Isolation and identification of a variant subtype G 2b porcine epidemic diarrhea virus and S gene sequence characteristic

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ABSTRACT

Porcine epidemic diarrhea (PED) which is caused by porcine epidemic diarrhea virus (PEDV), is an intestinal communicable disease. In recent years, though pigs have been immunized with the vaccines in pig farms, PED still broke out and caused severe economic losses to the swine industry in the northeast China. In this study, the sample was positive for PEDV variant strains via the nano-nest PCR. The strain was successfully isolated from positive samples and was serially passaged in Vero-E6 cells. In addition, the strain was identified via electron microscopy observation, indirect immunofluorescence assay and infection experiment in newborn piglets and named PEDV CH/JLDH/2016 strain (Accession No. MF346935). Phylogenetic analysis of the S gene showed that the CH/JLDH/2016 strain was clustered into G2b subgroup. Comparing with the CV777 vaccine strain, amino acid sequence analysis of CH/JLDH/2016 strain showed that 15 nucleotides were inserted and 9 were absent in S gene, whose amino acid sequence it educed insertions of 5 amino acids([58]NQGX[61] and [145]N) and absences of 3 amino acids([164]RD[165] and [1204]Y). Our strain, in the SS2 epitope have no amino acid, variant while in SS6 epitope, Y changed into S in 776th amino acid. The results indicated that PEDV G2b variant strains have been emerged in Jilin province. The identification of new types of PEDV variant strains would stimulate the development of effective vaccines for the prevention and control of PED. The novel vaccines that based on these newly identified PEDV variant strains may contribute to the control of PED outbreaks in China.

1. Introduction

Porcine epidemic diarrhea (PED) which is caused by porcine epidemic diarrhea virus (PEDV), is an intestinal communicable diseases. PED is characterized by watery diarrhea, dehydration and weight loss in adult and lethal in piglets (Debouck and Pensaert, 1980; Pijpers et al., 1993). Since the first report in England in the 1970s (Choudhury et al., 2016; Pensaert and Martelli, 2016), PED has been reported in some countries (Pensaert et al., 1981; Takahashi et al., 1983). In the 1980s, PEDV was first identified in China (Sun et al., 2018). Until 2000, PEDV has been widely spread in 26 provinces in China, which made a largescale of economic losses in breeding industry. PED which is caused by variant PEDV, was re-emerged in China since October 2010 and caused devastating economic losses to the piggeries. The variant PEDV stains with five amino acids (aa) insertions at about 56–60 aa and one aa insertion at 141 aa of S protein comparing with CV777-based vaccines.

PEDV belongs to the member of the order Nidovirales, the family Coronaviridae and the genus Coronavirus (Pensaert and de Bouck, 1978). It is spherical 80–220 nm in diameter, with envelope. And it also has 20 nm rodlike fiber and a symmetrical spiral nucleocapsid. The approximately 28 kb genome is an infectious, single-stranded, positive-sense RNA, which 5′ → 3′ is in the order of 5′UTR-Replicase-S-ORF3-E-M-N-3′ UTR (Chen et al., 2013). The transmembrane S glycoprotein takes an important part in virus binding, with receptors, fusing cells and neutralizing antibodies development (Lai et al., 2007; Lee et al., 2010). S protein is implicated in virulence, which was found out in the other studies that S protein can be variant readily while receiving immune pressure (Gerber et al., 2014). Classifying PEDV with S gene can help us to understand its evolution rule easier. Therefore, S protein is used to study the viral relationships between different areas. It is an effective tool in PED epidemiologic investigations, also as diagnostic

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identification to distinguish between wild strain and cells accommodation attenuated strain (Park et al., 2011). In this study, PEDV CH/JLDH/2016 variant strain was isolated and identified, phylogenetic relationship between domestic and globally emerging PEDV isolates were also studied. The results would be helpful to find out the reason of the continuously outbreaks of PEDV in China and provide more information about the origin, evolution and diversity of PEDVs. Based on these newly identified PEDV variants, the novel vaccines may contribute to the control of PED outbreaks.

2. Materials and methods

2.1. Clinical samples collection and preparation

The samples (intestine tissues), were collected from a piggery in Dehui, Jilin province, China. There are 200 sows managed as four batches of 50 sows weaning approximately 550 piglets every five weeks. The herd was composed of 3 separate buildings on the same site with approximately 15–20 m distance between them (farrowing, nursery and fattening). 6-day-old piglets were exhibited watery diarrhea in clinical manifestation. Expansion small intestine, tenuous content and attenuation wall of intestine were showed after necropsy examination. The morbidity rate was evaluated about 80%, a total of 26 porcine samples including faeces and intestine tissues were collected. The piglets were characterized by vomiting, severe watery diarrhea, and dehydration, therefore suspected to be infected with PEDV. The samples were homogenized and diluted 1:10 with phosphate-buffered saline (PBS). The homogenate was freeze and thawed three times to release virus and further vortexed for 10 min and centrifuged at 12000 rpm/min at 4 °C for 15 min. Then supernatants were used for RNA extraction immediately or stored at −80 °C refrigerator for further usage.

2.2. RNA extraction, reverse transcription and nano-nest PCR detection

Total RNA was extracted using a Simply P Total RNA Extraction Kit (Bioer Technology Co., Ltd., China). Reverse transcription was performed using a TransScript First-Strand cDNA Synthesis Kit (Beijing TransGen Biotech Co., Ltd., China). The nano-nest PCR assay, which can distinguish between classical and variant strains of PEDV, was performed using a procedure described previously (Wang et al., 2017a). In brief, it was constructed with a nanoPCR kit (GREDBIO, China) and a mixture containing 0.4 μL cDNA, 12.5 μL nanoPCR mixture, 0.4 μL nanoTaq DNA polymerase, 1.5 μL each outer prime and nuclease-free water up to 25 μL. Thermocycling conditions of the first-round PCR consisted of a 5-min hold at 95 °C for denaturation, 35 cycles of 40 s at 94 °C, 1 min at 57 °C and 1 min at 72 °C with a final extension cycle 10 min at 72 °C. The second-round PCR were similar to the first, except the template was 3 μL of a 50-fold dilution of the first PCR products. The second nano-nest PCR thermocycling conditions consisted of 30 cycles of 40 s at 94 °C, 40 s at 58 °C and 40 s at 72 °C, with a final hold of 10 min at 72 °C. The amplicons were electrophoresed on a 1% agarose gel. Primer information was shown in the Table 1.

Table 1
Sequences of primers used in this study.

Primer name	Sequence (5′–3′)	Product size (bp)
P1-S-F	TTTAGGCGGTTCTTTTCA	817
P1-S-R	TTACAAACRCCATCSATC	
P2-S-F	CAGTTTCCHAGCATYAAA	295
P2-S-R	TACCATCCTCACCAGCAC	

F: forward primer; R: reverse primer.

2.3. Virus isolation

After incubated with Penicillin-Streptomycin solution (Gibco, USA), PEDV-positive sample solution filtrated in 0.22 μm filter. 30 μL 0.05% trypsogen was added into 2% culture bottle volume of sample, then incubated at 37 °C for 1 h. The viruses were confluented in Vero-E6 cells in monolayer, incubated in dulbecco's modified eagle medium (DMEM) (supplemented with 10 μg/mL trypsogen) which without serum at 37 °C for 1.5 h. After all cells were fed the DMEM medium mentioned above, all of those incubated at 37 °C and observed cytopathic effect (CPE).

2.4. Electron microscopy observation

This assay was performed using a procedure described previously (Wang et al., 2017b). Vero-E6 cells were infected with field isolates in a 25 cm² flask, and the cultures were observed closely for CPE. Approximately 16 h after the infection, the cell culture medium was decanted, and the monolayer was overlayered with cold 2.0% paraformaldehyde and 2.5% glutaraldehyde. The flasks were held for 1 h and then stored at 4 °C for a few days. Post-fixation was performed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 1–2 h. Grid staining was performed with 2% aqueous uranyl acetate and lead citrate. The samples were observed under an electron microscope.

2.5. Ultrathin section observation

This assay was performed using a procedure described previously (Oka et al., 2014). In brief, for imaging virions in infected Vero-E6 cells, Vero-E6 cells culture media was removed and replaced with fixative containing 3% glutaraldehyde, 1% paraformaldehyde in 0.1 M potassium phosphate buffer pH 7.2 (PB). Cells were fixed for 4 h at room temperature, then washed three times with excess PB, and post-fixed with 1% osmium tetroxide, 1% uranyl acetate in PB for 1 h. After three washes with distilled water, cells were collected, embedded in 0.6% low melting agarose, dehydrated through a graded ethanol-propylene oxide series, and embedded in EM Bed812 resin (Polysciences, USA). Ultra-thin-sections were prepared using a UC65 ultra-microtome (Leica, Germany). After staining with 3% aqueous uranyl acetate for 20 min, followed by Reynolds' lead citrate for 10 min, sections were imaged using a CJEM-1200EXII transmission electron microscope (Hitachi, Japan).

2.6. Indirect immunofluoresence assay (IFA)

This assay was performed using a procedure described previously (Wang et al., 2017b). Transformed the cultured cells into 24-well plates, after digesting and infected cells when they were cultured to monolayer. 24 h later, dropped the supernatant, and 80% cold acetone was added, in −20 °C for 2 h. Then, the fixative was removed and the cells were washed three times with PBST. Dropped the PBST. Fresh PEDV hyper-immune serum (supplemented with 50 times diluted 1 BSA) was added, and the infected cells were incubated at 37 °C. After 1 h, the cells were washed five times with PBST, then, 100 times diluted FITC-labeled rabbit anti-pig IgG (Sigma, USA) and 300 times diluted Evans blue were added, which were incubated at 37 °C for 1 h. Afterwards, the cells were washed with PBST and observed in fluorescence microscope.

2.7. TCID$_{50}$ assay

Vero-E6 cells were seeded in 96-well plates at a density of 3.5×10^5 cells per well and were grown at 37 °C for 36 h. Upon infection, the medium was removed, and 0.2 mL of 10-fold serial virus dilutions in Opti-MEM was added to each well. Eight wells containing a monolayer of cells were infected with 50 μL of each virus dilution, and the CPE was examined under a microscope every 12 h for 5–7 days post-infection.

The CPE was recorded, and the virus titer was calculated as the 50% tissue culture infective dose ($TCID_{50}$) using the Reed-Muench method.

2.8. Experimental design of PEDV infection in newborn piglets

Six newborn piglets were randomly allotted into two groups (3 piglets per group). Each inoculated group was separately housed in cages. Pigs were fed with liquid milk and had free access to water. Piglets in experimental group was separately inoculated with 3 mL of DMEM containing $1.0 \times 10^{4.29}$ $TCID_{50}$ of PEDV CH/JLDH/2016 strains. Piglets in control group were mock infected with 3 mL of DMEM. All virus inoculation was done via the oculonasal route. After inoculation, all pigs were monitored daily for clinical signs of disease, including diarrhea and vomiting. At necropsy, intestinal tissues and contents were grossly tested. Additionally, a portion of the jejunum and ileum were fixed in 10% neutral buffered formalin for histopathology.

2.9. Histopathologic examination

Tissue samples were collected and set in 10% buffered formalin solution, processed and embedded in paraffin for histopathological analysis. Three-mm-thick sections were stained with haematoxylin and eosin (H&E). Histopathologic changes were evaluated in 5 sections from each sample. Three samples from each group were analyzed.

2.10. Amplification and sequencing of S gene

The PEDV RT-PCR was established with a 25 μL reaction, which consisted of 3 μL cDNA template, others of 3 μL PCR buffer mixture, 2 μL dNTP Mixture, 0.5 μL each of primers, 0.5 μL Taq polymerase (TaKaRa) and top up of 25 μL ddH$_2$O. Primer sequences: S11-F: 5′ GGTAAGTTGCTAGTGCGTAA3′, S11-R: 5′CAGGGTCATCACAATAAA GAA3′; S12-DM-F: 5′TTTCTGGACCATAGCATC3′, S12-DM-R: 5′CTGCC AGATTTACAAACACC3′; S2-F: 5′GAGTTGCCTGGTTTCTTC3′, S2-R: 5′ TATAATTGCGCCTCAAAG3′. Reaction conditions: predenaturation at 95 °C for 5 min and denaturation at 94 °C for 40 s, 30 cycles of annealing for 1 min, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. 5 μL RT-PCR products were subjected to electrophoresis on a 1% agarose gel electrophoresis. RT-PCR-amplified target fragments were purified and recycled, connected with pMD18-T, transfused into competent cells. The positive plasmids were sequenced by Sangon Biotech (Shanghai) Co., Ltd. To obtain the consensus sequence, at least three independent sequencing for each amplicon were performed. Phylogenetic analyses were performed using Molecular Evolutionary Genetics Analysis software (MEGA) 6.06. Neighbor-joining method including the Bootstrap value of 1000 repetitions was used for construction of the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site.

3. Results

3.1. Results of nano-nest PCR identification

To determine the causative agent of the diarrhea outbreaks, pigs with severe diarrhea was euthanized using nano-nest PCR. Based on the classical and variant PEDV strains, the same sequence designed a fragment of 817 bp outer primers and the different region set inner primers of 295 bp. When the outer primers were simultaneously amplified, the classical and variant strains of PEDV displayed conserved regions. However, the inner primer only identified the variant strain and did not amplify the classical strain. Results showed that the sample was positive for PEDV variant strains (Fig. 1). Of 26 specimens with diarrhea symptom, 23 samples were detected as PEDV positive, and the positive rate was 88.46% (23/26). All of the PEDV-positive samples ($n = 23$), the positive rate of the PEDV variant strains was 80.77% (21/
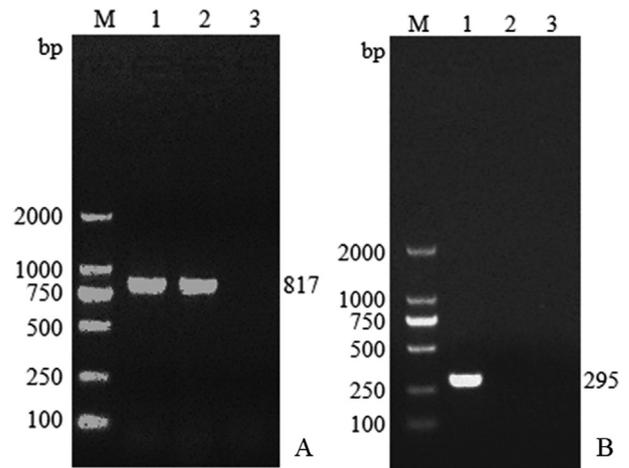

Fig. 1. Amplification results of nano-nest PCR. Size of the first products (A) and second products (B) of the PEDV nano-nest PCR was distinguished the classical strains and variant strains of PEDV by gel electrophoresis. M: DL2000; 1: Variant strains of PEDV; 2: Classical strains of PEDV (CV777); 3: Negative control.

26).

3.2. Results of virus isolation and culture

The viruses were successfully propagated in Vero-E6 cells, the CPE are shown in (Fig. 2B). The Vero-E6 cells that infected by PEDV of round shaped and shedding. These viruses can be passaged on Vero-E6 cells stably and named as PEDV CH/JLDH/2016 (Accession No.MF346935).

3.3. Result of Vero-E6 cells electron microscopy observation

After conventional negative staining, the complete virus with a typical coronavirus structure were showed under the transmission electron microscope. The virus had petal-like fibrils on the surface, and the diameter of the virus (including the fibrous) was about 100 nm–120 nm (Fig. 3A). The process of replication and release of PEDV virus particles in the endoplasmic reticulum was observed under electron microscope. As the pictures showed that PEDV which is oral or round shape, likes crown, surrounding with fiber and was pictured at 20 h after infected that entered the cells by endocytosis (Fig. 3B). No virions were observed in Vero-E6 cells without PEDV infection. The PEDV propagation successfully in the Vero-E6 cells cultures was confirmed by electron microscopy observation.

3.4. Result of indirect immunofluorescence assay (IFA)

The IFA method was used on Vero-E6 cells. Specific green fluorescence was visible in Vero cells 24 h post-infection with CH/JLDH/2016 isolated (Fig. 4A), but no fluorescence was detected in control cultures (Fig. 4B). The results showed that PEDV were successfully cultured in the Vero-E6 cells.

3.5. Clinical signs in piglets were experimentally reproduced by PEDV isolates

On the 3rd day, all piglets in experimental group inoculated exhibited typical clinical signs of PEDV infection, including watery diarrhea (Fig. 5A). Core body temperatures remained within normal limits. Piglets in the uninfected control group had no clinical signs. Histopathological examination showed the small intestine was thin-walled and contained soft to watery contents (Fig. 5B) in infected piglets. No
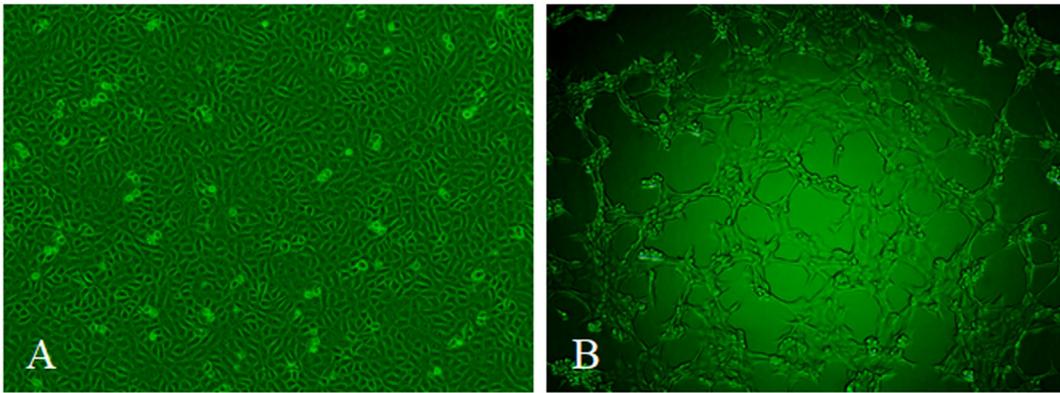
Fig. 2. The result of PEDV infected Vero E6 cells. A: Normal Vero E6 cells. B: PEDV infected Vero E6 cells. PEDV CH/JLDH/2016 induced an obvious CPE at 24 h post-inoculation, the Vero E6 cells that infected by PEDV were changed to round shaped and shedding.

intestinal lesions were found in uninfected piglets. Compared with the control group (Fig. 5C), small intestine of experimental group contained only short blunted villi variably lined by flattened squamoid-to-cuboidal epithelial cells (Fig. 5D).

3.6. S gene sequence analysis

A phylogenetic analysis based on the partial S gene of CH/JLDH/2016 and the reference strains gave two clusters. CH/JLDH/2016 was distributed into G2b, together with most of the other Chinese strains, and one American strain (KF468754) and one Canada strain (KM189367) (Fig. 6).

3.7. The alignment of amino acid sequence

S gene of CH/JLDH/2016 strain is in 4158 bp. As one of the dominant structural genes of PEDV, S gene codes S protein which plays an important role in invading cells and inducing the hosts to generate neutralizing antibodies. Comparing with vaccine strain, there are 15 bases insertions and 9 bases deletions in S gene that amino acid sequence educed insertions of 5 amino acids(^{58}NQGX^{61} and ^{145}N)and absences of 3 amino acids(^{164}RD^{165} and ^{1204}Y)of our strain (Fig. 7A, D). Containing: NQGX inserted in 58th–61th, N inserted in 145th, RD deleted in 164th–165th, Y deleted in 1204th. Our strain, in the SS2

epitope have no amino acid, variant while in SS6 epitope, Y changed into S in 776th amino acid (Fig. 7C).

4. Discussion

In Asia, the first time PED outbroke in Shanghai, China in 1973 (Sun et al., 2016). Since then, PEDV has made a severe influence in Asia, especially in China, Japan and Korea, which frequently leads to high mortality in piglets. PED as a fatal intestinal disease, made a large scale of economic losses in breeding industry. Many of researchers are trying to obtain the PEDV adaptive strains by culturing PEDV in vitro with many methods. However, there are few cells suit for culturing PEDV in vitro and low titer of multiplication. Moreover, complicated culture conditions are also the important reason for curbing the prevention and treatment of PEDV. We referenced to the method that isolating virus with trypsin as previous reports (Hofmann and Wyler, 1988; Kusanagi et al., 1992; Lee et al., 2015; Li et al., 2015; Zhang et al., 2018). Then, a strain of PEDV, CH/JLDH/2016, was isolated and identified in our study. These results suggest that variant PEDV strains have been existed in Jilin province, China.

PEDV has only a single serotype. As the difference between the amino acids of N-terminal domain in S gene, PEDV can be divided into two groups, G1 (G1a and G1b) group and G2 (G2a and G2b) group (Kocherhans et al., 2001). Most of the PEDV in Europe and Asia were
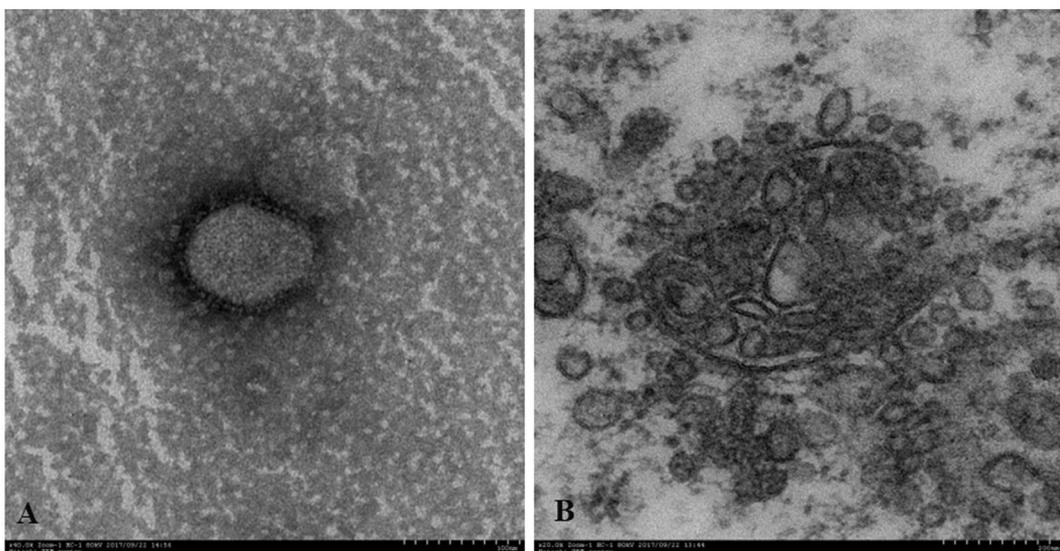

Fig. 3. Result of Vero E6 cells electron microscopy observation. A: Result of electron microscopy observation (×40 K). B: Result of Vero E6 cells ultrathin section electron microscopy observation (×20 K), there are lots of viruses in the cytoplasm.
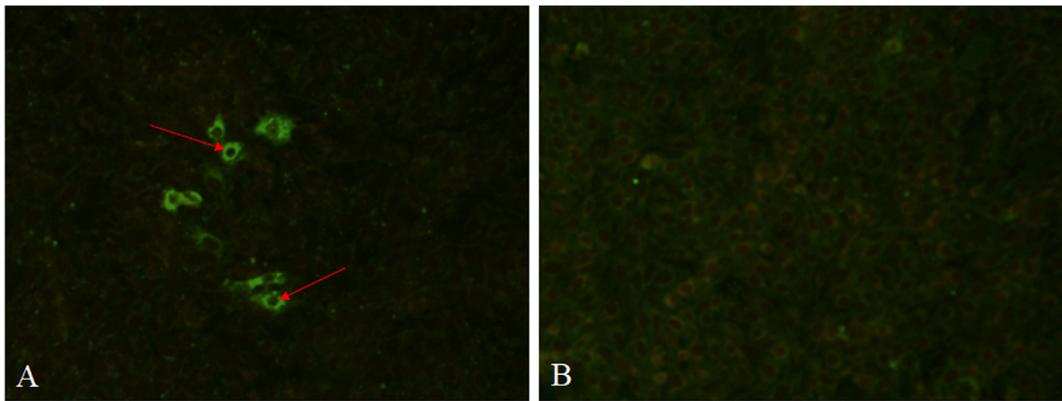
Fig. 4. Result of CH/JLDH/2016 indirect immunonfluscent assay. A: Vero E6 cells infected by CH/JLDH/2016 (10×). Green fluorescence can be seen in endochylema of Vero E6 cells after isolated strain stained by IFA. B: Negative control (10×). Dark red of Vero E6 cells of negative control was showed in fluorescence microscope Negative control (10×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

G1a strains before 2010, including classic strain and vaccine strain like CV777 and DR-1y231. In North America and Europe, G1a and G2b strain of gene U21a, which are also called S-IN DEL strain, were reported (Pensaert and Martelli, 2016). The genetic characteristic of classic G1a strain was contained in S gene, which is similar to classic G1a strain and epidemic G2b strain that is also called non-S-IN DEL or epidemic strain (Puranaveja et al., 2009). PED was outbroken in a large scale in Asia since 2010. As the article described, higher mortality in piglets of PED than before, which virus was mainly in PEDV G2b. After 2013, the PEDV G2b strain, which was similar to the PEDV strain
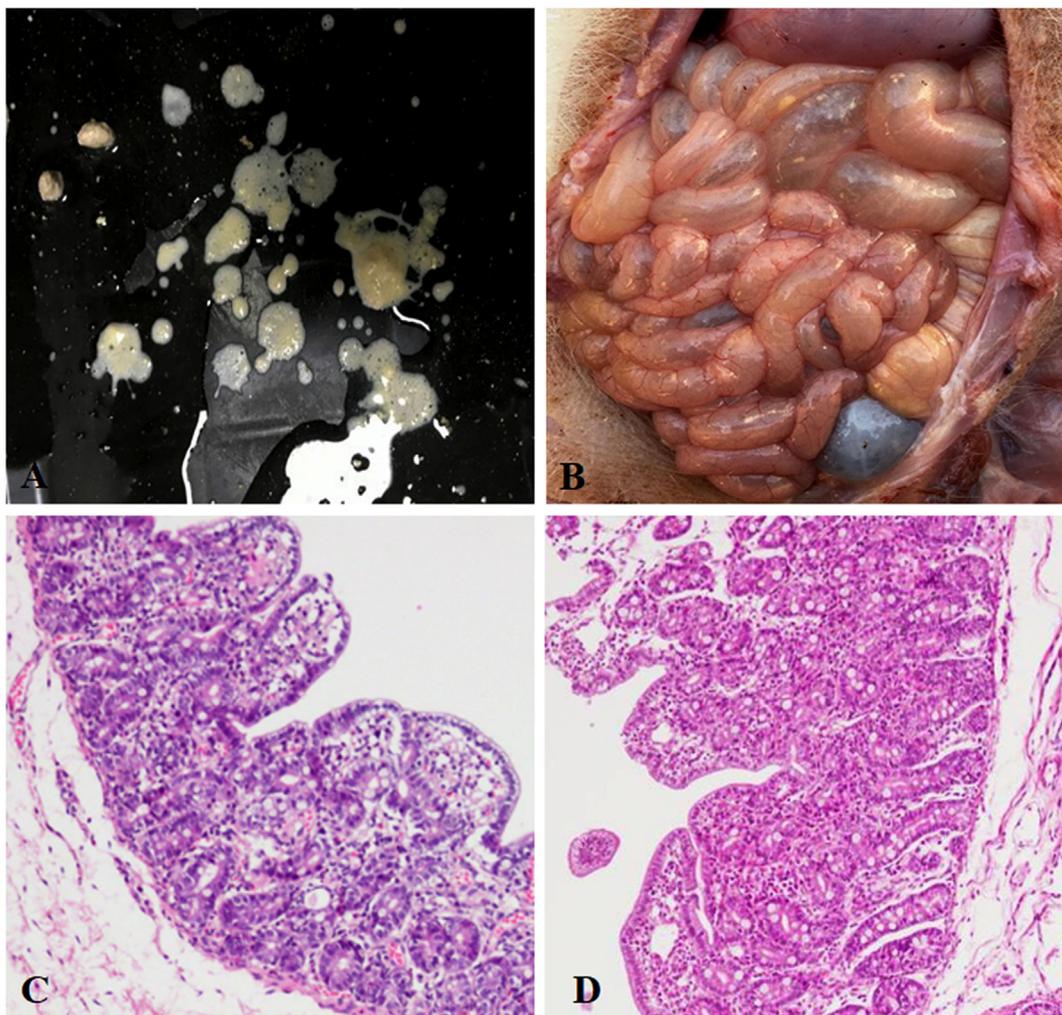

Fig. 5. The replication and pathogenesis of PEDV in newborn piglets. A-D: Necropsy examinations of the intestine of piglets inoculated with PEDV-JLDH and control medium. A: watery diarrhea. B: the small intestine was thin-walled and contained soft to watery contents. C: No lesions were found in the intestines of uninfected piglets. D: Severe villous atrophy of the ileum. The villous changes were associated with extensive intestinal epithelial degeneration and necrosis. All divisions of small intestine contained only short blunted villi variably lined by flattened squamoid-to-cuboidal epithelial cells.
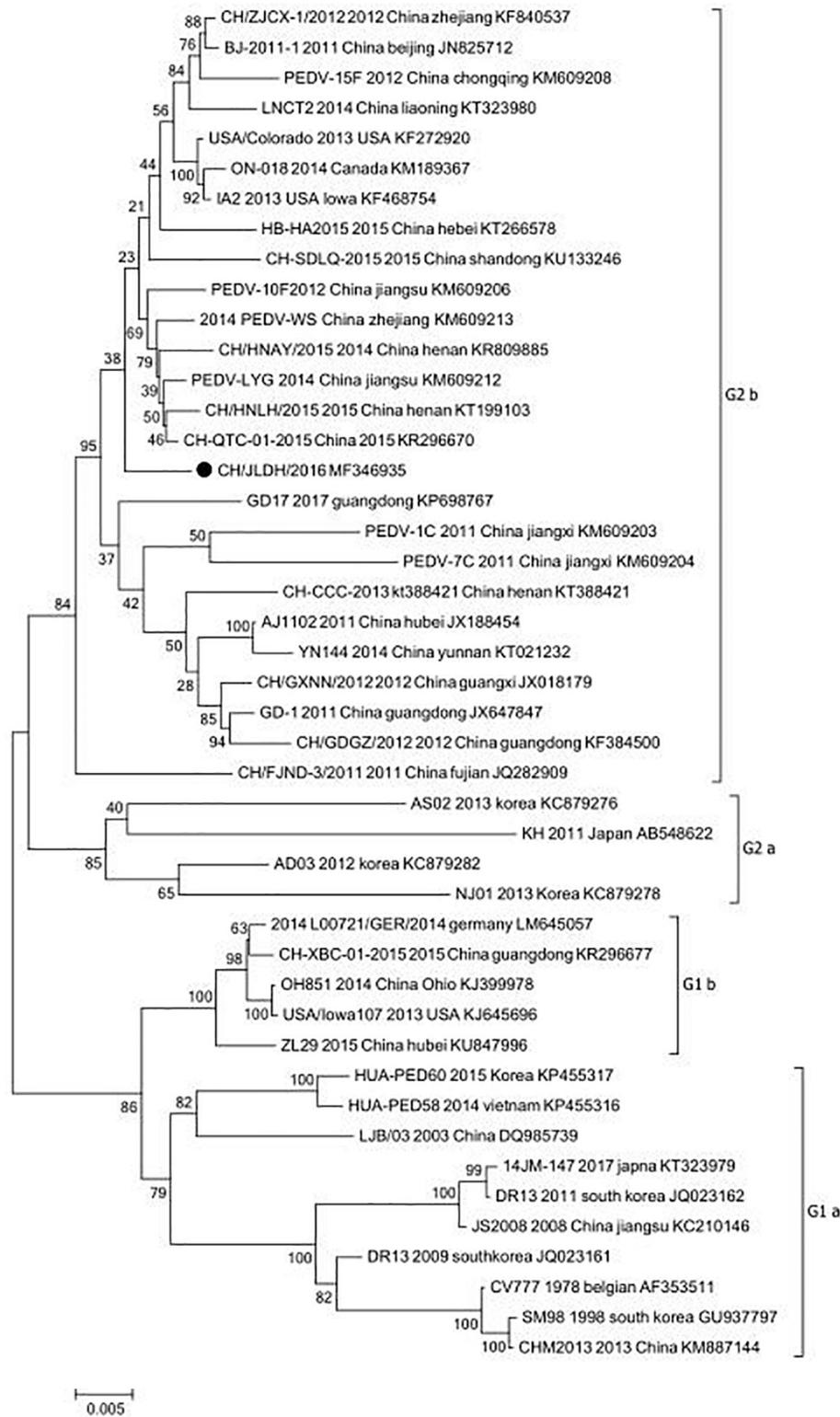
Fig. 6. phylogenetic tree based on the nucleotide sequences of the entire S genes of PEDV CH/JLDH/2016 strain identified in this study and other global reference strains. The tree was constructed by neighbor-joining method in the MEGA V7.0 program. Numbers at nodes represent the percentages of 1000 bootstrap replicates.

prevalent in China since 2010, was spread to the pig breeding areas in the mid-western American. The PEDV G2b was spread across to America, then was spread to Canada and South America (Takahashi et al., 1983). Coexisting with PEDV classic strain, the prevalent of PED is based on PEDV variant strain. To deal with the complex PED prevalent circumstance, we have S gene of our strain carried on sequence analysis. Results showed that PEDV variant strain was PEDV G2b strain.

It was first reported in Jilin province, China.

In the early 1900s, CV777 vaccine strain were used widely through the pig breeding industry in China, that less PED cases were reported in a large scale. However, PED outbroke in some pig produced provinces in the late 2010. In the same time, the PEDV variant strain was first reported in China (Wen et al., 2018). Moreover, the pigs that were inoculated vaccine CV777 still have a high mortality in the PED
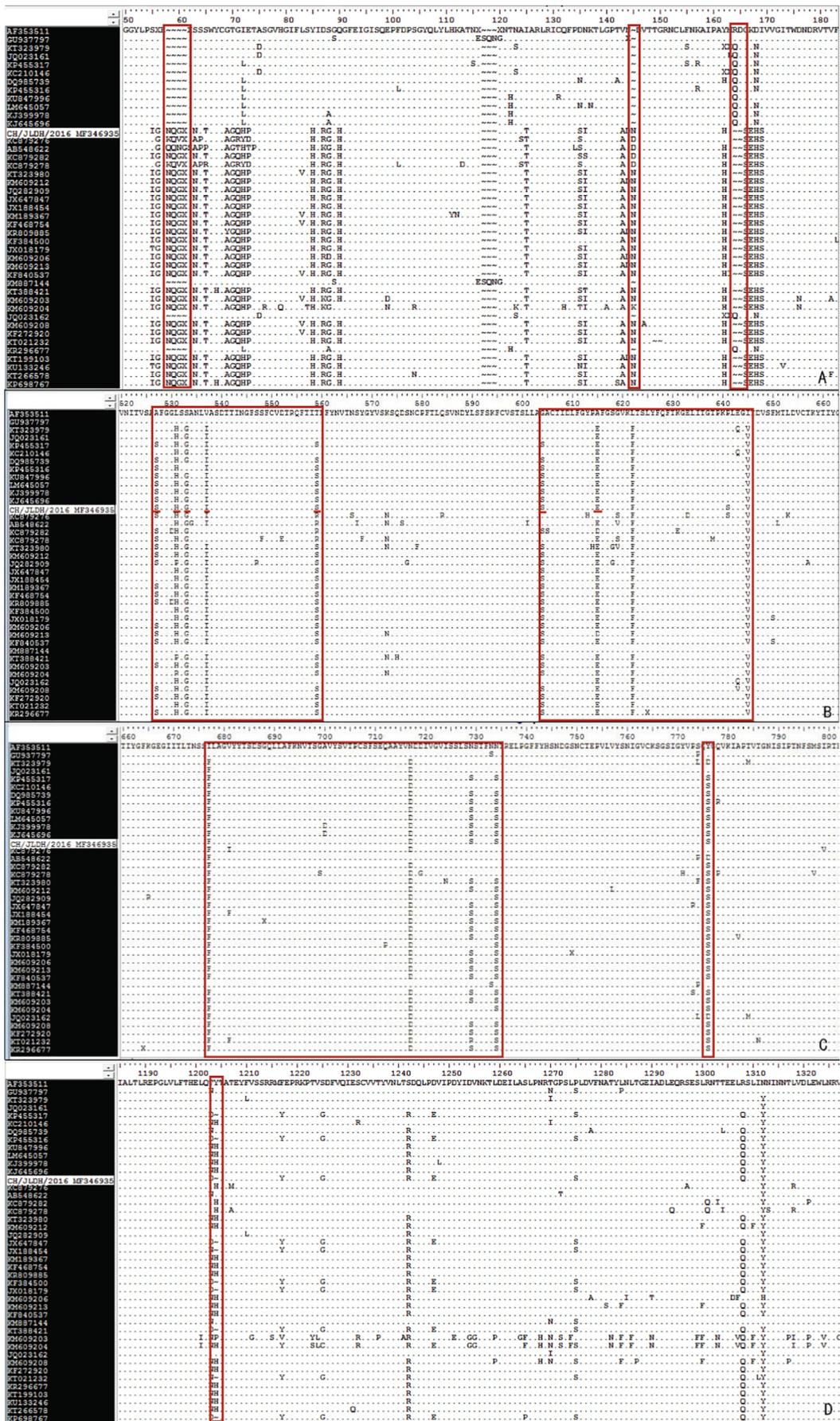
(caption on next page)

Fig. 7. The alignment of amino acid sequence of CH/JLDH/2016 strains and reference strains. The dashes (~) indicate the deletions and insertions. Deletions, insertions and variants in PEDV isolates are shown in red boxes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

outbreaks. Since then, PED was continued to be reported in different areas in China. Significantly, the samples were collected from 15 provinces in China from 2012 to 2017, in a study, which in all strains, G1a, G1b, G2a and G2b clusters accounted for 1.9%, 9.6%, 32.2% and 56.3%, respectively, namely G2a and G2b were the two most prevalent clusters in China (Wen et al., 2018). As the evidence showed that the PEDV strains of China have genetic diversity.

In this study, the CH/JLDH/2016 strain belongs to the different branches with the vaccine strains and the earlier epidemic strains of China, that cause a far relative among them. While sharing the same branch with the recent epidemic strains, the CH/JLDH/2016 strain has a close relative with them. The reason PED still epidemically outbreaks in the immune pigs may be the differences between recent epidemic strains and earlier epidemic vaccine strains. Comparing to vaccine strain CV777, there are 15 nucleotides insertions and 9 deletions in the S gene of our strain which of a massive amout of bases variant, 3 epitopes (527-559aa, 604-645aa, 677-734aa) induced by neutralizing antibody were in S1 section. As a study suggests that the SS2 epitope ([748]SNIGVCK[755]) in S1 region of classic strains and variant strains is highly conservative rather amino acids mutate in SS6 epitope ([764]QDGQVKI[771]) and porcine aminopeptidase N (pAPN) receptor binding domain (490~615aa) (Wang et al., 2016b). Our strain, in the SS2 epitope have no amino acid, variant while in SS6 epitope, Y changed into S in 776th amino acid, which have a homogeneity in the changes of amino acid of variant strain that reference presents. The epitope existence in conservative region and 7 amino acid mutants in pAPN receptor binding domain (aa490-615) in our strain that possesses the concordant result with CV777 vaccine in providing part of crossing immune protection for some epidemic PEDV strains in China. The changes of amino acids in the antigenic epitope may change the immunogenicity of the virus, which is in line with previous studies (Lin et al., 2016). Based on these finding, we speculated that variant subtype G 2b PEDV may be circulating in pig herds of Northeast China.

It is confirmed that piglets could not get the immune protection from the commercial vaccine made by the classical strains CV777 and DR13. The attenuated CV777 strain belongs to the G1b subgroup, which is not genetically closely related with our variant strain. The CV777 vaccine strain failed to provide sufficient protection against the PEDV variants due to limited cross-protection, all of which have been reported in other studies (Li et al., 2012; Sun et al., 2012; Vlasova et al., 2014; Wang et al., 2013, 2014, 2016a). While the inactivated antigen produced by the YC2014 strain could provide immune protection for piglets (Lin et al., 2016), which can infer that the potential reason PED outbroke in some immune piggery are that bases inserted and deleted in S gene and the changes of neutralizing epitope amino acids. Our variants were different from G1 group PEDVs, which helps to explain why the CV777 vaccine strain could not provide sufficient protection against the PED outbreaks in some pig farms nowadays. It is contributed to understand the mutated circumstances of prevalent strain in order to provide reference material of PED preventive treatment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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