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DR. YONGCHANG CAO (Orcid ID : 0000-0002-0311-5474)

MR. ZHICHAO XU (Orcid ID : 0000-0002-5697-5734)

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Isolation and characterization of A Highly Pathogenic Strain of *Porcine enteric alphacoronavirus* Causing Watery Diarrhea and High Mortality in Newborn Piglets

Zhichao Xu[1], Yun Zhang[1], Lang Gong[1], Licheng Huang[1], Ying Lin[1], Jianru Qin[1], Yunping Du[2], Qingfeng Zhou[2], Chunyi Xue[1], and Yongchang Cao[1*]

[1]State Key Laboratory of Biocontrol, School of Life Science, Sun Yat-sen University, Guangzhou, 510006, China, [2]Guangdong Wen' s Group Academy, Guangdong Wen' s Foodstuffs Group Co., Ltd, Xinxing, Guangdong, 527400, China

[*]School of Life Science, Sun Yat-sen University, Higher Education Mega Center, Guangzhou, 510006, China

Tel: 86-(20)-3933-2938. Fax: 86-(20)-3933-2841. Email: caoych@mail.sysu.edu.cn

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Running title: PEAV strain GDS04 is highly pathogenic to newborn piglets

Key words：*Porcine enteric alphacoronavirus* (PEAV), pathogenicity, newborn piglets

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 diarrhea in neonatal piglets. Viral RNA was detected in feces 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.

Importance

In this work, a PEAV strain GDS04 was successfully isolated from a case of piglet diarrhea in Guangdong, China. Newborn piglets orally challenged with PEAV GDS04 developed typical

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clinical symptoms as watery diarrhea 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 virologic and serological assays, as well as vaccine development.

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 (Stohlman SA, 1982, Tsunemitsu H, 1995, Pan, 2012, Rihtaric et al., 2010, Felippe et al., 2010). Porcine CoVs are significant enteric and respiratory pathogens of swine. Six porcine CoVs have so far been identified: transmissible gastroenteritis virus (TGEV) (Doyle, 1946), porcine respiratory coronavirus (PRCV) (Wesley RD, 1990), porcine epidemic diarrhea virus (PEDV) (Pensaert MB, 1978), and porcine enteric alphacoronavirus (PEAV) (Gong L, 2017) in the *Alphacoronavirus* genus; porcine hemagglutinating encephalomyelitis virus (PHEV) (Sasseville AM, 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 diarrhea-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 L, 2017).

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PEAV is an enveloped, single-stranded, positive-sense RNA virus with a genome of appropriately 27 kb in length (Gong L, 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 et al., 2008, Woo et al., 2010). GDS04 strain of PEAV has the smallest S protein among all coronaviruses (Gong L, 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, diarrhea, dehydration, and mortality rate as high as 90% in piglets (Gong L, 2017, Pan et al., 2017, Zhou et al., 2018). Since the new bat-HKU2-like coronavirus (PEAV) was detected in pigs with severe diarrhea (Gong L, 2017), another two swine enteric HKU2-related CoV (SADS-CoV and SeACoV) strains were identified in the same region, which reproduced clinical diarrheal 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 L, 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

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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 diarrhea and death in neonatal piglets in the field case.

Materials and Methods

PEAV-positive specimens

In early February 2017, an outbreak of PEAV was reported in swine herds in Guangdong, China (Gong L, 2017), with a mortality rate ranging up to 90% (Zhou et al., 2018). To increase the virus titers for isolation, fresh excreta from infected PEDV-vaccinated newborn piglets were inoculated into 5-day-old non-vaccinated piglets as described previously (Gong L, 2017). Intestinal contents were collected from an inoculated piglet developing symptoms of severe diarrhea 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).

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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% fetal 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 et al., 2015, Pan et al., 2017, Oka et al., 2014). 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 microliters of the filtered inoculums, together with 300 μL maintenance medium was added to each well. After adsorption for 1.5 h at 37°C in 5% CO_2, cells were washed 3 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℃ 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-h postinoculation and 4 d.p.i. were tested by PEAV specific RT-PCR as described previously (Gong L, 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 h at 37 °C in 5% CO_2 and then the maintenance medium was discarded, followed by overlaying 2 mL

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maintenance medium containing 1.25% Agarose LM GQT (TaKaRa, Dalian) to immobilize the virus. After 24 h, 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 ℃ and thawed twice. The cells and supernatant were harvested together to determine viral titers.

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 microliter 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 to 7 days, and virus titer were calculated using the Reed-Muench method (LJ Reed, 1938) and expressed as $TCID_{50}$ per milliliter.

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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 h 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)-labeled goat anti-mouse secondary antibody (KPL, USA) (1:1000) for 1 h. Then the stained cells were observed with a fluorescence microscope (LEICA DMi8, Germany).

Electron microscopic observation

Electron microscopy (EM) was conducted to observe virus samples as described previously with some modifications (Kong, 2010, Hu et al., 2015a, Alsaad et al., 2018). For visualization of the viral particles in infected-cell culture medium, PEAV-infected Vero cells were frozen at -80℃ and thawed twice, and the cell culture was centrifugated at 7000 × *g* for 30 min at 4 ℃. The supernatant was supplemented with 6% PEG6000 for 12 h at 4℃. The mixture was centrifuged at 12000 × *g* for 1 h at 4℃, and the pellet was resuspended in sterile 1 × PBS (pH 7.4) buffer, followed by equilibrium in 8 mL non-linear 20%-60% sucrose-TNE gradients by centrifugation at 110000 × *g* for 2 h at 4℃ with an ultracentrifuge (Hitachi Koki himac CP 100WX, Japan). After purification by sucrose gradient centrifugation, purified virions were

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diluted with sterile 1 × PBS (pH 7.4) buffer and the sucrose was removed by centrifugation at 7000 × *g* for 2 h at 4℃ 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-□ 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 post fixation in 1% osmium tetraoxide for 1 h. 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℃ for 24 hours. Tissue semithin sections (1 μm) were prepared, stained by Toludin blue stain for 5 minutes 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).

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 synthesizedd by reverse transcription using RT-PCR kit (TaKaRa, Dalian). A total of 18 primer pairs based upon the PEAV GDS04 (GenBank accession

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no. MF167434.1; Supplemental Table 1) 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 analyzed using the DNASTAR prograom. 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 neighbor-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.

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 with 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 et al., 2015b, Saeng-Chuto et al., 2017, Jeong et al., 2009). 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

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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, diarrhea, lethargy, and body condition. Diarrhea 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, cecum, and colon) were collected and then formalin-fixed. The fresh samples were stored at -80℃ for viral RNA distribution analysis and formalin-fixed samples were used for histopathology and immunohischemistry analysis. In addition, the mortality of newborn piglets in each group was recorded daily.

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 plasmid concentration for quantification. Briefly, the homogenates from serum, various tissues and the supernatants of rectal swab from each piglet were centrifuged at 6000 × *g* for 5 min, espectively. 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:

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5'-GTAGCAGGTTCTTTGTTAC-3'), and probe (5'-FAM-TCCTCACGCAGATGCTCCTT-TAMRA-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.

Histology and immunohistochemistry

At necropsy, tissue samples of heart, lung, spleen, liver, kidney, stomach, duodenum, jejunum, ileum, cecum, and colon of the piglets from the challenged and control groups were collected separately and routinely fixed in 10% formalin for 36 h at room temperature (Hu et al., 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 hematoxylin and eosin (H&E), the slides were examined and analyzed with conventional light microscopy. Sections (5 μm) of formalin-fixed

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paraffin-embedded tissues were placed onto positively charged glass slides and the slides were air dried for 120 min at 60□. 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 h at 4℃. They were then incubated with peroxidase-labeled 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). Hematoxylin was used for counterstaining. Tissues of piglets from negative control groups were used as negative samples.

Results

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 L, 2017). As shown in Figure 1B, one inoculated cell monolayers 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 L, 2017). After

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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.

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 L, 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 (Fig 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

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SeACoV belong to the same subclade (Fig 3B&C). Therefore, these data suggest that the GDS04 strains was closely relative to SADS-CoV and SeACoV strains from Guangdong, China.

Clinical manifestations of newborn piglets challenged with PEAV GDS04 P12

In order to determine whether PEAV GDS04 was the causative agent for diarrhea, 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 diarrhea from 1 d.p.i. to 4 d.p.i., and all developed severe watery diarrhea, together with vomiting, and dehydration from 5 d.p.i. to 12 d.p.i., as compared with controls (Fig 4A-E), indicating a role of PEAV as an important causative agent for severe watery diarrhea in newborn piglets. Furthermore, four piglets from group 2 as cohabitation controls also developed watery diarrhea from 5 d.p.i. to 12 d.p.i. (Fig 4E). Since PEAV GDS04 caused severe watery diarrhea in newborn piglets, we also recorded the mortality of newborn piglets in each group. As shown in Fig 8, in PEAV-inoculated groups, except that two piglets were necropsied at 7 d.p.i., all remaining 10 piglets died from 5 d.p.i. 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 d.p.i., 6 d.p.i., 7 d.p.i., 8 d.p.i., 9 d.p.i., 10 d.p.i. and 12 d.p.i., respectively. In addition, 2/4 piglets from group 2 as cohabitation controls also died at 7 d.p.i. 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.

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Fecal shedding and virus distribution in newborn piglets challenged with PEAV GDS04 P12

Since PEAV GDS04 caused watery diarrhea in newborn piglets, we explored the fecal viral shedding in PEAV-challenged piglets. As shown in Fig 5A, the PEAV RNA was detected by qRT-PCR in fecal swabs collected from orally inoculated piglets from 1 d.p.i. to 11 d.p.i., and peaked on 4 d.p.i. and kept up until 11 d.p.i.. We also examined the fecal viral shedding in piglets from group 2 as cohabitation controls. PEAV RNA was detected in rectal samples collected from these piglets from 3 d.p.i. to 13 d.p.i. (Fig 5A), 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 Fig 5B, the PEAV RNA was detected in all collected samples of duodenums, jejunums, ileums, cecums, and colons. The virus was also detected in 2/2 hearts, 2/2 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.

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 (Fig 6B) and full virus particles in the intercellular space (Fig 6C) with typical crown-shape of 80-160 nm in diameter and spiky surface projections of CoV, indicating that PEAV GDS04 strain could

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replicate in the jejunum of pig. In addition, the virus-infected cells showed atrophied, ruptured cell morphology (Fig 6B&C). No virus particles or pathological lesions were detected in the jejunum from the control piglets (Fig 6A). Taken together, these results show that PEAV GDS04 could replicate and cause jejunum lesions in newborn piglets.

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 (Fig 7B). No lesions were observed in any other organs of the PEAV-challenged piglets (data not shown) or the organs in the negative control piglets (Fig 7A), indicating that intestinal tract is the target organ of PEAV infection. Microscopic lesions were also analyzed. As shown in Figure 7 H-L, abruption of intestinal villus was observed, whereas the intestinal in negative control was normal (Fig 7C-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 (Fig 7O&P). Taken together, these results indicate that PEAV GDS04 could cause intestinal lesions in newborn piglets.

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Discussion

The widely distributed CoVs could be isolated from a variety of animal hosts and products with animal origin (Stohlman SA, 1982, Tsunemitsu H, 1995, Pan, 2012, Rihtaric et al., 2010, Felippe et al., 2010), as well as from human (Larson HE, 1980). Bats are thought to be the natural reservoir of a range of CoVs (Cui J, 2007). The pathogenicity of five porcine CoVs has so far been confirmed (Doyle, 1946, Sasseville AM, 2002, Pensaert MB, 1978, Wesley RD, 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 L, 2017). Although PEAV has been detected in piglets with severe diarrhea (Gong L, 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 diarrhea in Guangdong, and showed high pathogenicity to newborn piglets. This PEAV strain can be further used for virologic and serological assay development, as well as vaccine development.

A stable African green monkey kidney cell line (Vero cells) (Rhim JS, 1969) is commonly used to isolate CoVs like PEDV or bat coronavirus HKU2 (Lee et al., 2015, Lau et al., 2007). Since PEAV was first detected by genomic analysis in pigs (Gong L, 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

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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 titer 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 determined 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 24790 to 24792, a 1-nt deletion (A) in nt 27071, a 1-nt deletion (T) in nt 27081, a 1-nt deletion (A) in nt 27089, a 3-nt insertion (TTG) in nt 4554 to 4556, 10-nt insertion (GACTAGAGCC) in nt 12483-12492, indicating that these mutation might be related to cellular adaptation. Based on the phylogenetic tree analysis of *s* genes, the PEAV strain GDS04 shares 36.23%–99.91% 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

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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 are 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 L, 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 diarrhea 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 diarrhea and vomiting in piglets by oral infection strongly suggest the pathogenicity of PEAV to the newborn piglets. Furthermore, PEAV RNA was detected from 1 d.p.i. to 11 d.p.i. in fecal 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 diarrhea and viral shedding, suggesting the possibility of fecal-oral transmission of PEAV. Results from this study may help guide future PEAV experimental designs as the clinical diarrhea and virus shedding patterns.

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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 were 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, ceca 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's 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 diarrhea in newborn piglets, we also recorded the mortality of newborn piglets from 1 d.p.i. 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 diarrhea and vomiting in pigs of 3 days old. However, SeACoV infection did not cause fatality in piglets in

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five 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, 1 piglet died in dpi 5, and all PEAV-inoculated piglets and half piglets from cohabitation contrast died in two 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 addtition, 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 is 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.

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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 diarrhea with a mortality of 100% in neonatal piglets. Collectively, these findings suggest that PEAV GDS04 is highly virulent in piglets.

Author contributions

YC and ZX conceived and designed the experiments; ZX, YL, LH and QZ performed the experiments; ZX analyzed 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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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict interst.

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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.

References

Alsaad, K. O., Hajeer, A. H., Al Balwi, M., Al Moaiqel, M., Al Oudah, N., Al Ajlan, A., AlJohani, S., Alsolamy, S., Gmati, G. E., Balkhy, H., Al-Jahdali, H. H., Baharoon, S. A., and Arabi, Y. M. (2018). Histopathology of Middle East respiratory syndrome coronovirus (MERS-CoV) infection - clinicopathological and ultrastructural study. *Histopathology,* 72, 516-524.

Chen, Q., Gauger, P., Stafne, M., Thomas, J., Arruda, P., Burrough, E., Madson, D., Brodie, J., Magstadt, D., Derscheid, R., Welch, M., Zhang, J. (2015). Pathogenicity and pathogenesis of a United States porcine deltacoronavirus cell culture isolate in 5-day-old neonatal piglets. *Virology,* 482, 51-59.

Cruz, D. J., Kim, C. J., and Shin, H. J. (2008). The GPRLQPY motif located at the carboxy-terminal of the spike protein induces antibodies that neutralize Porcine epidemic diarrhea virus. *Virus research,* 132, 192-196.

Cui, J., Han, N., Streicker, D., Li, G., Tang, X., Shi, Z., Hu, Z., Zhao, G., Fontanet, A., Guan, Y., Wang, L., Jones, G., Field, H. E., Daszak, P., Zhang, S. (2007). Evolutionary Relationships between bat coronaviruses and their hosts. *Emerging Infectious Diseases,* 13, 10.

Dong, N., Fang, L., Yang, H., Liu, H., Du, T., Fang, P., Wang, D., Chen H. and Xiao, S. (2016). Isolation, genomic characterization, and pathogenicity of a Chinese porcine deltacoronavirus strain CHN-HN-2014. *Veterinary microbiology,* 196, 98-106.

Doyle, L. P., Hutchings, L. M. (1946). A transmissible gastroenteritis in pigs. *Journal of the American Veterinary Medical Association,* 108, 257-259.

This article is protected by copyright. All rights reserved.

Accepted Article

Felippe, P. A., da Silva, L. H., Santos, M. M., Spilki, F. R., and Arns, C. W. (2010). Genetic diversity of avian infectious bronchitis virus isolated from domestic chicken flocks and coronaviruses from feral pigeons in Brazil between 2003 and 2009. *Avian diseases,* 54, 1191-1196.

Gong, L., Li, J., Zhou, Q., Xu, Z., Chen, L., Zhang, Y., Xue, C., Wen, Z., Cao, Y. (2017). A New Bat-HKU2-like Coronavirus in Swine, China, 2017. *Emerging Infectious Diseases,* 23, 9.

Hu, H., Jung, K., Vlasova, A. N., Chepngeno, J., Lu, Z., Wang Q., and Saif, L. J. (2015a). Isolation and characterization of porcine deltacoronavirus from pigs with diarrhea in the United States. *Journal of clinical microbiology,* 53, 1537-1548.

Hu, H., Jung, K., Vlasova A. N., and Saif, L. J. (2016). Experimental infection of gnotobiotic pigs with the cell-culture-adapted porcine deltacoronavirus strain OH-FD22. *Archives of virology,* 161, 3421-3434.

Hu, X. Jr., Li, N. Jr., Tian, Z. Jr., Yin, X. Jr., Qu, L., and Qu, J. (2015b). Molecular characterization and phylogenetic analysis of transmissible gastroenteritis virus HX strain isolated from China. *BMC veterinary research,* 11, 72.

Jeong, Y. J., Park, S. I., Hosmillo, M., Shin, D. J., Chun, Y. H., Kim, H. J., Kwon, H. J., Kang, S. Y., Woo, S. K., Park, S. J., Kim, G. Y., Kang M. I., and Cho, K. O. (2009). Detection and molecular characterization of porcine group C rotaviruses in South Korea. *Veterinary microbiology,* 138, 217-224.

Kong, Q., Xue, C., Ren, X., Zhang, C., Li, L., Shu, D., Bi, Y., and Cao, Y. (2010). Proteomic analysis of purified coronavirus infectious bronchitis virus particles. *Proteome Science,* 8, 29.

Larson, H. E., Reed, S. E., Tyrrell, D. A. (1980). Isolation of rhinoviruses and coronaviruses from 38 colds in adults. *Journal of Medical Virology,* 5, 221-229.

Lau, S. K., Woo, P. C., Li, K. S., Huang, Y., Wang, M., Lam, C. S., Xu, H., Guo, R., Chan, K. H., Zheng, B. J., and Yuen, K. Y. (2007). Complete genome sequence of bat coronavirus HKU2 from Chinese horseshoe bats revealed a much smaller spike gene with a different evolutionary lineage from the rest of the genome. *Virology,* 367, 428-439.

Lee, S., Kim, Y., and Lee, C. (2015). Isolation and characterization of a Korean porcine epidemic diarrhea virus strain KNU-141112. *Virus research,* 208, 215-224.

LJ Reed. (1938). A simple method of estimating fifty per endpoints. *The American Journal of Hygiene,* 27, 493-497.

Oka, T., Saif, L. J., Marthaler, D., Esseili, M. A., Meulia, T., Lin, C. M., Vlasova, A. N., Jung, K., Zhang, Y., and Wang, Q. (2014). Cell culture isolation and sequence analysis of genetically diverse US

This article is protected by copyright. All rights reserved.

Accepted Article

porcine epidemic diarrhea virus strains including a novel strain with a large deletion in the spike gene. *Veterinary microbiology,* 173, 258-269.

Pan, Y., Tian, X., Qin, P., Wang, B., Zhao, P., Yang, Y. L., Wang, L., Wang, D., Song, Y., Zhang, X., and Huang, Y. W. (2017). Discovery of a novel swine enteric alphacoronavirus (SeACoV) in southern China. *Veterinary microbiology,* 211, 15-21.

Pan, Y., Tian, X., Li, W., Zhou, Q., Wang, D., Bi, Y., Chen, F., and Song, Y. (2012). Isolation and characterization of a variant porcine epidemic diarrhea virus in China. *Virology Journal,* 9, 195.

Pensaert, M. B., de, Bouck. P., (1978). A new coronavirus-like particle associated with diarrhea in swine. *Archives of virology,* 58, 243-247.

Rhim, J. S., Schell, K., Creasy, B., Case, W. (1969). Biological characteristics and viral susceptibility of an African green monkey kidney cell line (Vero). *Proceedings of the Society for Experimental Biology and Medicine,* 132, 670-678.

Rihtaric, D., Hostnik, P., Steyer, A., Grom, J., and Toplak, I. (2010) Identification of SARS-like coronaviruses in horseshoe bats (Rhinolophus hipposideros) in Slovenia. *Archives of virology,* 155, 507-514.

Saeng-Chuto, K., Lorsirigool, A., Temeeyasen, G., Vui, D. T., Stott, C. J., Madapong, A., Tripipat, T., Wegner, M., Intrakamhaeng, M., Chongcharoen, W., Tantituvanont, A., Kaewprommal, P., Piriyapongsa, J., and Nilubol, D. (2017). Different Lineage of Porcine Deltacoronavirus in Thailand, Vietnam and Lao PDR in 2015. *Transboundary and emerging diseases,* 64, 3-10.

Sasseville, A. M., Boutin, M., Gelinas, A. M., Dea, S. (2002). Sequence of the 3'-terminal end (8.1 kb) of the genome of porcine haemagglutinating encephalomyelitis virus: comparison with other haemagglutinating coronaviruses. *Journal of General Virology,* 83, 2411-2416.

Stohlman, S. A., Brayton, P. R., Fleming, J. O., Weiner, L. P., Lai, M. M. (1982). Murine coronaviruses: isolation and characterization of two plaque morphology variants of the JHM neurotropic strain. *Journals of General Virology,* 63, 265-275.

Tsunemitsu, H., el-Kanawait, Z. R., Smith, D. R., Reed, H. H., Saif, L. J. (1995). Isolation of coronaviruses antigenically indistinguishable from bovine coronavirus from wild ruminants with diarrhea. *Journal of clinical microbiology*, 3264-3269.

Wesley, R. D., Woods, R. D., Hill, H. T., Biwer, J. D. (1990). Evidence for a porcine respiratory coronavirus, antigenically similar to transmissible gastroenteritis virus, in the United States. *Journal of Veterinary Diagnostic Investigation,* 2, 312-317.

This article is protected by copyright. All rights reserved.

Accepted Article

Woo, P. C., Huang, Y., Lau, S. K., and Yuen, K. Y. (2010). Coronavirus genomics and bioinformatics analysis. *Viruses,* 2, 1804-1820.

Woo, P. C., Lau, S. K., Lam, C. S., Lau, C. C., Tsang, A. K., Lau, J. H., Bai, R., Teng, J. L., Tsang, C. C., Wang, M., Zheng, B. J., Chan, K. H., and Yuen, K. Y. (2012). Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *Journal of virology,* 86, 3995-4008.

Zhang, J. (2016). Porcine deltacoronavirus: Overview of infection dynamics, diagnostic methods, prevalence and genetic evolution. *Virus research,* 226, 71-84.

Zhou, P., H. Fan., T. Lan., X. L. Yang., W. F. Shi., W. Zhang., Y. Zhu., Y. W. Zhang., Q. M. Xie., S. Mani., X. S. Zheng., B. Li., J. M. Li., H. Guo., G. Q. Pei., X. P. An., J. W. Chen., L. Zhou., K. J. Mai., Z. X. Wu., D. Li., D. E. Anderson., L. B. Zhang., S. Y. Li., Z. Q. Mi., T. T. He., F. Cong., P. J. Guo., R. Huang., Y. Luo., X. L. Liu., J. Chen., Y. Huang., Q. Sun., X. L. Zhang., Y. Y. Wang., S. Z. Xing., Y. S. Chen., Y. Sun., J. Li., P. Daszak., L. F. Wang., Z. L. Shi., Y. G. Tong and J. Y. Ma. 2018: Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. *Nature,* 556, 255-258.

Figure legends

Fig.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 h postinoculation, an immunofluorescence assay (IFA) was performed.

Fig.2. Electron micrographs of PEAV inoculated Vero cells.

Crown-shaped spiked of PEAV are visible (arrows). The sample was negatively stained with 3% phosphotungstic acid.

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Fig.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 neighbor-joining method in the MEGA software package, version 5 (http://www.megasoftware.net). Bootstrap resampling (1000 replication) was performed, and bootstrap values are indicated for each node. Reference sequence obtained from GenBank are indicated by strain name. The scale bar represents 1, 0.2, or 0.5 nucleotide substitutions per site.

Fig.4. Reproduction of watery diarrhea and fecal viral shedding in newborn piglets inoculated with PEAV GDS04 strain P12 via oral feeding.

(A&C) Newborn piglets uninfected as control. (B&D) Watery diarrhea (indicated by arrows) were observed at 3 d.p.i. and 7 d.p.i. with PEAV infection. (E) Average diarrhea scores after PEAV infection.

Fig.5. 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 fecal swabs and viral RNA shedding in fecal swabs after PEAV inoculation or mock inoculation. (B) Virus distribution at 7 d.p.i. in newborn piglets challenged with PEAV.

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Fig.6. 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..

Fig.7. 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) Hematoxylin 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..

Fig.8. The survival rate of newborn piglets post infection with PEAV GDS04 P12.

The mortality of newborn piglets in each group was recorded from 1 d.p.i. to 14 d.p.i.

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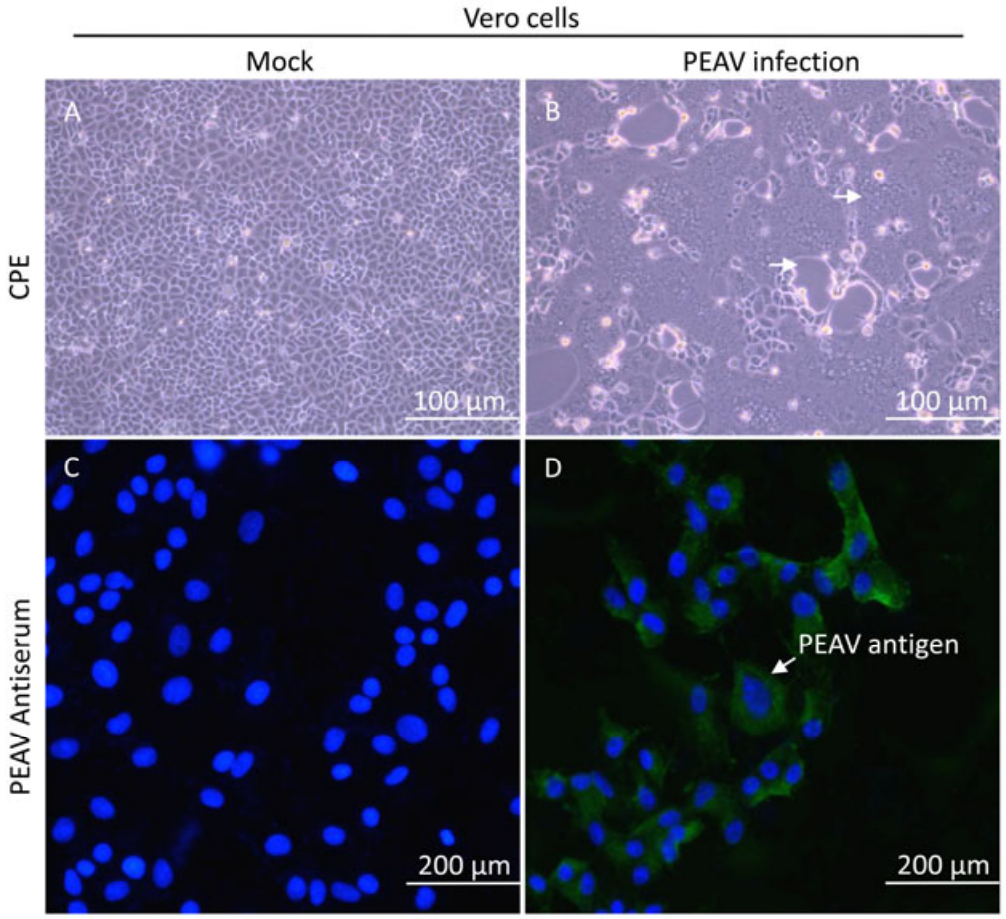

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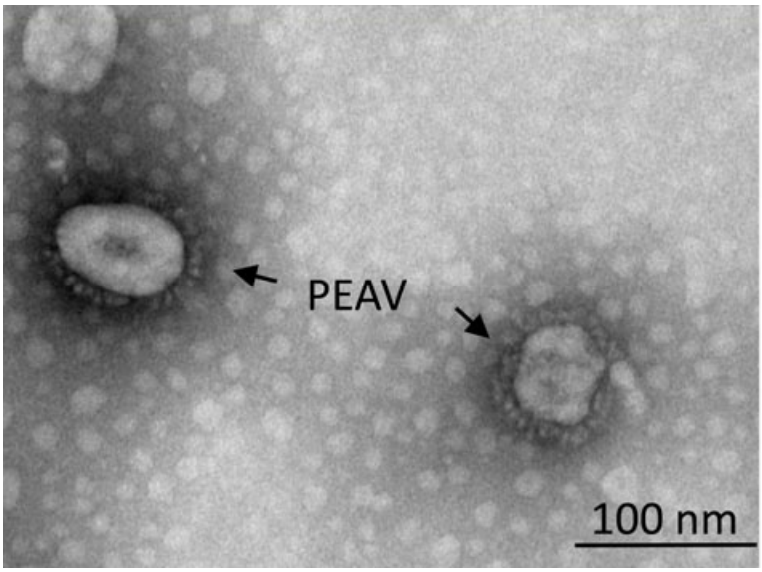

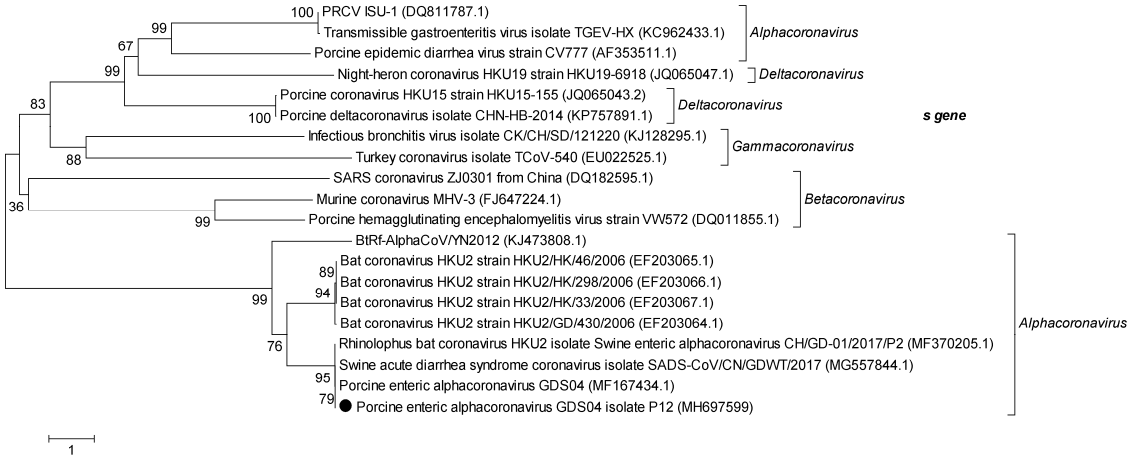

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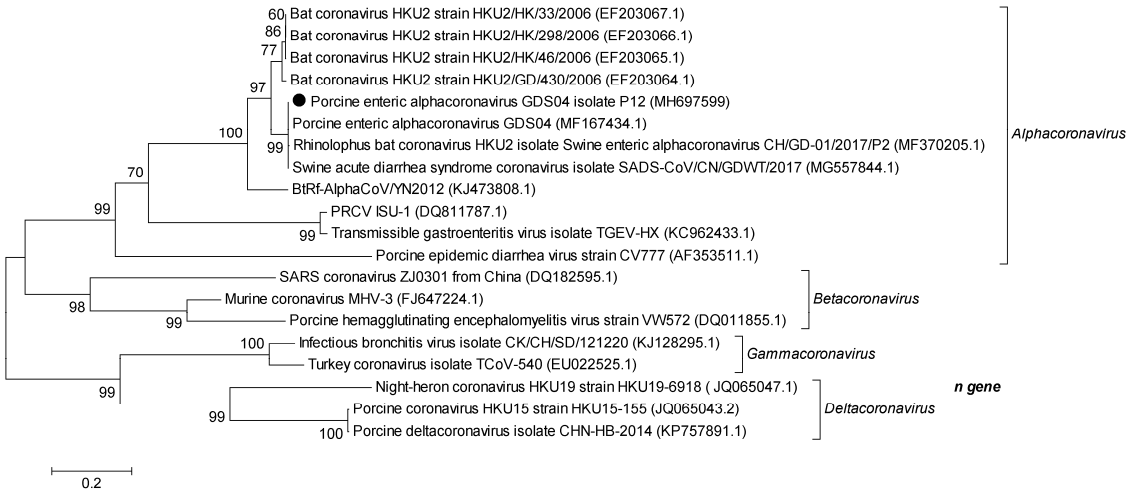

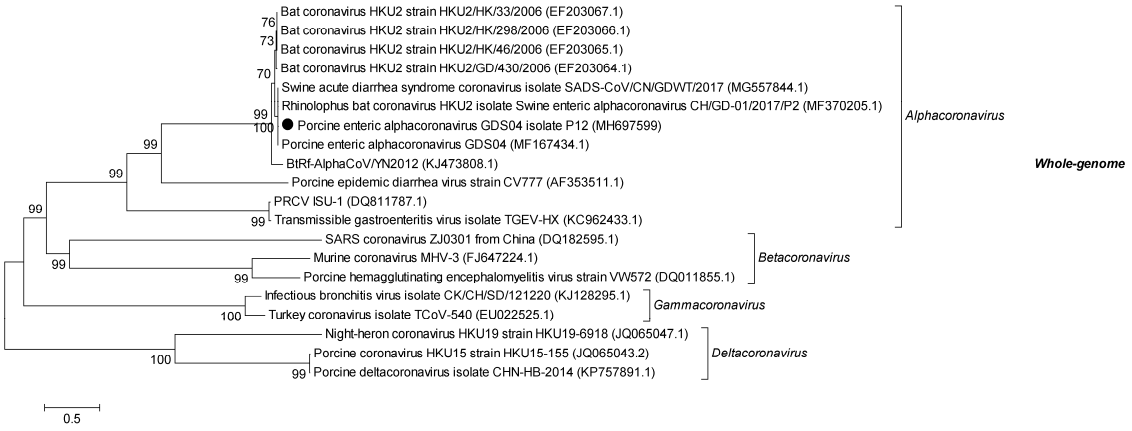

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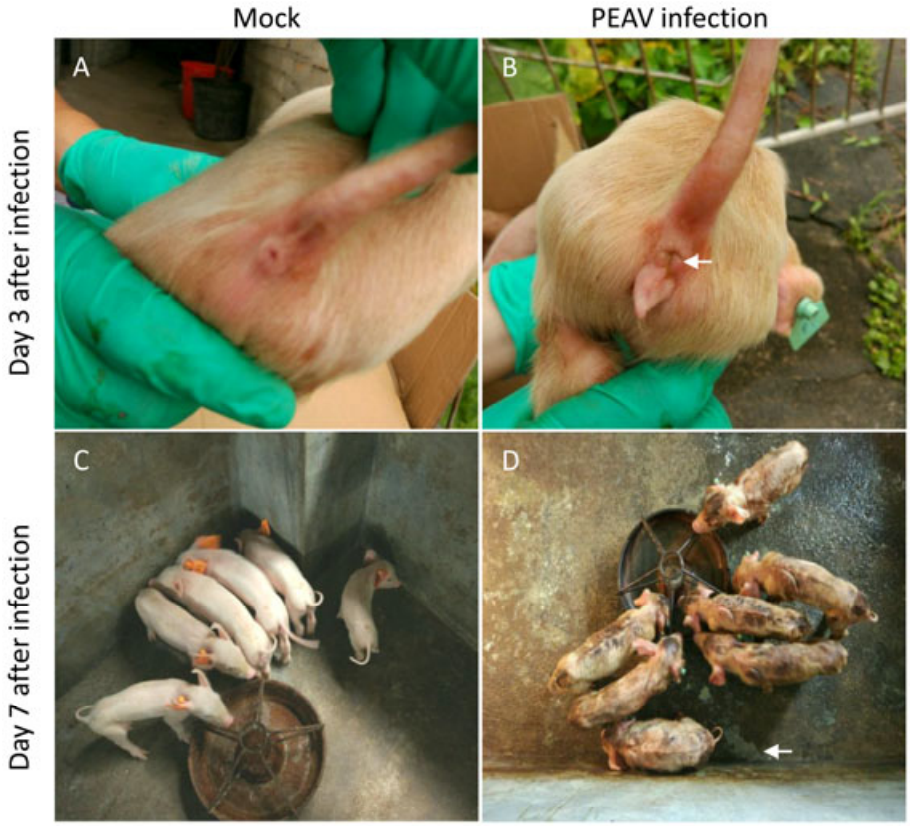

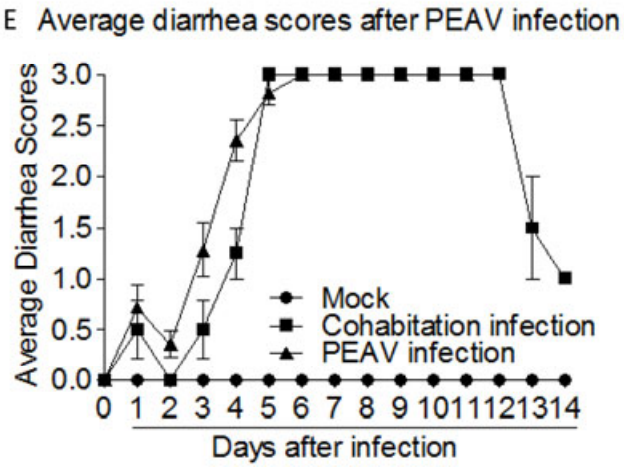

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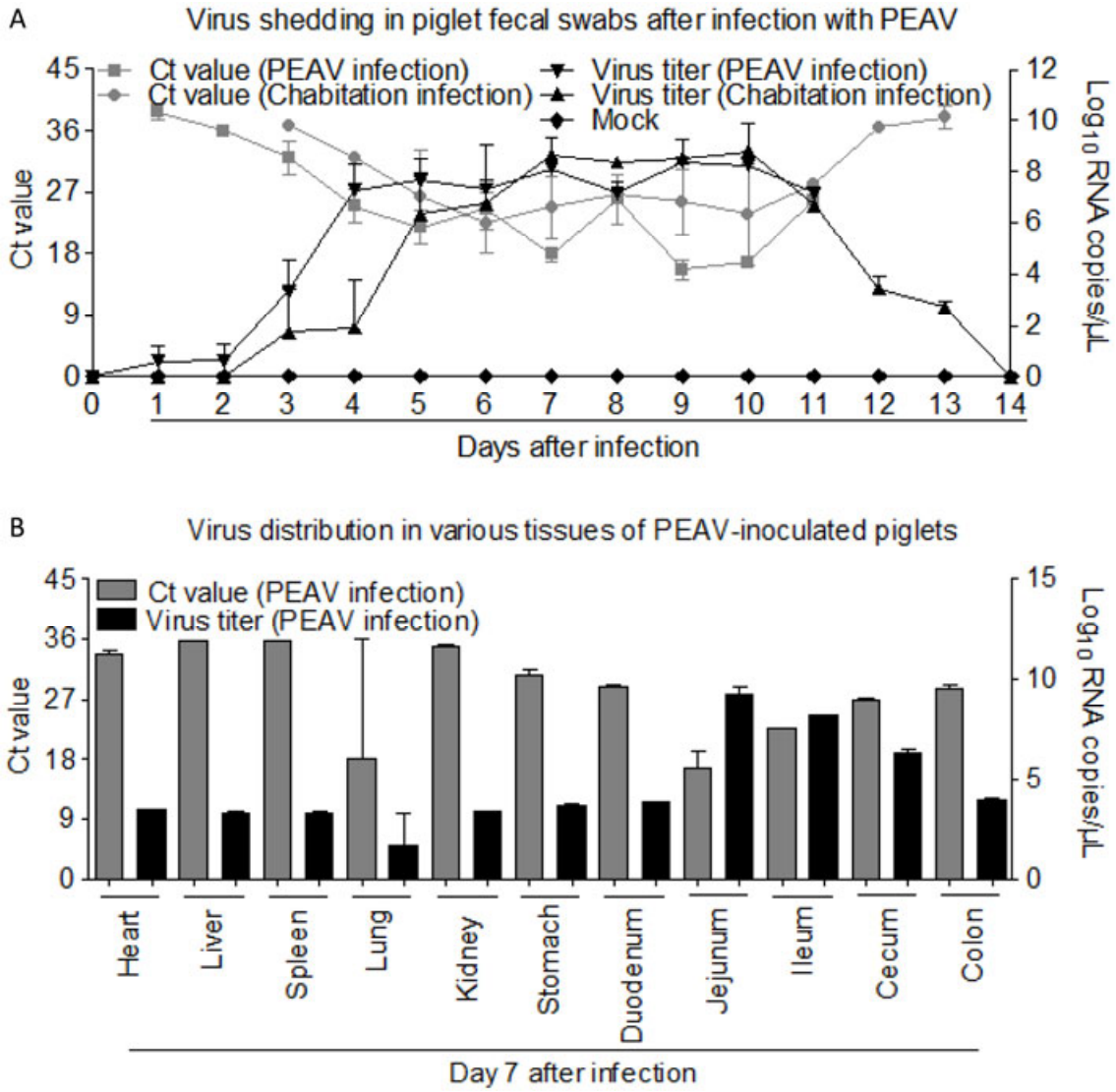

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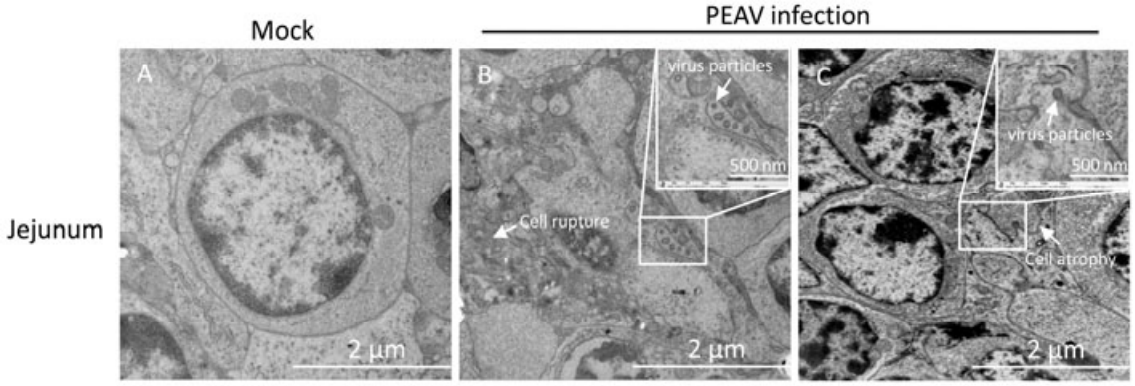

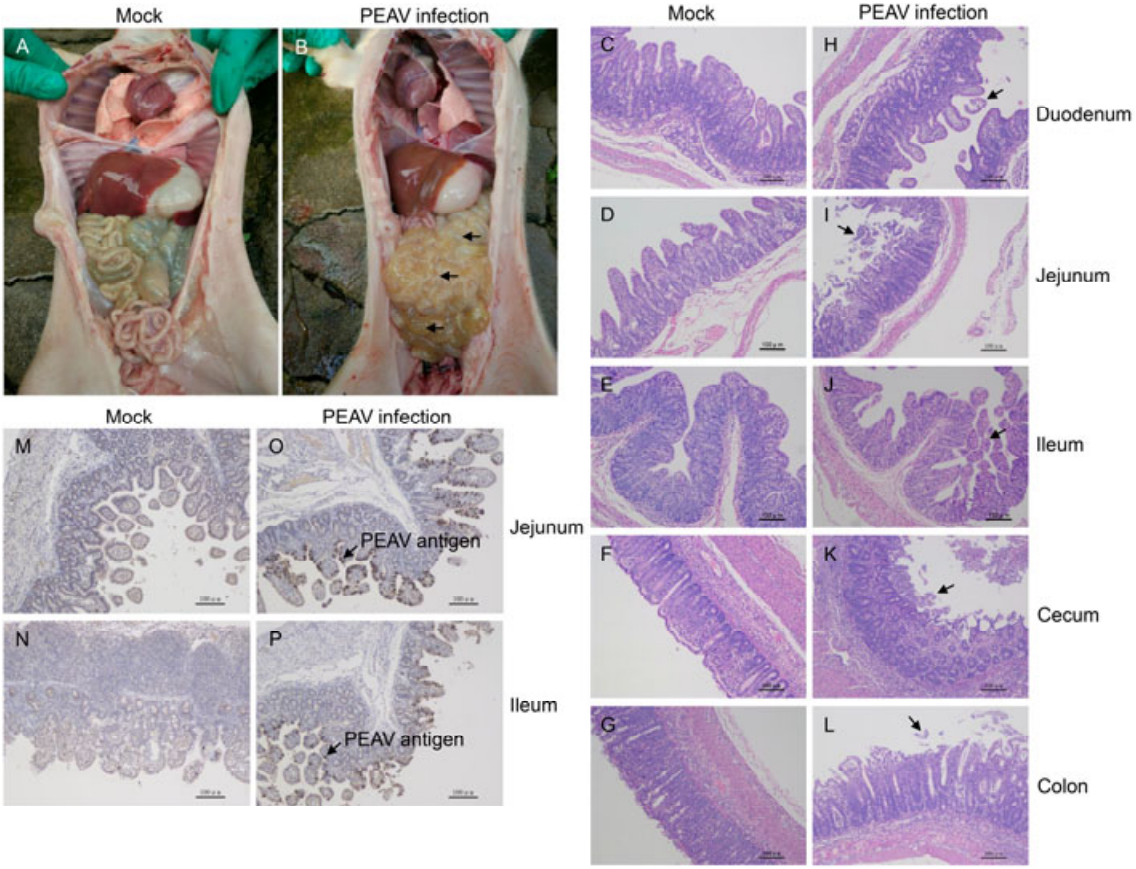

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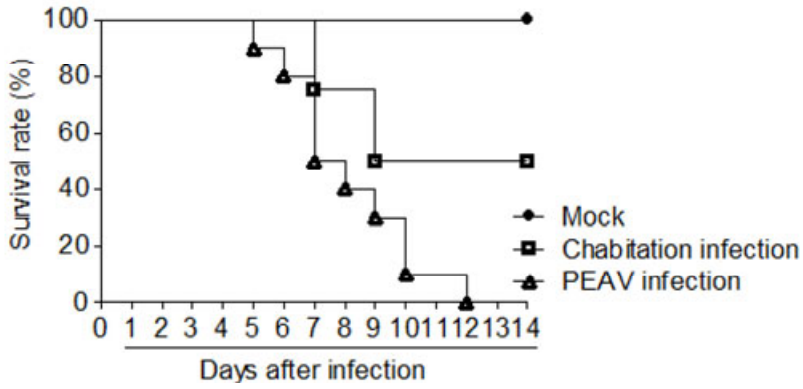
The survival rate of newborn piglets post infection with PEAV



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