10 or 5 LD₅₀ of wild PEDV. The clinical signs of diarrhea and mortality of challenged piglets were observed for two weeks.

2.4. ELISA

For the preparation of antigen, the cell adapted PEDV was concentrated with polyethylene glycol (PEG, MW 6000) as described [12]. The PEG treated viral solution was then precipitated and resuspended at 1/10th of original volume with TEN buffer (0.01 M Tris, 0.001 M EDTA, 0.1 M NaCl, pH 7.4). The purified virus was then used for the ELISA. The procedures for ELISA were basically the same with a previous study [13]. Briefly, the dilution of antigen and second antibodies were adjusted to the optical density (OD) around 0.2 (A 490) using the negative porcine sera. Usually each well in 96-well microplate (Costar) was coated with 1–2 μg of protein in 50 mM carbonate buffer (pH 9.6) at 5°C overnight, followed by the blocking with 1% skim milk at 37°C. The 1/400 diluted porcine sera in PBS with 0.01% Bovine serum albumin (BSA) and 0.05% Tween 20 (PBST) were reacted at 37°C for 30 min and then washed extensively with PBST three times at 5-min intervals. The reacted plate was washed again at the same condition and incubated with 2000-fold diluted horseradish peroxidase (HRP) labelled anti-porcine IgG (KPL) for 1 h at 37°C. The plate was developed in *O*-phenylenediamine (OPD) at room temperature for 20 min. The reaction was stopped with 2 M H₂SO₄ before measuring OD at 490 nm.

2.5. Safety experiment in pregnant sows

A total of 63 pregnant sows were inoculated intramuscularly with 1 ml of the virus containing 10^{7.0} TCID₅₀/ml. Twenty-three pregnant sows received one injection 3–4 weeks prior to farrowing. In another farm, 40 pregnant sows received two injections at 2–3-week intervals before farrowing. The average number of the litters were compared with the data of uninoculated pregnant sows at the corresponding farm during same period of time.

2.6. Nucleotide sequence accession numbers

The sequence accession number for M protein of attenuated KPEDV-9 is GeneBank accession number AF015888.

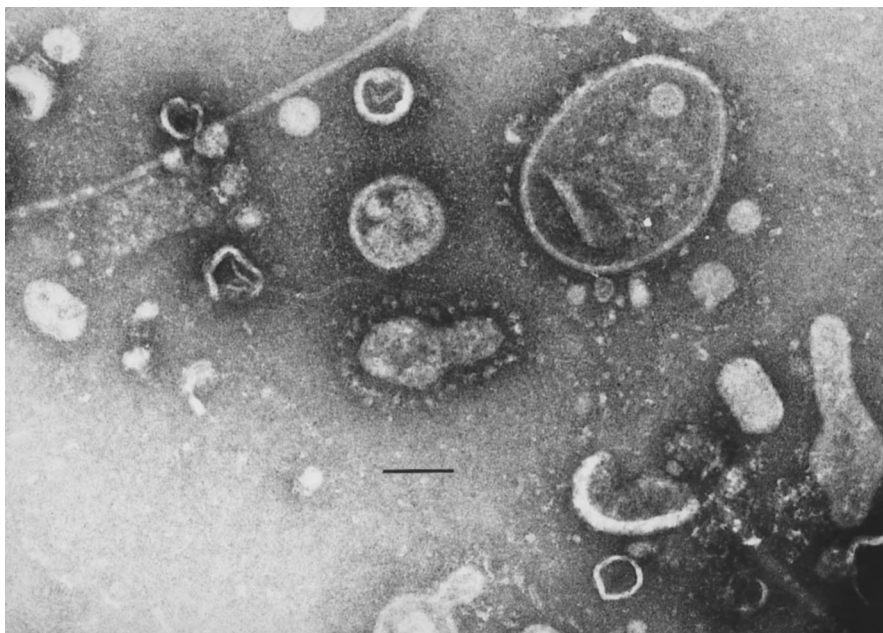


Fig. 1. Transmission electron microscopy of cell adapted KPEDV-9 strain after staining with 2% uranyl acetate. Bar represents 100 nm.

3. Results

3.1. Derivation of high passage level of PEDV in Vero cell culture

The PEDV was continuously passaged in Vero cells. Sequential passage of virus regularly conducted every 4–5 days postinfection in cells. The supernatant was harvested and used for next inoculation in Vero cells up to 93 passages. However, cytopathogenic effect (CPE) in Vero cells was not so clear that the culture supernatant of virus infected cell was subjected to morphological and genetic characterization. When the culture supernatant of virus infected cell was examined by the transmission electron microscopy, characteristic shape of coronavirus with diameter of 100–150 nm was possible to identify (Fig. 1). In addition, the com-

parison of M gene of cell passaged virus showed the 98.97% in nucleotide and 98.24% in amino acid identity with previously reported PEDV strain (Fig. 2). No adventitious viral contaminations were detected in the final stock of PEDV.

3.2. Pathogenicity of cell attenuated PEDV

The pathogenicity of attenuated virus was tested in the 1-day-old piglets before taking colostrum. Six separate litters of 53 piglets and 15 litters of 111 piglets were inoculated intramuscularly with virus of $10^{7.0}$ and $10^{6.0}$ TCID₅₀/ml, respectively. In this experiment, all the inoculated piglets failed to show signs of diarrhea and symptoms related to PEDV infection.

In order to avoid any possible effects from the maternal immunity through the colostrum and detect the

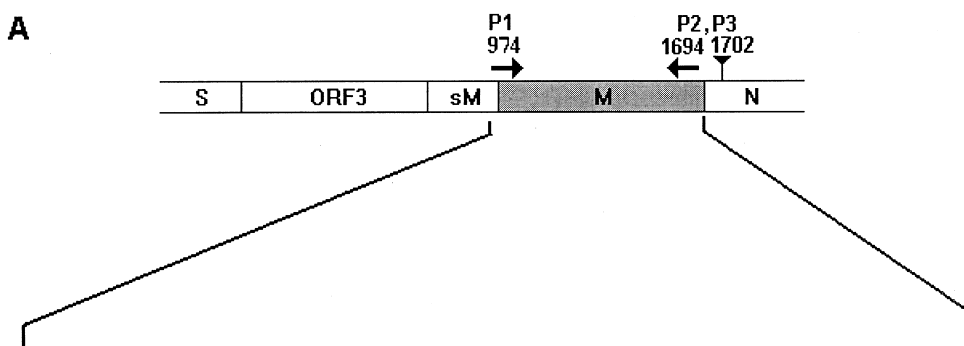


Fig. 2. Detection of PEDV M fragment identified by RT-PCR. (a) Diagram of PEDV genome according to Duarte et al. The sites for PCR primers (P1 and P2) with the directions of the extension and cDNA synthesis (P3). Nucleotide sequence of cDNA corresponding M gene of PEDV. (b) Sequence and amino acid comparison between B: Br1/87 [7] and V: cell adapted KPEDV-9 strain, respectively. Difference of nucleotide and amino acid are indicated.

B

1		16
	Met Ser Asn Gly Ser Ile Pro Val Asp Glu Val Ile Glu His Leu Arg	
	BATG TCT AAC TGT TCT ATT CCC GTT GAT GAG GTG ATT GAA CAC CTT AGA	
	VATG TCT AAC TGT TCT ATT CCC GTT GAT GAG GTG ATT CAA CAC CTT AGA	
		↓
		Gln
1		48
17		32
	Asn Trp Asn Phe Thr Trp Asn Ile Ile Leu Thr Ile Leu Leu Val Val	
	AAC TGG AAT TTC ACA TGG AAT ATC ATA CTG ACG ATA CTA CTT GTA GTG	
	AAC TGG AAT TTC ACA TGG AAT ATC ATA CTG ACG ATA CTA CTT GTA GAG	
		↓
		Glu
49		96
33		48
	Leu Gln Tyr Gly His Tyr Lys Tyr Ser Val Phe Leu Tyr Gly Val Lys	
	CTT CAG TAT GGC CAT TAC AAG TAC TCT GTG TTC TTG TAT GGT GTC AAG	
	CTT CAG TAT GGC CAT TAC AAG TAC TCT GCG TTC TTG TAT GGT GTC AAG	
		↓
		Ala
97		144
49		64
	Met Ala Ile Leu Trp Ile Leu Trp Pro Leu Val Leu Ala Leu Ser Leu	
	ATG GCT ATT CTA TGG ATA CTT TGG CCT CTT GTG TTG GCA CTG TCA CTT	
	ATG GCT ATT CTA TGG ATA CTT TGG CCT CTT GTG TTG GCA CTG TCA CTT	
145		192
65		80
	Phe Asp Ala Trp Ala Ser Phe Gln Val Asn Trp Val Phe Phe Ala Phe	
	TTT GAC GCA TGG GCT AGC TTC CAG GTC AAC TGG GTC TTT TTC GCT TTC	
	TTT GAC GCA TGG GCT AGC TTC CAG GTC AAC TGG GTC TTT TTC GCT TTC	
193		240
81		96
	Ser Ile Leu Met Ala Cys Ile Thr Leu Met Leu Trp Ile Met Tyr Phe	
	AGC ATC CTT ATG GCT TGC ATC ACT CTT ATG CTG TGG ATA ATG TAT TTT	
	AGC ATC CTT ATG GCT TGC ATC ACT CTT ATG CTG TGG ATA ATG TAT TTT	
241		288

Fig. 2 (continued)

97		112
Val Asn Ser Ile Arg Leu Trp Arg Arg Thr His Ser Trp Trp Ser Phe		
GTC AAT AGC ATT CGG TTG TGG CGC AGG ACA CAT TCT TGG TGG TCT TTC		
GTC AAT AGC ATT CGG TTG TGG CGC AGG ACA CAT TCT TGG TGG TCT TTC		
289		336
113		128
Asn Pro Glu Thr Asp Ala Leu Leu Thr Thr Ser Val Met Gly Arg Gln		
AAT CCT GAA ACT GAC GCG CTT CTC ACT ACT TCT GTG ATG GGC CGA CAG		
AAT CCT GAA ACT GAC GCG CTT CTC ACT ACT TCT GTG ATG GGC CGA CAG		
337		384
129		144
Val Cys Ile Pro Val Leu Gly Ala Pro Thr Gly Val Thr Leu Thr Leu		
GTC TGC ATT CCA GTG CTT GGA GCA CCA ACT GGT GTA ACG CTA ACA CTC		
GTC TGC ATT CCA GTG CTT GGA GAC CCA ACT GGT GTA ACG CTA ACA CTC		
	↓ ↓ Asp	
385		432
145		160
Leu Ser Gly Thr Leu Leu Val Glu Gly Tyr Lys Val Ala Thr Gly Val		
CTT AGT GGT ACA TTG CTT GTA GAG GGC TAT AAG GTT GCT ACT GGC GTA		
CTT AGT GGT ACA TTG CTT GTA GAG GGC TAT AAG GTT GCT ACT GGC GTA		
433		480
161		176
Gln Val Ser Gln Leu Pro Asn Phe Val Thr Val Ala Lys Ala Thr Thr		
CAG GTA AGT CAA TTA CCT AAT TTC GTC ACA GTC GCC AAG GCC ACT ACA		
CAG GTA AGT CAA TTA CCT AAT TTC GTC ACA GTC GCC AAG GCC ACT ACA		
481		528
177		192
Thr Ile Val Tyr Gly Arg Val Gly Arg Ser Val Asn Ala Ser Ser Gly		
ACA ATT GTC TAC GGA CGT GTT GGT CGT TCA GTC AAT GCT TCA TCT GGC		
ACA ATT GTC TAC GGA CGT GTT GGT CGT TCA GTC AAT GCT TCA TCT GGC		
529		576

Fig. 2 (continued)

193		208
Thr Gly Trp Ala Phe Tyr Val Arg Ser Lys His Gly Asp Tyr Ser Ala		
ACT GGT TGG GCT TTC TAT GTC CGG TCA AAA CAC GGC GAC TAT TCA GCT		
ACT GGT TGG GCA TTC TAT GTC CGG TCA AAA CAC GGC GAC TAC TCA GCT		
577		624
209		224
Val Ser Asn Pro Ser Ala Val Leu Thr Asp Ser Gly Lys Val Leu His		
GTG AGT AAT CCG AGT GCG GTT CTC ACA GAT AGT GAG AAA GTG CTT CAT		
GTG AGT AAT CCG AGT GCG GTT CTC ACA GAT AGT GAG AAA GTG CTT CAT		
625		672
225		
Leu Val ***		
TTA GTC TAA		
TTA GTC TAA		
673		

Fig. 2 (continued)

potential pathogenicity of the attenuated virus, eight piglets of 4 days old were infected orally with 10 ml of virus stock, which contained virus of $10^{8.0}$ TCID₅₀/ml, and were artificially fed with dairy milk. Although three piglets showed signs of anorexia and mild signs of diarrhea in two or three days after inoculation, nevertheless, the signs seemed to be transient.

In fact, all piglets recovered in the next 2–3 days. However, in the groups of piglets fed with wild virus before cell passages, all the piglets developed symp-

toms of watery diarrhea in 2–3 days, and the mortality reached up to 10–100%, depending on the dilution of virus within one week as shown in Table 1.

3.3. Immunogenicity of attenuated virus

When the collected sera were tested for the presence of the antibodies by ELISA, all inoculated sows showed the rising ELISA titers (Fig. 3). On the other hands, the antibody titers of control pigs decreased at

Table 1
Comparison of attenuated virus and wild virus in new-born piglets

Virus	Age (days)	No. of piglets	Inoculation		Clinical signs ^c and No. of death
			doses ^a (TCID ₅₀ /ml)	route ^b	
Cell attenuated	4	8	$10^{8.0}$	O	-/+ , 0/8
	1 ^d	53	$10^{7.0}$	IM	-, 0/53
	1	111	$10^{6.0}$	IM	-, 0/111
Wild type ^e	4	5	10%	O	+, 5/5
	4	5	1%	O	+, 5/5
	4	5	0.5%	O	+, 1/5

^a 1 ml.

^b O means oral and IM intramuscular, respectively.

^c - means no signs of diarrhea, -/+ signs of mild diarrhea and + diffuse diarrhea.

^d Inoculation before taking the colostrum.

^e Ground intestines of piglets before cell adaptation.

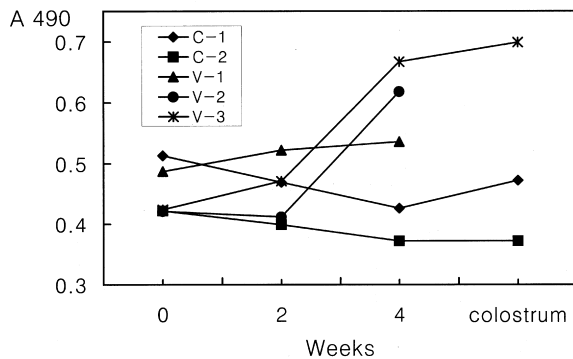


Fig. 3. Immune responses of pregnant sows inoculated with cell attenuated KPEDV-9 strain. Animals were inoculated twice at 2-week intervals and serum samples were tested by ELISA.

the time of delivery. In addition, colostrum at delivery showed higher or similar level of antibodies of corresponding sows. After challenge exposures mortality of piglets were compared with uninoculated control. Although the mortality of piglets after challenge with 10 LD₅₀ of wild PEDV was reduced to 20% compared to 100% in control litter, all piglets survived in the litters after challenge experiment with 5 LD₅₀ of virus compared to 60% in control (Table 2). However, mild signs of diarrhea were also detected in one litter of piglets 2 days after challenge, but they recovered the next day.

3.4. Safety experiment of attenuated PEDV on pregnant sow

The safety test of the attenuated virus in pregnant sow was conducted in two separate farms. One farm has not had any history of epidemic diarrhea in last few years and another farm had the experience of PEDV outbreak in the previous year. A total of 63 pregnant sows were inoculated once or twice before farrowing. As shown in Table 3, all the inoculated sows farrowed the same average numbers of litters of uninoculated control group without any clinical problems.

Table 2
Survival of piglets from attenuated KPEDV-9 vaccinated sows (V) and unvaccinated control (C) after challenge exposure

Treatment	Dose of challenge (LD ₅₀)	No. of survival/piglets ^a
C	10	0/10
V-1	10	2/10
C	5	4/10
V-2	5	8/8
V-3	5	7/7

^a One week after challenge exposure.

Table 3
Farrowing data in pregnant sows inoculated with attenuated KPEDV-9 strain

Farm	No. of inoculation (10 ^{7.0} TCID ₅₀ /ml)	Route ^a	No. of pregnant sows (Mean No. of litters)	
			control	inoculated
A	1 ^b (1 ml)	IM	28 (8.0)	23 (8.0)
B	2 ^c (1 ml)	IM	40 (10.7)	40 (10.7)

^a Intramuscular.

^b 2–3 weeks before farrowing.

^c 4–5 weeks and 2–3 weeks before farrowing, respectively.

4. Discussion

In this study, we investigated the attenuation of PEDV through serial passages in Vero cell cultures and its prophylactic effect in pregnant sows. After serial passages in Vero cells, the growth of virus was rather trypsin-independent and the detection of cytopathic effect (CPE) was rather variable, depending on clones of Vero cell lines (data not presented here). Since the SPF or gnotobiotic piglets were not used in this experiment, it might not reasonable to figure out the exact differences in pathogenicity between the wild and the attenuated virus. Nevertheless, when compared with the wild PEDV, the animals inoculated with the high passage level of virus did not show any severe signs of diarrhea or death in piglets, supporting attenuation.

It is known that PEDV replicates mainly in the villi of small intestines [14]. Like attenuated TGEV, the replication of attenuated PEDV may be limited to the small portion of intestine with short duration of secretion compared to virulent virus [15]. In fact, the detection of signs of diarrhea from piglets inoculated orally with attenuated virus delayed at least by two days and lasted one day compared with the signs from piglets inoculated with wild virus.

When we tested the immunoprophylactic effect in pregnant sows, it was demonstrated that the vaccinated swine resulted in reduced piglet mortality after challenge experiment (20–100% in vaccinates compared with 0–40% in controls), indicating that the attenuated PEDV could induce the status of immunity in pregnant pigs, providing protection in piglets like other enteric disease in swine. Previously, we found that PEDV infections were related to more than 20% of diarrheal cases in the neonatal pigs, thus causing a considerable losses in pig industry [13]. In fact, field application of attenuated virus as vaccine resulted in the overall reduction of mortality of neonatal pigs (2–

52% compared with before vaccination) in the farms having PEDV outbreak [16]. Nevertheless, it is worthy to note that the efficacy of protection was rather complicated and conflicting according to the challenge dose like other enteric diseases. Although the pregnant pigs inoculated with attenuated virus showed the increased immune status by ELISA as compared to uninoculated control, it is difficult to explain the exact relation to mucosal immunity for protection. Since it was well confirmed that the mechanisms of the passive immunity are extensively related to the presence of IgA and IgM antibodies [17], further experiments, including detection of antibody secreting cells of IgA and IgM, may give the practical information for immunoprophylaxis against PEDV.

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