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Original article

Isolation and genetic analysis of a variant porcine epidemic diarrhea virus in China

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Abstract

Porcine epidemic diarrhea virus (PEDV) is having a severe effect on the pig breeding industry in central China. The mucosa and the content of the small intestine from newborn pre-weaned piglets with diarrhea were tested for the presence of PEDV by molecular and morphologic methods, and found to be positive. Negative-staining electron microscopy (EM) revealed the presence of coronavirus-like particles in the samples. The result of molecular detection by nested RT-PCR based on the amplification of the M gene was positive. Using a novel alternative method we successfully propagated the PEDV strain (CH/QX-2) in Vero cells, confirmed by ultrathin sections of the cells and Immunofluorescence assay (IFA). Phylogenetic analysis based on the partial S gene showed that the CH/QX-2 isolate was genetically closer to strains more commonly found in China, but differed genetically from two domestic strains (CH/S, 1986 and LZC, 2007), Korean strains (DR13, 2007), and the vaccine strain (CV777 vs) currently being used in China. CH/QX-2 formed a unique clade in the derived phylogenetic tree indicating that the CH/QX-2 strain currently circulating in central China is a new variant of PEDV. This study extends current knowledge on the diversity and epidemiology of PEDV.

Key words: isolation, genetic, variant, porcine epidemic diarrhea virus

Introduction

As a member of the family *Coronaviridae,* porcine epidemic diarrhea virus (PEDV) can induce an acute and highly contagious enteric disease of swine with a high mortality in piglets, which is characterized by severe enteritis, vomiting, and watery diarrhea (Ducatelle et al. 1981). The disease was first recognized in England in 1971 (Pensaert et al. 1978), and since then, outbreaks have been reported in Europe and Asia (Bridgen et al. 1993, Puranaveja et al. 2009, Chen et al. 2010), and more recently in the United States (Wang et al. 2014). In China, although a periodic vaccination strategy has been applied nationwide to control the disease in pig farms, PEDV outbreaks have continued to occur causing large financial losses in the

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Table 1. The primers used in this study.

Primer names	Nucleotide sequence (5'-3')	Size of product (bp)
PEDV-Out-F[a]	ACACCTATAGGGCGCCTGTA	853
PEDV-Out-R[a]	AACCCTAAGAGGGGCATAGA	
PEDV-in-F[b]	GGGCGCCTGTATAGAGTTTA	412
PEDV-in-R[b]	AGACCACCAAGAATGTGTCC	
PEDV-sF	TTCTGAGTCATGAACAGCCAAT	894
PEDV-sR	CATACTAAAGTTGGTGGGAAT	

[a] outer primer pairs used for nested-RT-PCR; [b] inner primer pairs used for nested-RT-PCR

Chinese swine industry. Since 2010, PEDV infection has become one of the most significant diseases affecting pig farming in central China (Sun et al. 2012). The PEDV genome contains at least seven open reading frames (ORFs), encoding four structural proteins [spike (S), envelope (E), membrane (M), and nucleocapsid (N)], and three non-structural proteins (replicases 1a and 1b and ORF3) (Song et al. 2012). The spike protein (S) is the major structural protein of PEDV and consists of 1383 amino acids. Similar to other coronaviruses, the S protein of PEDV can be divided into three domains, including a large outer domain, a transmembrane domain and a short cytoplasm domain at the carboxy terminus. It has been demonstrated to have four neutralizing epitopes on the surface of S protein (aa 499-638, 748-755, 764-771, and 1,368-1,374) (Sun et al. 2008), which are pivotal in receptor binding and cell entry, host-cell fusion, as well as the induction of neutralizing antibodies (Godet et al. 1994, Chang et al. 2002, Sun et al. 2007). Therefore, the S protein is a suitable candidate for examining the epidemiological status in the field, strain diversity and the association between gene mutations and viral antigenicity of PEDV (Park et al. 2007).

Molecular techniques have been commonly used diagnostically for identifying the presence of the PEDV genome. However, morphological methods are needed to confirm the identity of the pathogen and to further investigate pathogenesis. Combining molecular detection and morphologic methods, the present study describes the isolation of a PEDV strain currently circulating in the central region of China by optimizing the isolation procedures. Based on a partial S gene sequence, phylogenetic analysis has indicated that the isolated PEDV strain may be a new variant. The amino acid mutations in the neutralizing epitope regions may be the basis of its altered antigenicity and consequent resistance to vaccination. These findings may be useful for understanding its epidemiology as well as for the design of new and more effective vaccines.

Materials and Methods

Specimen source

An outbreak was observed in a swine farm from the central region of China, and characterized by acute watery diarrhea and high mortality rates among neonatal piglets. The specimens (small intestine and its contents) were sent to the Key Laboratory of Animal Immunology of the Ministry of Agriculture (Henan Academy of Agricultural Sciences, Zhengzhou, China) for the detection of PEDV.

Morphologic observation of virus by TEM

Samples were prepared for electron microscopy using negative staining, following a previously described procedure with slight modifications (Derbyshire 1988). The scraped-off mucosa and the content from the small intestine were pooled, diluted 1:5 in phosphate-buffered saline (PBS; 0.1 M, pH 7.2) to make 10% (V/V) suspensions. The suspensions were vortexed and after freezing and thawing three times clarified by centrifugation at 10,000 x g for 5 min. Clarified supernatant fluids were placed on parlodion-coated grids in 50 µl aliquots. After adsorption for 5 min, the grids were negatively stained with 2% phosphotungstic acid (pH 6.6) for 2 min and subsequently viewed with an H-7500 transmission electron microscope (TEM).

Nested RT-PCR for the detection of PEDV in the samples

The mucosal scrapings and the small intestine contents were pooled, diluted 1:5 in phosphate-buffered saline (PBS; 0.1 M, pH 7.2), and homogenized by ultrasonication. The suspensions were vortexed and after freezing and thawing three times clarified by centrifugation for 10 min at 4,800×g. PEDV RNA was

Table 2. PEDV strains used in this study for sequence alignment and phylogenetic analysis.

Reference Strains	Accession No	Origin	Reference Strains	Accession No	Origin
ZJCZ4	JX524137.1	China, 2011	GD-1	JX647847.1	China, 2011
LC	JX489155.1	China, 2012	AH2012	KC210145.1	China, 2012
CHGD-01	JX261936.1	China, 2012	USA/Colorado/2013	KF272920.1	USA, 2013
AJ1102	JX188454.1	China, 2012	CH/ZMDZY/11	KC196276.1	China, 2011
CH/FJZZ-9/2012	KC140102.1	China, 2013	BJ-2011-1	JN825712.1	China, 2011
GD-A	JX112709.1	China, 2012	CV777	AF353511.1	Belgium, 2001
DR13	JQ023161.1	Korea, 2008	LZC	EF185992.1	China, 2007
JS-HZ2012	KC210147.1	China, 2012	CH/FJND-3/2011	JQ282909.1	China, 2011
GD-B	JX088695.1	China, 2012	CH/S	JN547228.1	China, 1986

extracted using TRIzol reagent in accordance with the manufacturer's instructions. Two pairs of oligonucleotide primers were designed for the amplification of membrane protein (M) gene of PEDV CV777 strain (GenBank No. AF353511) and were used for the nested RT-PCR in two amplification steps. The nucleotide sequences of the primers and the expected size of the amplified DNA fragments were shown in Table 1.

The PCR mixture contained 2.5 µl of 10×Taq DNA polymerase buffer, 3 mM of $MgCl_2$, 2.0 µl of dNTPs (2.5 mM/L), 0.5 µl of each specific primer (10 pmol), 1 µl of Taq DNA polymerase, 2 µl cDNA and brought to 25 µl with autoclaved, filtered (0.2 µm) distilled water. The amplification step was carried out using the following thermal cycles: the denaturation at 94 for 4 min was followed by 30 cycles of the amplification step (denaturation at 94 for 1min, annealing at 50 for 40s, and extension at 72 for 1 min), a final extension step at 72 for 5 min. The product of the first reaction (2 µl) was used as a template for the second amplification; the following thermal cycles were carried out: the denaturation step at 94 for 4 min followed by 30 cycles of the amplification step (denaturation at 94 for 30 s, annealing at 55 for 30 s, and extension at 72 for 40s), a final extension step at 72 for 5 min. The size of the amplified products in the first and the second reaction was 853 bp and 412 bp, respectively.

Virus isolation

Propagation of PEDV was attempted in Vero cells (Vero-E6) as previously described with modifications (Hofmann et al. 1988). Vero cells were cultured and maintained in Minimum Essential Medium (MEM) supplemented with 10 % fetal bovine serum, 2 mM L-Glutamine, 0.05 mg/ml Gentamicin, 10 unit/ml penicillin, 10 g/ml Streptomycin, and 0.25 g/ml Amphotericin. First, the monolayers were washed twice with PBS and then inoculated with virus suspension pre-treated with trypsin [10 µg of trypsin (1:250) was added to 1 ml of diluted and filtered virus suspension at 37 for 30 min]. After adsorption at 37 for 2 h at room temperature, maintenance medium [MEM; supplemented with 0.3% tryptose phosphate broth and 0.02% yeast extract, and containing 2.5 µg of trypsin (1:250) per ml] was added without removing the viral inoculum, and the cultures further incubated at 37. Control cultures were mock inoculated with the same volume of maintenance medium instead of viral inoculum and further treated in the same manner as virus-inoculated cultures.

Ultrathin section and IFA

Vero cells infected with the CH/QX-2 isolate were trypsinized at 24 h post infection and centrifuged for 5 min at 800×g. The cell pellets were resuspended in 0.01M PBS (pH 7.2-7.4) and centrifuged for 5 min at 800×g. The resulting pellets were fixed in 2.5% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide for 90 min. The samples were dehydrated in an ascending ethanol series, followed by propylene oxide and embedded in Eponate 12™. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined by TEM (H- 7500).

IFA was used to detect PEDV in the fixed-methanol infected Vero cells utilizing a 1:1000 dilution of mouse anti-S monoclonal antibody specific for PEDV (Cat No: 9191, MEDIAN Diagnostics Inc, Korea) and a 1:100 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Cat no: F0257, Sigma). Vero cells without virus were similarly treated and used as an antigen negative control. The results were obtained using a fluorescence microscope.

Cloning and sequence analysis of the partial S gene for PEDV

Viral RNA extraction was performed with 50 µl of virus isolates propagated in the Vero cell cultures

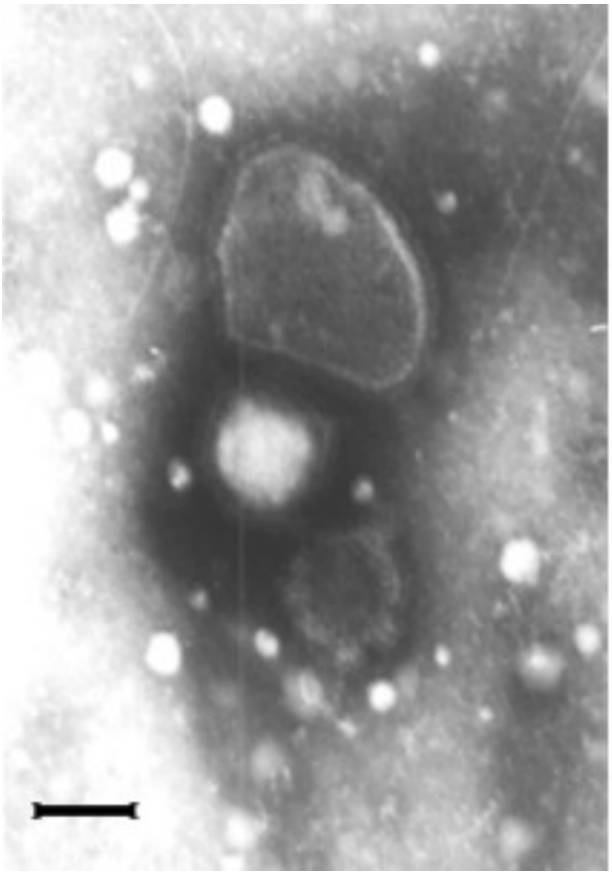

Fig. 1. Negatively stained virus particles in the small intestine homogenates from ill piglet with diarrhea. Some virus particles with crown-shaped spikes were viewed by TEM. (Bar=100 nm).

using the TRIzol reagent following the manufacture's instruction. Synthesis of the first-strand cDNA of the partial S gene was carried out by reverse transcription using TAKARA Reverse Transcription Kit (Code No. RR014A). The primers for amplifying partial S gene were designed based on the genome of PEDV CV777 (GenBank No. AF353511), and the size of the final fragment containing the three neutralizing epitopes of PEDV was 894 bp. Two sets of primers were synthesized by Sangon Biotech, China. The RT-PCR primers were given in Table 1. The partial S gene was amplified under the following conditions: the denaturation at 95 for 5 min was followed by 30 cycles at 95 for 1 min, 55 for 1 min, 72 for 1 min, and a final extension at 72 for 10 min. The positive clones were sent to Sangon Biotech Company, China, for sequencing. All sequencing reactions were performed in duplicate.

Multiple-sequencing alignments were generated with Clustal W method by Meg-align 4.0 program in DNASTAR. Phylogenetic tree was constructed by Molecular Evolutionary Genetics Analysis (MEGA) software (version 5.2.1) with the neighbor-joining method based on nucleotide sequences of the partial S gene (Tamura et al. 2011). To assess the relative support for each clade, bootstrap values were calculated from 1,000 replicate analyses. All strains used for phylogenetic analysis were presented. B-cell epitope prediction was performed by BepiPred 1.0 Server (http://www.cbs.dtu.dk/services/BepiPred/) (Larsen et al. 2006).

Results

Morphologic characterization of virus

TEM of the negatively stained preparations from the small intestinal contents revealed the presence of virus particles of approximately 100-140 nm in diameter carrying a number of ca 20 nm surface projections, in accord with the morphological characteristics of *Coronaviridae* members (Fig. 1).

Molecular detection by nested-RT-PCR

First 853-bp product was amplified using the outer primer pairs PEDV-out-F and PEDV-out-R. The second amplification, using nested or internal primer pairs PEDV-in-F and PEDV-in-R, produced a 412-bp PCR product. These were consistent with the expected fragment in length. Control reaction failed to produce a detectable amplification product (Fig. 2).

Virus isolation and characterization

Virus isolation was attempted on 40 PEDV-PCR positive intestine homogenates on Vero cells. One PEDV isolate designated as CH/QX-2 obtained from the small intestines of a 3-old day sucking piglets was successfully serially propagated in the Vero cell cultures.

Ultrathin sections of Vero cells after the ninth serial passage were examined by TEM. The cytoplasm of Vero cells infected with the CH/QX-2 isolate contained masses of virus particles with distinctive crown-shaped projections. Some virus particles were also seen at the surface of the cytoplasm membrane. No virions were observed in the Vero cells without CH/QX-2 infection (Fig. 3a). The Vero cells infected by CH/QX-2 also tested positive by IFA, whereas no fluorescence was observed in the control cultures (Fig. 3b).

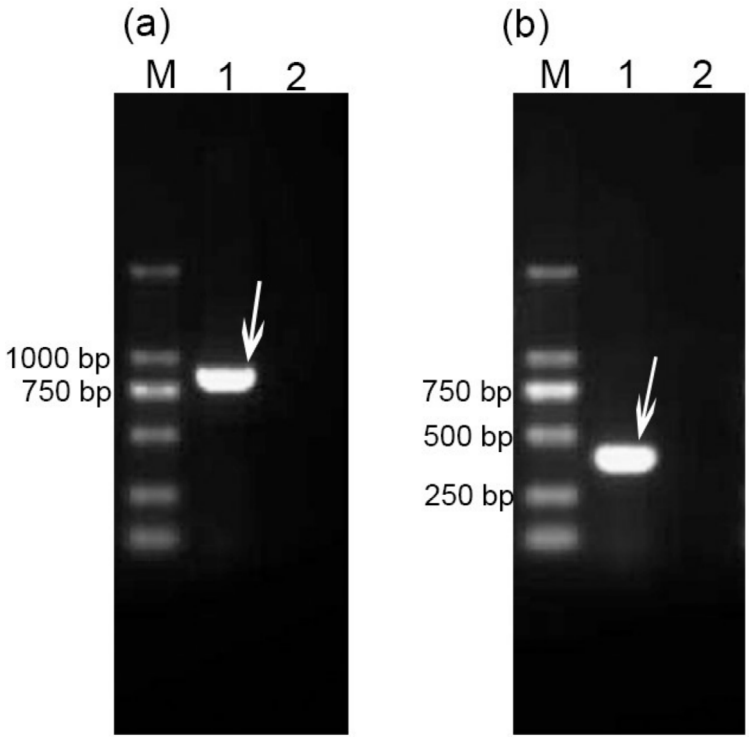

Fig. 2. Identification of PEDV in the content from small intestine of piglet with diarrhea by nested RT-PCR. (a) A specific fragment of about 853 bp length was amplified using outer primer pairs of PEDV-Out-F: and PEDV-Out-R, which was indicated by white arrow. (b) A specific fragment of about 412 bp length was amplified using inner primer pairs of PEDV-in-F, and PEDV-in-F, which was indicated by white arrows. M, DNA Marker DL 2000; lane 1, PEDV positive; Lane 2, Negative control (double distilled water).

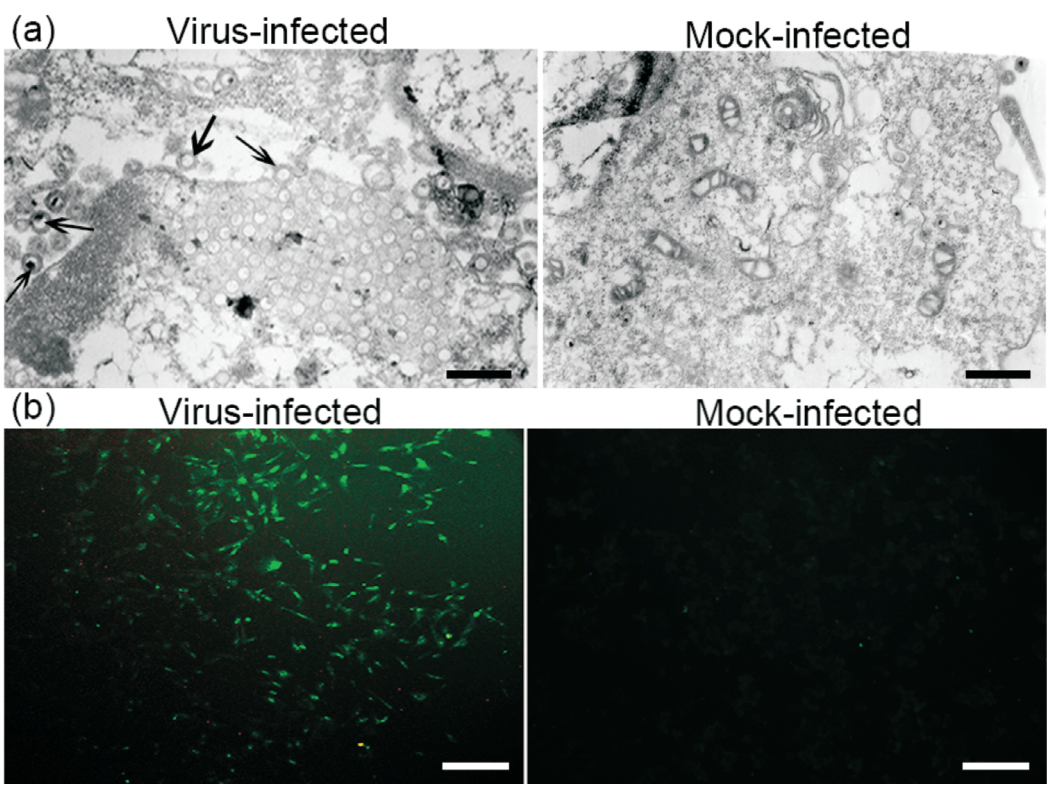

Fig. 3. The PEDV propagation successfully in the Vero cell cultures was confirmed by cell ultrathin section and IFA. (a) Multiply virus particles in the cytoplasm of Vero cells infected by CH/QX-2 were observed by TEM (*left*). No virions were viewed in the mock-infected cells (*right*). (Bar=333 nm). (b) Specific green fluorescence was visible in Vero cells 24 h post-infection with CH/QX-2 isolate (*left*), but no fluorescence was detected in control cultures (*right*). (Bar=100 μm).

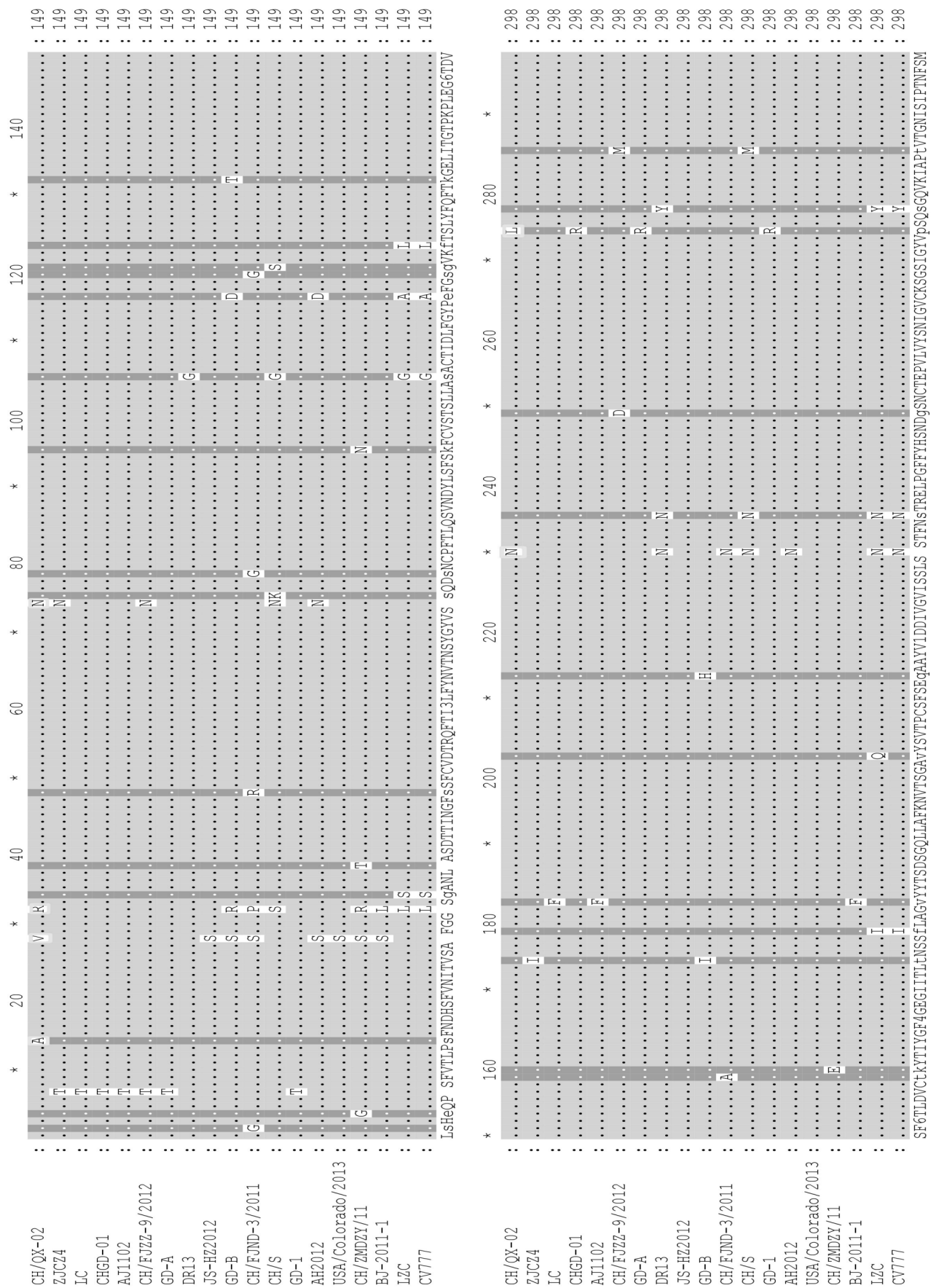

Fig. 4. Alignment of the partial S amino acid sequences of CH/QX-2 and PEDV reference strains by using Clustal W. The positions of the amino acid mutations in the partial S protein of CH/QX-2 isolate are marked by yellow square frame.

Cloning and phylogenetic analysis of the partial S gene for PEDV

The partial S gene amplified from the CH/QX-2 isolate gave an 894 nt fragment. The sequence of the partial S gene, encoding a 298 amino acid polypeptide, was deposited in GenBank under the accession numbers KF484735, and the polypeptide contained three neutralizing epitopes (7-146, 245-252, 271-278), with 94.7% nucleotide and 94.3% deduced amino acid sequence similarity to the reference strains. Furthermore, the deduced amino acid sequences analysis showed that there was no amino acid change in the epitope aa 245-252 on the S protein of CH/QX-2. However, compared with the vaccine strain CV777, some variants were observed in the epitope regions aa 7-146 and 271-278, including S→A at 14, A→V at 28, H→R at 32, K→N at 74, P→L at 274. The predicted B-cell epitope of partial S protein at aa 7-146 was based on the amino acid sequence of CV777 by BepiPred 1.0b Server. The results showed that there were three B-cell epitopes with high scores which located at positions aa 22-30, 32-40, 64-75, respectively. The amino acid mutations at positions aa 28, 32, and 74 of the S protein of CH/QX-2 were distributed within the epitope regions predicted above, respectively (Fig. 4), which indicated that these changes might result in antigenicity difference between CH/QX-2 and the vaccine strain CV777.

A phylogenetic analysis based on the partial S amino acid sequence of CH/QX-2 and the reference strains gave two clusters. CH/QX-2 was distributed into G1, together with most of the other Chinese strains (GD-1, GD-A, CHGD-01, LC, AJ1102, ZJCZ4, CH/FJZZ-9/2012, JS-HZ2012, BJ-2011-1, GD-B, CH/ZMDZY/11, CH/FJND-3/2011, and AH2012), and one American strain (USA/Colorado/2013). Noticeably CH/QX-2 differed from two earlier domestic strains (CH/S, 1986 and LZC, 2007), the Korean strains (DR13, 2007), and the current vaccine strain (CV777 vs). Interestingly, CH/QX-2 formed an ndividual unique clade in phylogenetic tree (Fig. 5).

Discussion

PEDV has been detected frequently in many provinces in China and has become one of the most economically important viral causes of diarrhea in piglets (Gao et al. 2013). At this time the losses caused by the continuing rate of PEDV infection is a serious problem for many pig farms in China. In this study, the samples were collected from a pig breeding farm in Henan province, China, where a severe acute diarrhea outbreak associated with high morbidity (80-100%) and mortality (60-100%) was observed in suckling piglets. For the whole-herd in this farm, a total number of 216 PEDV positive swine cases have been diagnosed in the following age groups: 93 suckling, 64 nursery, 36 grower/finisher, 23 sow/boar. Cleanliness, sanitation and disinfection measures were taken immediately following whole-herd exposure in preparation for the recovery phase post-infection. These actions reduce environmental load of virus and play a significant part in the restriction of viral transmission and biosecurity.

At present, RT-PCR based on the amplification of ORF3, M, S, and N gene has been used for the clinical diagnosis of PEDV (Lee et al. 2003, Park et al. 2008, Ogawa et al. 2009). However, morphological methods are still useful for the identification of an unknown virus. We initially examined the ultrastructure of lesions in the small intestine after an initial infection by PEDV. There was a loss of cell organelles and the terminal web. The tight junctions were lost, and microvilli had become fragmented or partially disappeared (date not shown).

It is necessary to diagnose rapidly and precisely in order to take steps to prevent the spread of PEDV. Due to the similarities in causative agents of diarrhea, the diagnosis of PED can not only depend on the clinical signs and histopathological lesions (Kweon et al. 1997). In this study nested RT-PCR was used for the detection of the PEDV based on a partial amplification of the M gene. We have applied this nested RT-PCR-based detection to clinical sample for diagnostic detection of PEDV and have found the positive rate of PEDV in all examined samples from 343 cases of diarrhea up to 78.6.

Trypsin is essential for the *in vitro* propagation of PEDV. The concentration of trypsin required in the culture medium is determined by the type of cell line and virus strain (Hofmann et al. 1988). In this study we found that treatment of the samples with 10 μg/ml of trypsin at 37 for 30 minutes before adding to the Vero cells was adequate to achieve an infection. Cytopathic effects (CPE) including cell fusion was subsequently observed in the infected Vero cells beyond the seventh passage (date not shown). Electron microscopy of infected cells at the ninth passage showed masses of virus particles distributed in a grid-like pattern in the cytoplasm (Fig. 3a), and the cells were positive by immunofluorescence (Fig. 3b). A sequence comparison with other PEDV strains selected from GenBank indicated that the partial S protein of CH/QX-2 shared highly homology with most Chinese strains and had a distant phylogenetic relationship with earlier Chinese strains (CH/S and LZC), the Korean strain (DR13), and the vaccine strain CV777. It is worth noting that the amino acid muta-

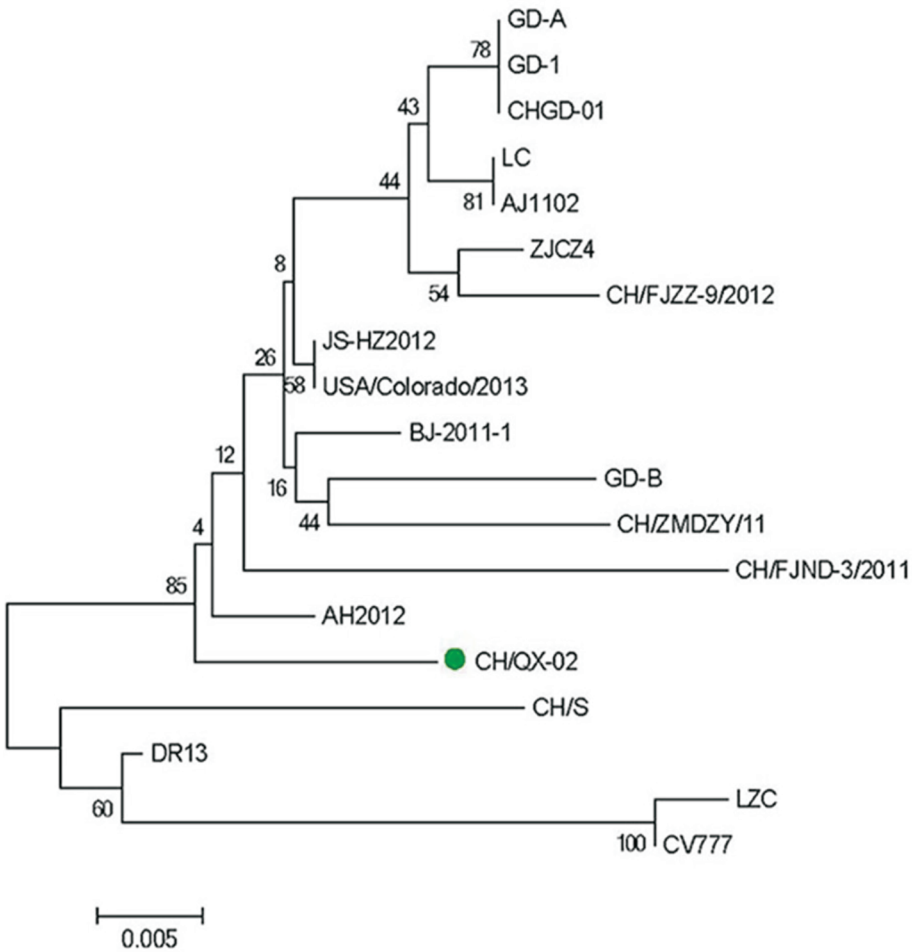

Fig. 5. Phylogenetic tree constructed based on the deduced amino acid sequences of the partial S genes from CH/QX-2 isolate and the referenced strains. The tree was generated by the neighbor-joining method with bootstrap analysis (1,000 replicates) and p-distance model. The CH/QX-2 isolate is highlighted with green triangle.

tions occurred within the B cell epitope regions and these could result in a change in the antigenicity of the new PEDV. Notably CH/QX-2 formed a unique clade in the phylogenetic tree which was closely related to AH2012 but did not belong to any subgroup (Fig. 5). Together, these findings indicate that CH/QX-2 is likely to be a new variant and most importantly may have a different antigencity to other strains and in particular to the strain used in the production of the current vaccine and hence has capacity to escape from immune surveillance and enhanced virulence which in turn causes immune response failure.

Using a novel alternative method we have successfully adapted PEDV to Vero cells allowing its proliferation by serial passage. This was confirmed by electron and immunofluorescence microscopy. Additionally, it was shown that although CH/QX-2 was genetically closer to most Chinese strains it differed from the earlier Chinese strains particularly the strain CV777 used for vaccine production. These findings indicate that the CH/QX-2 is a new variant of PEDV. There is an urgent need to study further the biologic characteristics, antigenicity, and mechanism of virulence variation of this new PEDV.

Acknowledgments

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