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Isolation and characterization of Chinese porcine epidemic diarrhea virus with novel mutations and deletions in the S gene

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ABSTRACT

Porcine epidemic diarrhea (PEDV) has raised growing concerns in the pig-breeding industry because it has caused significant economic losses. To better understand the molecular epidemiology and genetic diversity of PEDV field isolates, in this study, the complete spike (S) and ORF3 genes of 17 PEDV variants in Zhejiang, China during 2014 to 2017, were characterized and analyzed. Phylogenetic analysis based on the S gene and ORF3 gene of these 17 novel PEDV strains and PEDV reference strains indicated that all the PEDV strains fell into two groups designated G1 and G2. Notably, the strains identified in 2014–2015 were in G2, while the other five strains identified from 2016 to 2017 were in G1. Sequencing and phylogenetic analyses showed that recently prevalent Chinese PEDV field strains shared higher identities with United States strains than with South Korean strains. Compared with classical vaccine strains, a series of deletions and frequently occurring mutations were observed in the receptor binding domains of our PEDV strains. Besides, we successfully isolated and reported the genetic characterization two novel PEDV strains, PEDV-LA1 and PEDV-LY4-98, found on the Chinese mainland, which had significant variations in the S gene. Meanwhile, the virulence of the new mutants may be changed, the PEDV-LY4-98 strain, which has multiple mutations in the signal peptide-encoding fragment of the S gene showed delayed cytopathic effects and smaller plaque size compared with strain PEDV-LA1, which lacks these mutations. Three unique amino acid substitutions (L7, G8, and V9) were identified in the SP-encoding fragment of the S1 N-terminal domain of the PEDV-LY4-98 S protein compared with the S proteins of all the previous PEDV strains. The animal experiment revealed that these two novel strains were high pathogenic to neonatal pigs. Whether these amino acids substitutions and the N-glycosylation site substitutions influence the antigenicity and pathogenicity of PEDV remains to be investigated. Meanwhile, amino acid substitutions in the neutralizing epitopes may have conferred the capacity for immune evasion in these PEDV field strains. This study improves our understanding of ongoing PEDV outbreaks in China, and it will guide further efforts to develop effective measures to control this virus.

1. Introduction

Porcine epidemic diarrhea (PED), which is caused by porcine epidemic diarrhea virus (PEDV), is an acute, highly contagious, and devastating swine disease that is characterized by enteritis and lethal watery diarrhea that results in dehydration, which frequently leads to high mortality in piglets (Coussement et al., 1982; Wood, 1977). PED was first reported in Belgium and the United Kingdom in 1978 (Kocherhans et al., 2001; Pensaert and de Bouck, 1978) and then emerged in a number of other European countries. Since 2010, more variant PEDV strains whose sequences differ from that of the classic European strain (CV777) have appeared recently in many Asian countries, including China (Bi et al., 2012; Li et al., 2012a; Pan et al., 2012; Song et al., 2015; Sun et al., 2015), South Korea, Japan, and Vietnam (Lee et al., 2010; Park et al., 2011). PEDV was first identified in the 1980 s in China, and since then, PED outbreaks have increased

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markedly and spread rapidly across countries. In October 2010, a large-scale outbreak of PED caused by a PEDV variant occurred in China, resulting in tremendous economic losses. New outbreaks associated with a novel PEDV strain that is genetically distant from the prototype PEDV strain, CV777, have been reported in China (Chen et al., 2012; Li et al., 2012a, b). PED is recognized worldwide for the dramatic changes observed in its epidemic character and pathogenic molecules. Since its initial recognition in May 2013, PEDV has rapidly spread across the United States (U.S.), resulting in high mortality in piglets in more than 17 states to date (Chen et al., 2014; Huang et al., 2013; Pasma et al., 2016).

PEDV is an enveloped, single-stranded, positive-sense RNA virus belonging to the order Nidovirales, the family Coronaviridae, the subfamily Coronavirinae, and the genus Alphacoronavirus (Huang et al., 2013). The approximately 28-kb genome contains a 5′ untranslated region (UTR), a 3′ UTR, at least seven open reading frames (ORFs), which encode four structural proteins, namely the spike (S), envelope (E), membrane (M), and nucleocapsid (N), and three non-structural proteins, designated as replicases 1a and 1ab and ORF3, which are arranged in the order of 5′ UTR-ORF1a/1b-S-ORF3-E-M-N-3′ UTR (Brian and Baric, 2005). Two long ORFs, ORF1a and ORF1b, occupy two-thirds of the genome and encode two nonstructural polyproteins (pp1a and pp1b) that direct genome replication, transcription and viral polyprotein processing.

The PEDV S protein is a key factor that mediates the entry of PEDV into host cells by binding to aminopeptidase N and/or glycan receptors through an unknown mechanism, thereby initiating the fusion of the virus envelope with the host cell membrane and the subsequent infection cycle (He et al., 2005; Ye et al., 2015). Thus, the S glycoprotein is the primary target for the development of vaccines against PEDV, as well as for understanding the genetic relationships between different strains and the epidemiological status of PEDV in the field. The PEDV S protein is a type I glycoprotein that consists of three segments - an ectodomain, a single-pass transmembrane anchor, and a short intracellular tail (Li, 2012). The ectodomain is often cleaved into a receptor-binding S1 subunit and a membrane-fusion S2 subunit, and the amino acid sequences of S1 subunits diverge across different genera, but are relatively conserved within each genus (Deng et al., 2016; Zheng et al., 2006). S1 can recognize a variety of host receptors, including proteins and sugars (Makadiya et al., 2016; Wang et al., 2016). induce most of the host immune responses, and may serve as subunit vaccines against coronavirus infections. The S protein is also the major envelope glycoprotein, contains four neutralizing epitopes named COE, SS2, SS6, and 2C10, and the regions of the aligned sequences that correspond to these regions are amino acids 499–638, 748–755, 764–771, and 1368–1374 (Chang et al., 2002; Li, 2015; Sun et al., 2008). Mutations, deletions and/or insertions in the S protein may change the pathogenicity and antigenicity. A previous report suggested that a field CHN/FL2013 strain with a 7 aa-earlier termination of S protein showed reduced virulence in cell culture (Zhang et al., 2015). Park et al. reported that Korean strain MF3809/2008 was identified with a large deletion (204 aa) at amino acid positions 713–916 of the S protein reduced its virulence and changed its tissue tropism from intestinal (Park et al., 2014). The highly virulent PEDV strains from China had an increased number of substitutions within the S1 domain compared with the highly virulent American strains, probably due to the longer circulation time of PEDV in pigs in China. N terminal domain. bioinformatics predicted that the S protein of highly virulent PEDV strains changed in primary/secondary structures, high-specificity N-glycosylation sites, potential phosphorylation sites, and palmitoylation sites (Chiou et al., 2017; Hao et al., 2014) These changes may affect viral antigenicity and change viral neutralizing activity. In recent years, new S mutant strains have been constantly found and this may be related to the immune pressure of the vaccines that are used, including those from vaccine strains CV777 and DR13.

Unlike the structural proteins, little is known about the functions of the accessory proteins. The ORF3 gene, which is the only accessory gene, encodes an ion channel protein that regulates virus production (Wang et al., 2012), and it has been suggested to be an important virulence determinant (Ye et al., 2015). A previous report suggested that piglets that were inoculated orally with the wild-type CV777 strain became sick and developed severe diarrhea. However, piglets inoculated with strain KPEDV-9, which was serially passaged in Vero cells, showed reduced disease and fewer lesions (Kweon et al., 1999). Different ORF3 variants could serve as markers of vaccine- and wild-type PEDVs, and they could be a valuable tool for studying the molecular epidemiology of PEDV (Kim et al., 2016; Li et al., 2016).

In this study, we found two novel PEDV field strain PEDV-LA1 and PEDV-LY4-98 with amino acid mutations and deletions in the protein encoded by the S gene. The animal experiment revealed that PEDV-LY4-98 strain were high pathogenic to neonatal pigs. This study showed that a PEDV strain with the new molecular characterizations and phylogenies was found in southern China. This will be useful to take into consideration in the control and prevention of this disease. Thereby, a comprehensive study is necessary to better understand the genetic variations and relationships between different strains, and would be helpful to find out the reason of the continuously outbreak of PEDV and develop new strategy to control and prevent PEDV infection.

2. Materials and methods

2.1. Sample collection

In this study, a total of Eighty-five porcine samples, consisting of feces or intestine tissues, were collected from 8 farms in Zhejiang provinces during 2014 to 2017. All samples were taken from pigs that exhibited severe watery diarrhea, vomiting, and dehydration. Seventeen of the 85 samples were confirmed as positive for PEDV, as determined by a PEDV N gene-based real-time RT-PCR. PEDV-positive intestine tissues were used to generate a 10% (wt/vol) homogenate in phosphate-buffered saline (0.1 M, pH 7.2). The suspension was centrifuged at 4500 × *g* for 10 min at 4 °C, filtered through a 0.22-μm-pore-size syringe filter and used as an inoculum for virus isolation. One-tenth gram of feces was suspended in 1 ml phosphate-buffered saline(PBS), vortexed for 5 min, and then centrifuged at 4500 × *g* for 5 min.

2.2. PEDV N gene-based real-time RT-PCR

Viral RNA extraction was performed with 50 μl of the small intestine homogenates using the Nucleic Acid Co-prep Kit (GeneReach Biotechnology Corp., Taichung, Taiwan) according to the manufacturer's instructions. The concentrations of the extracted RNAs were measured by a Synergy H1 microplate reader (Bio Tek, Winooski, VT, USA), and then eluted into 90 μl of elution buffer. Primers and probes for real-time RT- PCR were designed and synthesized to target conserved regions of the PEDV N gene. RT-PCR was conducted using 50–200 ng of extracted RNA, a forward primer (5′–TTGGTGGTAATGT GGCTGTTCGTG–3′), a reverse primer (5′–ATCCACCTGTGAAACAAGA AGCTCAAC–3′), and the One-Step RT-PCR Kit (TaKaRa, Shiga, Japan) according to the manufacturer's protocol.

2.3. PCR amplification and sequencing

Viral RNAs were extracted from PEDV-positive sample suspensions as described above. RT was conducted using random hexamer primers, and first-strand cDNA was synthesized using the ReverTra Ace® qPCR RT Kit (Toyobo, Tokyo, Japan) at 42 °C for 60 min and then at 98 °C for 5 min to inactivate the Moloney murine leukemia virus reverse transcriptase, followed by incubation 4 °C for 5 min. The cDNA was immediately used for amplification or stored at −20 °C. The S and ORF3 genes were amplified by RT-PCR using KOD-Plus Neo (Toyobo, Tokyo, Japan) DNA polymerase with primers targeting sequences that are

conserved among the CV777 strain and recently prevalent Chinese PEDV field strains **(data not shown)**. The S gene was amplified using primers S-F and S-R under the following conditions: denaturation at 94 °C for 2 min, 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C for 2.5 min, followed by a final extension at 68 °C for 7 min. The ORF3 gene was amplified using primers ORF3- F and ORF3-R (**data not shown**) under the following conditions: denaturation at 94 °C for 2 min, 35 cycles of 98 °C for 10 s, 53 °C for 30 s, and 68 °C for 2.5 min, followed by a final extension at 68 °C for 7 min. PCR products were excised from 1.0% agarose gels and purified using the AxyPrepTM DNA Gel Extraction Kit (Axygen Scientific, Inc., Union City, CA, USA) and then cloned into pMD18-T vector. Three recombinant DNA clones of each PEDV strain were sequenced by Auspicious Biological Co., Ltd. (Shanghai, China).

2.4. Electron microscopy

Samples were prepared for negative-stain and thin-section examination by electron microscopy (EM) following previously described procedures with modifications. The ISU13-22038 small-intestine homogenate was centrifuged at 4200 × *g* for 10 min, and the supernatants were subjected to ultracentrifugation at 30,000 × *g* for 30 min to pellet the virus particles, which were then negatively stained with 2% phosphotungstic acid (PTA; pH 7.0) and examined with a FEI Tecnai G2 BioTWIN electron microscope (FEI Co., Hillsboro, OR).

2.5. Phylogenetic analysis

Phylogenetic analysis was performed using the amino acid sequences of the 17 PEDV viruses from this study as well as 16 other China PEDV strains and 11 non-China PEDV strains with complete S gene sequences available in GenBank. Phylogenetic trees were constructed using the spike gene and a hypothetical protein gene (ORF3) gene sequences. The trees were constructed using the distance-based neighbor-joining method of MEGA6.0 software. Bootstrap analysis was carried out on 1000 replicate data sets.

2.6. Virus isolation and propagation

Vero E6 cells were seeded in a T-25 flask at a density of 1×10^7 cells, and they were grown at 37 °C for 18 h. Upon infection, the medium was removed, and then the cells were washed twice with phosphate-buffered saline and infected with 1 ml of inoculum. After 1 h of adsorption with constant shaking, PEDV maintenance media (Dulbecco's modified Eagle's medium supplemented with 0.3% tryptose phosphate broth, 2 μg/ml trypsin, and 1% penicillin–streptomycin) was added without removing the inoculum, and then the infected cells were incubated at 37 °C for 5 d. When extensive cytopathic effects (CPEs) were observed, the supernatant and cells were harvested by three freeze-thaw cycles, followed by centrifugation at 1500 × *g* at room temperature for 15 min. The final supernatants were collected, aliquoted, and stored at −80 °C. Subsequently, the supernatant was used to infect new Vero E6 cells until T-75 flasks were used for viral propagation.

2.7. Genetic stability of PEDV strains in cell culture

Confluent Vero E6 cells in T25 flasks were infected by PEDV strains at an MOI of 0.1. When extensive CPE was observed, the cell culture supernatant was harvested and used for the next passage in Vero E6 cells. The PEDV strains were repeatedly passaged 10 times in Vero E6 cells. At each passage, the S gene was amplified by RT-PCR and sequenced.

2.8. Viral replication kinetics in Vero-E6 cells

This study was performed using a procedure described previously (Sun et al., 2014). Confluent Vero-E6 cells in 35-mm dishes were infected by PEDV-LA1 or PEDV-LY4-98 at an MOI of 0.1. After adsorption for one hour, the inoculum was removed, then the cells were washed three times with phosphate-buffered saline (PBS). Fresh DMEM medium (supplemented with 2% FBS) was added, and the infected cells were incubated at 37 °C. At different time points post-infection, the supernatant and cells were harvested followed by centrifugation at 1500 × *g* at room temperature (RT) for 15 min. Virus titer was determined by plaque assay in Vero-E6 cells.

2.9. $TCID_{50}$ assay

This TCID50 assay was performed using a procedure described previously (Sun et al., 2014). Vero-E6 cells were seeded in 96-well plates (Corning, Lowell, MA) at a density of 10^5 cells per well and were grown at 37 °C for 18 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 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.

2.10. Plaque assay for PEDV detection

The histology study was performed using a procedure described previously (Sun et al., 2014). Vero cells were seeded in six-well plates at a density of 2×10^6 cells per well. After incubation for 18 h, the medium was removed, and cell monolayers were infected with 400 μl of a 10-fold dilution series of each virus. After incubation at 37 °C for 1 h with agitation every 10 min, the cells in each well were overlaid with 2.5 ml of Eagle's minimum essential medium containing 0.8% agarose, 0.3% tryptose phosphate broth, 5000 UI/ml penicillin–streptomycin, and 2 mg/ml trypsin. The plates were incubated at 4 °C for 30 min to solidify the overlaid medium. Then, the cells were grown at 37 °C and 5% CO_2 to allow for plaque formation. After incubation for 3 d, the cells were fixed in 10% (vol/vol) formaldehyde for 2 h, and the plaques were visualized by staining with 0.05% (wt/vol) crystal violet.

2.11. Experimental design of PEDV infection in newborn piglets

Nine newborn piglets were randomly allotted into three 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 group 1 and group 2 were separately inoculated with 3 ml of DMEM containing $1.0 \times 10^{6.5}$ $TCID_{50}$ of PEDV-LY4-98 and PEDV-LY1 strains. Piglets in group 3 were mock infected with 3 ml of DMEM and served as uninfected controls. All virus inoculation was done via the oculonasal route. After inoculation, all animals were monitored daily for clinical signs of disease, including diarrhea and vomiting. For sampling, we chose different time points to collect samples, one piglet was euthanized at 48 h post-inoculation (hpi), and the other two piglets were euthanized at 54 h and 60 h, respectively. At necropsy, intestinal tissues and contents were grossly evaluated. Additionally, a portion of the jejunum and ileum were fixed in 10% neutral buffered formalin for histopathology.

2.12. Histology

The histology study was performed using a procedure described previously (Sun et al., 2014). After euthanization, the intestinal tissue of each piglet were removed, inflated, and fixed in 4% neutral buffered formaldehyde. Fixed tissues were embedded in paraffin and sectioned

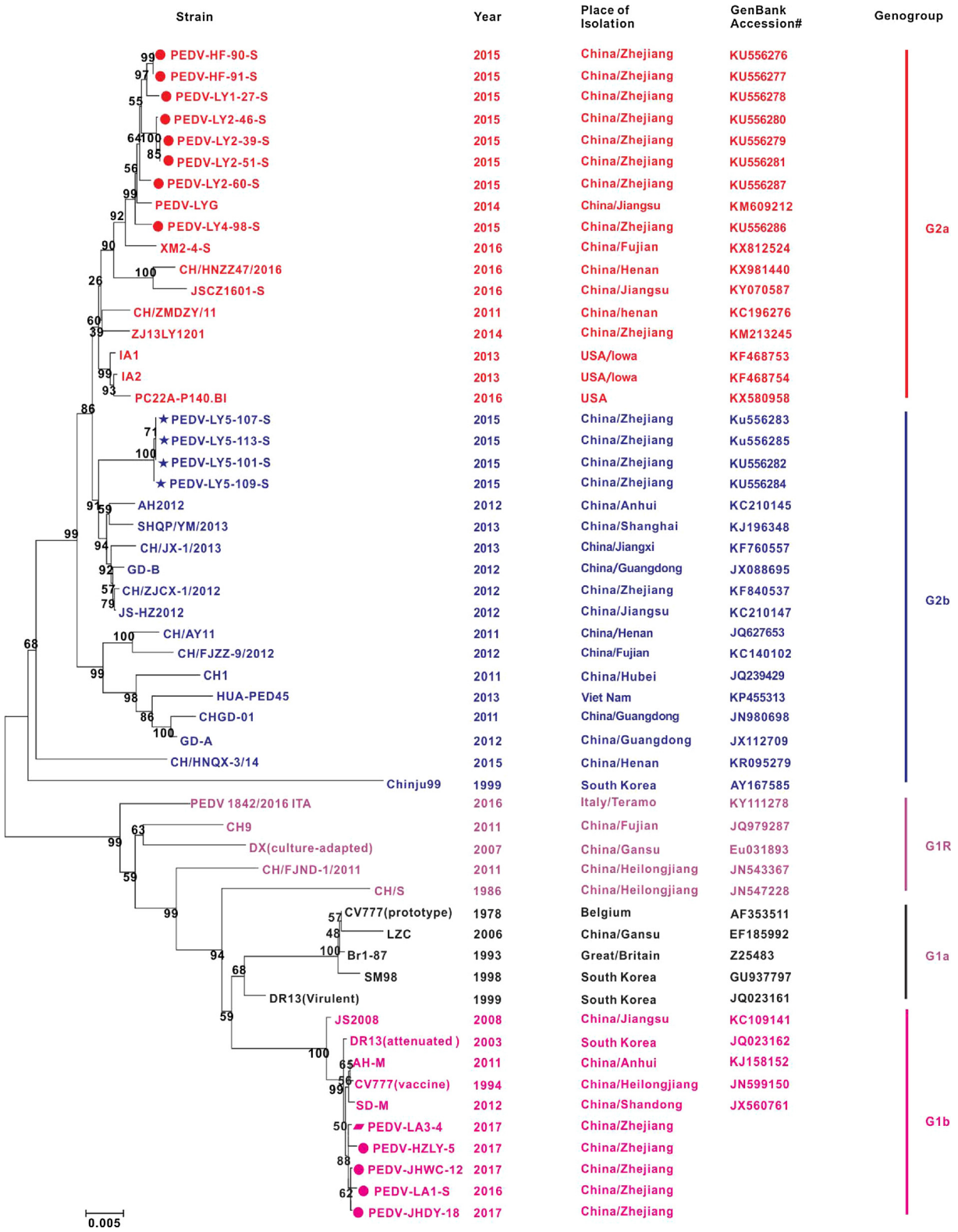

Fig. 1. Phylogenetic analysis of PEDV isolates and reference strains based on the S gene.
The phylogenetic tree based on the S gene of PEDV was constructed by the neighbor-joining method using the MEGA 6 program. The scale bar indicates the estimated number of substitutions per 20 amino acids. Bootstrap values are indicated for each node from 1000 resamplings. The names of the strains, years and places of isolation, GenBank accession numbers, and genogroups and subgroups proposed in this study are shown. Red solid circles, the eight PEDV strains in subgroup 2a from our study; Blue solid circles, the four PEDV strains in subgroup 2b from our study. Pink solid circles, the four PEDV strains in subgroup 2b from our study. All the PEDV strains fell into two groups designated G1 and G2. At least three subgroups (1a, 1b, and 1R) were classified as genogroup G1, while two subgroups (2a and 2b) were classified as genogroup G2. The strains identified in 2014–2015 were in G2, while the other five strains identified from 2016 to 2017 were in G1.

at 5 μm. Slides were then stained with hematoxylin and eosin.

3. Results

3.1. PEDV-LY4-98 and PEDV-LA1 viruses were isolated

To determine the causative agent of the diarrhea outbreaks, pigs with severe diarrhea was euthanized, and fecal, intestinal content, liver, kidney, lung, and blood samples were collected. Total RNA was extracted from these samples and subjected to reverse transcription (RT)-PCR. Results showed that these samples were positive for PEDV. Feces and intestinal contents were filtered and subsequently analyzed by electron microscopy. Typical coronavirus like particles with diameters ranging from 60 to 180 nm were observed **(data not shown)**. No other viral particles were observed. Subsequently, the filtered duodenum contents were subjected to deep sequencing. No other known viral sequences were identified, except for PEDV. The virus identified was named PEDV-LY4-98 and PEDV-LA1.

3.2. PEDV-LY4-98 and PEDV-LA1 are new variant strains

The nt and deduced amino acid identities of the S genes and S proteins of the 17 strains from this study (PEDV-LY1-27, PEDV-LY2-39, PEDV-LY2-46, PEDV-LY2-51, PEDV-LY2-60, PEDV-HF-90, PEDV-HF-91, PEDV-LY4-98, PEDV-LY5-101, PEDV-LY5-107, PEDV-LY5-109, and PEDV-LY5-113, PEDV-LA3-4, PEDV-HZLY-5, PEDV-JHWC-12, PEDV-LA1, PEDV-JHDY-18) that are shared with PEDV reference strains are analysed. These 17 strains shared 94% to 95% nt sequence identities and 92% to 93% deduced amino acid identities with each other. The 12 isolates (PEDV-LY1-27, PEDV-LY2-39, PEDV-LY2-46, PEDV-LY2-51, PEDV-LY2-60, PEDV-HF-90, PEDV-HF-91, PEDV-LY4-98, PEDV-LY5-101, PEDV-LY5-107, PEDV-LY5-109, and PEDV-LY5-113) identified in 2014–2015 shared more homology with U.S. strains than South Korean strains and European strains. Compared with CV777 vaccine strain, the 12 isolates shared 94% nt sequence identities and 92% to 93% amino acid identities. While the five isolates (PEDV-LA3-4, PEDV-HZLY-5, PEDV-JHWC-12, PEDV-LA1, PEDV-JHDY-18) identified in 2016–2017 shared 99–100% homology with CV777 vaccine strain. Besides, the identities shared between the five isolates and the U.S. stains was only 92–93%. Our PEDV strains shared higher identity with U.S. strains than South Korean strains **(data not shown)**. ZJ13LY1201 (a Chinese PEDV strain) shared the highest DNA sequence identity (99%) with our PEDV-LY2-60, PEDV-LY4-98, and PEDV-HF-90/91 strains, exhibiting only 34-, 35-, and 39-nt differences, respectively, across the entire S gene. Comparisons of the nt and amino acid sequences of the ORF3 genes and ORF3 proteins of the PEDV reference strains and PEDV isolates are demonstrated. The nt sequences of five strains in the study (PEDV-HF-90, PEDV-LY4-98, PEDV-LY5-101, PEDV-LY5-109, and PEDV-LY5-122) shared 99.0% to 100.0% DNA sequence and deduced amino acid identities with one another. Compared with the reference strains, PEDV-LYG (a Chinese strain isolated from Jiangsu) shared the highest DNA sequence identity (99%) and deduced amino acid identity (greater than 99%). However, our PEDV strains shared only 94.0% DNA sequence identity and 86% deduced amino acid identity with the CV777 vaccine strain.

Sequences of the partial ORF3 genes of nine classical PEDV field isolates were determined. We identified a total of 27 nt differences between our strains and the consensus sequence, which resulted in eight amino acid changes. Previously reported attenuated-type PEDVs had 17 specific deduced amino acid sequence deletions (at positions 82–98), which were produced by 51 nt sequence deletions (at positions 245–295), that were not found in wild-type PEDVs. A comparison of the nt and deduced amino acid sequences of the ORF3 genes of wild- and attenuated-type PEDVs showed that this large deletion was not seen in our five PEDV isolates identified in 2014–2015, which further showed that these isolates are new variant strains, rather than the vaccine strain **(data not shown).**

3.3. PEDV-LY4-98 and PEDV-LA1 belongs to G2a and G1b genogroup

Phylogenetic analysis based on the S gene and ORF3 gene of these 17 novel PEDV strains and PEDV reference strains indicated that all the PEDV strains fell into two groups designated G1 and G2. At least three subgroups (1a, 1b, and 1R) were classified as genogroup G1, while two subgroups (2a and 2b) were classified as genogroup G2. Notably, the strains identified in 2014–2015 were in G2, while the other five strains identified from 2016 to 2017 were in G1. Sequencing and phylogenetic analyses showed that recently prevalent Chinese PEDV field strains shared higher identities with United States strains than with South Korean strains. Compared with classical vaccine strains, a series of deletions and frequently occurring mutations were observed in the receptor binding domains (RBD) of our PEDV strains (Fig. 1).

In addition, a phylogenetic analysis of the ORF3 gene showed that strains IA1 and IA2 shared 100% identity, while strain CV777 shared 96% identity, with our PEDV strains (excluding PEDV-HF-90). The phylogenetic relationship of the ORF3 genes indicates that these new PEDV variants differ genetically from European field strains (CV777 and Br1/87) and that they have a closer phylogenetic relationship to U.S. strains than some South Korean strains **(data not shown)**.

3.4. Three unique amino acid substitutions were found in S protein of PEDV-LY4-98

The S genes of all 17 PEDV strains contained 1387 amino acid, which is 3-aa longer than that of the CV777 strain. Unique genetic characteristics were observed among our PEDV strains based upon a sequence analysis of their S genes. Notably, four strains (PEDV-LY5-101, PEDV-LY5-107, PEDV-LY5-109, and PEDV-LY5-113) had a 1-amino acid mutation (T) at position 20, whereas all other strains had a F residue at this position. The phylogenetic analysis also showed that these four isolates all fell into genogroup G2b, whereas the others fell into other groups **(data not shown)**. These unique amino acids can potentially serve as genetic markers to discriminate U.S. strains from other strains. Interestingly, a sequence alignment analysis of S1 (one subunit of the S protein) showed that a total of 42 unique amino acid changes were found between the PEDV-LY5-101, PEDV-LY5-107, PEDV-LY5-109, and PEDV-LY5-113 strains and the CV777 strain. Specifically, these strains all share ten additional unique amino acids (T20, V82, N139, Y184, S222, S240, D525, V533, L554, and V614) with the CV777 strain and other new isolates. Strains PEDV-LY2-39, PEDV-LY2-46, and PEDV-LY2-51 share two additional unique amino acids (N117 and V152) with the other isolates and the CV777 strain. In particular, compared with the CV777 strain, the 12 variant strains shared a 4-amino acid insertion at positions 59–62, a 1-amino acid insertion at position 140, and a 2-amino acid deletion at positions 163 and 164 of the S protein. Three unique amino acid substitutions (L7, G8, and V9) were identified in the SP-encoding fragment of the S1 N-terminal domain of the PEDV-LY4-98 S protein compared with the S proteins of all the previous PEDV strains **(data not shown)**. The first 18 amino acids in the S protein constitute a signal peptide-encoding fragment (SP), which contributes to viral protein release. The large mutation in the SP of the S protein may play an important role in viral protein release, which might affect the virulence of the virus.

3.5. COE antigen variants were found in neutralizing epitopes in PEDV isolates

Compared with the CV777 strain, six mutations (S522 A, G528S, S599 G, E610 A, I640 V, and S771Y) were found in all 12 PEDV strains, and three additional unique amino acids (A532, T554, and G615) were found in the COE neutralizing epitopes of the PEDV-LY5-101, PEDV-LY5-107, PEDV-LY5-109, and PEDV-LY5-113 strains. Specifically, one

amino acid (V599) in the COE epitope was unique to PEDV-LY2-46, compared with the other isolates and the CV777 strain. In addition, one mutation (Y9S) was also found in the SS6 neutralizing epitope of all 12 PEDV strains. However, no mutations were found in the SS2 neutralizing epitope (data not shown). Notably, strain PEDV-LY2-60 had a 6-amino acid deletion at positions 1413–1418 in the S protein, whereas all of the other strains shared the same sequence at these positions. Similar to other coronavirus S proteins, the PEDV S protein is a glycoprotein peplomer (surface antigen) on the viral surface, and it plays an important role in the induction of neutralizing antibodies, specific receptor binding, and cell membrane fusion. Thus, this gene is important for understanding the genetic relatedness of PEDV field isolates, as well as the epidemiological status of the virus and vaccine development. Further research is needed to determine whether the amino acid changes in the PEDV strains from our study result in any pathogenicity or antigenicity changes.

3.6. PEDV-LY4-98 strain causes cell lesions in cell culture

Two PEDV strains (PEDV-LA1 and PEDV-LY4-98) were successfully propagated in Vero E6 cells. The CPEs of these two PEDV isolates are shown in Fig. 2**A**. PEDV-LY4-98 induced an obvious CPE at 24 h post-inoculation, while the CPE caused by PEDV-LA1 was significantly delayed. As shown in Fig. 2**B and C**, both the PEDV-LA1 and PEDV-LY4-98 strains formed plaques, and the plaque sizes of the former strain were significantly smaller than those of the latter in a direct agarose overlay plaque assay, and PEDV-LA1 had a 6- to 12-hour delay in viral growth compared to PEDV-LY4-98, which is consistent with the above finding that strain PEDV-LY4-98 caused more pronounced cell lesions.

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

The replication and pathogenesis of PEDV were determined in newborn piglets. All animals were monitored daily for clinical signs of disease, including diarrhea and vomiting. At necropsy, intestinal tissues and contents were grossly evaluated. Additionally, a portion of the jejunum and ileum were fixed in 10% neutral buffered formalin for histopathology.

(i) Clinical signs. During the acclimation period, the clinical signs were monitored. At 24 hpi, all piglets in two challenge groups inoculated exhibited typical clinical signs of PEDV infection, including watery diarrhea, vomiting. Core body temperatures remained within normal limits. Respiratory signs (coughing and nasal discharge) were not observed. In spite of the diarrhea and progressive dehydration, appetite was maintained. Piglets in the uninfected control group had no clinical signs.

(ii) Histological findings. At 48 hpi, Necropsy examinations showed that two challenge groups which separately inoculated with PEDV-LY4-98 and PEDV-LY1 displayed typical PED-like lesions. No intestinal lesions were found in uninfected piglets (Fig. 3A), Severe hyperaemia was present in the mesentery (Fig. 3B), the small intestine was thin-walled and contained soft to watery contents (Fig. 3C), while. Histopathological examination showed severe villous atrophy of the duodenum, jejunum, and ileum was apparent. No lesions were found in the intestines of uninfected piglets (Fig. 3D), 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. 3E); Many mucosal epithelial cells were necrotic and lysed; nuclear changes consistent with cell death included pyknosis, karyorrhexis, and karyolysis (Fig. 3F) (Ma et al., 2015).

4. Discussion

Since 2010, more variant PEDV strains have appeared recently in southern China and immediately spread throughout the country. In this study, 17 PEDV variants were characterized and analyzed. Our genetic analyses showed that the PEDV strains fell into two genogroups, designated G1 and G2. Notably, all the strains determined from 2014 to 2015 fell into genogroup G2, while the other five strains identified from 2016 to 2017 clustered into genogroup G1. The study of the full-length S and ORF genes revealed a more comprehensive distribution profile that reflects the current PEDV status in pig farms in Zhejiang, China, including the presence of strains that are similar to U.S. strains. A genetic analysis based on the complete S gene showed that these new variant strains share higher identities with U.S. strains than with strains from South Korea and southern China. According to the sequence and phylogenetic analysis of the complete S gene, the PEDV strains in our study (except strains PEDV-LA3-4, PEDV-LA1, PEDV-HZLY-5, PEDV-JHWC-12, and PEDV-JHDY-18) are highly homologous to the strains AH-2012 and JS-HZ2012, even though they were isolated in different provinces at different times. Although our isolates and strain ZJ13LY1201 were isolated in the same province, their shared sequence identities were lower. These results indicate that the Chinese PEDV isolates were genetically diverse.

Based on sequence comparison and phylogenetic analysis, it appears that the eight new PEDV strains (PEDV-HF-90, PEDV-HF-91, PEDV-LY1-27, PEDV-LY2-39, PEDV-LY2-46, PEDV-LY2-51, PEDV-LY2-60, and PEDV-LY4-98) belonging to subgroup G2a were genetically closely related to some PEDV strains that were circulating in America, while the other four PEDV strains (PEDV-LY5-101, PEDV-LY5-107, PEDV-LY5-109 and PEDV-LY5-113) belonging to subgroup G2b were genetically closely related to some PEDV strains that were circulating in China in 2011 to 2012. A previous study also reported that the Chinese AH2012 strain was possibly transmitted to the eastern China regions and then transported to the United States. However, the exact source of the origin is difficult to identify at this point (Huang et al., 2013). Basing on the sequence and phylogenetic analysis, both two Chinese strains, PEDV-LYG and XM-2-4, which were identified from two adjacent provinces to Zhejiang Province, together with United States strains and AH2012 strain, are most closely related to the PEDV-LY4-98 strain. The data suggested that AH2012-related PEDV has probably spread into this area in China. Taken together, the available sequence and phylogenetic data strengthen that the PEDV circulating in China and the US have at least a possible common ancestor. Moreover, The information presented in this study provided new evidence for the ongoing spread of AH2012-related PEDV in Zhejiang Province. Besides, the finding that the novel PEDV strains in our study share unique genetic features compared with the highly virulent American strains, probably due to the immune pressure of the vaccines or longer circulation time of PEDV in pigs in China. Thus, urgent action is needed to prevent the continued transmission of this virus, including the development of novel vaccines for prevention.

We attempted PED virus isolation from 17 intestine homogenates samples which were confirmed as positive for PEDV, and only 2 isolates (PEDV-LA1 and PEDV-LY4-98) were obtained (success rate, 12%). Although the sample quality could have been a contributing factor to the low success rate, the isolation procedures also need to be further improved. The two isolates were serially propagated in cell culture and characterized. By examining the CPE development, EM, infectious virus titers, and partial genome sequences, we clearly demonstrated that the two PEDV isolates are phenotypically (titers, growth characteristics) and genetically stable, during at least the first 10 serial passages in cell culture. Availability of the U.S. PEDV isolates provides an important tool for PEDV pathogenesis investigation, virological and serological assay development, and vaccine development. We are continuing serial passages of these viruses in efforts to develop a live attenuated PEDV vaccine that could potentially be used to vaccinate nursery pigs and

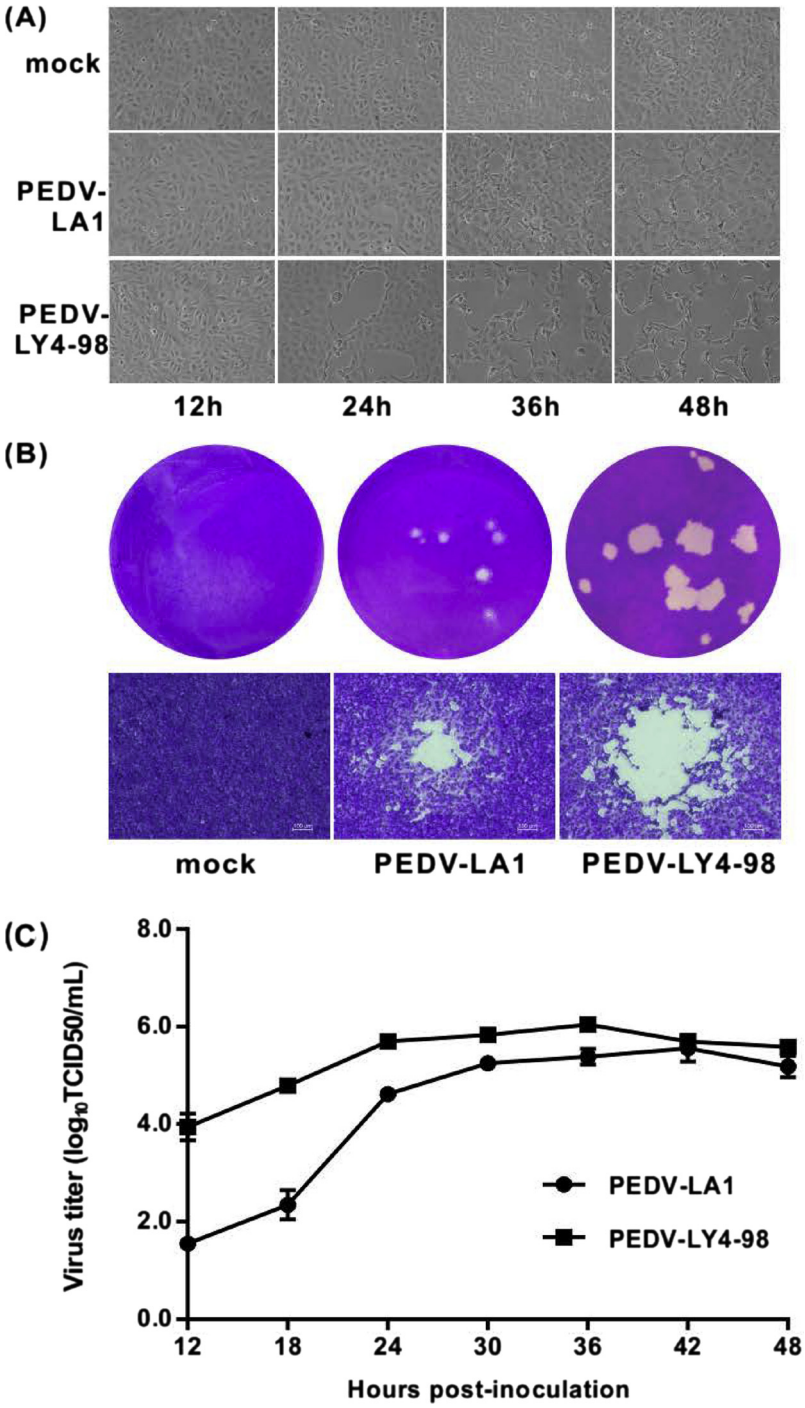

Fig. 2. Virus isolation and detection in Vero E6 cells.
(A) Cytopathic effects (CPEs) caused by PEDV isolates. Vero E6 cells were seeded into T-25 flasks and infected at an MOI of 0.1 with PEDV-HF-90 and PEDV-LY5-101, respectively. CPE was monitored every 12 h. Pictures were taken at 12 h, 24 h, 36 h and 48 h post-infection. (B) Plaque morphology of PEDV isolates in our study. An agarose overlay plaque assay was performed in monolayer Vero E6 cells. Viral plaques were developed at day 4 post-infection. The cells were fixed in 10% formaldehyde, and the plaques were visualized by staining with crystal violet. (C) Viral replication kinetics in Vero-E6 cells. Vero-E6 cells in 35-mm dishes were infected with each PEDV isolate at an MOI of 0.1. After adsorption for 1 h, the inoculum was removed, and the infected cells were washed three times with Opti-MEM. Then, fresh Opti-MEM containing 2% FBS was added, and cells were incubated at 37 °C for various time periods. Aliquots of the cell culture fluid were removed at the indicated intervals. Viral titer was determined by TCID50 assay inVero-E6 cells.

pregnant sows to mitigate the negative impact caused by PEDV infection. Viruses at selected passages will be inoculated into pigs to evaluate their virulence/attenuation phenotypes so that the genetic changes potentially associated with virus attenuation can be identified.

In this study, unique genetic characteristics were observed among our PEDV strains based upon the sequence analysis of their S genes. Large mutations were found in the SP-encoding fragment of the S gene, and they may play an important role in viral protein release, which could affect the virulence of the virus. Compared with classical and vaccine strains, a series of amino acid insertions and mutations in the S gene may play an important role in the pathogenicity and antigenicity of the new PEDV variants. Both cell culture and plaque assay studies confirmed this view. The antigenicity analysis of the S gene showed that the four neutralizing epitopes (COE, SS2, SS6, and 2C10) share unique genetic features compared with the CV777 strain. A previous study report showed that five highly virulent PEDV strains (JY5C, JY6C, JY7C, YJ3 F, and YJ7C) detected in Gansu Province, China had eight mutations (A517S, S523 G, V527I, T549S, G594S, A605E, L612 F, and I635 V) in the neutralizing epitope COE (Lin et al., 2016), these similar mutations were also found in the PEDV-LY4-98 strain and the United States strains, but not present in South Korean strains. A two-amino-acid substitution (N58 and S59) was found in the S1 N-terminal domain, and this mutation completely eliminated a potential N-glycosylation site that was present in the S proteins of the other strains. Moreover, three unique amino acid substitutions (L7, G8, and V9) were identified in the SP-encoding fragment of the S1 N-terminal domain of the PEDV-LY4-98 S protein compared with the S proteins of all the PEDV reference strains. Whether these amino acids substitutions and

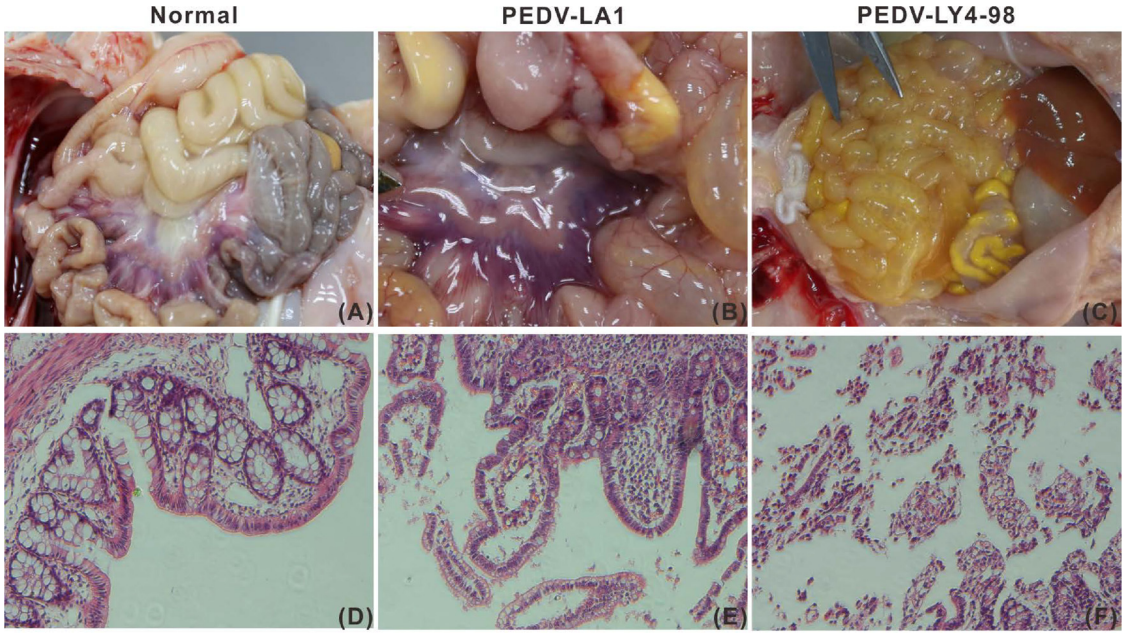

Fig. 3. The replication and pathogenesis of PEDV in newborn piglets.
(A–C) Necropsy examinations of the intestine of piglets inoculated with PEDV-LY4-98 and PEDV-LY1 and control medium. (A) no intestinal lesions were found in uninfected piglets. (B) Severe hyperaemia was present in the mesentery. (C) the small intestine was thin-walled and contained soft to watery contents. (D–F) Histopathological examinations of the intestine of piglets inoculated with PEDV-LY4-98 and PEDV-LY1 and control medium. (D) No lesions were found in the intestines of uninfected piglets. (E) Severe villous atrophy of the duodenum. 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. (F) Many mucosal epithelial cells were necrotic and lysed; nuclear changes consistent with cell death included pyknosis, karyorrhexis, and karyolysis.

the N-glycosylation site substitutions influence the antigenicity and pathogenicity of PEDV remains to be investigated.

In summary, here we reported the genetic characterization of two novel PEDV strain, PEDV-LA1 and PEDV-LY4-98, found on the Chinese mainland, which had significant variations in the S gene. In recent years, new S mutant strains have been constantly found and this may be related to the immune pressure of the vaccines that are used, including those from vaccine strains CV777 and DR13. Meanwhile, the virulence of the new mutants may be changed, and amino acid substitutions in the neutralizing epitopes may have conferred the capacity for immune evasion in these PEDV field strains. The monitoring of PEDV variations will be useful in the control and prevention of PED. However, the exact origins of our PEDV strains are difficult to identify at this point. The information presented in this study will guide current control measures designed to stop the ongoing spread of PEDV, and it will also provide important clues for the development of an effective vaccine against emergent PEDV strains.

5. Statistical analysis

All experiments were repeated at least three times. Data were analyzed using the two-tailed homoscedastic Student's *t*-test. Differences with P values < 0.05 were considered as statistically significant.

Ethics statements

All animal care and use protocols were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. The protocol was approved by the Institutional Animal Care and Use Committee of Zhejiang A&F University (Permit Number: ZJAFU/IACUC_2011-10-25-02).

Author contributions

XW and HS conceived the study. JS, QL, HH, CS, SJ, SB, LS, and LW carried out experiments. JS, QL, XW, YW, and HH analyzed data. JS and QL drafted the manuscript and all authors contributed to this study prepared the final version of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.05.021.

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