# Pathogenicity and immunogenicity of a new strain of porcine epidemic diarrhea virus containing a novel deletion in the N gene

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## ABSTRACT

Since late 2010, highly virulent PEDV G2-genotype strains have emerged globally extracting heavy losses on the pork industries of numerous countries. We investigated the characteristics of a field strain of PEDV (PEDV strain SH) isolated from a piglet with severe diarrhea on a farm in Shanghai China. Whole genome sequencing and analysis revealed that the SH strain belonged to subtype G2b and has a unique 12-aa deletion (aa 399–410) including the antigenic epitope NEP-1C9 (aa 398–406) of the N protein. PEDV SH strain is highly pathogenic to challenged newborn piglets, resulting in 100 % morbidity and mortality. Pathological examination revealed significant villus atrophy in the jejuna of infected piglets. Mice inoculated with inactivated PEDV SH produced antibodies against the N protein, but no antibodies against the deletions. These results illustrated that deletion of the NEP-1C9 epitope had no effect on the immunogenicity or pathogenicity of PEDV, providing evidence of the necessity to monitor the genetic diversity of the virus. Our study also contributes to development of candidate for vaccines and diagnostics that could differentiate pigs seropositive due to vaccination by conventional strains from wild virus infection.

## 1. Introduction

Porcine epidemic diarrhea virus (PEDV) was first described in 1978 by de Bouck and Pensaert as the etiologic agent of an enteric disease of swine termed "epidemic viral diarrhea" that had been recognized in early 1970's in Europe. Since then, porcine epidemic diarrhea (PED), as the disease is now called, has been reported throughout Europe and Asia, and more recently the United States, Canada, and Mexico (Hoang et al., 2013; Marthaler et al., 2013; Mole, 2013; Ojkic et al., 2015; Stevenson et al., 2013). PED is characterized by severe watery diarrhea, vomiting, and dehydration, resulting in 100 % morbidity and 80–100 % mortality in suckling piglets, and a tremendous economic burden to the swine industry (Chasey and Cartwright, 1978; Debouck and Pensaert, 1980; Pensaert and de Bouck, 1978; Singh, 1999; Sueyoshi et al., 1995). Outbreaks of PED in China were sporadic (Chen et al., 2010) until late 2010, when PED broke out in South China, and subsequently spread throughout the country inflicting a huge blow to the Chinese pig industry(Ge et al., 2013; Sun et al., 2012). Numerous studies have revealed that new variant strains of PEDV were the primary pathogens in these outbreaks (Chen et al., 2012; Hoang et al., 2013; Li et al., 2012a, b; Marthaler et al., 2013).

PEDV is an enveloped positive-sense single-stranded RNA virus belonging to the genus *Alphacoronavirus* in the *Coronaviridae* family. The PEDV genome is approximately 28 kb in length (Chen et al., 2010), consisting of 5′- and 3′- untranslated region (UTR), and seven open reading frames (ORFs) encoding polyproteins 1a and 1b (PP1a and PP1b), spike (S), ORF3, envelope (E), membrane (M) and nucleocapsid (N) proteins (Song and Park, 2012). The S protein is a type I transmembrane glycoprotein containing two functional subunits, S1 and S2, which are responsible for viral binding and fusion respectively. The S protein also plays a role in the induction of neutralizing antibodies, and the virus adaptability in cells (Bosch et al., 2003; Park et al., 2007). Genome comparisons between the prototype strain CV777 and PEDV variants showed that the differences were mainly concentrated in the S1 subunit which is important for studying the genetic relationships among different PEDV strains and for epidemiological investigations (Lin et al., 2017).The N protein is a highly conserved phosphoprotein, only a few point mutations have been reported to date. It has multiple

**Table 1**
Primers used for amplification of the PEDV SH strain. The locations correspond to positions within the CV777 genome.

| Primer names | Forward primers | Reverse primers | Fragment length(bp) |
|---|---|---|---|
| 194-2039 | TCCGTGCGCCTTCTACATA | GACAACGTCAGCATCAGC | 1846 |
| 1907-2564 | AGCCAATGTGAATCTCGTC | TGCTACAGTGACAACAATGC | 658 |
| 2440-3146 | TGCTTGAAGAGATTGATGGTG | ATACTCAACAACATCGTCCCA | 707 |
| 3065-4076 | TGAGACTATTATGGCTGTGC | CTTTATCTGATGACGACCA | 1012 |
| 3991-5181 | TTATAGGCAAGGATAGTGGTC | CGTAGAAACGCTCCACAT | 1191 |
| 5112-6353 | TATTCCAAGTTGTCCCACCTC | TTTTAGCTTGAAGAAGACACCC | 1242 |
| 6129-7443 | ACTATGAATTATGCATCGGAA | AACTTGACAGGTCTTTGCC | 1315 |
| 7336-9244 | TAGTGGACTCAGCGTTGTTG | GTGTCGTTGTGATTAGAAGC | 1909 |
| 9086-10188 | TGCCAACTCTATCTCAACTG | GATTAACGCCATACATTTGA | 1103 |
| 10019-10839 | TGATTGCTTTTCTATTCTTGC | CGTAGAAGCAACAGGTAAAA | 821 |
| 10665-11949 | TGTGCTATGACACTATTTGC | GTGCCTTACCTTCTCCAA | 1285 |
| 11839-13163 | TTGGGTGTGAGCGTATTG | TTGCATCACAGAACTTAACG | 1325 |
| 13065-14355 | TTGACCCTGTTGAAAATGAA | GTAGTACCAATAACAACCGAAG | 1291 |
| 14148-15399 | AACAGGATGAACTTTATGC | CAAACAACACAAAGCCCT | 1252 |
| 15175-16557 | TGCCTACCCGTTATCTAAGC | TAGCTGATGCGCTGATTTATG | 1383 |
| 16370-18195 | TCACGCATCATACCACAG | TCCGTTACGGCACATCTA | 1826 |
| 18055-19095 | ATTTATTGGTAATGAGGCTGTT | AATCGCTCTAATGAACCAAC | 1041 |
| 18888-19998 | TGTATGCCAAGCGTAAGGTA | AACCAACGTCGTAAGACAGC | 1111 |
| 19884-22021 | GTCAATATCTCAATAGCACCAC | CACAATAAAGAATACGCTGA | 2148 |
| 21837-22999 | TGGTGATGTTTATGTCAATGG | TCATACTAAAGTTGGTGGGAA | 1163 |
| 22899-24017 | TAAATCTGGCAGTATTGGCT | TAACGCTGAGATTGCGATTT | 1119 |
| 23922-26242 | TGCTCAAACCCTCACTAA | GACTGAACGACCAACACG | 2321 |
| 26098-27978 | TGTAACGCTAACACTCCTT | TACCTGGCACGCTCTTCC | 1881 |
| 5′ -UTR-R |  | CAGAGCAAGCGAGGTCGTCTTTGGA |  |
| 3′ -UTR-F | AGGACCTCACGCCGACCTCCGCTAT |  |  |

functions in viral replication, assembly, and pathogenesis, for example, it can block nuclear factor-κB nuclear translocation, antagonizing interferon-λ production (Shan et al., 2018) and may also be a promising target for vaccine development research due to its antigenicity.

Phylogenetic analysis based on the full-length genome and the S gene have suggested that PEDV can be divided into three genotypes, G1 (classical strains), G2 (variant strains) and S INDEL (recombinant strains). G1 and G2 can be further divided into G1a, G1b, and G2a, G2b, respectively. The G2 group comprises the post-2010 global epidemic isolates including mutations mainly in the N terminal domain of S1 (S1-NTD) (Fan et al., 2017). These mutations affect the conformational structure and N-linked glycosylation of S1-NTD, which may alter the pathogenicity of the variants (Chen et al., 2019). With the increased severity and prevalence of PED, an integrated understanding of the genetic diversity of PEDV is needed to facilitate the development of new vaccine therapies.

In this study, a PEDV field strain PEDV SH, was isolated from an infected piglet in Shanghai, China. We found that this strain contained a consecutive 12-aa deletion including an antigenic epitope, NEP-1C9, in the N protein. Our experimental results showed PEDV SH to be highly pathogenic to suckling piglets, and that the antigenicity of the N protein was not impaired by the deletion of NEP-1C9. Vaccines developed from SH, or other gene-deletion strains, were proved to be useful to distinguish pigs seropositive due to vaccination versus those seropositive due to wild infections.

## 2. Materials and methods

### 2.1. Clinical samples, cells, and antibodies

Tissue samples from the small intestine of a pig suffering severe diarrhea were collected in October 2016 in Shanghai China. The tissues were found to be PEDV positive, and TGEV and RV negative by RT-PCR. The tissues were homogenized in phosphate buffer saline (PBS, pH7.2), subjected to three rounds of freeze/thaw, then centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was collected and filtered through a 0.22 μm filter and used as an inoculum for virus propagation and isolation. Vero cells (ATCC CCL-81) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Corning, USA) containing 10 % heat-

inactivated fetal bovine serum (Lonsera, Uruguay) and 1 % penicillin-streptomycin (Sigma, USA), and maintained in a humidified 5 % $CO_2$ atmosphere at 37 °C. Monoclonal antibodies (mAbs) against PEDV N protein were prepared and stored in our laboratory.

### 2.2. Virus isolation and propagation

When Vero cells seeded into 6-well plates reached 100 % confluence the monolayers were washed twice with sterile PBS. Subsequently, 1 mL of the PEDV-positive inoculum, supplemented with 8 μg/mL trypsin, was inoculated onto the cells. After incubation for 1 h at 37 °C, growth medium (DMEM containing 8 μg/mL trypsin, 1 % penicillin-streptomycin) was added to each well without removing the inoculum. When observed cytopathic effect (CPE) was > 90 %, the plate was subjected to two cycles of freezing and thawing. The mixture of cells and culture medium was centrifuged and the supernatant was aliquoted and stored at − 70 °C. After three rounds of plaque isolation the virus was considered clonal and designated PEDV SH (Lee et al., 2017). The virus culture was scaled up over five generations; $TCID_{50}$ was then measured and a one-step growth curve was performed (Fan et al., 2017; Sun et al., 2018).

### 2.3. Indirect immunofluorescence assay (IFA)

At 18 h post infection, the cells infected with SH (MOI 1) were fixed with 4 % paraformaldehyde for 20 min at room temperature (RT). After three washes with PBS, the cells were permeabilized using 0.1 % Triton X-100 for 20 min at RT. Subsequently, the cells were blocked with 2 % bovine serum albumin (BSA) and then successively incubated with mouse anti-PEDV N mAb, 3F10 and Alexa Fluor®488-conjugated goat anti-mouse IgG (Abcam, Britain) diluted in 2 % BSA for 1 h at 37 °C. After washing, the cells were visualized with a fluorescence microscope (ZEISS, Germany).

### 2.4. Genome cloning and sequencing

To obtain the entire sequence of SH, the genome was separated into 21 overlapping fragments and amplified by RT-PCR. All primers were designed using Primer Premier 5.0 software (http://www.

**Table 2**
Information on the reference strains used in this study.

| Strains | Origins | Years | GenBank No. | Strains | Origins | Years | GenBank No. |
|---------|---------|-------|-------------|---------|---------|-------|-------------|
| BJ-2011-1 | Beijing, China | 2011 | JN825712 | USA/Minnesota269/2014 | USA | 2014 | KR265783 |
| LZW | Beijing, China | 2014 | KJ777677 | USA/Ohio126/2014 | USA | 2014 | KJ645702 |
| CH/BJ9/2015 | Beijing, China | 2015 | MG546687 | PEDV/USA/Texas134/2015 | USA | 2015 | KU982966 |
| CHN/SH-2012-5/2012 | Shanghai, China | 2012 | MG837011 | USA/OK10240-6/2017 | USA | 2017 | MG334554 |
| PEDV-15 F | Chongqing, China | 2012 | KM609208 | CV777 | USA | 1987 | AF353511 |
| JS2008 | Jiangsu, China | 2008 | KC109141 | MEX/104/2013 | Mexico | 2013 | KJ645708 |
| JS-HZ2012 | Jiangsu, China | 2012 | KC210147 | ON-018 | Canada | 2014 | KM189367 |
| PEDV-LYG | Jiangsu, China | 2014 | KM609212 | GER/L00719/2014 | Germany | 2014 | LM645058 |
| ZJCZ4 | Zhejiang, China | 2011 | JX524137 | Virulent DR13 | South Korea | 1999 | JQ023161 |
| PEDV-WS | Zhejiang, China | 2014 | KM609213 | SM98 | South Korea | 2011 | GU937797 |
| CHGD-01 | Guangdong, China | 2011 | JX261936 | KNU-1305 | South Korea | 2013 | KJ662670 |
| GD-A | Guangdong, China | 2012 | JX112709 | KNU-141112-feces | South Korea | 2014 | KR873431 |
| GD-B | Guangdong, China | 2012 | JX088695 | OKN-1/JPN/2013 | Japan | 2013 | LC063836 |
| CH/HNYF/14 | Henan, China | 2014 | KP890336 | MYG-1/JPN/2014 | Japan | 2014 | LC063838 |
| CH/HNAY/2015 | Henan, China | 2015 | KR809885 | IWT-2/JPN/2014 | Japan | 2014 | LC063839 |
| LZC | Gansu, China | 2006 | EF185992 | HUA-14PED96 | Vietnam | 2014 | KT941120 |
| CH/FJND-3/2011 | Fujian, China | 2011 | JQ282909 | TGEV AHHF | China | 2015 | KX499468 |
| AJ1102 | Hubei, China | 2011 | JX188454 | TGEV HE-1 | China | 2015 | KX083668 |
| AH2012 | Anhui, China | 2012 | KC210145 | HCoV NL63 | France | 2008 | KY862070 |
| CH/JX-1/2013 | Jiangxi, China | 2013 | KF760557 | FIPV DF-2 | Hungary | 2012 | JQ408981 |
| SD2014 | Shandong, China | 2014 | KX064280 | SARS MA-15 | USA | 2007 | DQ497008 |
| CH/SXYL/2016 | Shanxi, China | 2016 | MF462814 | PRCV ISU-1 | USA | 2016 | DQ811787 |
| USA/Iowa106/2013 | USA | 2013 | KJ645695 | HEV VW572 | Belgium | 2006 | DQ011855 |
| USA/Colorado/2013 | USA | 2013 | KF272920 | MHV A59 | USA | 2004 | AY700211 |
| IA1 | USA | 2013 | KF468753 | BCoV_2014_13 | France | 2014 | KX982264 |
| USA/Colorado/2013 | USA | 2013 | KF272920 | TCoV-540 | USA | 2008 | EU022525 |
| USA/NC/2013/35140 | USA | 2013 | KM975735 | IBV Mass41 1985 | USA | 1985 | FJ904723 |
| NPL-PEDV/2013 | USA | 2013 | KJ778615 | PDCoV/CHJXNI2/2015 | China | 2015 | KR131621 |
| OH851 | USA | 2014 | KJ399978 | PDCoV CH-01 | China | 2016 | KX443143 |

**Table 3**
Sequences of the synthetic polypeptides.

| Names | Sequences | Reference strain |
|-------|-----------|------------------|
| Polypeptide I | H EEAIYDDVGAPS | CV777 |
| Polypeptide II | N EEAIYDDVGVPS | YZ |

premierbiosoft.com/) and are listed in Table 1. Total RNA from passage 1 SH infected cell cultures was extracted using an E.Z.N.A.® Total RNA Kit I (OMEGA Bio-Tek, USA), according to manufacturer's instructions. The RNA was then reverse transcribed into cDNA using HiScript® II Reverse Transcriptase (Vazyme, China), and stored at −20 °C for later use. PCR was performed using PrimeSTAR®HS DNA Polymerase (Takara, Japan). The 5′-UTR and 3′-UTR were amplified using a SMARTer® RACE 5′/3′ Kit (Clontech, China). Thermal cycling conditions were as follows: 98 °C for 1 s; followed by 35 cycles of 98 °C for 10 s, 55 °C for 5 s and 72 °C for 2 min; then a final extension at 72 °C for 5 min. After agarose gel electrophoresis and purification, the PCR products were cloned into Zero Background TOPO-Blunt vector (Aidlab, China), the recombinant plasmid was sequenced by commercial sequencing service (GenScript, China).

### 2.5. Nucleotide and amino acid sequence analyses

The overlapping part of each fragment was spliced to obtain the whole genome sequence of PEDV SH. Multiple sequence alignments of PEDV SH and the reference strains (Table 2) were generated using the ClustalW algorithm of MEGA 7 (https://www.megasoftware.net/). Phylogenetic trees based on the full-length genome and S gene were constructed using MEGA7. The distance-based neighbor-joining method and the bootstrap values were calculated based on 1000 replicates. The amino acid sequence comparisons were obtained using BioEdit v7.1.3 (http://www.mbio.ncsu.edu/BioEdit/page2.html). The identity analysis was performed using DNAstar (https://www.dnastar.com/). Potential N-glycosylation sites (NGSs) were identified using the online

version of NetNGlyc v1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/). RDP4 was used to evaluate the potential recombination events in PEDV SH.

### 2.6. Pig challenge experiment

Ten three-day-old suckling pigs that tested seronegative for PEDV by virus neutralization assay were obtained from a farm that had no PED outbreak or PEDV vaccination. Pigs were randomly divided into two groups ($n = 5$), piglets in group A were challenged orally with 3 ml of PEDV SH ($1.0 \times 10^5$ TCID$_{50}$/mL), piglets in group B were orally inoculated with 3 ml DMEM. Piglets were assessed daily for mental and physical health status, rectal temperature and weight were measured, and anal swabs were collected and feces were scored as follows: 0, solid; 1, pasty; 2, semiliquid (mild diarrhea); and 3, liquid (severe diarrhea) as described previously (Hou et al., 2017). At 4 days post-infection, all pigs were euthanized and examined for pathology. Intestinal tissues were collected and either soaked in paraformaldehyde for subsequent paraffin embedding or stored at −70 °C for quantification of PEDV by qPCR.

During the experiment, the piglets were housed in cages that allowed free-walking and were fed commercially purchased cow milk. Animal care and experiments were conducted in accordance to the guidelines of the Animal Care and Ethics Committee of Nanjing Agricultural University (permit number IACECNAU20161201). We endeavored to guarantee the best animal welfare.

### 2.7. Pathological examination

Macroscopic examination was conducted during the necropsies. Intestinal tissues were collected and fixed with 4 % paraformaldehyde for 24 h. Following the procedures described in (Zhang et al., 2016a, b) the fixed intestinal tissues were dehydrated, cleared in xylene, embedded in paraffin wax, then sectioned and mounted on slides, which were then stained with hematoxylin and eosin for observation using standard light microscopy (Nikon).

**A**



**B**
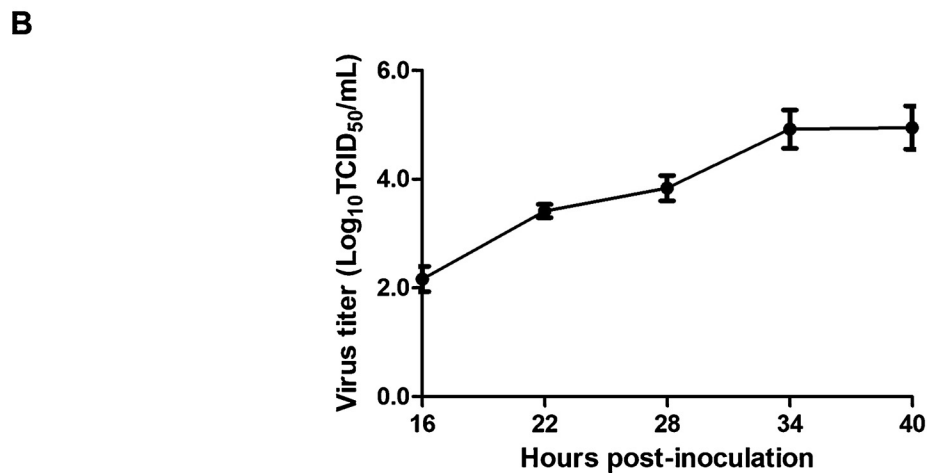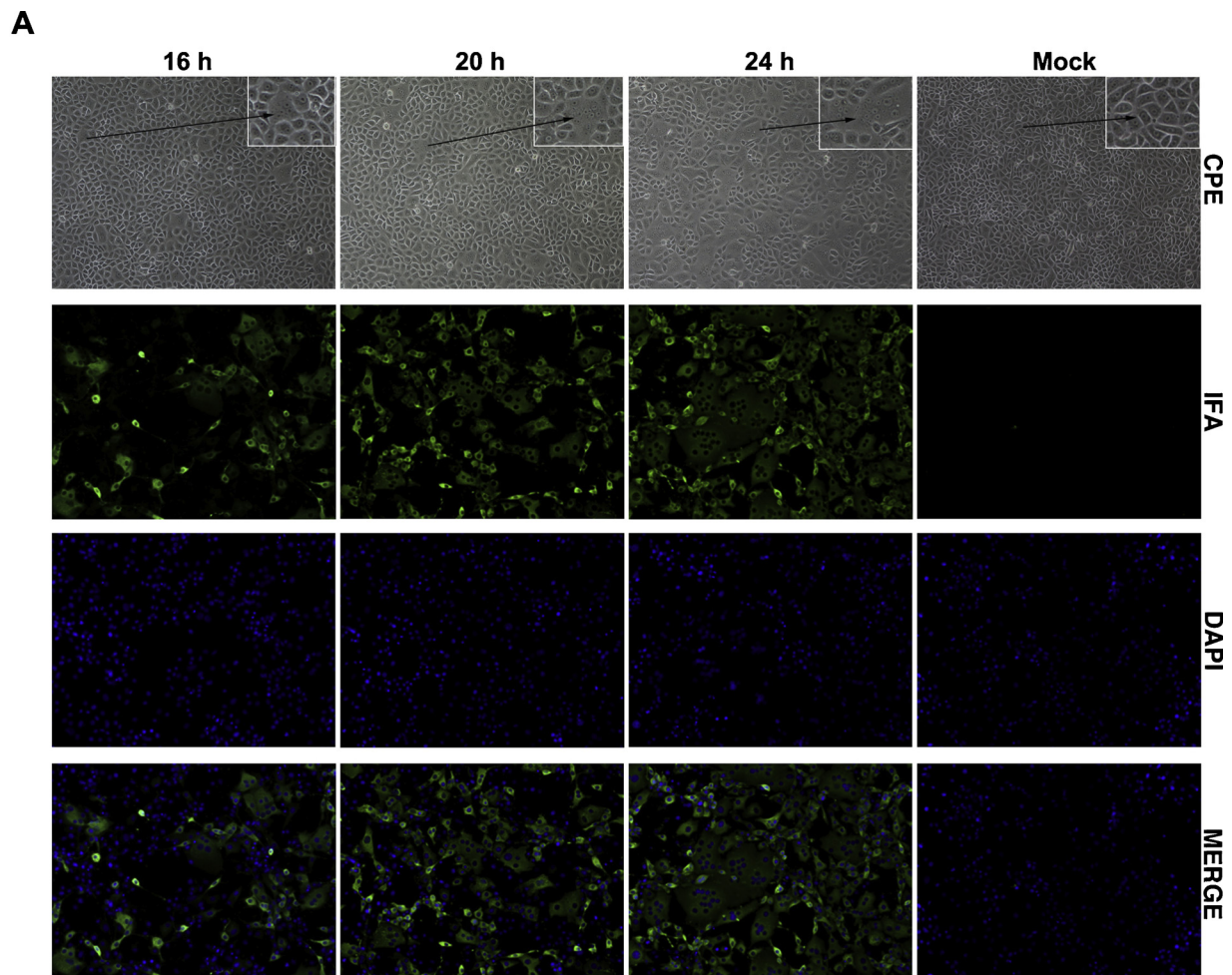


**Fig. 1.** Replication and Vero cell adaption of PEDV SH. (A) CPE formation over time in Vero cells infected with 5th passage virus (magnification, $\times 200$). The arrows indicate enlarged syncytium (upper panel). The corresponding immunofluorescent images are shown in lower panels. Infected cells were fixed at the indicated hpi then probed with PEDV N protein-specific mAb 3F10 and Alexa Fluor®488-conjugated goat anti-mouse IgG (magnification, $\times 200$). (B) Growth curve of 5th passage PEDV SH. ($TCID_{50}$/mL) was measured every 6 h. Results are based on the results of three independent experiments.

**Fig. 2.** Amino acid analysis of the deduced N protein. The shadings indicate functional signals. The amino acids that bind to NPM1 (148–294), are underlined with black. Epitopes NEP-D6 and NEP-1C9 are shown by black boxes. The deletion in PEDV is highlighted in black.

Immunohistochemistry, to determine the distribution of PEDV in the intestine samples, was done as follows. Sections were washed three times in 10 mM PBS for 10 min each and then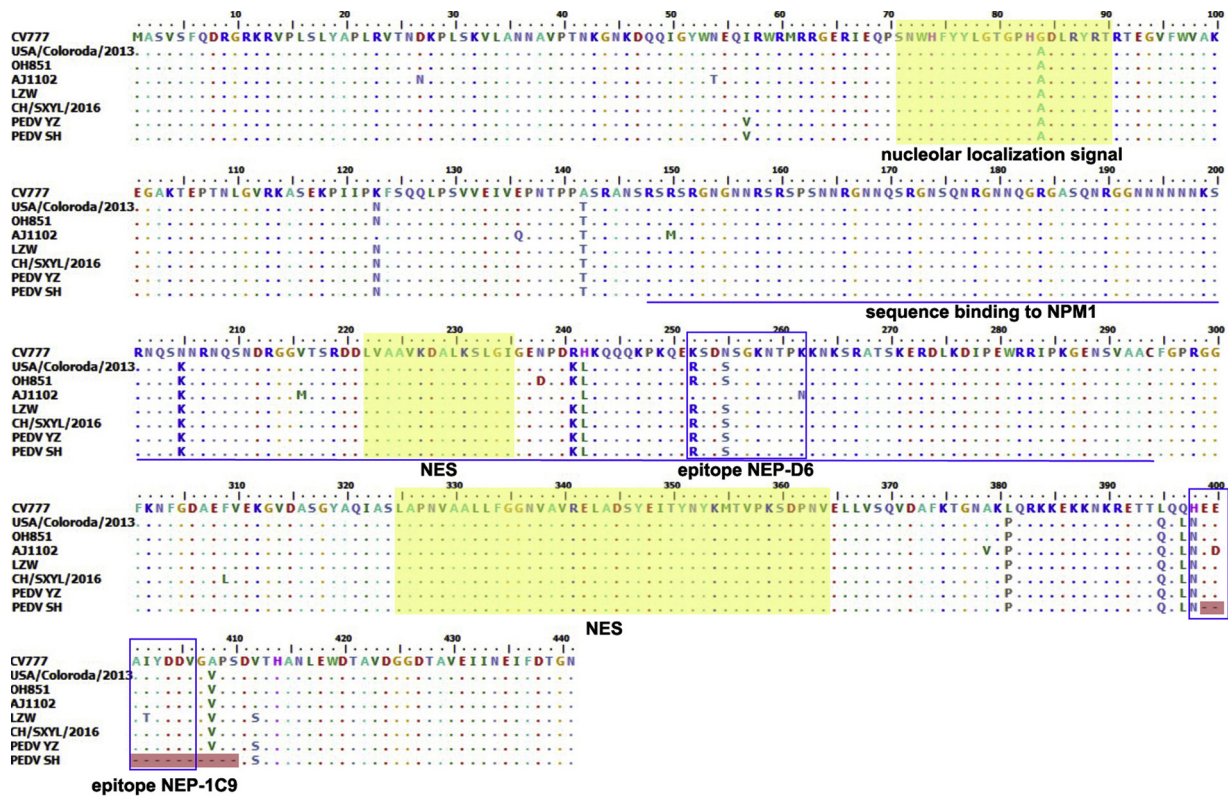 treated for 20 min with 0.25 % trypsin in methanol for antigen retrieval, then for 30 min with 3 % $H_2O_2$ in methanol to quench endogenous peroxidase activity in the tissue. After three 10-min washes with PBS, tissues were blocked for 30 min with 5 % goat serum to reduce non-specific binding of the secondary antibody, then incubated with anti-PEDV N protein-specific polyclonal antibody (obtained from Shandong Agricultural University) followed by HRP-labeled goat anti-rabbit IgG antibody (1:50 dilution) (Beyotime, China). Coloration and counterstaining were performed using an Enhanced HRP-DAB Substrate Coloring Kit (TIANGEN, China) and hematoxylin. After dehydration and mounting, the sections were observed using standard light microscopy (Nikon).

### 2.8. Quantitative RT-PCR

The PEDV in samples was quantified using RT-qPCR. Primers were designed with Primer Premier 5.0 software, using the sequence of CV777 (GenBank No. AF353511) as template, then synthesized by GenScript. The primer sequences are: F: TTCTTGTTTCACAGGTGGATG and R: GCTGCTGCGTGGTTTCA. For each sample, 1 g of intestinal tissue or feces was homogenized with 3 mL of sterile PBS, subjected to three cycles of freeze-thaw then centrifuged at 12,000 rpm for 15 min. Total RNA was extracted from 250 μL of supernatant using an E.Z.N.A.® Total RNA Kit I (OMEGA Bio-Tek, USA), according to the manufacturer's instructions. The extracted RNA was resuspended in 20 μL of diethylpyrocarbonate treated water. Reverse transcription was performed in a total volume of 10 μL, containing 8 μL of RNA and 2 μL of HiScript® II Reverse Transcriptase (Vazyme, China). The reverse transcription conditions were 50 °C for 15 min; 85 °C for 5 s.

qPCR was performed in a final volume of 20 μL, containing 10 μL of SYBR Green Master Mix, 0.4 μL of ROX Reference Dye 1, 0.4 μL of 10 mM forward primer, 0.4 μL of 10 mM reverse primer, 6.8 μL of ddH₂O,

and 1 μL of template. The cycling conditions were: initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 10 s, and annealing and extension at 60 °C for 35 s. Reactions were performed in single tubes in an ABI 7300 Real-Time Thermocycler (Applied Biosystems, USA). The standard curve for SYBR Green real time qPCR was generated using pTOPO-N, which contains the N gene of PEDV, 10-fold serially diluted from $10^7$ to $10^1$ copies/μL.

### 2.9. Mouse vaccination

To assess the effect of the deleted NEP 1C9 epitope ($H^{398}$EEAIYDDG$^{406}$) on the immunogenicity of PEDV SH, mice were inoculated with PEDV SH and PEDV YZ (no deletion) and the levels of circulating antibodies were accessed by I-ELISA. Viruses were inactivated with 0.2 % formaldehyde for 28 h at 37 °C, then emulsified 1:1 with ISA 206 adjuvant by 15 min of agitation at 200 rpm at 37 °C. Twenty-four mice were randomly divided into 3 groups ($n = 8$), then inoculated by multi-point subcutaneous injection along the back, with $10^5$ TCID$_{50}$/mouse inactivated PEDV SH, PEDV YZ, or PBS. Mice were housed in separate cages and provided with adequate feed and water. Mice were boosted at 14, 28, and 42 days post initial inoculation (dpi), with $10^5$ TCID$_{50}$/mouse of the appropriate inoculum. At 56 dpi, samples of serum were collected, I-ELISA was used to detect circulating antibodies.

### 2.10. Indirect ELISA assay

From sequence alignments of the N protein region deleted in SH, two polypeptides containing the complete epitope from CV777 (H EEAIYDDVGAPS) and from variant strains (N EEAIYDDVGVPS), were synthesized by GenScript (Table 3). Wells of 96-well flat-bottom microplates were coated with 32 μg/mL of polypeptide(s) or 2 μg/mL of *E. coli* expressed N protein for 2 h at 37 °C. Wells were then washed three times with PBS and blocked with 5 % BSA for 2 h. Primary antiserum
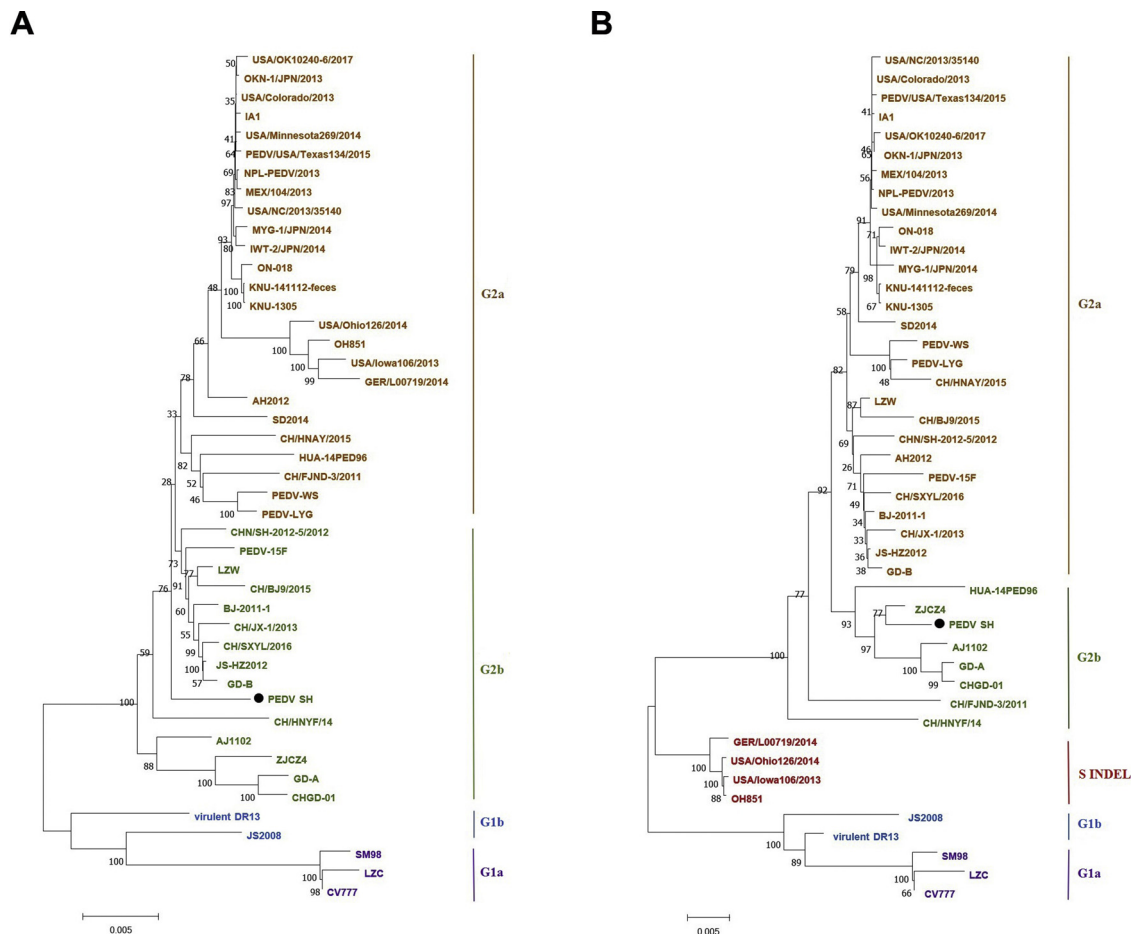
## A



## B



**Fig. 3.** Phylogenetic analysis based on the nucleotide sequences of the S gene (A) and full-length genome (B) of PEDV SH and representative strains. Multiple sequence alignments were performed with ClustalW. The neighbor-joining method in MEGA 7 software was used for the reconstruction of the phylogenetic trees. Numbers at each branch represent the bootstrap values. The scale bar indicates the nucleotide substitutions per position. The information on reference strains is in Table 2.

(100 μl of a 1:50 dilution) was aliquoted into the wells and incubated for 2 h at 37 °C. Wells were washed again with PBS, then HRP-labeled goat anti-mouse IgG was aliquoted into each well and incubated for 1 h at 37 °C. OD$_{450}$ values were read after 15 min of color development using tetramethylbenzidine (TMB, Beyotime, China).

### 2.11. Statistical analysis

All data analysis was performed with GraphPad Prism 5 software. Data are expressed as the mean ± standard error. The differences between groups were analyzed using one-way analysis of variance (ANOVA). Differences were determined as statistically significant when the probability ($p$) value was < 0.05.

## 3. Results

### 3.1. Replication and Vero cell adaptation of PEDV SH

Vero cells were inoculated with PEDV-positive supernatants from homogenized small intestines, and incubated in DMEM supplemented with 8 μg/mL trypsin. At forty-eight hours post inoculation, visible CPEs were observed. By the 5th passage, CPEs in the form of syncytia and vacuole formation could be detected from 16 h.p.i. and well advanced at 24 h.p.i. The mock-infected group showed no signs of CPE. IFA was also performed to detect the 5th passage virus with anti-PEDV N mAb followed by Alexa Fluor®488-conjugated goat anti-mouse IgG. At 16 h.p.i. approximately 20 % of the cells were infected (green

fluorescence), by 20 and 24 h.p.i. the number of infected cells increased to approximately 50 % and 90 %, respectively. No green fluorescence was observed in uninfected cells (Fig. 1A). The TCID$_{50}$ of the 5th passage virus was $10^{5.33}$ TCID$_{50}$/mL (Fig. 1B).

### 3.2. Genomic and amino acid sequence analyses

The complete genome of the PEDV SH strain is 28,002 nucleotides, barring the polyA tail in the 3′UTR, and is composed of the following UTRs and genes: 5′UTR (292 nt), ORF1a (12,309 nt), ORF1b (8037 nt), S (4161 nt), ORF3 (675 nt), E (231 nt), M (681 nt), N (1290 nt) and 3′UTR (334 nt) (GeneBank No. MK841494). Based on the whole genome sequence alignments, strains SH and LZW were the most highly related, sharing 99.3 % nucleotide identity. The S, ORF3, and N protein sequences of the SH strain shared the highest identities with those of the U.S. and Asian strains, versus those of the classical strains (data not shown). A specific continuous 36-nt deletion in the N gene was unique to the SH strain. This deletion resulted in a 12-aa deletion (aa 399–410) at the N-terminus of the highly conserved N protein. In addition to this unique deletion, there were two amino acid substitutions (252K→R and 255N→S) within epitope NEP-D6 (R$^{252}$SDSSGKNTPK$^{262}$) of SH N protein that have been identified in the LZW strain and the USA/Colorado/2013 strains (Wang et al., 2016a, b). Other differences between CV777 and the SH strain are an amino acid substitution (84G→A) within the nuclear localization signal (aa 71–90) (no mutation was found within nuclear export signal however (aa 222–235, aa 325–364)), and five substitutions (205N→K, 241R→K, 242H→L, 252K→R and 255N→S)
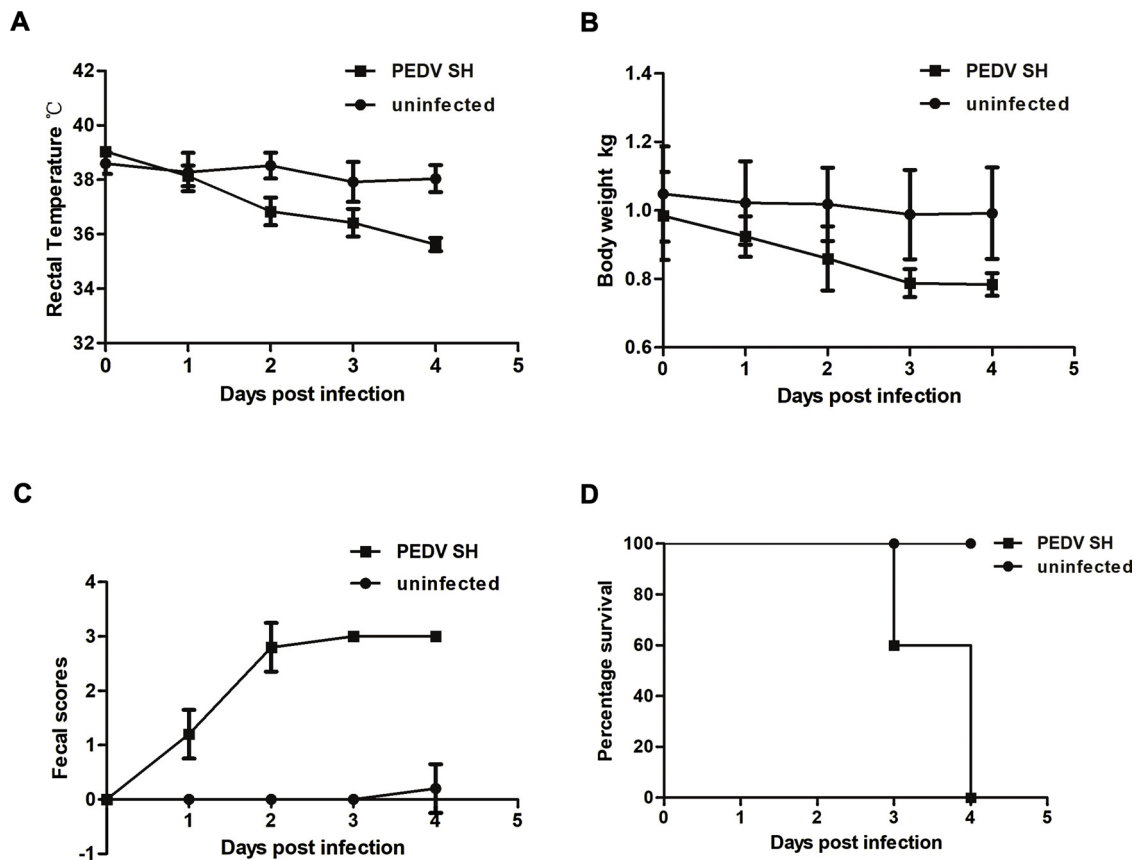
**Fig. 4.** Rectal temperature, weight, fecal score and survival of challenged and unchallenged piglets. Data are shown as the mean ± SD. (A) Rectal temperatures of piglets infected with PEDV SH or DMEM (negative control). (B) Weights of piglets. (C) Fecal scores of each group. (D) Time course of survival of challenged and unchallenged piglets.

within the region of PEDV SH, that binds nucleophosmin (aa 48–294). The five substitutions were shared by PEDV SH and other reference strains except AJ1102 (Fig. 2).

PEDV SH contains point mutations in the S gene compared with CV777, which are not seen in the other variant strains. Sequence analysis showed that these mutations were three substitutions (N57SS→NST, N341LS→NFS and N723NT→NST), two insertions (N118AT and N1196HT) and three deletions (N127KT, N230CT and N1006IT) within the PEDV SH strain (data not shown). Phylogenetic analysis of the S gene and complete gene (Fig. 3) showed that the PEDV SH strain belonged to the G2b subtype and had a closer phylogenetic relationship with Japanese and South Korean strains than with U.S. strains. In addition, recombination detection analysis indicated no potential recombination events occurred in PEDV SH (data not shown).

### 3.3. Pathogenicity of PEDV SH in suckling piglets

#### 3.3.1. Clinical symptoms

Suckling piglets infected with PEDV SH developed mild diarrhea from 18 hpi, subsequently diarrhea and vomiting until severe dehydration, hypothermia, and death at 69–84 hpi. Unchallenged piglets presented no clinical symptoms. During the time-course of the experiment, both the rectal temperatures and the body weights of the challenged piglets gradually decreased. The temperatures and weights of unchallenged piglets remained steady throughout the experiment (Fig. 4A and B). The fecal scores of challenged piglets were elevated (score 1) from 24 h post challenge, and rose to score 3 by the second day post challenge. Fecal scores of unchallenged piglets remained at 0 throughout the experiment (Fig. 4C). Two challenged piglets died on 3d.p.i., and the other 3 piglets died on 4 d.p.i. No unchallenged piglets

died during the experiment (Fig. 4D).

#### 3.3.2. Pathological examination and IHC

Gross pathological examination showed that the small intestines of infected pigs were dilated and filled with a yellow-white liquid or milky substance, the walls were thinned, and the mesentery was congested (Fig. 5B) compared to uninfected piglets. HE staining showed lesions characterized by significant atrophy of intestinal villi, a decreased ratio of villi length to crypt depth, and capillary congestion in the jejunum of challenged pigs (Fig. 5D). IHC results revealed that specific staining (brown) for viral antigens were mainly distributed in the jejunum villus epithelial cells of challenged animals (Fig. 5F).

#### 3.3.3. Intestinal and fecal viral load

RT-qPCR was used to quantify the PEDV SH present in the feces and intestinal tissues of challenged piglets. In feces, viral load reached the highest level at 2 d.p.i, then declined somewhat at 3 d.p.i. It should be noted that 2 challenged piglets died on 3 d.p.i. (Fig. 6). PEDV in the tissues of the duodenum, jejunum, and ileum were quantified. The greater viral load was shown in the jejuna and the ilea (Table 4). No viral RNA was detected in the feces or the intestine tissues of the uninfected piglets.

### 3.4. Deletion of NEP-1C9 does not affect the immunogenicity of PEDV SH

Mice were inoculated for four times with inactivated PEDV SH (NEP-1C9 deletion), PEDV YZ, or PBS, respectively. I-ELISA was used to detect and quantitate circulating antibodies produced against N protein and NEP-1C9 polypeptides I (CV777, H EEAIYDDVGAPS), and II (variant strains, N EEAIYDDVGVPS) (Fig. 7 B). The circulating anti-N
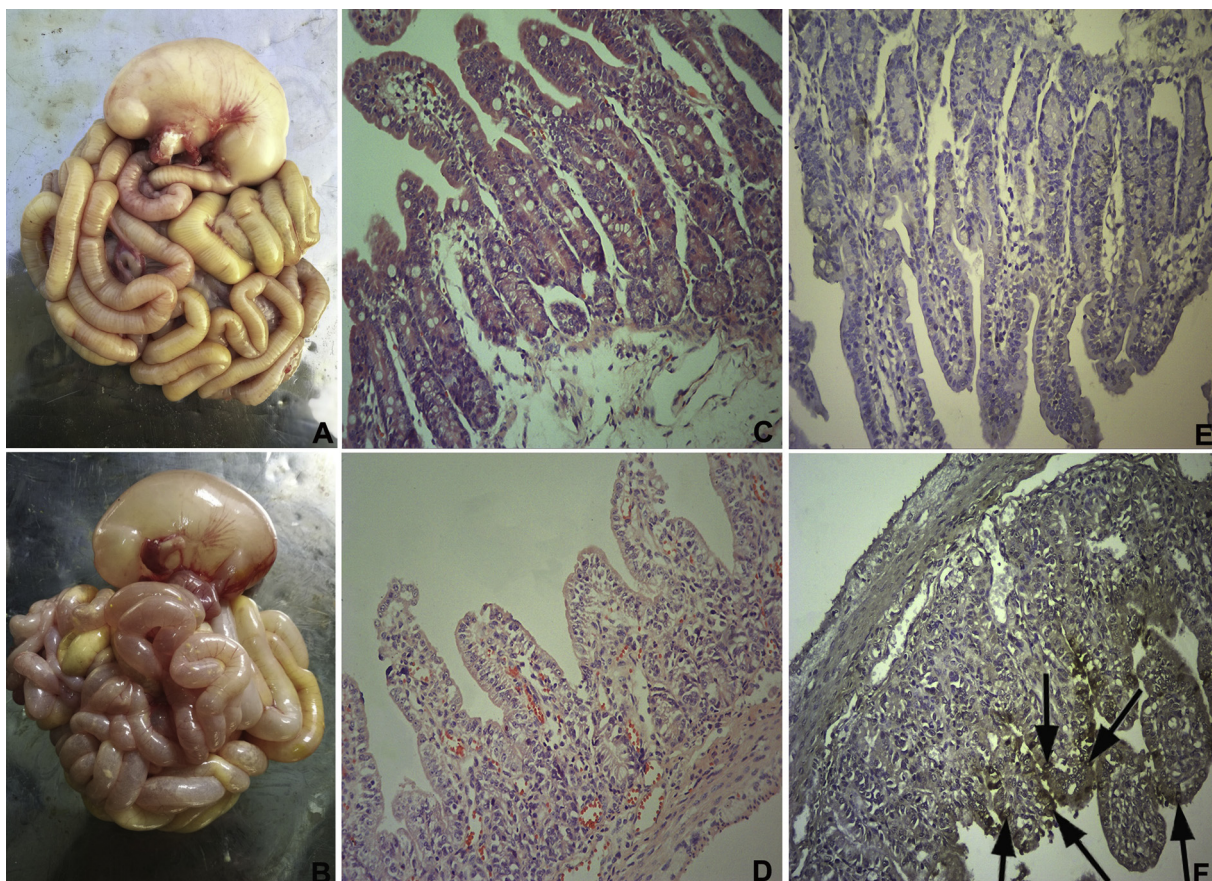
**Fig. 5.** Pathological changes and PEDV detection in the intestine of piglets. Panels A, C, and E are representative of the unchallenged group. Gross examination of the small intestines of infected pigs shows intestinal dilatation and wall thinning (B). Histological examination revealed distinct villus atrophy and capillary congestion in the small intestinal tissue of challenged pigs (D). IHC revealed that PEDV specific antigens (brown stain) were distributed in the jejunum villus epithelial cells of challenged pigs (F). Magnification, ×200.
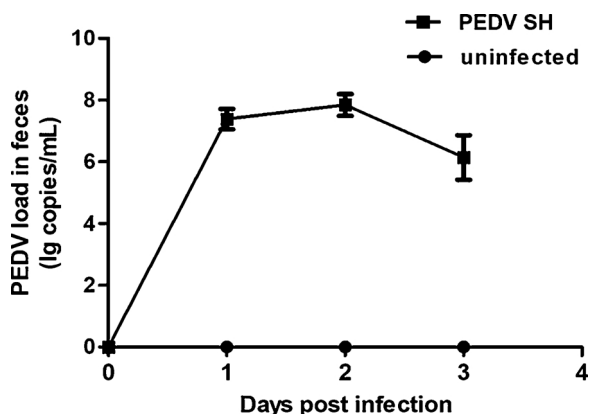


**Fig. 6.** Viral load (log10 copies/mL) in daily feces quantitated by RT-qPCR. Data are shown as the mean ± SD.

antibody were elevated to the same level in SH- and YZ-inoculated mice. Anti-polypeptide I antibodies were elevated only in YZ inoculated mice, and anti-polypeptide II antibodies were elevated in both SH- and YZ-mice while YZ-inoculated mice showed a significantly higher level.

## 4. Discussion

Since 2010, the highly pathogenic variants of PEDV-induce PED has caused a 80–100 % mortality rate among piglets in China. PED outbreaks have subsequently been reported in Korea, Thailand, and North America, resulting in an increasingly serious threat to the swine industry worldwide. At present, most of the prevalent strains in China are highly pathogenic variants (Lin et al., 2016). While there are many commercially available PEDV vaccines, they are based on the classical strains CV777, SM98, and DR13, and vaccinated pigs are not well protected from the Chinese highly pathogenic variants infection, regardless of the inoculum strain or type (activated/killed or attenuated)

**Table 4**
PEDV load by intestinal region.

| Tissue | Group | Virus load (log copies/g, n = 5) | | | | | Average |
|--------|-------|------|------|------|------|------|---------|
| Duodenum | PEDV SH | 6.00 | 7.29 | 7.08 | 8.06 | 7.85 | 7.26 ± 0.72[b] |
| | uninfected | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0[c] |
| Jejunum | PEDV SH | 8.88 | 8.45 | 7.95 | 9.40 | 9.29 | 8.79 ± 0.54[a] |
| | uninfected | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0[c] |
| Ileum | PEDV SH | 7.78 | 8.05 | 8.46 | 7.35 | 6.72 | 7.67 ± 0.6[a] |
| | uninfected | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0[c] |

Means with the same letter are not significantly different (P > 0.05).

**A**



**B**



**Fig. 7.** Amino acid sequence and immunogenicity of the NEP-1C9 epitope (deleted in PEDV SH). (A) Amino acid sequences of the NEP-1C9 epitope in different coronaviruses. (B) Immunogenicity of the NEP-1C9 epitope. The serum antibody levels in mice inoculated with inactivated PEDV SH, PEDV YZ, or PBS were detected by I-ELISA using polypeptide I and II of NEP-1C9, and recombinant N protein as coating antigens. Data are shown as the mean $OD_{450} \pm SD$.

(Wang et al., 2016a, b; Ayudhya et al., 2012). The differences in genetic component between the current epidemic strains and commercial vaccine strains may be the main reason for this lack of immune protection. Because of the genetic and antigenic diversity of PED isolates, it is necessary to monitor emerging epidemic strains with genomic surveillance programs (Sun et al., 2019). From a molecular epidemiological survey of PEDV done in our lab in 2016, we found a PEDV strain, from one clinical sample of small intestine, that had a specific continuous 36-nt deletion in the N gene. This PEDV strain, originated from a farm in Shanghai, is named PEDV SH. After virus isolation, PEDV SH was plaque purified three times then grown out over 5 generations in Vero cells. The N gene sequence of the purified isolate, after 5 generations, was the same as the parent virus originally isolated from the intestinal tissue. We then commenced characterizing the full genome, pathogenicity, and immunogenicity of the 5th generation of this new strain.

The pathological lesions caused by PEDV SH, including severe villi atrophy, are similar to those induced by the highly virulent US strain, PEDV PC22A (Lin et al., 2016). To understand whether the deletion of the NEP-1C9 epitope impacts SH pathogenicity and immunogenicity, neonatal piglets were infected with the SH strain and mice were inoculated with the inactivated SH. All piglets challenged with SH presented typical symptoms of diarrhea, vomiting, weight loss, and

ultimately death at 3- and 4-d.p.i., demonstrating that PEDV SH was highly pathogenic to suckling piglets. To study the effect of the gene deletion on the immunogenicity of PEDV SH, the sera from the mice inoculated with PEDV SH were detected by ELISA. All mice immunized with PEDV SH produced circulating antibodies against N protein to the same extent as mice immunized with PEDV YZ, indicating that the deleted epitope didn't significantly affect the overall immunogenicity of PEDV SH.

PEDV and TGEV belong to two distinct species of the *Alphacoronavirus* genus but induce similar clinical symptoms and pathological lesions in newborn piglets. The N proteins of these viruses exhibit considerable antigenic cross-reactivity that has been attributed to their N terminal residues, 58–68 and 78–85 respectively (Xie et al., 2019; Lin et al., 2015). There are differences between the N proteins of PEDV and TGEV in residues 398–406, although the C terminal of neither the PEDV N protein (aa 302–441) nor the TGEV N protein reacts with anti-NEP-1C9 monoclonal antibody (not shown here). These results remind us that the NEP-1C9 polypeptide may act as a candidate antigen in differential diagnosis assays to distinguish not only the circulating antibodies to PEDV from that to TGEV, but also the conventional strains of PEDV from SH strain. Of course, to fully understand the effect of the NEP-1C9 deletion, the N gene from SH should be cloned to

study its expression, localization, and stability. Additionally, the effect of the NEP-1C9 deletion on viral virulence could be tested by constructing infectious cDNA.

PEDV N protein plays important roles in the course of PEDV infection, not only modulating cell cycle (Xu et al., 2013), but also regulating secretion of cytokines and immunomodulatory factors (Ding et al., 2014). Previous reports have shown that PEDV N of the cell-adapted G1 strain is likely to be cleaved by 3C-like protease during replication, leaving only the first 380 amino acids after cleavage (Jaru-Ampornpan et al., 2017). This cleavage motif is present in the majority of cell culture-adapted PEDV strains but absent in the emerging field isolates. This protein cleavage is possibly a marker of PEDV adaptation to cell culture. The C-terminal region of the N protein may not be essential for virus replication. Because the PEDV 3Cpro cleavage site is at residue 382 which is in front of the deleted NEP-1C9 epitope, we deduced that the deletion might have no effect on virus replication in vitro. In addition to the unique deletion found in N protein, we noted some amino acid substitutions in N protein of SH strain also occurred in most wild PEDV. These amino acid substitutions may compensate for the deletion of NEP-1C9 to facilitate virus replication, which needs further study using infectious cDNA techniques.

Of the four structural proteins of PEDV, the S protein has the maximum variability, playing a critical role in the induction of neutralizing antibodies, binding to host cell receptors, and mediating membrane fusion and virus invasion (Bosch et al., 2003). The S protein of the PEDV SH isolate has characteristics shared by all emerging strains, and similar mutations to the Korean strains, in which the base insertions and deletions were firstly found, compared with CV777 (Lee et al., 2010). The multiple point mutations at the N-terminal of the PEDV SH S gene result in a decrease in the number of N-glycosylation sites and positional changes, compared with CV777, but the effect of them on the function of the S protein has not been clarified. It has been reported that a 5 % amino acid difference in the S1 protein of infectious bronchitis virus led to poor cross-immune protection (Cavanagh, 2003). Based on the S gene, PEDV strains are divided into three genotypes: G1 (classical strains), G2 (variant strains) and S-INDEL (recombinant strains). At present, the most prevalent PEDV strains worldwide, especially in China, belong to G2 strains (Lin et al., 2016). G2 strains are further divided into 2 subgroups: G2a mainly occurrs in Asia, while G2b is primarily endemic to Asia and North America (Sun et al., 2019). Liu et al. (2019) reported that G2a strain-based inactivated vaccine candidates were more promising than G2b-based candidates for the development of an effective vaccine against the current highly virulent pandemic PEDV strains. Phylogenetic analysis shows that the SH strain belongs to group G2b, as do the other field isolates in China (Chen et al., 2019; Liu et al., 2019). SH strain has a closer phylogenetic relationship with strains isolated from southern China in 2011–2012, than with other Chinese strains, which share a closer phylogenetic relationship with Asian strains than with U.S. strains. We also note that the S gene of the SH isolate has three asparagine substitutions, (N57SS→NST, N341LS→NFS, and N723NT→NST), two insertions (N118AT and N1196HT), and three deletions (N 127KT, N230CT and N1006IT). The effects of these mutations on viral virulence and immunity should be studied in the future.

Altogether, PEDV SH, a G2b genotype, has potential as a candidate for vaccine which not only provides effective immune protection against emerging variant strains, but also the vaccine-induced antibodies could be distinguished from field-induced antibodies by using NEP-1C9 polypeptide as coating antigen.

## 5. Conclusion

We report a novel deletion in the N protein of a field isolated PEDV; a continuous 12-aa (aa 399–410) deletion encompassing the epitope NEP-1C9 (aa 398–406). This isolate (PEDV SH) is a highly virulent G2a subtype. The NEP-1C9 polypeptide is specific to PEDV and can be used

as an antigen to distinguish the antibody induced by the classic or variant PEDV, or by TGEV. Thus, this study provides a candidate PEDV strain for vaccine development and a new tool for diagnosis of classic and variant PEDV and TGEV.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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