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Isolation and characterization of a highly pathogenic strain of *Porcine enteric alphacoronavirus* causing watery diarrhoea and high mortality in newborn piglets

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Abstract

Porcine enteric alphacoronavirus (PEAV) was first discovered in China in February 2017, and the origin and virulence of this novel porcine coronavirus were not fully characterized. Here, we isolated a strain of PEAV, named GDS04 that is identified by immunofluorescence and typical crown-shaped particles observed with electron microscopy. Genomic analysis reveals that PEAV GDS04 shares a close relationship with SADS-CoV and SeACoV. Furthermore, newborn piglets orally challenged with PEAV GDS04 developed typical clinical symptoms as watery diarrhoea in neonatal piglets. Viral RNA was detected in faeces and various tissues of the infected piglets. Moreover, macroscopic and microscopic lesions in whole intestinal tract were observed, and viral antigen could be detected in the small intestines by immunohistochemical staining and electron microscopy. Importantly, the mortality rate of inoculated-newborn piglets was 100% and half of the cohabiting piglets died. Collectively, we demonstrate that PEAV is highly pathogenic in newborn piglets.

KEYWORDS
newborn piglets, pathogenicity, *Porcine enteric alphacoronavirus* (PEAV)

1 | INTRODUCTION

Coronaviruses (CoVs), belonging to the subfamily *Coronavirinae* in the family *Coronaviridae* within the order *Nidovirales* (Zhang, 2016), are found in a wide variety of animals (Felippe, da Silva, Santos, Spilki, & Arns, 2010; Pan et al., 2012; Rihtaric, Hostnik, Steyer, Grom, & Toplak, 2010; Stohlman, Brayton, Fleming, Weiner, & Lai, 1982; Tsunemitsu, el-Kanawait, Smith, Reed, & Saif, 1995). Porcine CoVs are significant enteric and respiratory pathogens of swine. Six porcine CoVs have so far been identified: transmissible gastroenteritis virus (TGEV) (Doyle & Hutchings, 1946), porcine respiratory coronavirus (PRCV) (Wesley, Woods, Hill, & Biwer, 1990), porcine epidemic diarrhoea virus (PEDV) (Pensaert & de Bouck, 1978), and porcine enteric alphacoronavirus (PEAV) (Gong et al., 2017) in the *Alphacoronavirus* genus; porcine haemagglutinating encephalomyelitis virus (PHEV) (Sasseville, Boutin, Gelinas, & Dea, 2002) in the *Betacoronavirus* genus; porcine deltacoronavirus (PDCoV) (Woo et al., 2012) in the *Deltacoronavirus* genus. PEAV as the newest member was first detected by our team by genomic analysis of samples collected from a diarrhoea-outbreak swine herds routinely vaccinated with PEDV vaccine in a farm in Guangdong, China in February 2017, and complete genome of the PEAV strain GDS04 was then sequenced (Gong et al., 2017).

Porcine enteric alphacoronavirus is an enveloped, single-stranded, positive-sense RNA virus with a genome of appropriately 27 kb in length (Gong et al., 2017). The genome organization of PEAV is similar to that of bat-like HKU2 strains of coronavirus, with an order of: 5′ untranslated region (UTR), open reading frame 1a/1b (ORF1a/1b), spike (S), nonstructural protein 3 (NS3), envelope (E), membrane (M), nucleocapsid (N), nonstructural protein 7a (NS7a), and 3′ UTR (Lau et al., 2007). The S protein of CoVs is the pivotal surface glycoprotein involved in virus attachment and entry, and induction of neutralizing

antibodies in vivo (Cruz, Kim, & Shin, 2008; Woo, Huang, Lau, & Yuen, 2010). GDS04 strain of PEAV has the smallest S protein among all coronaviruses (Gong et al., 2017).

The clinical symptoms in newborn piglets from pig farm with reported PEAV are similar to that by other porcine enteric pathogens such as PEDV and TGEV, which include vomiting, diarrhoea, dehydration, and mortality rate as high as 90% in piglets (Gong et al., 2017; Pan et al., 2017; Zhou et al., 2018). Since the new bat-HKU2-like coronavirus (PEAV) was detected in pigs with severe diarrhoea (Gong et al., 2017), another two swine enteric HKU2-related CoV (SADS-CoV and SeACoV) strains were identified in the same region, which reproduced clinical diarrhoeal disease by experimentally infecting piglets with isolated SADS-CoV and SeACoV strains (Pan et al., 2017; Zhou et al., 2018).

Although PEAV GDS04 was detected by genomic analysis in pigs (Gong et al., 2017), detailed information remains unclear. In this study, we isolated a PEAV strain from Guangdong province of China using Vero cells, characterized its genome based on *s* genes, *n* genes, and whole-genome, and investigated its pathogenicity in 5-day-old conventional pigs by clinical assessment, virus shedding, virus distribution, histological test, immunohistochemical study, and the mortality rate of inoculated piglets. The results suggest that the isolate of PEAV GDS04 is closely related to SADS-CoV and SeACoV but caused 100% mortality in neonatal piglets, indicating its potential role as pathological agent responsible for severe watery diarrhoea and death in neonatal piglets in the field case.

2 | MATERIALS AND METHODS

2.1 | PEAV-positive specimens

In early February 2017, an outbreak of PEAV was reported in swine herds in Guangdong, China (Gong et al., 2017), with a mortality rate ranging up to 90% (Zhou et al., 2018). To increase the virus titres for isolation, fresh excreta from infected PEDV-vaccinated newborn piglets were inoculated into 5-day-old nonvaccinated piglets as described previously (Gong et al., 2017). Intestinal contents were collected from an inoculated piglet developing symptoms of severe diarrhoea and vomiting, and stored at −80°C until further use. Prior to virus isolation, intestinal contents were diluted one time using sterile 1× phosphate buffer saline (PBS) (pH 7.4). The supernatants were then collected by centrifugation at 6000× *g* for 5 min at 4°C, and filtered through 0.22-μm-pore-size filters (Millipore, USA).

2.2 | Virus isolation, plaque purification, and propagation in Vero cells

Vero cells were obtained from ATCC (ATCC number: CCL-81) (USA) and were used to isolate PEAV from the intestinal contents of piglet. Vero cells were cultured in DMEM (Hyclone, USA) supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), and 10% foetal bovine serum (FBS) (BOVOGEN, Australia). The maintenance medium for PEAV propagation was DMEM supplemented with 10 μg/ml trypsin (Gibco, USA).

Virus isolation, plaque purification, and propagation were performed as previously described with some modifications (Lee, Kim, & Lee, 2015; Oka et al., 2014; Pan et al., 2017). Briefly, for the first inoculation, Vero cells were cultured in 6-well plates, and washed three times with sterile 1× PBS (pH 7.4) at a confluency of 90%. Two hundred microlitres of the filtered inoculums, together with 300 μl maintenance medium was added to each well. After adsorption for 1.5 hr at 37°C in 5% CO_2, cells were washed three times with the maintenance medium, and then 2 ml maintenance medium was added. The cells were cultured continuously at 37°C in 5% CO_2 for cytopathic effect (CPE) observation. The plates were frozen at −80°C and thawed twice around 4 day postinoculation (d.p.i). The cells and supernatant termed as "passage 1 (P1)" were harvested together. Samples collected at 0-hr postinoculation and 4 d.p.i. were tested by PEAV-specific RT-PCR as described previously (Gong et al., 2017). The RT-PCR positive samples were used as seed stocks for the next passage and plaque purification. For virus plaque purification, supernatants from virus-infected cells were serially diluted and used to infect Vero cells in the maintenance medium for 1.5 hr at 37°C in 5% CO_2 and then the maintenance medium was discarded, followed by overlaying 2 ml maintenance medium containing 1.25% Agarose LM GQT (TaKaRa, Dalian) to immobilize the virus. After 24 hr, cells were fixed and visualized with 2 ml maintenance medium containing 1.25% Agarose LM GQT and 0.01% Neutral red solution (Sigma, USA). The plaques were picked by using sterile pipette tips, and the agarose plaque was placed into a microcentrifuge tube containing 0.5 ml maintenance medium. The selected plaques of PEAV were named GDS04 and used for viral propagation. Vero cells were cultured in T175 flasks, and washed three times with sterile 1× PBS (pH 7.4) at a confluency of 90%. One mL of PEAV together with 50 ml maintenance medium was added into the flask. The cell pellets and supernatant were cultured continuously at 37°C in 5% CO_2 to observe CPE. When CPE was evident in the inoculated cell monolayers (around 1 d.p.i.), the plates were frozen at −80°C and thawed twice. The cells and supernatant were harvested together to determine viral titres.

2.3 | Infectious-virus titrations by a $TCID_{50}$ assay

Vero cells were seeded on 96-well plates and cultured overnight before washed two times with sterile 1× PBS (pH 7.4). One hundred microlitre of 10-fold dilutions of PEAV was inoculated in eight replicates per dilution, then the cells were cultured continuously at 37°C in 5% CO_2. Viral CPE was observed for 5–7 days, and virus titre was calculated using the Reed-Muench method (Reed, 1938) and expressed as $TCID_{50}$ per millilitre.

2.4 | Immunofluorescence assay (IFA)

Immunofluorescence assay was conducted to observe PEAV-infected Vero cells as described previously with some modifications (Dong et al., 2016). Briefly, Vero cells (1×10^5) were seeded on 24-well plates

and cultured overnight, then infected with PEAV at a multiplicity of infection (MOI) of 1. At 24 hr after inoculation, the cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized with 0.2% Triton X-100 for 15 min at room temperature. The cells were then blocked with 1% bovine serum albumin (BSA), and incubated with PEAV-specific mouse antisera (Guangdong Wen' s Foodstuffs Group Co., Ltd, China) (1:250), followed by fluoresceinisothiocyanate (FITC)-labelled goat anti-mouse secondary antibody (KPL, USA) (1:1,000) for 1 hr. Then, the stained cells were observed with a fluorescence microscope (LEICA DMi8, Germany).

2.5 | Electron microscopic observation

Electron microscopy (EM) was conducted to observe virus samples as described previously with some modifications (Alsaad et al., 2018; Hu, Jung et al., 2015; Kong et al., 2010). For visualization of the viral particles in infected-cell culture medium, PEAV-infected Vero cells were frozen at −80°C and thawed twice, and the cell culture was centrifugated at 7,000× *g* for 30 min at 4°C. The supernatant was supplemented with 6% PEG6000 for 12 hr at 4°C. The mixture was centrifuged at 12,000× *g* for 1 hr at 4°C, and the pellet was resuspended in sterile 1× PBS (pH 7.4) buffer, followed by equilibrium in 8 mL nonlinear 20%–60% sucrose-TNE gradients by centrifugation at 110,000× *g* for 2 hr at 4°C with an ultracentrifuge (Hitachi Koki himac CP 100WX, Japan). After purification by sucrose gradient centrifugation, purified virions were diluted with sterile 1× PBS (pH 7.4) buffer and the sucrose was removed by centrifugation at 7,000× *g* for 2 hr at 4°C with centrifugal filter units (Millipore, USA). The purified virus pellets were resuspended in sterile 1× PBS (pH 7.4) buffer and negatively stained with 3% phosphotungstic acid. After blotting and drying, the grids were examined with a JEM-100 CX-II electron microscope (JEOLLTD, Japan).

For visualization of the viral particles in jejunum of PEAV-inoculated piglets, portion of jejunum was fixed in 5% glutaraldehyde fixative prepared in sterile 1× PBS (pH 7.4) for 4 weeks and underwent postfixation in 1% osmium tetraoxide for 1 hr. The samples were washed twice by sterile 1× PBS (pH 7.4) and dehydrated using increasing concentrations of acetone. The samples were then embedded in Araldit 520 resin and polymerized in oven at 90°C for 24 hr. Tissue semithin sections (1 μm) were prepared, stained by Toludin blue stain for 5 min and inspected under conventional light microscope. Ultrathin sections (70 nm) were prepared using Leica EM UC7 ultramicrotome, collected on 200 mesh copper grid (PELCO) and stained with uranyl acetate for 15 min and lead citrate for 5 min. The ultrathin sections were screened by 120-kV JOEL1230 transmission electron (TEM) (Akishima, Japan) and images were obtained using side-mounted digital camera (Gatan 780AJ03FA, Pleasanton, CA, USA).

2.6 | Genomic cloning and phylogenetic analysis of the s genes, n genes, and whole-genome

Total RNA was prepared from the isolated virus using a RNeasy kit (Magen, China) and was treated with DNase I. The cDNA was synthesized by reverse transcription using RT-PCR kit (TaKaRa, Dalian). A total of 18 primer pairs based upon the PEAV GDS04 (GenBank accession no. MF167434.1; Supporting information Table S1) were designed to amplify the complete genome of PEAV GDS04 "passage 12 (P12)". The PCR products were cloned into the pMD19-T (TaKaRa, Dalian) and sequenced to determine the consensus sequence. The sequences were assembled and analysed using the DNASTAR program. Sequence alignment analysis was performed using the Clustal W program implemented in DNAStar software Lasergene 7.0. A phylogenetic tree was then constructed by the neighbour-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software version 5 (http://www.megasoftware.net/) based on the *s* genes, *n* genes, and whole-genome from PEAV GDS04 strain P12 together with other different CoVs (alpha, beta, gamma, and delta), like PEDV, PDCoV, and TGEV.

2.7 | Experimental infection with the PEAV GDS04 strain in newborn piglets

The animal study was approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University and performed in accordance with regulation and guidelines of this committee. Twenty-four 4-day-old conventional newborn piglets were randomly divided into two groups (8 piglets in group 1 and 16 piglets in group 2). Piglets were fed a mixture of skim milk powder (Inner Mongolia Yi Li Industrial Group Co., Ltd, China) with warm water. Prior to inoculation, piglets were confirmed negative for the major porcine enteric viruses (PDCoV, PEDV, TGEV, PRoV) by testing of rectal swabs using specific RT-PCR according to previously described method (Hu, Li et al., 2015; Jeong et al., 2009; Saeng-Chuto et al., 2017). After 1-day acclimation, piglets in group 1 were orally inoculated with 5 ml of maintenance medium and served as uninfected controls. Twelve piglets in group 2 were orally challenged with 5 ml of maintenance medium containing 5×10^5 $TCID_{50}$ of the PEAV GDS04 P12 and the remaining four piglets served as cohabitation control.

All piglets were observed daily for clinical signs of vomiting, diarrhoea, lethargy, and body condition. Diarrhoea severity was scored with the following criteria (Chen et al., 2015): 0 = normal, 1 = soft (cowpie), 2 = liquid with some solid content, 3 = watery with no solid content.

Rectal swabs were collected daily from each piglet from 1 d.p.i. to 14 d.p.i. and were submerged into 1 ml sterile 1× PBS (pH 7.4) immediately after collection. Two piglets from each group were necropsied at 7 d.p.i. At necropsy, the fresh samples (serum, heart, liver, spleen, lung, kidney, stomach, duodenum, jejunum, ileum, caecum, and colon) were collected and then formalin-fixed. The fresh samples were stored at −80°C for viral RNA distribution analysis and formalin-fixed samples were used for histopathology and immunohistochemistry analysis. In addition, the mortality of newborn piglets in each group was recorded daily.

2.8 | Real-time RT-PCR analysis

Rectal swabs, serum, and various tissues were tested by a PEAV *n* gene-based real-time RT-PCR including viral standards with known

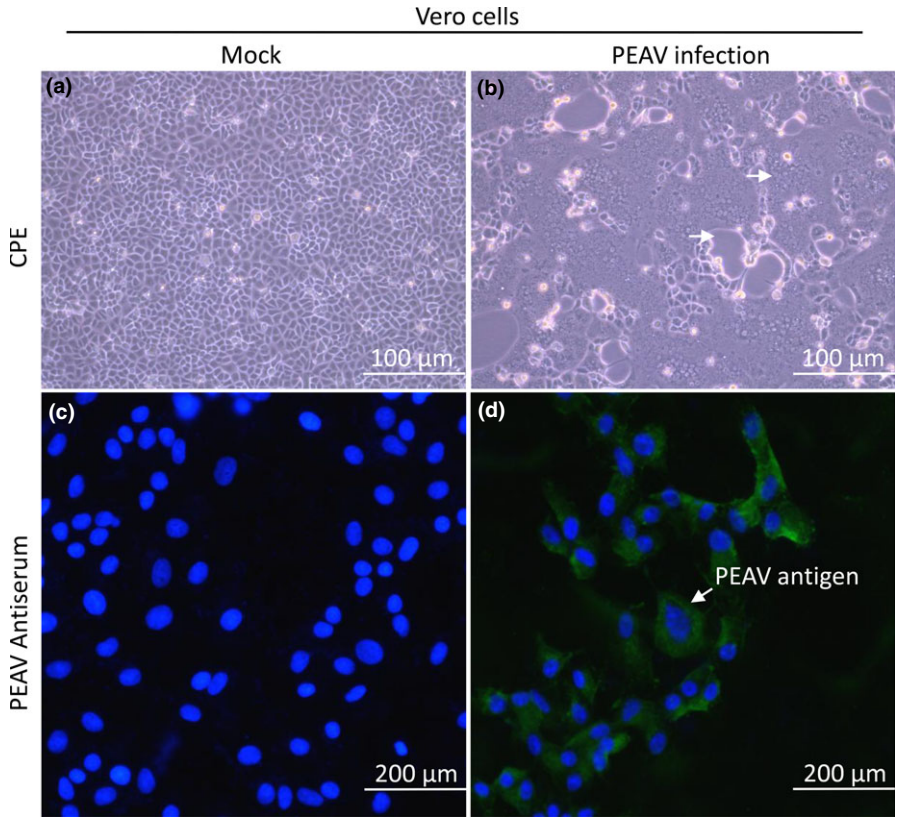

FIGURE 1 Cytopathic effects (CPE) and IFA staining on PEAV-inoculated Vero cells. (a) Mock-inoculated Vero cell culture showing normal cells. (b) PEAV-inoculated Vero cells at 1 d.p.i. showing syncytium and cells detachment (indicated by arrows). Vero cells were mock-inoculated (c) or inoculated with PEAV GDS04 (d). At 24 hr postinoculation, an immunofluorescence assay (IFA) was performed [Colour figure can be viewed at wileyonlinelibrary.com]

plasmid concentration for quantification. Briefly, the homogenates from serum, various tissues, and the supernatants of rectal swab from each piglet were centrifuged at 6,000× *g* for 5 min, respectively. Total RNA was prepared and used for cDNA synthesis as described above. Specific primers for the nucleocapsid (N) gene of PEAV (sense: 5′-GCACTTTTATTACCTTGGTA-3′; antisense: 5′-GTAGCAGGTTCTTTGTTAC-3′), and probe (5′-FAM-TCCTCACGCAGATGCTCCTTTAMRA-3′) were designed according to reference sequence (GenBank, Accession no: MF167434.1) and synthesized by TaKaRa (Dalian, China). The real-time PCR assay was carried out with an Applied Biosystem 7500 Fast instrument (Life Technologies, USA). The PCR was performed in a 20-μl volume containing 1 μl of cDNA, 10 μl of Thunderbird Probe qPCR Mix, 0.04 μl 50× Rox reference dye (TOYOBO, Shanghai), 0.2 μM of probe, and a 0.3 μM of each gene-specific primer. The thermal cycling parameters were as follows: 95°C for 20 s; 40 cycles of 95°C for 3 s, 60°C for 30 s. The standard curve was generated by construction of plasmids. Briefly, the *n* gene was amplified from PEAV GDS04 P12 strain using the specific primers as described above, and the PCR products were cloned into the pMD19-T (TaKaRa, Dalian). The known plasmid concentration was 10-fold serially diluted for generating a standard curve in each plate. The quantity of PEAV viral RNA in tested samples was calculated based on the cycle threshold (Ct) values for the standard curve.

2.9 | Histology and immunohistochemistry

At necropsy, tissue samples of heart, lung, spleen, liver, kidney, stomach, duodenum, jejunum, ileum, caecum, and colon of the piglets from the challenged and control groups were collected separately and routinely fixed in 10% formalin for 36 hr at room temperature (Hu, Jung, Vlasova, & Saif, 2016), and then dehydrated in graded ethanol, embedded in paraffin, cut in 5-μm sectioned, and mounted onto glass slides. Afterwards, the sections were deparaffinized, rehydrated, and stained with haematoxylin and eosin (H&E); the slides were examined and analysed with conventional light microscopy. Sections (5 μm) of formalin-fixed paraffin-embedded tissues were placed onto positively charged glass slides and the slides were air-dried for 120 min at 60°C. The tissue sections were deparaffinized, and then rinsed and incubated with target retrieval solution (Servicebio, China). After being blocked with 1% BSA (Solarbio, China), the sections were incubated with PEAV-specific mouse antisera (1:400) as the primary antibody for 12 hr at 4°C. They were then incubated with peroxidase-labelled goat anti-mouse IgG secondary antibody (Dako, Denmark) for 50 min at room temperature, and the samples were finally visualized with a 3, 3′-diaminobenzidine (DAB) chromogen kit (Dako, Denmark). Haematoxylin was used for counterstaining. Tissues of piglets from negative control groups were used as negative samples.

3 | RESULTS

3.1 | A strain of PEAV was isolated from an inoculated-newborn piglet with fresh excreta from ill newborn piglets

We attempted to isolate PEAV, a new bat-HKU2-like coronavirus from the positive samples since its first detection in China (Gong et

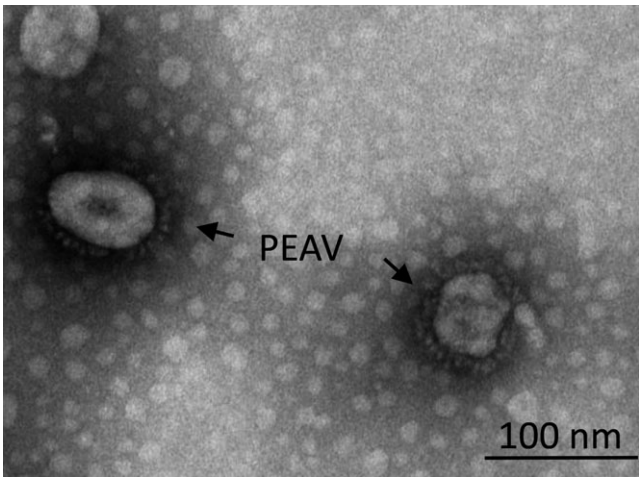

FIGURE 2 Electron micrographs of PEAV-inoculated Vero cells. Crown-shaped spikes of PEAV are visible (arrows). The sample was negatively stained with 3% phosphotungstic acid

al., 2017). As shown in Figure 1b, one inoculated cell monolayer with the supernatant from virus-infected cells of passage 10 (P10) showed visible CPE in the form of syncytium and detachment at 1 d.p.i. as compared with the control in Figure 1a. To confirm PEAV replication in Vero cells, viral RNA was extracted from the inoculated cells at 1 d.p.i. and tested by specific RT-PCR. This cell culture-passaged sample was positive for PEAV but negative for other porcine enteric coronaviruses (data not shown). PEAV from the first passage in Vero cells was named GDS04 (Gong et al., 2017). After blindly passaged in Vero cells for a total of 20 passages, the PEAV GDS04 could still be detected by specific RT-PCR (data not shown), indicating that the proliferation ability of the strain in Vero cells. Plaque-purified PEAV in Vero cells was further confirmed by IFA with PEAV-specific mouse antisera. As shown in Figure 1d, PEAV-specific immunofluorescence was detected in infected cells, as compared to the control (Figure 1c). To characterize the morphology and size of the virus particles, the PEAV GDS04 virus purified from infected Vero cells were examined with EM. Typical crown-shaped particles with spiky surface projections as in other coronaviruses were observed by negative staining on EM. The size of the viral particles was 80–160 nm in diameter (Figure 2). Taken together, these results suggest that a PEAV strain was successfully isolated from the intestinal contents of a newborn diarrheic piglet in China.

3.2 | Phylogenetic analysis of the s genes, n genes, and whole-genome of PEAV GDS04 P12

Since complete genome of the GDS04 was fully described (Gong et al., 2017), to further understand the origin of the virus, phylogenetic trees of complete *s* genes, *n* genes, and whole-genome of PEAV GDS04 strains together with other CoVs were constructed. Phylogenetic analysis of the *s* genes shows that the PEAV GDS04 strains along with four bat coronavirus HKU strains from Hong Kong, a BtRf-AlphaCoV strain from Beijing clustered in one group. Furthermore, the PEAV GDS04 strains, SADS-CoV, and SeACoV clustered into a subclade between bat coronavirus HKU strains and BtRf-AlphaCoV strain (Figure 3a). Consistent with the results of the *s* genes, phylogenetic analysis of the *n* genes and whole-genome of the PEAV GDS04 strains and other CoVs reveals that PEAV GDS04 strains, SADS-CoV, and SeACoV belong to the same subclade (Figure 3b&c). Therefore, these data suggest that the GDS04 strains were closely relative to SADS-CoV and SeACoV strains from Guangdong, China.

3.3 | Clinical manifestations of newborn piglets challenged with PEAV GDS04 P12

In order to determine whether PEAV GDS04 was the causative agent for diarrhoea, we experimentally infected newborn piglets with the isolated virus. Twelve newborn piglets inoculated with GDS04 P12 at a dose of 5×10^5 $TCID_{50}$/head via oral feeding showed mild diarrhoea from 1 to 4 d.p.i., and all developed severe watery diarrhoea, together with vomiting, and dehydration from 5 to 12 d.p.i., as compared with controls (Figure 4a–e), indicating a role of PEAV as an important causative agent for severe watery diarrhoea in newborn piglets. Furthermore, four piglets from group 2 as cohabitation controls also developed watery diarrhoea from 5 to 12 d.p.i. (Figure 4e). Since PEAV GDS04 caused severe watery diarrhoea in newborn piglets, we also recorded the mortality of newborn piglets in each group. As shown in Figure 5, in PEAV-inoculated groups, except that two piglets were necropsied at 7 d.p.i., all remaining 10 piglets died from 5 to 12 d.p.i. And the PEAV-inoculated piglets had 1, 1, 3 (+2 euthanized), 1, 1, 2, and 1 death (s) at 5, 6, 7, 8, 9, 10 and 12 d.p.i., respectively. In addition, 2/4 piglets from group 2 as cohabitation controls also died at 7 and 9 d.p.i. No piglets (6/6) died in control group except that two piglets were necropsied at 7 d.p.i. Taken together, these results suggest that PEAV GDS04 is highly pathogenic to the newborn piglets.

3.4 | Faecal shedding and virus distribution in newborn piglets challenged with PEAV GDS04 P12

Since PEAV GDS04 caused watery diarrhoea in newborn piglets, we explored the faecal viral shedding in PEAV-challenged piglets. As shown in Figure 6a, the PEAV RNA was detected by qRT-PCR in faecal swabs collected from orally inoculated piglets from 1 to 11 d.p.i., and peaked on 4 d.p.i. and kept up until 11 d.p.i. We also examined the faecal viral shedding in piglets from group 2 as cohabitation controls. PEAV RNA was detected in rectal samples collected from these piglets from 3 to 13 d.p.i. (Figure 6a), indicating that these piglets may be infected by the PEAV-challenged piglets. No PEAV RNA was detected in the negative control piglets during the study. To examine the distribution of the PEAV virus in different tissues in PEAV-challenged piglets, two piglets from each group were necropsied at 7 d.p.i. As shown in Figure 6b, the PEAV RNA was detected in all collected samples of duodenums, jejunums, ileums, caecum, and colons. The virus was also detected in 2/2 hearts, 2/2

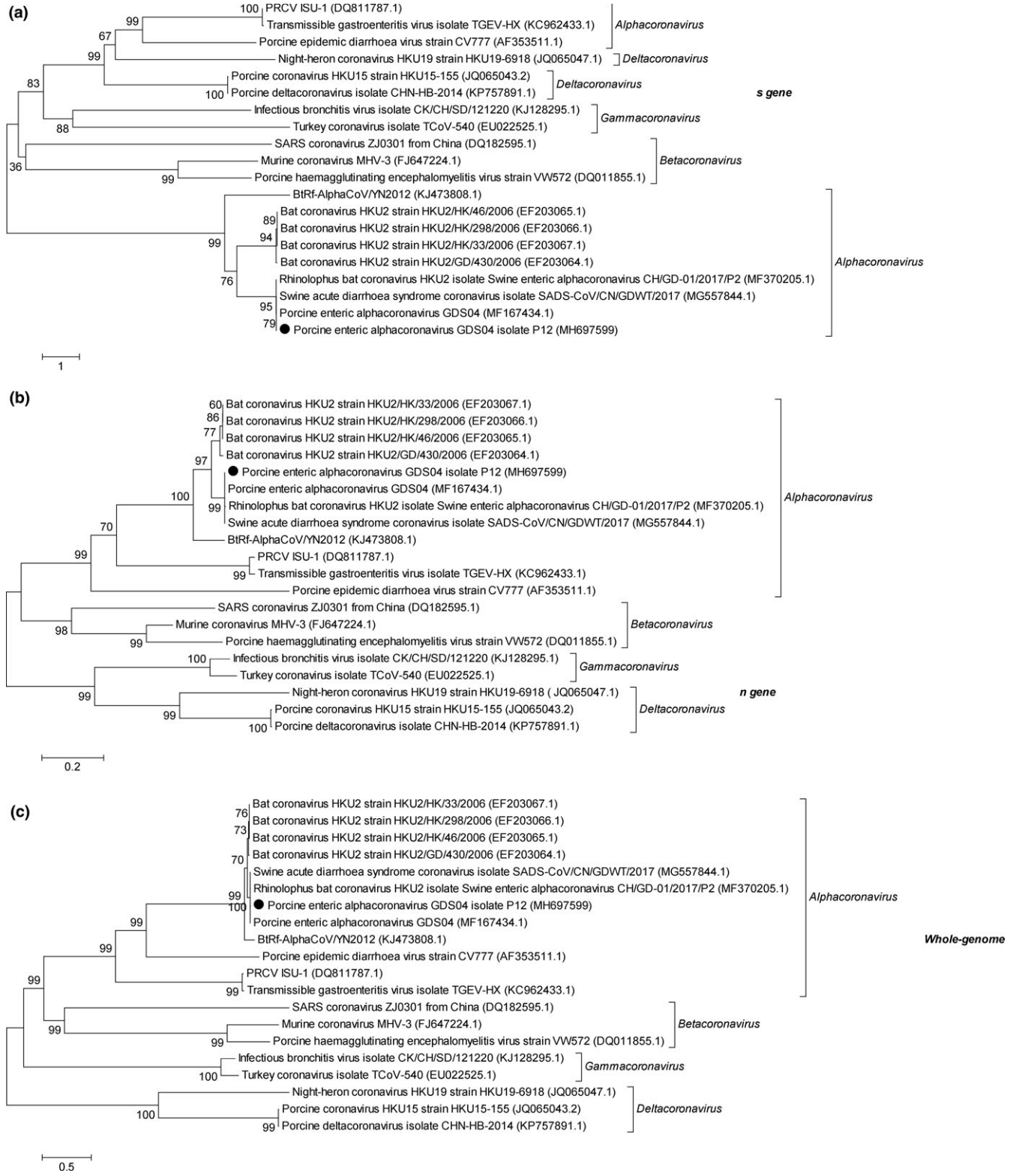

FIGURE 3 Phylogenetic trees constructed on the basis of the *s* genes, *n* genes, and whole-genome nucleotide sequences of PEAV GDS04 or other coronaviruses (CoVs). (a) Phylogenetic tree of the *s* gene. (b) Phylogenetic tree of the *n* gene. (c) Phylogenetic tree of the whole-genome. The dendrogram was constructed using the neighbour-joining method in the MEGA software package, version 5 (http://www.megasoftware.net). Bootstrap resampling (1,000 replication) was performed, and bootstrap values are indicated for each node. Reference sequence obtained from GenBank is indicated by strain name. The scale bar represents 1, 0.2, or 0.5 nucleotide substitutions per site

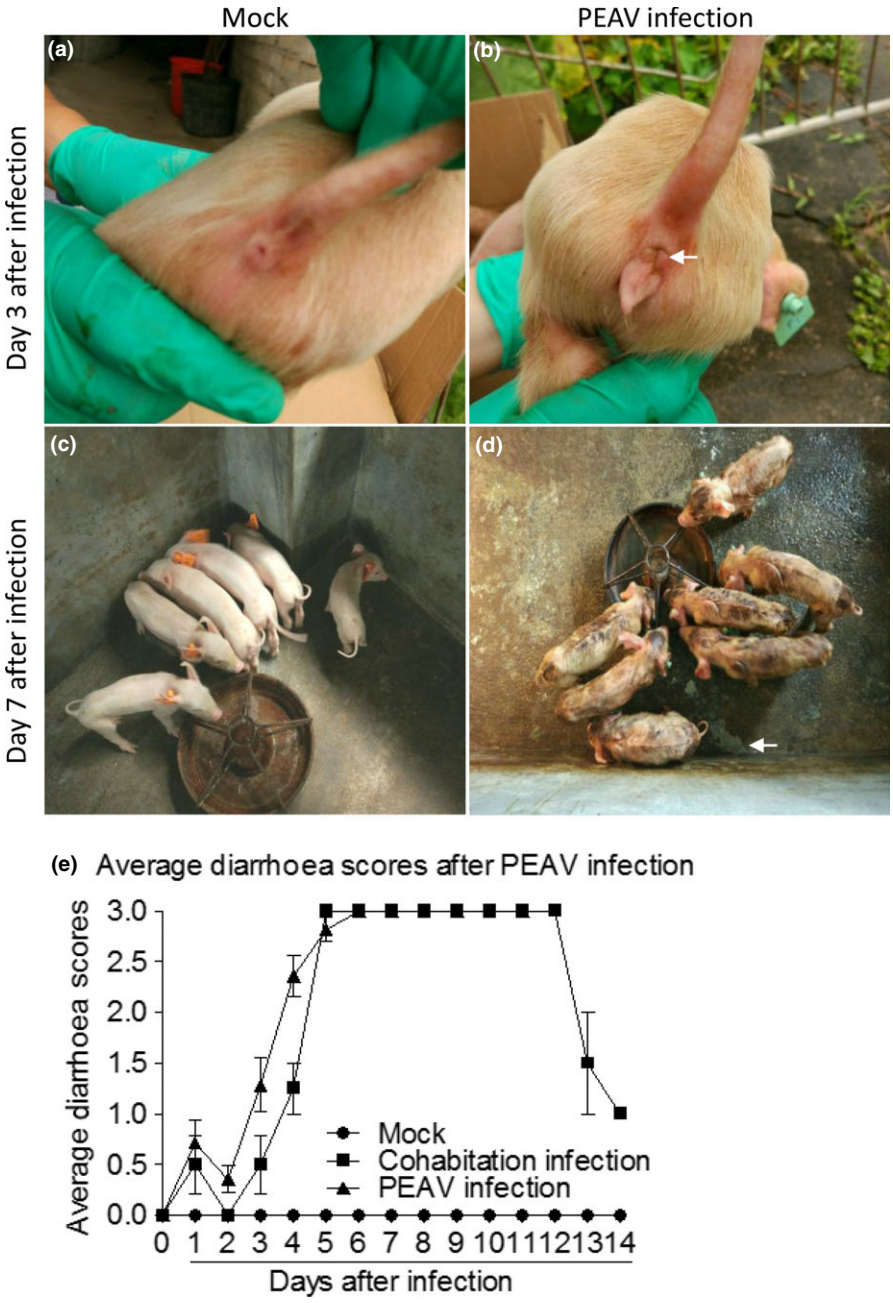

FIGURE 4 Reproduction of watery diarrhoea and faecal viral shedding in newborn piglets inoculated with PEAV GDS04 strain P12 via oral feeding. (a & c) Newborn piglets uninfected as control. (b & d) Watery diarrhoea (indicated by arrows) was observed at 3 and 7 d.p.i. with PEAV infection. (e) Average diarrhoea scores after PEAV infection [Colour figure can be viewed at wileyonlinelibrary.com]

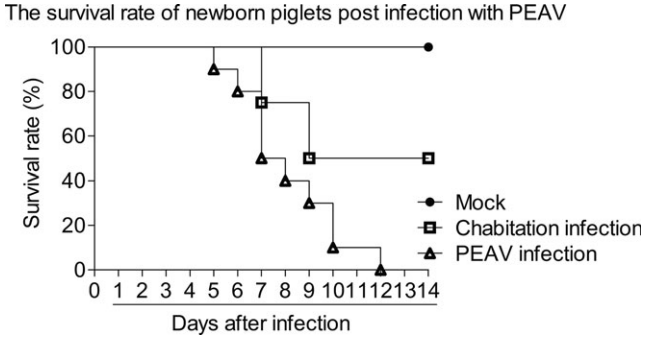

FIGURE 5 The survival rate of newborn piglets postinfection with PEAV GDS04 P12. The mortality of newborn piglets in each group was recorded from 1 to 14 d.p.i

livers, 2/2 spleens, 2/2 kidneys, 2/2 stomachs, and 1/2 lungs, but no viral RNA was detected in blood. No PEAV RNA was detected in the tissue samples from the control piglets. Taken together, these results demonstrate that PEAV GDS04 strain could be widely distributed in different tissues, but mainly concentrated in the intestines of pig.

3.5 | Virus particles in jejunum of inoculated-PEAV piglets

To observe virus particles in vivo, the jejunum of piglets from each group was examined with EM. EM demonstrated that the virion without membrane in the vesicle (Figure 7b) and full virus particles in the intercellular space (Figure 7c) with typical crown-shape of

80–160 nm in diameter and spiky surface projections of CoV, indicating that PEAV GDS04 strain could replicate in the jejunum of pig. In addition, the virus-infected cells showed atrophied, ruptured cell morphology (Figure 7b&c). No virus particles or pathological lesions were detected in the jejunum from the control piglets (Figure 7a). Taken together, these results show that PEAV GDS04 could replicate and cause jejunum lesions in newborn piglets.

3.6 | Gross pathology, histopathology, and immunohistochemistry in newborn piglets infected with PEAV GDS04

To determine the gross pathological and histological changes in piglets infected with the PEAV GDS04 strain, two piglets from each group were necropsied at 7 d.p.i. Gross findings were similar in both piglets orally inoculated with PEAV GDS04. The whole intestinal tract, where yellow watery contents accumulated, were transparent, thin-walled, and gas-distended (Figure 8b). No lesions were observed in any other organs of the PEAV-challenged piglets (data not shown) or the organs in the negative control piglets (Figure 8a), indicating that intestinal tract is the target organ of PEAV infection. Microscopic lesions were also analysed. As shown in Figure 8h–l, abruption of intestinal villus was observed, whereas the intestinal in negative control was normal (Figure 8c–g). Consistent with the histopathological results, PEAV antigen was detected in the cytoplasm of the villous enterocytes of the PEAV-challenged piglets by immunohistochemical analysis (Figure 8o&p). Taken together, these results indicate that PEAV GDS04 could cause intestinal lesions in newborn piglets.

4 | DISCUSSION

The widely distributed CoVs could be isolated from a variety of animal hosts and products with animal origin (Felippe et al., 2010; Pan et al., 2012; Rihtaric et al., 2010; Stohlman et al., 1982; Tsunemitsu et al., 1995), as well as from human (Larson, Reed, & Tyrrell, 1980). Bats are thought to be the natural reservoir of a range of CoVs (Cui et al., 2007). The pathogenicity of five porcine CoVs has so far been confirmed (Doyle & Hutchings, 1946; Pensaert & de Bouck, 1978; Sasseville et al., 2002; Wesley et al., 1990; Woo et al., 2012). In February 2017, a new bat-HKU-like porcine coronavirus (PEAV) was detected by genomic analysis in swine herds (Gong et al., 2017). Although PEAV has been detected in piglets with severe diarrhoea (Gong et al., 2017), little information is known regarding the pathogenicity of PEAV strains in animals. In the present study, we reported that a PEAV strain was successfully isolated from a case of piglet diarrhoea in Guangdong, and showed high pathogenicity to

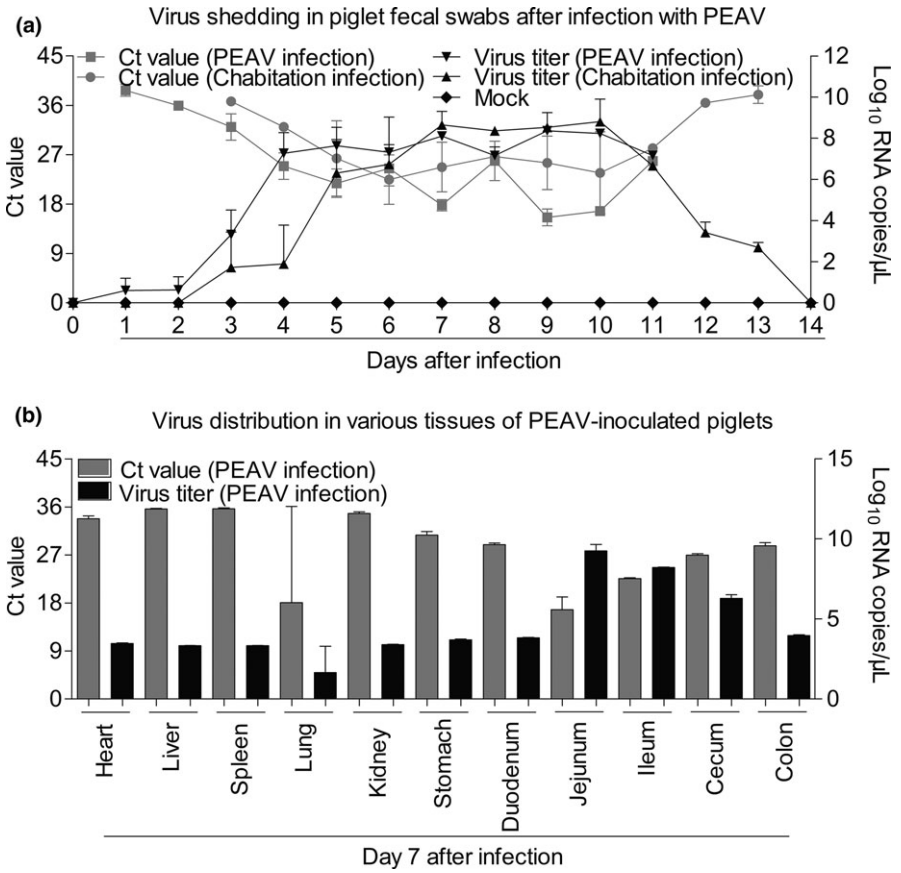

FIGURE 6 Virus shedding in rectal swabs and various tissues of PEAV-inoculated piglets. (a) Ct values of group PEAV inoculation or as cohabitation contrast newborn piglet faecal swabs and viral RNA shedding in faecal swabs after PEAV inoculation or mock inoculation. (b) Virus distribution at 7 d.p.i. in newborn piglets challenged with PEAV

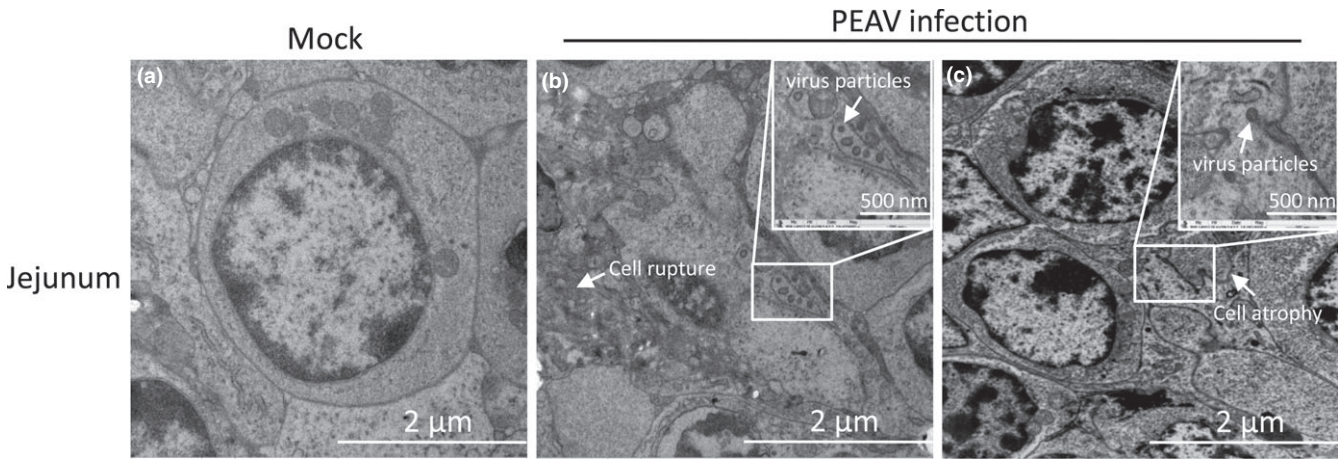

FIGURE 7 Electron micrographs of PEAV on jejunum of PEAV-inoculated newborn piglet. (a) Electron micrographs of jejunum of a control newborn piglet at 7 d.p.i. (b & c) Electron micrographs of PEAV (indicated by arrows) on jejunum of a PEAV-challenged newborn piglets at 7 d.p.i

newborn piglets. This PEAV strain can be further used for virological and serological assay development, as well as vaccine development.

A stable African green monkey kidney cell line (Vero cells) (Rhim, Schell, Creasy, & Case, 1969) is commonly used to isolate CoVs like PEDV or bat coronavirus HKU2 (Lau et al., 2007; Lee et al., 2015). Since PEAV was first detected by genomic analysis in pigs (Gong et al., 2017), we attempted to isolate virus from PEAV-positive samples using Vero cells. The virus could only be isolated in circumstance of inoculation with fresh homogenate in piglets. The difficulty to isolate PEAV from positive samples might be associated with the fact that positive samples characterized by RT-PCR may contain noninfectious or low amount of virus. During virus isolation, the DMEM supplemented with 10 μg/ml trypsin performed better than 7 μg/ml in PEAV propagation with more evident CPE, which indicates that the amount of trypsin in Vero cells might also contribute to the successful PEAV isolation. CPE was firstly observed in inoculated Vero cells until the passage 10. After plaque purification and several passages, the viral titre reached 5.13×10^5 $TCID_{50}$/ml, showing that the PEAV GDS04 strain was highly replicative in Vero cells. The plaque-purified PEAV strain in Vero cells was further verified by IFA with PEAV-specific mouse antisera. The characteristic crown-like particles of the purified PEAV GDS04 strain was observed by EM. Although there were many PEAV-positive samples by genomic analysis, only GDS04 was isolated, indicating that the success rate of isolation PEAV strains was very low. Thus, further attempts are needed to improve PEAV isolation.

To determine the complete genome of P12 of GDS04 strain, we amplified and sequenced the complete genome of PEAV GDS04 P12 by RT-PCR. We found that PEAV GDS04 P12 strain (accession no. MH697599) shares 99.79% nucleotide identity with PEAV GDS04 strain (accession no. MF167434), and compared to the complete genome of the virus in the original small intestinal homogenate, the PEAV GDS04 P12 strain possesses 35 point mutations, a 1-nt deletion (A) in nt 77, a 3-nt deletion (GTA) in nt 24,790–24,792, a 1-nt deletion (A) in nt 27,071, a 1-nt deletion (T) in nt 27,081, a 1-nt deletion (A) in nt 27,089, a 3-nt insertion (TTG) in nt 4,554–4,556, 10-nt insertion (GACTAGAGCC) in nt 12483-12492, indicating that these mutations might be related to cellular adaptation. Based on the phylogenetic tree analysis of *s* genes, the PEAV strain GDS04 shares 36.23%–99.66% nucleotide identity with other 18 CoVs in GenBank. Notably, the PEAV GDS04 strains can be clustered into one clade with four bat coronavirus HKU strains from Hong Kong, a BtRf-AlphaCoV strain from Beijing, SADS-CoV strain from Guangdong, and SeACoV strain from Guangdong, indicating a close relationship of these strains. In addition, the PEAV GDS04 strains, SADS-CoV, and SeACoV clustered into a subclade, indicating that these three viruses might have similar origins. Consistent with the results of the *s* genes, phylogenetic analysis of the *n* genes and whole-genome of the PEAV GDS04 and other CoVs revealed that PEAV GDS04, SADS-CoV, and SeACoV belong to the same subclade. Previous studies have shown that the *s* gene, the most variable region in the CoV genomes, belongs to type I membrane glycoproteins family (Woo et al., 2010) and is involved in receptor binding and viral entry (Woo et al., 2010). Sequencing of *s* genes revealed that GDS04 had the smallest S protein among all coronaviruses (Gong et al., 2017). Compared to the *s* gene of the SeACoV and SADS-CoV strains, the PEAV GDS04 possesses 11 point mutations, 3 point mutations with SeACoV and SADS-CoV, respectively. Whether these unique variations contribute to the efficiency of viral replication and virulence needs to be further investigated.

We further investigated whether the PEAV GDS04 strain was responsible for causing clinical symptoms as severe diarrhoea and death in piglets. As a result, we infected 5-day-old newborn piglets with the PEAV GDS04 strain P12 via oral feeding. The sequential severe diarrhoea and vomiting in piglets by oral infection strongly suggest the pathogenicity of PEAV to the newborn piglets. Furthermore, PEAV RNA was detected from 1 to 11 d.p.i. in faecal of GDS04 challenged piglets, while no RNA was detected in the negative control piglets. Four piglets from group 2 as cohabitation contrast also developed profuse watery diarrhoea and viral shedding, suggesting the possibility of faecal–oral transmission of PEAV. Results from this study may help guide future PEAV experimental designs as the clinical diarrhoea and virus shedding patterns.

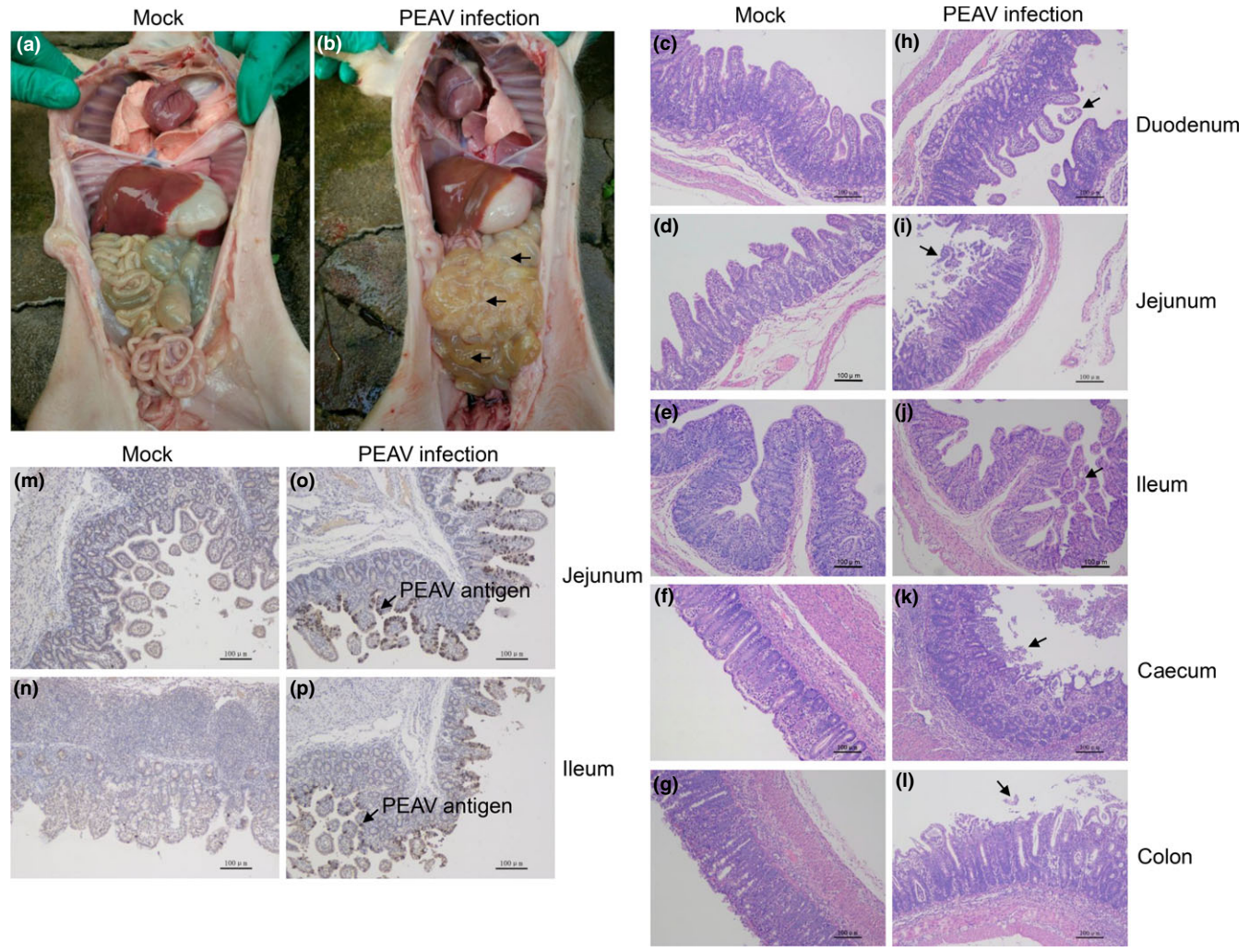

FIGURE 8 Intestinal changes in newborn piglets inoculated with PEAV strain GDS04 P12. (a) Macroscopic picture of a control piglet at 7 d.p.i. (b) Thin-walled intestinal tract (indicated by arrows) of a PEAV-challenged newborn piglets at 7 d.p.i. (c-g) Haematoxylin and eosin (H & E)-stained intestinal tissue section of a control piglet at 7 d.p.i. (h-l) H&E-stained intestinal tissue section of a PEAV-challenged piglet at 7 d.p.i. (Blunt intestinal villus was indicated by arrows). (m&n) Immunohistochemically stained jejunum or ileum tissue section of a control piglet at 7 d.p.i. (o&p) Immunohistochemically stained jejunum or ileum tissue section of a PEAV-challenged piglet at 7 d.p.i [Colour figure can be viewed at wileyonlinelibrary.com]

In addition, the viral RNA distribution in the PEAV-challenged piglets was also tested. The intestines contained higher levels of viral RNA copies compared with the other tissues, and no PEAV viral RNA was detected in the blood, indicating that PEAV might infect multiple organs in pigs, but the intestinal tract is the major target organ of PEAV. Furthermore, we confirmed that PEAV could replicate in pig intestines by EM. These results suggested that PEAV has similar infection process with other porcine CoVs like PEDV or PDCoV. Gross lesions by virus infection were obviously observed in the small intestines, caeca and colons of the 5-day-old piglets at necropsy at 7 d.p.i., similar to observations in PEDV or PDCoV infection (Chen et al., 2015; Lee et al., 2015). While microscopic lesions were observed in the jejunum and ileum infected by PDCoV in previous report (Chen et al., 2015), we found microscopic lesions distributed in the whole intestinal tract in GDS04 infected piglets, suggesting a more deteriorative effect by PEAV than that caused by PDCoV. What is more, no microscopic lesions were observed in any other organs of the PEAV-challenged piglets (data not shown), similar to observations in PDCoV infection (Chen et al., 2015). Consistent with the histopathological results, the PEAV antigen was detected in the cytoplasm of the villous enterocytes of challenged piglets by immunohistochemical analysis. This information is useful for choosing appropriate tissues for PEAV diagnostic investigations.

Since PEAV GDS04 caused severe watery diarrhoea in newborn piglets, we also recorded the mortality of newborn piglets from 1 to 14 d.p.i.. The pathogenicity of a SeACoV strain CH/GD/01/2017/P2 and SADS-CoV in neonatal piglets were described (Pan et al., 2017; Zhou et al., 2018). Results showed that SeACoV and SADS-CoV caused severe diarrhoea and vomiting in pigs of 3 days old. However, SeACoV infection did not cause fatality in piglets in 5 days (Pan et al., 2017), while 50% died after SADS-CoV infection within the same period (Zhou et al., 2018). When infected with PEAV

GDS04, one piglet died in dpi 5, and all PEAV-inoculated piglets and half piglets from cohabitation contrast died in 2 weeks, indicating that PEAV GDS04 strain and SADS-CoV were more pathogenic than SeACoV CH/GD/01/2017/P2. In addition, compared to the complete genome of the SeACoV CH/GD/01/2017/P2, the PEAV GDS04 P12 possesses 82 point mutations, a 1-nt deletion (A) in nt 177, 10-nt insertion (GACTAGAGCC) in nt 12483-nt 12492, a 1-nt insertion (A) in nt 27068, a 1-nt insertion (T) in nt 27078, and a 1-nt insertion (A) in nt 27086, presenting a clue of different pathogenicity of the two strains. And compared to the complete genome of the SADS-CoV, the PEAV GDS04 P12 possesses 41 point mutations, a 1-nt deletion (A) in nt 176, a 1-nt insertion (A) in nt 27067, a 1-nt insertion (T) in nt 27077, and a 1-nt insertion (A) in nt 27085. In addition, the PEAV strain GDS04 P12 shares 99.58%, 99.66% nucleotide identity with SeACoV and SADS-CoV, respectively, indicating that PEAV has more similarities with SADS-CoV than SeACoV. The details of genome comparison might also help explain the high mortality rate as high as 90% in piglets in PEAV-reported swine herds (Zhou et al., 2018). Together, all these results confirm that the PEAV GDS04 strain isolated in this study could cause enteric diseases and death in newborn piglets. However, there are still several important questions needed to be addressed. For instance, what is the prevalence of PEAV as a new CoV in herds? What are the molecular mechanisms of pathogenesis of PEAV infection? How to prepare effective vaccines against the PEAV? Elucidation of these questions will elevate our understandings of the pathogenicity of PEAV infection and help to develop better strategies to control PEAV.

In summary, we isolated a field strain of PEAV from the intestinal content of an inoculated-newborn piglet with fresh excreta from ill newborn piglets. Genomic analysis shows that the isolate manifests close relationship with SADS-CoV and SeACoV apart from several unique genetic characteristics. Remarkably, inoculation of newborn piglets with PEAV GDS04 P12 by oral feeding reproduced clinical symptoms, including vomiting, dehydration, and severe diarrhoea with a mortality of 100% in neonatal piglets. Collectively, these findings suggest that PEAV GDS04 is highly virulent in piglets.

5 | IMPORTANCE

In this work, a PEAV strain GDS04 was successfully isolated from a case of piglet diarrhoea in Guangdong, China. Newborn piglets orally challenged with PEAV GDS04 developed typical clinical symptoms as watery diarrhoea and high mortality, confirming PEAV is potential role of important pathogen in newborn piglets. The isolation and characterization would lay solid foundation for understanding this virus and the strain GDS04 can be further used for virological and serological assays, as well as vaccine development.

AUTHOR CONTRIBUTIONS

YC and ZX conceived and designed the experiments; ZX, YL, LH, and QZ performed the experiments; ZX analysed the data; YC, YZ, LG, LH, YL, JQ, YD, and QZ contributed reagents/materials/analysis tools; ZX and YZ wrote the paper; YC and CX revised the paper.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

The animal study was supervised by the Institutional Animal Care and Use Committee of Sun Yat-sen University (IACUC DD-17-1003) and used in accordance with regulation and guidelines of this committee.

INFORMED CONSENT

Informed consent was obtained from all individual participants included in the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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