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ORIGINAL RESEARCH ARTICLE

# Isolation and identification of a highly pathogenic strain of porcine epidemic diarrhea virus

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## Abstract

Porcine epidemic diarrhea virus (PEDV) causes acute watery diarrhea and high mortality in neonatal piglets, resulting in substantial economic losses to the global swine industry. Here, we successfully isolated a PEDV strain, designated CHN-CQ-2021, from PEDV-positive diarrheic samples collected from a pig farm in Chongqing, China. Electron microscopy observation revealed that the CHN-CQ-2021 strain exhibited typical coronavirus morphology and could be recognized by PEDV-specific antibodies. Phylogenetic analysis of its full-length genome and *S* gene further classified this isolate as a G2b variant strain. Importantly, 1-day-old newborn piglets were orally challenged with CHN-CQ-2021 at $2 \times 10^6$ 50% tissue culture infectious dose/mL. Compared with the control group, the infected piglets developed severe diarrhea with 100% mortality. In addition, viral RNA was detected in rectal swabs and multiple tissues, including the intestinal tract and brain, with macroscopic/microscopic intestinal lesions and viral antigen distribution confirmed using histopathology and immunohistochemistry. These findings demonstrate the presence of a pathogenic PEDV strain in Chongqing, China, capable of causing severe neonatal piglets' enteric disease.

***Keywords:*** Porcine epidemic diarrhea virus; Isolation and identification; Pathogenicity; Newborn piglets; China

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## 1. Introduction

Porcine intestinal infectious diseases pose significant threats to the swine industry and have exhibited an increasing prevalence in multiple regions across global pig farming countries in recent years. Various pathogens, including viruses (such as norovirus, astrovirus, and rotavirus) and bacteria (such as *Salmonella*, *Campylobacter*, and *Escherichia coli*), can cause diarrhea in both humans and animals.[1-6] Among various pathogens, viral-induced enteric diseases are particularly severe,[7] resulting in substantial economic losses to the global swine sector. At present, clinically significant viral swine enteric pathogens include transmissible gastroenteritis virus, porcine rotavirus, porcine epidemic diarrhea

virus (PEDV), porcine deltacoronavirus, and swine acute diarrhoea syndrome coronavirus.[8-11] Despite the availability of vaccines for immunization control, frequent outbreaks of viral enteric diseases continue to occur, with more than 50% of cases attributed to PEDV infections.[12] Notably, PEDV exhibits a high mutation rate, enabling variant strains to evade immune protection conferred by existing vaccines derived from original strains.[13] This highlights the necessity for continuous monitoring and analysis of the biological characteristics of circulating PEDV strains to optimize prevention strategies.

PEDV is an enveloped, single-stranded positive-sense RNA virus belonging to the *Alphacoronavirus* genus, with a genome length of approximately 28 kb.[14] The 5' end of its genome contains a cap structure in the untranslated region, while the 3' end contains a polyadenylated tail.[15] The PEDV genome contains seven open reading frames (ORFs): ORF1a, ORF1b, and ORF2–6, encoding a total of four structural proteins (spike [S], envelope [E], membrane [M], and nucleocapsid [N]) and 16 non-structural proteins (nsp1–nsp16).[16] Porcine epidemic diarrhea (PED) was first reported in the United Kingdom in 1971, and the pathogen PEDV was first identified and isolated in Belgium in 1978, with the isolate named CV777.[17] Between 1970 and 1980, PEDV spread across Europe, causing significant mortality among piglets.[18] From 1980 to 2010, the disease occurred sporadically, with occasional reports from several countries, including the United Kingdom, Germany, and Italy.[18] In China, PED was first reported in 1973.[19] In October 2010, a large-scale PED outbreak emerged across pig farms nationwide, with mortality rates among neonatal piglets reaching 50–100% in affected farms, causing substantial economic losses to the Chinese swine industry.[19] Subsequent investigations revealed that variant PEDV strains had evolved to evade the immune protection conferred by CV777-derived vaccines,[20] indicating the importance of monitoring circulating PEDV strains and characterizing their biological properties to inform effective control strategies.

To investigate the biological characteristics of current circulating PEDV strains in Chinese pig farms, this study isolated viruses from diarrheic piglets obtained from a swine facility in Chongqing in 2021. The isolates underwent comprehensive genomic characterization and *in vivo* pathogenicity assessment. These findings enhance our understanding of PEDV pathogenesis and establish the foundation for vaccine development.

## 2. Materials and methods

### 2.1. Clinical samples collection

In early March 2021, an outbreak of PEDV was reported in swine herds in Chongqing, China, with mortality rates reaching up to 100%. Intestinal luminal contents were collected from PEDV-positive piglets exhibiting clinical signs of severe diarrhea and vomiting, cryopreserved at −80°C, and processed for viral isolation.

### 2.2. Virus isolation, plaque purification, and propagation in Vero cells

Before virus isolation, intestinal luminal contents were homogenized in an equal volume of sterile 1× phosphate-buffered saline (PBS, pH 7.4; Solarbio, China). Following homogenization, particulate debris was removed through centrifugation (11,000 *g*, 4°C, 10 min; 5804 R, Eppendorf, Germany), and supernatants were clarified through sterile 0.22-μm syringe filters (Millipore, United States of America). Vero cells (ATCC number: CCL-81; ATCC, USA) were utilized for PEDV isolation.

Virus isolation, plaque purification, and propagation were conducted as previously described with some modifications.[21] Briefly, Vero cells were cultured in a six-well plate until reaching 90% confluency. The cells were washed three times with sterilized 1× PBS buffer. Subsequently, 350 μL of Dulbecco's Modified Eagle Medium (DMEM; Solarbio, China) maintenance medium (serum-free DMEM medium supplemented with 7 μg/mL trypsin (Sigma, USA) and 200 μL of filtered sample were added to each well. Control wells received an equivalent volume of DMEM maintenance medium. The plates were incubated at 37□ in a 5% $CO_2$ atmosphere (Thermo, USA). Cells were observed daily for cytopathic effects (CPE). When CPE reached 80%, the plates were subjected to two freeze-thaw cycles. The supernatant was collected after centrifugation at 11,000 × *g* for 10 min at 4□, and stored at −80□, followed by further examination using quantitative real-time (RT)-polymerase chain reaction (PCR) as described below.

For virus plaque purification, supernatants from virus-infected Vero cells were serially diluted and inoculated onto fresh Vero cell monolayers. Cells were incubated with the diluted virus in maintenance medium for 1.5 h at 37°C under 5% $CO_2$. After incubation, the medium was aspirated, and cells were overlaid with 2 mL of maintenance medium containing 1.25% low-melting-point (LM), genetically quality-tested (GQT) agarose (Gibco, USA) to immobilize viral particles. Following a 24-h incubation, cells were stained with 2 mL of maintenance medium supplemented with 1.25% LM GQT Agarose and 0.01% Neutral Red solution (Sigma, USA). Plaques were isolated using sterile pipette tips and transferred into microcentrifuge tubes containing 0.5 mL of maintenance medium.

The purified plaques, named as CHN-CQ-2021, were propagated in Vero cells. Vero cells were cultured in T175 flasks until reaching 90% confluency, washed 3 times with

sterile 1× PBS (pH 7.4), and inoculated with 1 mL of PEDV CHN-CQ-2021 diluted in 50 mL of maintenance medium. Cells and supernatant were maintained at 37°C under 5% $CO_2$ and monitored for CPE. When CPE became evident (approximately 2 days post-infection), the cultures were subjected to two freeze-thaw cycles. The resulting cell lysates and supernatants were harvested for viral titer determination as described below.

### 2.3. Immunofluorescence assay (IFA)

The IFA experiment was performed as previously described with some modifications.[22] Briefly, Vero cells were seeded in 12-well plates and cultured overnight, followed by infection with PEDV (CHN-CQ-2021) at a multiplicity of infection (MOI) of 0.1. At 16–24 h post-PEDV infection, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 10 min at room temperature, and blocked with 3% bovine serum albumin (BSA). Subsequently, cells were sequentially incubated with PEDV-specific antiserum (M100048, Zoonogen, China) and Cy3-conjugated secondary antibody (A10521, Thermo, USA) in the dark at room temperature for 1 h. After three washes with 1× PBS, nuclei were counterstained with 4',6-diamidino-2-phenylindole (Solarbio, China) for 5 min. Fluorescence signals were visualized using an inverted fluorescence microscope (Leica, Germany).

### 2.4. Electron microscopic observation

Virus morphology was observed using transmission electron microscopy (TEM; JEM-1400, JEOL, Japan) as previously described, with some modifications.[22] Briefly, PEDV was propagated in Vero cells. Cell debris was removed through centrifugation at 11,000 × *g* (4□, 30 min). The clarified supernatant was mixed with 7% PEG6000 (Solarbio, China) and incubated overnight at 4□ with continuous agitation. The mixture was then centrifuged at 11,000 × *g* (4□, 1 h), and the pelleted virions were resuspended in 2 mL of 1× PBS and layered onto a discontinuous sucrose gradient (20–60%). After ultracentrifugation at 177,600 × *g* (4□, 2 h) using an ultracentrifuge (Himac CP 100WX, Hitachi Koki, Japan), distinct viral bands were carefully collected. Viral band was diluted in 1× PBS and subjected to additional ultracentrifugation (177,600 × *g*, 4□, 2 h) for sucrose removal. Purified virions were resuspended in minimal sterile 1× PBS and negatively stained with 3% (w/v) phosphotungstic acid (pH 6.8; Sigma, USA) for 2 min. Excess liquid was blotted using filter paper, and grids were air-dried before observation under TEM at 80 kV.

### 2.5. Infectious-virus titrations by a 50% tissue culture infectious dose ($TCID_{50}$) assay

Vero cells were seeded into 96-well plates and cultured overnight, followed by two washes with sterile 1× PBS. A 10-fold serially diluted PEDV (100 μL/well) was inoculated onto the cell monolayer, with eight replicates per dilution. Cells were maintained in a humidified incubator at 37°C with 5% $CO_2$ for 5–7 days. Viral titers were quantified by observing CPE and calculated using the Reed–Muench method, expressed as 50% tissue culture infectious dose ($TCID_{50}$)/mL.[23] Plaque-forming units (PFUs) were derived using Equation I as previously described,[24] and PFU values were used to determine the MOI.

$$PFU = 0.7 \times TCID_{50} \quad (I)$$

### 2.6. Measurement of PEDV (CHN-CQ-2021) growth

Vero cells were seeded into 35-mm cell culture dishes and infected with PEDV at an MOI of 0.1 when the cell density reached 90%. Cells cultured with DMEM maintenance medium without viral inoculation served as controls. Every 4 h post-inoculation (hpi), PEDV-infected cells and control cells were observed, and then, the cells were harvested and stored at −80□. After two freeze-thaw cycles, supernatants were harvested to determine viral titers as described above, and a growth curve of PEDV CHN-CQ-2021 was generated.

### 2.7. Genomic cloning and phylogenetic analysis of the whole genome and *S* genes

Viral RNA was extracted from PEDV cell lysates using an RNeasy kit (R4111-03, Magen, China) according to the manufacturer's instructions, followed by DNase I treatment to remove genomic DNA contamination. The specific primers (Sangon Company, China; Table 1) for amplification of the PEDV whole genome were designed with reference to the published sequence (GenBank: JX647847) and synthesized. The reaction mixture (50 μL total volume) contained 1 μL PrimeScript 1-Step Enzyme Mix, 25 μL 2× One-Step Buffer, 2.5 μM each of forward and reverse primers, 1 μg viral RNA, and RNase-free $H_2O$. The PCR reaction conditions were as follows: 50°C for 30 min; 94°C for 2 min; followed by 34 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2.5 min; with a final extension at 72°C for 10 min. Subsequently, the PCR products were subjected to 1% agarose gel electrophoresis, and target bands were excised and purified using a DNA gel extraction kit (UE-GX-50, US Everbright, China). Purified gene fragments were then sent for sequencing (Sangon Biotech, China). Viral genome sequences were assembled using DNAStar Lasergene 7.0 (Version 7.0, DNASTAR Inc., USA). The *S* gene and the whole genome of CHN-CQ-2021 were aligned with representative PEDV strains retrieved from the National Center for Biotechnology Information. Phylogenetic trees were constructed by the neighbor-joining method using MEGA 5 software available online (http://www.megasoftware.net/).

### 2.8. Experimental infection of conventional newborn piglets with PEDV CHN-CQ-2021 strain

The animal experiments were conducted in compliance with the ethical guidelines and regulations of the Institutional Animal Care and Use Committee of Sun Yat-sen University, and all procedures were approved by the committee. Ten 1-day-old healthy crossbred conventional piglets (Duroc × Landrace × Large White) were obtained from Wen's Foodstuffs Group Co., Ltd. (China). All piglets were confirmed negative for PEDV antigen by RT-PCR on rectal swabs and negative for PEDV antibodies (immunoglobulin [Ig]A/IgG) by enzyme-linked immunosorbent assay on serum samples.[25] All piglets were randomly divided into two groups ($n = 5$): one experimental group and one control group. Each group was housed in a separate isolation room. The experimental group was orally inoculated with 2 mL of the PEDV CHN-CQ-2021 strain containing $2 \times 10^6$ $TCID_{50}$, while the control group received 2 mL of DMEM maintenance medium. Rectal swabs of piglets were collected daily post-infection, and diarrhea was scored according to Chen *et al.*[26] Piglets that succumbed to infection were immediately necropsied, and fresh tissues (jejunum, ileum, cerebrum, cerebellum, and brainstem) were collected and fixed in 4% paraformaldehyde (Thermo, USA) for subsequent histopathological and immunohistochemical analysis. At the end of the experiment, piglets in the control group were euthanized and subjected to the same necropsy and tissue collection procedures.

### 2.9. RT-PCR analysis

The supernatants from rectal swabs or tissue homogenates were centrifuged at 6,010 × *g* for 5 min. Total RNA was extracted from the supernatants using an RNeasy kit (R4111-03, Magen, China) according to the manufacturer's instructions, followed by DNase I treatment. The specific primers (Table 2) and probe for the nucleocapsid (*N*) gene of PEDV were designed based on a previous publication[27] and synthesized by Sangon Company (China). RT-PCR was performed on a thermocycler (Applied Biosystems 7500 Fast instrument, Life Technologies, USA) in a 20 μL reaction volume containing 1 μg RNA, 10 μL 2 × Hifair V C58P2 MP Buffer, 0.8 μL of Hifair V C58P2 Enzyme Mix (Shanghai Yeasen Biotechnology Co., Ltd., China), 0.2 μmol/L probe, and 0.4 μmol/L of each primer. Thermal cycling conditions were as follows: 50□ for 20 min, 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. A standard curve was generated using a plasmid construct. Briefly, the *N* gene was amplified from the PEDV CHN-CQ-2021 strain using specific primers (Table 2) designed based on the whole genome of PEDV CHN-CQ-2021. The PCR product was cloned into the pMD19-T vector (Takara, Japan). The plasmid was serially diluted 10-fold to generate a standard curve for each plate. Viral RNA quantities in test samples were calculated based on cycle threshold values relative to the standard curve.

**Table 1. Primers of porcine epidemic diarrhea virus for whole-genome amplification**

| Primer name | Position | Primer sequences (5'→3') |
|---|---|---|
| PEDV-1F | 190–209 | GGCGTTCCGTCGCCTTCTAC |
| PEDV-1R | 2751–2729 | GCAAGTGCCTTCCAGATTCCTGT |
| PEDV-2F | 2663–2684 | GTATTATGCCACCAGTGTCCCA |
| PEDV-2R | 4957–4938 | CAGTTGCCAGCAGGCACTGT |
| PEDV-3F | 4887–4906 | ACCAGCGGTGCATTGCTTGA |
| PEDV-3R | 7475–7453 | CAATGTGCTCTTGCAATCCTGCA |
| PEDV-4F | 7327–7350 | CTGTTAAGTTAGTGGACTCAGCGT |
| PEDV-4R | 9875–9856 | ACTAGCGCCTTCAACTTGCA |
| PEDV-5F | 9712–9731 | GCGCTTGTGGTTCACCTGGT |
| PEDV-5R | 12259–12240 | GGATCCACAGCGAAAGCGCA |
| PEDV-6F | 12182–12202 | ACGCTTGCAGGCTGGTAAACA |
| PEDV-6R | 14462–14442 | TGGGCAGTGCTCTATCGCACT |
| PEDV-7F | 14322–14341 | ATACTAGGGGCGCTTCGGTT |
| PEDV-7R | 16780–16760 | GTCAGGGTGCACAGGAATGAA |
| PEDV-8F | 16662–16684 | GTATGTGTGCCCTTAAGCCTGAT |
| PEDV-8R | 19002–18980 | GTAAGTGGACGTTCGGCTTCATA |
| PEDV-9F | 18874–18898 | CGTAGCTTTTGAGTTGTATGCCA |
| PEDV-9R | 21330–21309 | GCAATTAGCTGTACAGGGTTCA |
| PEDV-10F | 21080–21101 | CCATTCCAGCTTATATGCGTGA |
| PEDV-10R | 23487–23465 | GTACATGTGAAGCTTCTCAGCGT |
| PEDV-11F | 23272–23292 | GTGTACGATCCTGCAAGTGGC |
| PEDV-11R | 25715–25694 | TCACCTCATCAACGGGAATAGA |
| PEDV-12F | 25535–25557 | TCGTCCAATTGGTTAATCTGTGC |
| PEDV-12R | 27840–27820 | TACCGTTGTGTGCAAGACCAA |

Abbreviation: PEDV: Porcine epidemic diarrhea virus.

**Table 2. Primers and probe for quantitative real-time polymerase chain reaction and full-length amplification of the porcine epidemic diarrhea virus nucleocapsid (*N*) gene**

| Primer name | Primer/probe sequences (5'→3') |
|---|---|
| PEDV-F | CGCAAAGACTGAACCCACTAATTT |
| PEDV-R | TTGCCTCTGTTGTTACTTGGAGAT |
| PEDV-probe | FAM-TGTTGCCATTGCCACGACTCCTGC-TAMRA |
| PEDV-N-CDS-F | ATGTCTGACGCAGAAGAGTG |
| PEDV-N-CDS-R | TTACATATACTTATACAGGCGAGC |

Abbreviations: FAM: 6-carboxyfluorescein; TAMRA: Carboxytetramethylrhodamine; PEDV: Porcine epidemic diarrhea virus.

### 2.10. Histological and immunohistochemical analysis

Tissue samples collected from piglets were subjected to histopathological and immunohistochemical analyses as previously described, with some modifications.[22] Briefly, samples were fixed in 4% paraformaldehyde for over 36 h, dehydrated through a graded ethanol series, embedded in paraffin, sectioned, and mounted on glass slides. For histopathological examination, sections (5 μm) were dewaxed, rehydrated, and stained with hematoxylin and eosin for observation under a conventional light microscope. For immunohistochemical analysis, sections were blocked with 1% BSA and incubated with a diluted PEDV-specific mouse antiserum (1:100; M100048, Zoonogen, China) at 4□ for 12 h. After washing, the sections were incubated in a diluted peroxidase-labeled goat anti-mouse IgG secondary antibody (1:200; SA00001-1, Proteintech, USA) at room temperature for 50 min. Finally, the sections were treated with 3,3'-diaminobenzidine chromogen kit (K3468, Dako, Denmark) and counterstained with hematoxylin. Stained sections were visualized and documented under a microscope. Tissues from piglets in control groups were used as controls.

### 2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (version 8.4.3, GraphPad Software Inc., USA). Data were presented as mean ± standard deviation or mean ± standard error of the mean, as appropriate. The normality of data distribution was assessed using the Shapiro–Wilk test. Comparisons of PFU and RNA copy numbers between the treatment and control groups were analyzed for statistical significance. For normally distributed data, one-way analysis of variance followed by Tukey's *post hoc* multiple comparison test was applied. For non-parametric data, the Mann–Whitney *U*-test was used. $p<0.05$ was considered statistically significant.

## 3. Results

### 3.1. Isolation of a PEDV strain from the intestinal contents of a piglet with diarrhea

To analyze the biological characteristics of the currently prevalent PEDV strains in pig farms, the PEDV-positive piglet pathological materials collected from a pig farm in Chongqing were inoculated into Vero cells. Compared to the control, the Vero cells inoculated with the diseased material showed significant cell membrane fusion. Over time, the area of cytopathic changes expanded, accompanied by cell detachment Figure 1A and B, consistent with the typical CPE of PEDV. To further confirm that PEDV caused the cytopathic changes, IFA was performed using PEDV-specific antibody serum. As shown in Figure 1C and D, specific red fluorescence was observed in the cells inoculated with plaque-purified virion, whereas no fluorescence signal was observed in the control group. These results indicate the isolation of a PEDV strain from a diarrheic pig, named as CHN-CQ-2021.

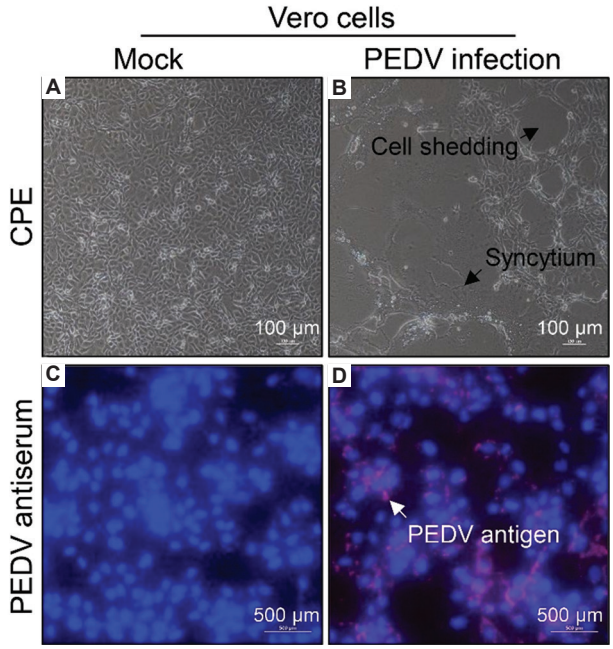


**Figure 1.** The cytopathic effect and immunofluorescence assay (IFA) analysis in porcine epidemic diarrhea virus (PEDV)-infected Vero cells. (A) Mock-inoculated Vero cells exhibited typical morphological integrity. Scale bar: 100 μm, magnification: 200×. (B) PEDV-infected Vero cells exhibited syncytia and cell shedding (indicated by arrows). Scale bar: 100 μm, magnification: 200×. (C) IFA performed at 24 h post-infection in mock-treated Vero cells. Scale bar: 500 μm, magnification: 40×. (D) IFA performed at 24 h post-infection in PEDV-infected cells, with arrows indicating specific PEDV antigen-positive signals. Scale bar: 500 μm, magnification: 40×.

To observe the morphology and size of the virus particles, the purified PEDV CHN-CQ-2021 strain was examined using TEM. Typical coronavirus morphology, with an envelope and spike proteins on its surface, was observed under the electron microscope (Figure 2). The diameter of the virus particles was approximately 80–120 nm. These observations further confirmed the isolation of a PEDV strain.

### 3.2. Stable proliferation of PEDV CHN-CQ-2021 strain in host cells

To analyze the proliferation kinetics of the PEDV CHN-CQ-2021 strain, the virus was inoculated into Vero cells. Samples were harvested at multiple time points, and viral titers were determined using the $TCID_{50}$ assay to generate the viral growth curve. Cell membrane fusion

was observed in Vero cells at 8 hpi. Prolonged incubation resulted in progressive expansion of the cytopathic area and the emergence of cell detachment (Figure 3A). Analysis of the virus proliferation curve further revealed that CHN-CQ-2021 reached its replication peak in Vero cells at 16 hpi (Figure 3B). These results demonstrate that the PEDV CHN-CQ-2021 strain can efficiently proliferate *in vitro*.

### 3.3. Phylogenetic analysis of whole-genome and *S* genes of PEDV CHN-CQ-2021

To investigate the genetic evolution of the strain, the complete genome of PEDV CHN-CQ-2021 was amplified using RT-PCR and compared with the full genome and *S* gene sequences of other reference strains retrieved from GenBank. Sequence alignment and phylogenetic analysis were performed using the neighbor-joining method to construct a phylogenetic tree, with bootstrap values applied to evaluate the reliability of the tree topology.[28] The PEDV CHN-CQ-2021 strain was classified within the G2b subgroup and displayed the closest genetic relationship to strain GD-1 (GenBank: JX647847). In contrast, it exhibited significant phylogenetic divergence from the G1-type classical strain branch represented by CV777 (Figure 4). These data suggested that the PEDV CHN-CQ-2021 strain was the most closely related to other PEDV strains from mainland China.

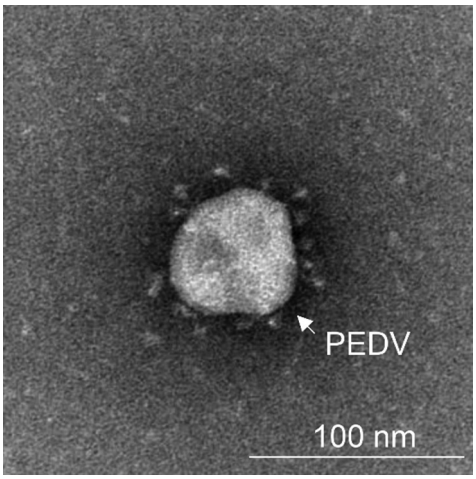


**Figure 2.** Electron micrograph of porcine epidemic diarrhea virus (PEDV) CHN-CQ-2021. The arrow indicates the crown-shaped spikes of PEDV CHN-CQ-2021. Scale bar: 100 nm, magnification: 30000×.

### 3.4. High pathogenicity of PEDV CHN-CQ-2021 strain in newborn piglets

To evaluate the pathogenicity of the PEDV CHN-CQ-2021 strain in piglets, newborn piglets were orally infected with the CHN-CQ-2021 strain. Compared to the control group, CHN-CQ-2021 strain-infected piglets exhibited typical clinical symptoms, including severe watery diarrhea and dehydration (Figure 5). Importantly, all CHN-CQ-2021 strain-infected newborn piglets died within 4 days post-inoculation (Figure 6). Further analysis of virus shedding and tissue tropism indicated that, compared to the control group, CHN-CQ-2021 strain-infected newborn piglets shed virus at varying levels during the 4-day observation period (Figure 7A), while viral nucleic acids were detected in both intestinal and brain tissues (Figure 7B). These results suggest that the PEDV CHN-CQ-2021 strain was highly pathogenic to newborn piglets.

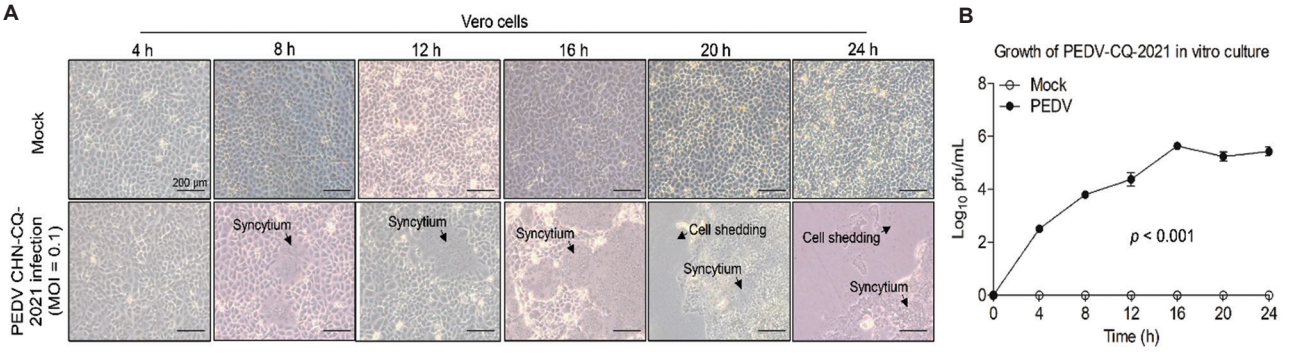


**Figure 3.** Measurement of PEDV CHN-CQ-2021 growth. Vero cells were seeded in 12-well plates and cultured until reaching 90% confluence. The cell monolayers were washed three times with sterile 1× phosphate-buffered saline (pH = 7.4), followed by infection with PEDV CHN-CQ-2021 (MOI = 0.1). The cell lysates and culture supernatants were collected at specified time points (0, 4, 8, 12, 16, 20, and 24 h post-inoculation) and stored at −80°C for subsequent viral titer quantification. (A) Microscopic images of Vero cells at specified time points (4, 8, 12, 16, 20, and 24 h post-inoculation) after mock or PEDV infection. The arrows indicate the CPE in PEDV-infected cells. Scale bar: 200 μm, magnification: 100×. (B) The growth of PEDV CHN-CQ-2021 in Vero cells. The data are presented as mean ± standard deviation ($n$ = 3), based on three independent experiments.
Abbreviations: CPE: Cytopathic effect; MOI: Multiplicity of infection; PEDV: Porcine epidemic diarrhea virus.

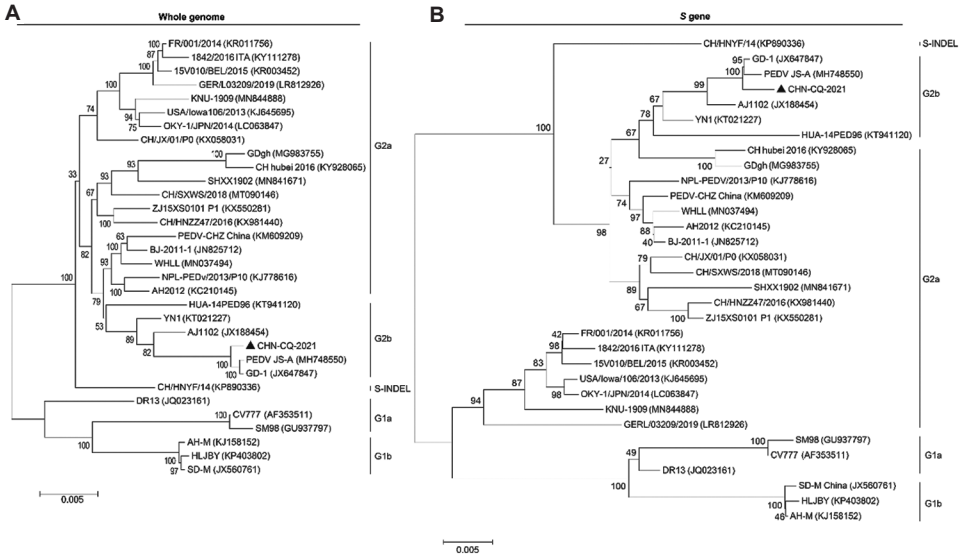


**Figure 4.** Phylogenetic trees of the whole genome and *S* gene of porcine epidemic diarrhea virus (PEDV) CHN-CQ-2021. (A) The genome-wide phylogenetic tree constructed using the neighbor-joining method in the MEGA software package (version 5, http://www.megasoftware.net/). (B) Phylogenetic tree of *S* genes of PEDV constructed using the neighbor-joining method in the MEGA software package (version 5, http://www.megasoftware.net/). Reference sequences, obtained from the GenBank database, are annotated with their respective strain names. The evolutionary distance scale bar corresponds to 0.005 nucleotide substitutions per site.

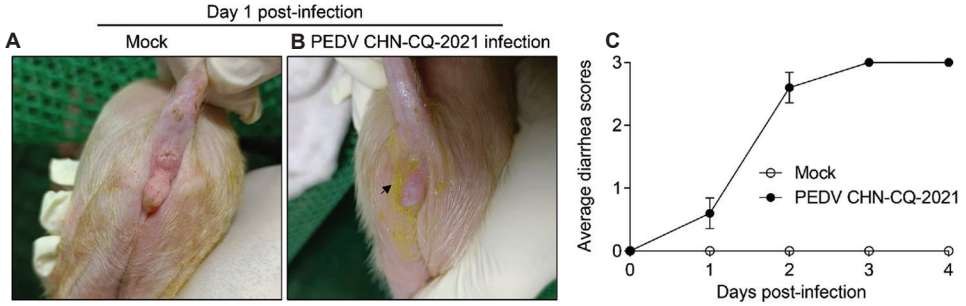


**Figure 5.** Investigation of watery diarrhea in piglets orally infected with porcine epidemic diarrhea virus (PEDV) CHN-CQ-2021. (A) Newborn piglets from the control group showed no clinical signs. (B) Watery diarrhea (indicated by the arrow) was observed on day 1 post-PEDV CHN-CQ-2021 infection. (C) Average diarrhea scores after PEDV infection.

### 3.5. Gross pathology, histopathology, and immunohistochemical in newborn piglets infected with PEDV CHN-CQ-2021

To further characterize the gross and histopathological changes of piglets after PEDV CHN-CQ-2021 infection, necropsy was performed on infected piglets, and the small intestine tissues were collected for histopathological and immunohistochemical analysis. The small intestines, which exhibited accumulation of yellow watery contents, displayed transparency, thinning of the intestinal wall, and gas distension (Figure 8B). No pathological changes were observed in other organs of PEDV-infected piglets or in the organs of the control group (Figure 8A), indicating that the small intestine is the primary target organ of PEDV infection. Microscopic lesions of the small intestine tissue were further analyzed. As shown in (Figure 8C-H), blunted intestinal villi were observed, whereas the intestinal structure in the control group remained normal. Immunohistochemical analysis confirmed the presence of PEDV antigen in the cytoplasm of villous enterocytes in PEDV-infected piglets (Figure 8L-N), consistent with the histopathological findings. In contrast, no PEDV antigen was detected in the control group (Figure 8I-K). Taken together, these results indicate that PEDV CHN-CQ-2021 infection causes diarrhea due to intestinal damage.

## 4. Discussion

PEDV was first reported in the United Kingdom in the last century; it has rapidly spread to numerous European and Asian countries,[17,18] posing a significant threat to the

sustainable development of the global swine industry. Notably, the emergence of a PEDV variant strain in China in 2010 resulted in substantial economic losses to pig farms nationwide,[29] primarily due to its ability to evade the immune protection conferred by existing vaccines.[30] Therefore, research on the biological characteristics of currently circulating PEDV strains is crucial for assessing the efficacy of existing vaccines and guiding vaccine development strategies. In this study, we isolated a highly pathogenic PEDV strain from the intestines of piglets with diarrhea. The genome of the isolated PEDV strain (CHN-CQ-2021) and pathogenicity were analyzed, which will help to understand the biological characteristics of prevalent PEDV strains in China.

The Vero cell line, derived from the kidney of an African green monkey in 1962,[31] has been widely used for the isolation of coronaviruses, such as PEDV and SARS-CoV-2.[21,27,32] In this study, we attempted to isolate PEDV from PEDV-positive clinical samples using Vero cells. Although multiple samples, including anal swabs and intestinal pathological tissues, were collected, successful viral isolation was exclusively achieved from fresh intestinal samples of diarrheic piglets. This finding suggests that intestinal pathological tissues with higher virus loads are easier to isolate viruses compared to anal swabs. Low viral titers resulting from suboptimal sample collection, transportation, or storage conditions may significantly hinder isolation efficiency. In addition, unfiltered samples, which were not sterilized by filtration, yielded higher isolation rates, likely because filtration removed a substantial number of viral particles. However, unfiltered samples required supplementation with high concentrations of antibiotics to suppress bacterial contamination and maintain cell viability. After three serial passages in Vero cells, the isolated virus induced significant CPE, which was subsequently confirmed as PEDV by indirect IFA and TEM observation. A viral growth curve generated by infecting Vero cells with CHN-CQ-2021 demonstrated robust viral proliferation, indicating its adaptability to this cell line and suitability for further mechanistic studies. Although we isolated the PEDV CHN-CQ-2021 strain, it is noteworthy that many clinical samples failed to yield viable virus. Our findings suggest that viral load in samples is a critical determinant for successful isolation. To improve isolation efficiency, pre-enrichment of viruses by passaging clinical materials through susceptible piglets before cell culture inoculation is strongly recommended.[26]

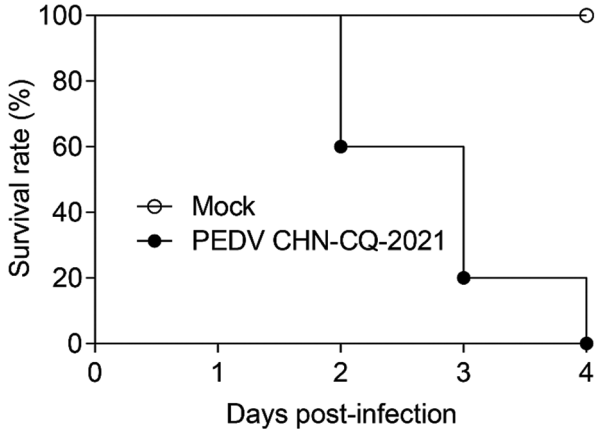


**Figure 6.** The survival rate of newborn piglets infected with porcine epidemic diarrhea virus (PEDV) CHN-CQ-2021. Mortality was monitored and recorded daily in each newborn piglet group from 1 to 4 days post-PEDV CHN-CQ-2021 infection.

To characterize the virus isolate, the whole genome of the CHN-CQ-2021 strain was sequenced and analyzed. Phylogenetic analysis revealed that the complete genomes of all PEDV strains retrieved from GenBank share high sequence similarity. Notably, the CHN-CQ-2021 strain isolated in Chongqing exhibited the closest genetic homology with PEDV strains AJ1102 and GD-1, which were previously isolated in Jiangsu and Guangdong, respectively. However, the mechanisms underlying their cross-regional transmission remain unclear and require further investigation. Many studies have confirmed that the

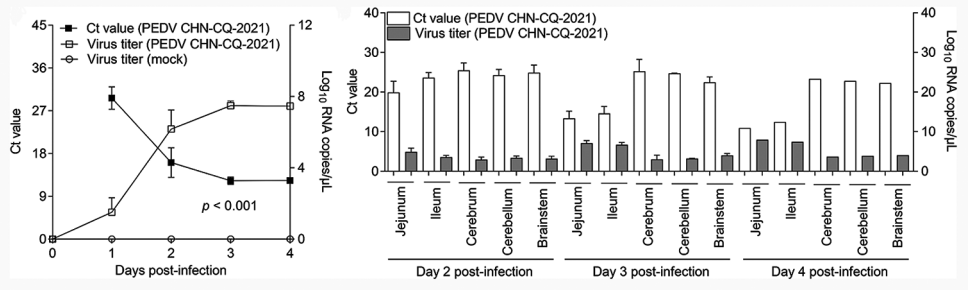


**Figure 7.** Viral shedding in rectal swabs and virus distribution in porcine epidemic diarrhea virus (PEDV)-inoculated piglets. (A) Cycle threshold (Ct) values from fecal swabs of PEDV-inoculated and mock piglets were measured to quantify viral RNA shedding. (B) Tissue tropism and viral distribution were assessed in newborn piglets at days 2, 3, and 4 post-PEDV CHN-CQ-2021 infection.

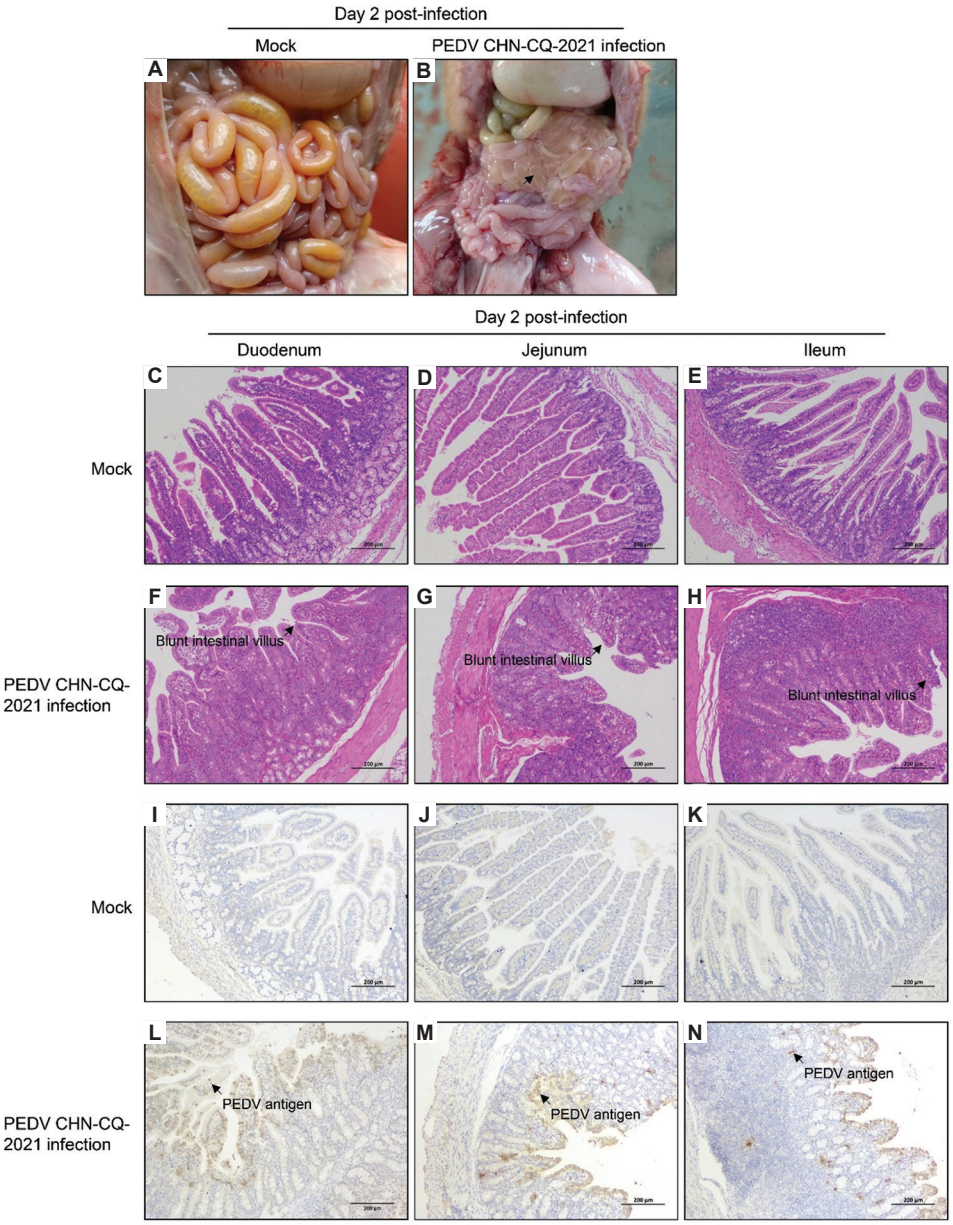


**Figure 8.** Intestinal pathological changes in newborn piglets inoculated with porcine epidemic diarrhea virus (PEDV) CHN-CQ-2021 strain. (A) Macroscopic examination of intestinal morphology in a control piglet at day 2 post-PEDV CHN-CQ-2021 infection (dpi). (B) Macroscopic observation of thin-walled intestinal tracts (arrow-indicated) in PEDV-challenged piglets at 2 dpi. (C-E) Histopathological analysis of hematoxylin and eosin (H&E)-stained intestinal tissue sections from a control piglet at 2 dpi. (F-H) H&E-stained intestinal tissue sections from a PEDV CHN-CQ-2021-challenged piglet at 2 dpi (The arrows indicate the blunt intestinal villi). (I-K) Immunohistochemical staining of intestinal tissue sections from a control piglet at 2 dpi. (L-N) Immunohistochemical staining of intestinal tissue sections from a PEDV CHN-CQ-2021-challenged piglet at 2 dpi (The arrows indicate the PEDV antigen).

*S* gene, which encodes the type I membrane glycoprotein, is the most genetically variable region in the coronavirus genome.[33] The S protein of coronavirus plays a critical role in receptor binding and virus entry.[33] Comparative analysis of the *S* gene sequence of the CHN-CQ-2021 strain revealed multiple nucleotide mutations compared to strains AJ1102 and GD-1. Whether these genetic variations contribute to the elevated virus replication efficacy and virulence requires further study.

It is noteworthy that numerous studies have already confirmed that PEDV is pathogenic to neonatal piglets, causing several symptoms, such as diarrhea, dehydration,

and death.[34,35] The PEDV GDS01 strain isolated in another study over a decade ago was initially highly pathogenic to newborn piglets.[36] However, its virulence has attenuated due to extensive *in vitro* passaging, which could affect vaccine evaluation and related studies. Orally inoculating 1-day-old newborn piglets with the CHN-CQ-2021 strain caused severe diarrhea and 100% mortality in piglets, indicating that this epidemic strain is highly pathogenic and poses a huge threat to pig farms. Viral nucleic acids could be detected in anal swabs, suggesting that fecal-oral transmission is a predominant route of PEDV dissemination. In addition, gross and histological examinations of intestinal tissues from infected piglets revealed extensive lesions. Microscopic observations showed severe cellular damage in the jejunum and ileum. Immunohistochemical analysis confirmed the predominant localization of PEDV antigens in the cytoplasm of enterocytes, consistent with previous studies.[36] These findings collectively demonstrate that PEDV CHN-CQ-2021 causes intestinal lesions, resulting in severe diarrhea. Interestingly, PEDV nucleic acids were also detected in the brains of virus-infected piglets. The mechanisms underlying PEDV entry into the brain and its potential neuropathogenicity remain to be elucidated. Importantly, we successfully isolated a highly pathogenic PEDV epidemic strain. However, several questions remain to be addressed in future research. For example, has the virulence of this strain increased compared to previously circulating strains? If so, what are the underlying mechanisms? Do current commercial vaccines confer protective immunity against this strain? If not, can this strain serve as a candidate for vaccine development? Clarifying these questions will aid in better controlling PEDV outbreaks.

## 5. Conclusion

A novel PEDV strain named CHN-CQ-2021 was isolated from the pathological samples of diarrheic piglets in this study. Phylogenetic analysis classified this strain as a G2b genotype variant, exhibiting the typical morphological features of coronavirus. Oral inoculation of newborn piglets with the CHN-CQ-2021 strain induced severe clinical symptoms, including watery diarrhea, dehydration, and high mortality, confirming its high pathogenicity. The isolation and characterization of the CHN-CQ-2021 strain provide a critical foundation for studying the pathogenic mechanisms of PEDV and developing prevention and control measures.

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## Conflict of interest

The authors declare that they have no competing interests.

## Author contributions

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*Resources:* Zhichao Xu, Chunyi Xue, Yongchang Cao
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*Validation:* All authors
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*Writing–original draft:* Xiaolu Li
*Writing–review & editing:* Zhichao Xu

## Ethics approval and consent to participate

The animal study was supervised by the Institutional Animal Care and Use Committee of Sun Yat-sen University (IACUC-2023-B0404) and conducted in accordance with the regulations and guidelines of this committee.

## Consent for publication

Not applicable.

## Availability of data

The data that support the findings of this study are available from the corresponding author on reasonable request.

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