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RESEARCH ARTICLE

Isolation and Characterization of a Vero Cell-Adapted Porcine Epidemic Diarrhea Virus in China

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ABSTRACT

Since early 2011, diarrhea was rather common among pig herds in China and the causative agent was proven to be virulent porcine epidemic diarrhea virus (PEDV). From the small intestine of a piglet showing mild diarrhea, we isolated an attenuated cell-adapted PEDV strain in a Vero cell line. The virus was designated as PEDV AH-M. The virus was recognized in infected cells by a monoclonal antibody against PEDV spike protein. After 10 passages in Vero cells, the virus grew stably to $10^{6.5}$ $TCID_{50}$/ml. Typical coronavirus particles were observed by electron microscopy. The complete genome sequence of PEDV AH strain was shown to be 27, 953 nucleotides in length. On the whole genome level, this virus strain shared highly nucleotide sequence identities with attenuated Korean DR13 (99%) and China SD-M strain (99%). These data implied that a cell-adapted attenuated PEDV was isolated. We infer that AH-M could be developed as a vaccine strain while immunogenicity and pathogenicity of this strain needs further confirmation.

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INTRODUCTION

Porcine epidemic diarrhea virus (PEDV), clinically causing vomiting, watery diarrhea and dehydration among pigs, is the pathogen of porcine epidemic diarrhea (PED). PEDV was first covered by the scientist of Belgium and UK during 1978. Since then, the disease has been discovered in numerous European countries, and nowadays in eastern Asia, *i. e.* China, Korea, Vietnam, Thailand, and Japan (Murakami *et al.*, 2015; Song *et al.*, 2015; Stadler *et al.*, 2015; Zafar *et al*., 2015).

PEDV, a single-stranded, positive-sense RNA virus, is a member of Group 1 of the genus *Alphacronavirus*, family *Coronaviridae*. The whole sequence of the virus is about 28 kb in size containing five genes (De Vries *et al.*, 1997). Belongin coronavirus, PEDV is composed of three corresponding principal structural proteins: S (~200 kDa), M (~30 kDa), and N (~56 kDa) protein (Oka *et al.*, 2014; Sun *et al.*, 2015). The S glycoprotein contains numerous important functions including receptor binding, neutralizing, cell fusion, and antibodies induction (Chen *et al.*, 2013; Wicht *et al.*, 2014). The M protein is mainly responsible for virus assembling (Nguyen and Hogue, 1997), and inducing antibodies which could inactivate virus with the company of complement (Fan *et al.*, 2012). The N protein of coronavirus participates in the formation of the viral core, and packaging of viral RNA (Li *et al.*, 2007). Unlike the proteins mentioned above, ORF3 is an accessory gene and could be used to differentiate the vaccine strains and the field isolates. Previous researchers found that there was a difference of ORF3 gene between the field strains and Vero-cell-adapted isolates. This discrepancy could be used as a convenient method to investigate epidemiologic situation of PEDV infection (Zhao *et al.*, 2012; Wang *et al.*, 2014).

In China, PEDV infection was first confirmed by serum neutralization assay and fluorescent antibody assay. Since then, it has been reported in most parts of China and threatens the whole pig industry as a common viral disease. At the beginning of 2011, the severe diarrhea outbreak occurred in many pig-producing areas in China with morbidity and mortality being rather high among suckling piglets. Although most of these herds have been injected with the PEDV vaccine (CV777), PEDV infection still occurred in some of the farms and the infected pigs exhibited anorexia and transient diarrhea.

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Thus PEDV field strains needed to be isolated and their genetic characteristics required to be further investigated to better understand and control PEDV prevalence in future. We isolate a cell-adapted PEDV strain surnamed AH-M in this study. Molecular characterization and genetic diversity of this isolate was performed and phylogenetic relationship with other PEDV reference strains was analyzed as well.

MATERIALS AND METHODS

Samples: Acute diarrhea outbreaks were observed at numerous pig farms in different provinces from January to October in 2011. The clinical symptoms of these infected pigs were watery diarrhea, dehydration and loss of appetite. Sows had been vaccinated with divalent inactivated TGEV and PEDV vaccine before delivery. From the farms located in 12 provinces of China, 455 samples collected from fecal, intestine, and milk were detected (Li *et al.*, 2012). The positive samples were used to isolate PEDV.

Vero cells were used to isolate the virus, as reported before (Kusanagi *et al.*, 1992) with some modifications. The positive samples of homologized intestinal suspension of PEDV were filtered through a syringe filter of 0.22-μm (Millipore, USA). Then the filtered liquid was prepared as inoculum. Dulbecco's modified Eagle's medium (DMEM) was used as the growth medium containing antibiotics and heat-inactivated fetal calf serum (10%). DMEM containing 10μg/ml trypsin was used as the Maintenance medium (MM) (Gibco, USA). GM used for cell cultures were removed. Then, the cells were washed three times by DMEM and inoculated with suspensions had been filtered before. After incubating at 37°C for 1 h, DMEM was used to wash the cells with MM being supplemented thereafter. The cells were observed in the next five days to search for typical cytopathic effect (CPE).

The PEDV field strain isolated was further verified though transmission electron microscopy (TEM) and immunofluorescence assay (IFA). The commercial mouse anti-S monoclonal antibody (Median Diagnostics, South Korea) of 1:500 dilution which was against PEDV and a fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Southern Biotech, USA) of 1:100 dilution were used for IFA. For preparing samples in the assay of TEM, viruses were ultra-centrifuged with the sucrose cushion (20%), then negatively stained by ammonium molybdate (2%), observed under TEM finally (FEI Tecnai G2 20, Oregon, USA).

Viral RNA was extracted from the supernatants of AH-M infected Vero cells by TRIzol LS Reagent. According to the genomic sequence of PEDV strain CV777, 12 pairs of primers, targeting different regions of AH-M, were designed. PCR product of the correct size was cloned into the vector of PMD18-T. Sequencing was conducted by an ABI 3730xl DNA Analyzer (Applied Biosystems, USA). Sequence assembly and analysis were performed by the software of lasergene (DNAstar Inc., USA). Alignments of several sequences were conducted through Clustal X 2.1 (Thompson *et al.*, 1997). MEGA 4 program were used to complete the phylogenetic analysis (Tamura *et al.*, 2007). Phylogenetic trees were built based on the neighbor-joining method and the bootstrapping was over 1, 000 replicates. All the genes information of PEDV strains collected in this study were listed in Table 1. Information about genome sequence of AH-M could be observed in GenBank (accession number KJ158152).

Table 1: PEDV strains investigated in this study

Strain/Isolate	Accession No, country	Strain/Isolate	Accession No, country
(a) Complete genome		Chinju99	EU792474, Korea
TGEV Miller M6	DQ811785, USA	M1763	HQ537438, Korea
HCoV-229E	AF304460, Germany	Br1-87	Z25483, France
CV777	AF353511, Belgium	CH/GSJIII/07	GU372743, China
SM98	GU937797, Korea	CH/HLJH/06	GU372732, China
Attenuated DR13	JQ023162, Korea	CH/HLJM/07	GU372735, China
Virulent DR13	JQ023161, Korea	CH/HNHJ/08	GU372736, China
BJ-2011-1	JN825712, China	CH/JL/08	GU372734, China
CH/FJND-3/2011	JQ282909, China	CH/JL/09	GU372741, China
CH/S	JN547228, China	(d)M gene	
CH/ZMDZY/11	KC196276, China	KPED-9	AF015888, Korea
SD-M	JX560761, China	Chinju99	DQ845249, Korea
CHGD-01	JX261936, China	CH/JSX/06	EU033967, Japan
AJ1102	JX188454, China	JMe2	D89752, Japan
(b) S gene		NIAH380_98	EU581712, Thailand
Br1-87	Z25483, France	NIAH1795_04	EU542415, Thailand
NK	AB548623, Japan	JS-2004-2	AY653205, China
MK	AB548624, Japan	LJB/03	AY608890, China
KH	AB548622, Japan	LZC	EF185992, China
83P-5,100th-passaged	AB548621, Japan	(e)N gene	
83P-5	AB548618, Japan	Chinju99	AF237764, Korea
Chinju99	AY167585, Korea	GDHSY	JN173275, China
KNU-0801	GU180142, Korea	JKA1	JN173296, China
KNU-0802	GU180143, Korea	JS-2004-2	AY653206, China
CH3	JQ239431, China	LJB/03	DQ072726, China
CH4	JQ239432, China	QY1	JN173297, China
JS-2004-2	AY653204, China	QY2	JN173298, China
LZC	EF185992, China	SWK1	JN173300, China
(c)ORF3 gene		SWK6	JN173301, China
CV777 truncated	GU372744, Belgium	XG1	JN173302, China
BI976	HQ537433, Korea	XG2	JN173303, China

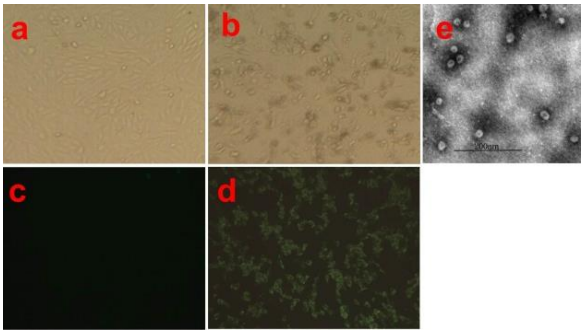

Fig. 1: Identification of the isolate by optical microscopy, IFA and electron microscopy. a) Mock-infected Vero cells. b) CPE in Vero cells infected with PEDV showing syncytia and multiple nuclei. c) IFA in non-infected Vero cells. (d) IFA in isolate infected Vero cells. Original magnification×200. e) Morphology of isolate under electron microscopy (negatively stained); bar=200nm.j

RESULTS

The typical cytopathic effect (CPE) including cell fusion, detachment and syncytium, can be detected after ten passages by microscope (Fig. 1b). The structural proteins of S, E, M and N and ORF3, were successfully cloned and sequenced. Our results exhibited that the fragments showed highly homology to PEDV reference strains. The virus isolate designated AH-M can be detected by IFA assay (Fig. 1d). And the viral particles with specific characteristics could be observed by TEM (Fig. 1e).

The whole genome of AH-M excluding the poly (A) tail was around 27, 953 nucleotides (nt). The genome and deduced amino acid (aa) sequences of the isolate AH-M were compared with other reported PEDV strains (Table 1). Alignment of the genome sequence with other PEDV reference strains acquired from the GenBank exhibited the highest similarity with the Chinese PEDV isolate SD-M (99%), a Vero Cell-Adapted strain isolated in 2012 and South Korea vaccine strain attenuated DR13 (99%). The Phylogenetic analysis result indicated that AH-M could form a cluster together with SD-M and attenuated DR-13 (Fig. 2a).

The S protein of AH-M was 4, 149 nucleotides long and encoded a 1, 383 amino acids protein. Comparing with the reference strain CV777, one deletion (in position 162) was observed (Fig. 3a). It displayed 92-99% aa identity with the reference isolates, and shared the highest similarity with isolates of SD-M, attenuated DR13, 83P-5 100th-passaged, parent 83P-5, CH3, CH4 (99%). Phylogenetic analyses based on S protein sequences showed that all PEDV isolates could be separated into two main branches (Fig. 2b). AH-M belonged to group 1, which also contained 6 Chinese strains (CH3, CH4, SD-M, LZC, CH/S, JS-2004-2), 3 Korea strains (SM98, attenuated DR13, virulent DR13), 3 Japanese strains (83P-5 100th-passaged, 83P-5, MK) and one French strain (Br1-87) (Fig. 2b).

Both the AH-M strain and CV777 vaccine strain (vs), SD-M, GSJIII/07 shared a deletion of 49 nt from nt 245 to 295, causing a shift of the ORF3 reading frame. And there was a TAG terminator at 274-276nt. As a result, ORF3 of AH-M was truncated and encodes a truncated protein of 91 amino acids. Phylogenetic analysis of the ORF3 gene confirmed that all the sequences can be grouped into two parts (Fig. 2c). Group 2 comprised AH-M, CV777 truncated, CH/GS JIII/07 truncated and SD-M. They were almost the same as each other with the amino acid similarity being 100%.

The highly conserved M protein of AH-M was 226 amino acids in length. And it shared 96-99% similarity with other strains. The highest similarity (99%) was with the strains of SD-M, attenuated DR13, CV777, CH/JSX/06, JMe2, AJ1102, CH/FJND-3/2011, GHGD-01, JS-2004-2. Comparing with CV777, there were two amino acid changes (S5F, V42A) for AH-M. Phylogenetic analyses based on M exhibited that the isolates mainly were divided into two separate groups (Fig. 3D). The AH-M strain was in Group 1, which was composed of 9 Chinese strains (CH/FJND-3/2011, CH/ZMDZY/11, BJ-2011-1, LJB/03, CH/S, LZC, SD-M, AH-M), 4 Korean strains (Chinju99, KPED-9, virulent DR13, attenuated DR13), two Japanese strains (JMe2, CH/JSDX/06) and one Belgium strain CV777. Group 2 contains two Chinese strains (AJ1102, CHGD-01) and two Thailand strains (NIAH380 98, NIAH 1795 04).

All the strains did not contain insertions or deletions in the N gene. The ORF corresponding to the N gene was 1, 326 nucleotides long and coded a protein of 441 amino acids. The N protein of AH-M showed 96-100% similarity with other PEDV strains. And the vaccine strain attenuated DR13 exhibited the highest identity (100%) with AH-M. Alignment analysis confirmed that the whole N protein was generally highly conserved. But comparing to CV777 9 amino acid substitutions could be observed. Based on the sequences of the N protein, all the isolates could be grouped into three unique branches (Fig. 2e). Between these groups, some unique amino acid changes could be detected (Fig. 3c). In Group 2, two amino acid mutations (A142T, H242L) could be observed; A145T only occurred in group 3; Group 2, 3 shared one specific amino acid changes (G84A).

DISCUSSION

In the present study, clinical signs with dehydration, typical watery diarrhea, and vomiting occurred on the pig farm. TGEV, RV and other pathogenic suspects were ruled out. Therefore PEDV was considered as the only possible cause of disease. Later, PEDV was successfully isolated and propagated in Vero cells, according to the method described before (Kusanagi, 1992). At passage 10, characteristic CPE was detected as early as 24 hours after inoculation. However this is not the same as reported before, Pan *et al*. observed a typical CPE at passage seven while Hofmann and Wyler reported a CPE even at passage one (Pan *et al.*, 2012). Different susceptibilities of Vero cells to different PEDV strains may just explain this discrepancy.

To further investigate the genetic characteristics of the strain, we sequenced the complete genome of AH-M and analyzed the genetic relationships at both the full sequence and separate protein levels. The S and ORF3 genes were most variable regions, and they both were important targets for understanding genetic differences among PEDV strains. S protein is an antigen on the surface of the virion that interacts with specific host-cell

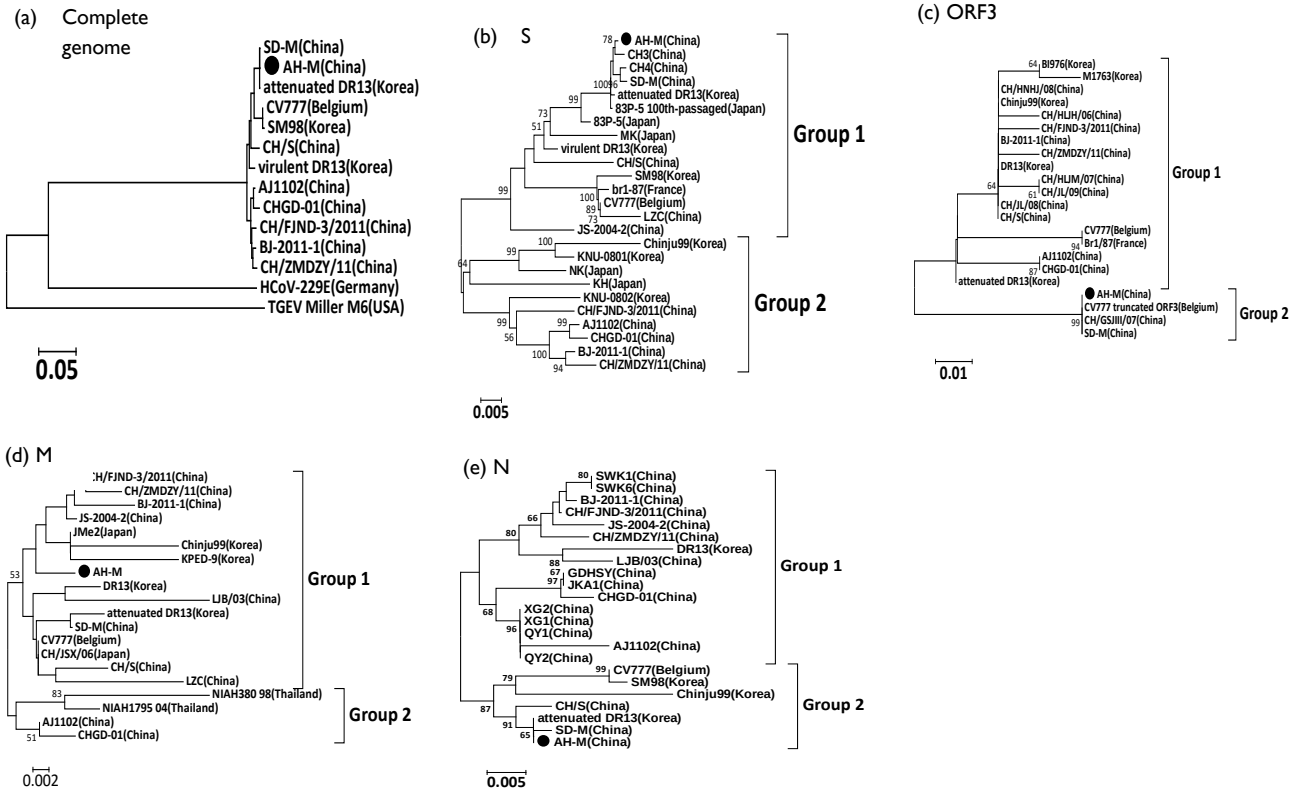

Fig. 2: Phylogenetic analysis of AH-M and reference isolates based on the nucleotide sequences of complete genome (a) and protein sequence of S (b), ORF3 (c), M (d), N (E). Phylogenetic trees were constructed using MEGA 5.02 software.●, isolated strain in this study. GenBank accession numbers of all strains examined in this study are listed in Table 1.

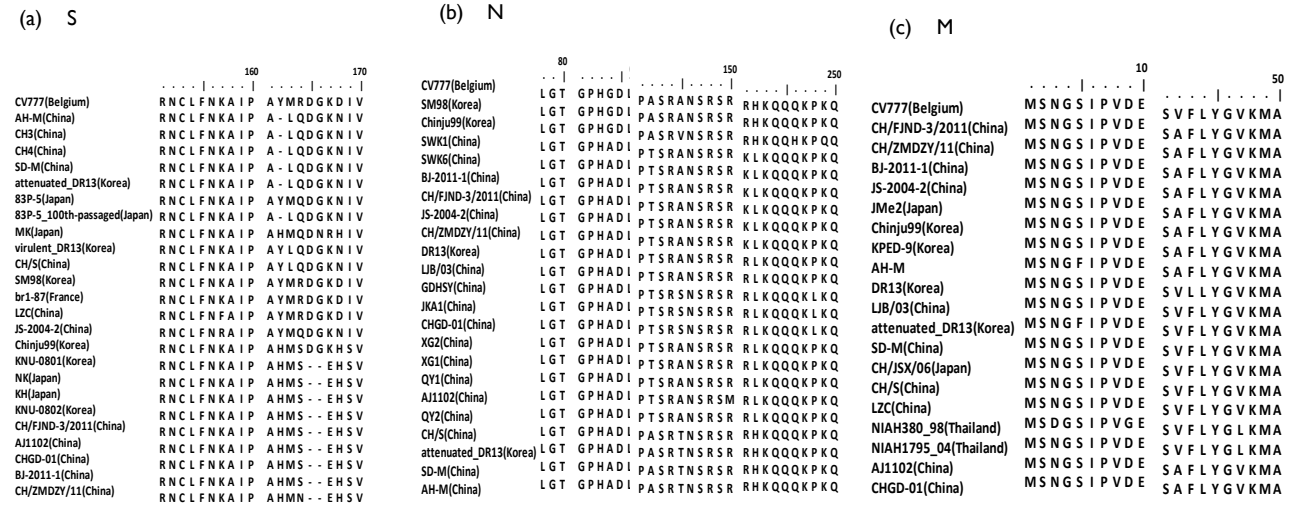

Fig. 3: Multiple alignments of the S (a), M (b), N (c) amino acid sequences of AH-M and reference strains.

receptor to mediate viral entry. This process involved with the entry of virus, cell fusion, and production of neutralizing antibodies (Shirato *et al.*, 2011; Zhang *et al.*, 2015). It also plays an important role in viral growth adaptation to cells and virulence attenuation (Sato *et al.*, 2011; Wicht *et al.*, 2014). Most PEDV reference isolates including AH-M were divided into the first group. Interestingly, all the cell-adapted isolates formed a unique cluster. In this study, comparing with CV777 the same amino acid deletion of 162Y could be found among the six strains (AH-M, CH3, CH4, SD-M, attenuated DR13, 83P-5 100th-pasaged). These results suggest that the deletion of 162Y may be an important change for cell-adapted PEDV strains.

ORF3 gene is located in the middle of S and E in the whole sequence. And the function of ORF3 remains unclear. The 49 nucleotides deletion of AH-M caused a truncated ORF3 of 91 amino acids. This deletion could also be observed in the vaccine isolate CV777 and field strains SD-M, CH/GSJIII/07(Chen *et al.*, 2010; Park *et al.*, 2011; Zhao, 2012). Attenuated DR13 also has a 51-nt deletion and finally encode a 208-aa-long ORF3 (Park *et al.*, 2008). Wild-type viruses containing DR13, Br1/87 and CV777 had maximum length of ORF3 genes, which

could only be observed in wild-type isolates. Taken together, all the results indicate that ORF3 may play a pivotal role *in vivo* virulence (Wang *et al.*, 2012). And the deletion caused by the serial propagation *in vitro*, could attenuate the pathogenicity of AH-M *in vivo*. However, further work of animal experiment will be required to confirm it.

AH-M has the highest sequence similarity with the isolates of attenuated DR13 and SD-M based on the genetic analysis. Although the question of how AH-M appeared remains unclear, one possible explanation is that it could just evolve from the vaccine virus. And it may also happen through recombination between strains in nature. Of the two possible causes, the last one is more convincible as the S, N and M proteins of AH-M are similar with the Vaccine strain attenuated DR13, while ORF3 gene is closer to CV777 vaccine strain. Both DR13 and CV777 were used as parent strains to develop PEDV vaccines. So it may be possible for us to develop AH-M as the PEDV vaccine in China.

Conclusions: This study exhibit a novel cell-adapted strain has been isolated. Phylogenetic analysis of S, ORF3, M, N genes confirmed that this isolate had highly similarity with the vaccine isolate CV777 and attenuated DR13. Hopefully, the information acquired in this research would help to control PED more efficiently in China.

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Author's contribution: MXR, HQG and LTF designed the experiment. HH and KXG helped with the manuscript. HH, LTF, LMY, CFZ and YSY executed the experiment and analyzed the data.

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