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Different intestinal tropism of the G2b Taiwan porcine epidemic diarrhea virus-Pintung 52 strain in conventional 7-day-old piglets

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ABSTRACT

The group 2b (G2b) porcine epidemic diarrhea virus (PEDV) that emerged in 2013 has since caused devastating diseases and economic loss. The full-length genome of the G2b Taiwan PEDV-Pintung 52 (PEDV-PT) strain and its intestinal tropism by evaluating the pathological changes in the original PEDV-PT infected field piglet and orally inoculation of either 10, 10^3, or 10^5 50% tissue culture infective dose/mL ($TCID_{50}$/mL) of the plaque-purified PEDV-PT-Passage 5 (P5) in 7-day-old conventional piglets were analyzed. Phylogenetic analysis of the full-length genome indicated that the G2b Taiwan PEDV-PT strain was closely related to the North American G2b PEDV strains. Some pathological features of the G2b Taiwan PEDV-PT infection, including the absence of lesions and antigen signal in the crypt epithelial cells of the jejunum and ileum and in the villus enterocytes of the duodenum and colon, were different from those of infections by the North American G2b PEDV strains. This difference in the intestinal tropism of the G2b Taiwan PEDV-PT strain highlights the importance of studying the pathogenicities of different PEDV variants. Moreover, similar distributions of PEDV antigens and lesions in the G2b Taiwan PEDV-PT infected field piglet and its plaque-purified isolate, PEDV-PT-P5, inoculated piglets indicating that the plaque-purified PEDV-PT-P5 viral stock could facilitate the preclinical evaluation of vaccines and other interventions aimed at preventing the G2b PEDV infection.

Introduction

The porcine epidemic diarrhea virus (PEDV) is the etiological agent of porcine epidemic diarrhea (PED), causing vomiting, watery diarrhea, and weight loss in all aged piglets (Huang et al., 2013). PEDV is an enveloped, single-stranded, positive-sense RNA virus belonging to the *Alphacoronavirus* genus of the family *Coronaviridae*. The genome of PEDV is approximately 28 kilobase pairs (kb) long and contains seven open reading frames (ORFs), including ORF1ab which encodes replicase, the spike region (S) which encodes structural proteins, ORF3, and the envelope (E), membrane (M), and nucleocapsid (N) genes (Gerdts et al., 2017).

According to phylogenetic analysis of the S gene, PEDV can be grouped into four major subgroups (G): G1a, G1b, G2a, and G2b (Huang et al., 2013; Gerber et al., 2016). Most PEDV strains that circulated in Europe and Asia prior to the 2010 outbreaks, such as the CV777 and DR13 strains, belong to the G1a group (Huang et al., 2013). After 2010, strains from PEDV G2a subgroup in Asia and strains of PEDV G2b, such as non S gene insertions and deletions strains (non S-INDEL), in North America and in some Asian countries (Huang et al., 2013; Zhang et al., 2015; Chen et al., 2016) have led to large-scale outbreaks with high rates of illness and death in naïve suckling piglets (Li et al., 2012). Later, G1b variants, such as S-INDEL PEDVs, in the United States and recent PEDV strains in Europe that cause milder disease in piglets have been identified (Lee, 2015; Chen et al., 2016; Hanke et al., 2017).

The pathogenicity and intestinal tropism of different PEDV strains have been evaluated. In general, the pathological findings of most PEDV-infected animals shared similar patterns of enteritis and villous atrophy, with viral antigens detected all over the small intestine, including the duodenum, jejunum, and

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ileum. However, variations in the distribution of PEDV antigens and lesions in the intestines were reported in different PEDV isolates (Appendix: Supplementary Table 1). It has been demonstrated that CV777 (Debouck et al., 1981) and most of G2b PEDVs, such as PC22A (Liu et al., 2015) and USA/IL20697/2014 (Chen et al., 2016) (Appendix: Supplementary Table 1), are able to infect and cause lesions in the colon and in crypt epithelial cells, whereas no detectable lesions in the colon and crypt were reported in SNUVR971496-infected 1-day-old cesarean-derived colostrum-deprived pigs (CD/CD) (Kim et al., 2003) and in USA/KSA/2013 strain-infected 4-week-old feeder piglets (Niederwerder et al., 2016). According to phylogenetic analysis of the S gene, the Taiwan PEDV-Pintung 52 (PEDV-PT) strain is closely related to the G2b strains and the US non-S-INDEL strains (Lin et al., 2014; Chang et al., 2017; Chiou et al., 2017). However, little is known about the phylogenetic relationship of the full-length G2b Taiwan PEDV-PT genome with other PEDV genomes. Additionally, information on the intestinal tropism and pathogenicity of the G2b Taiwan PEDV-PT strain is lacking.

The aims of this study were to perform phylogenetic analysis of the full-length Taiwan PEDV-PT genome in relation to other PEDV genomes, characterize the pathology of the original PEDV-PT infected field piglet and the pathogenicity of PEDV-PT experimental infection in conventional 7-day-old piglets by assessing clinical symptoms, fecal viral shedding, microscopic lesions, and distribution of immunohistochemistry signals.

Materials and methods

Virus isolation

The parental PEDV-PT strain was obtained in early 2014 from the intestine homogenate of a 7-day-old suckling pig in Taiwan (Chang et al., 2017). The piglet had presented with watery diarrhea and severe dehydration and was submitted for necropsy and viral isolation. The parental virus was plaque-purified at the third passage and propagated three times in Vero C1008 cells (American Type Culture Collection (ATCC) No. CRL-1586). A PEDV-PT passage 5 (P5) was prepared at a titer of 10^5 tissue culture infective dose/mL ($TCID_{50}$/mL) as previously described (Chang et al., 2017).

Comparative sequence analysis and phylogenetic analysis

The full-length genome sequence (GenBank accession number KY929405.1) of the PEDV-PT stock (Chang et al., 2017) excluding the polyadenosine tail was aligned with 25 published full-length PEDV genome sequences. The sequences used included those of the strains USA/KSA/2013 (KJ184549.1), USA/IN19338/2013 (KF650371), USA/IA49379/2013 (KM975736.1), MN (KF468752.1), USA/Iowa/18984/2013 (KF804028.1), USA/NC/2013/49469 (KM975737), TTR-2/JPN/2014 (LC063828), PC21A (KR078299.1), PC22A (KY499262.1), USA/NC/2013/35140 (KM975735), IA1 (KF468753.1), OKN-1/JPN/2013 (LC063836), USA/Colorado/2013 (KF272920.1), USA/IL20697/2014 (KT860508), CH/ZMDZY/11 (KC196276), USA/IL20697/2014 (KT860508.1), FL2013 (KP765609), attenuated DR13 (JQ023162.1), SM98 (GU937797.1), and CV777 (KT323979.1) were summarized in Appendix: Supplementary Table 2. Alignment was performed using Lasergene 7.1 (DNASTAR, Madison, WI, USA) and MEGA7 (Kumar et al., 2016). Phylogenetic trees were constructed using the distance-based neighbor-joining method and the maximum likelihood method in MEGA7. Bootstrap analysis was carried out with 1000 replicates.

Animals and study design

Twelve 7-day-old, fecal PEDV RNA-negative and PEDV seronegative Large White × Duroc crossbred pigs were acquired from a conventional pig farm with no known history of PED. These pigs were convenience sampling into four groups, including three groups (G1–G3, $n = 9$) inoculated with different titers of the virus and one control group ($n = 3$). Piglets in G1, G2, and G3 were orally inoculated with 1 mL of 10, 10^3, and 10^5 $TCID_{50}$/mL of PEDV-PT-P5 stock, respectively. Each group was housed in a separate room. Each pig in the control group orally received 1 mL of post-inoculation (PI) medium, which contained Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with tryptose phosphate broth (0.3%), yeast extract (0.02%), and 10 μg/mL of trypsin. All pigs were sacrificed three days post-infection (DPI). The animal experiment, rescue protocols, welfare/pain/health monitoring protocols, and animal welfare end-points were reviewed and approved by the Institutional Animal Care and Use Committee of National Taiwan University (Taipei, Taiwan, NTU105-EL-00087; Appendix: Supplementary file).

Clinical assessment and biological sample collection

Each group of pigs with clinical diarrhea was scored for fecal consistency using the following criteria: 0: normal, 1: loose, 2: semi-fluid, and 3: watery/liquid contents (Jung et al., 2015). Fecal scores were recorded every six hours (h) during the first 2 days and once on day 3. Rectal swabs were collected every day to monitor the duration of viral shedding using real-time RT-PCR performed as previously described (Chang et al., 2017).

Gross and histopathological examination

All piglets in the experimental groups at 3 DPI were humanely euthanized by electrocution and subsequent exsanguination for gross and histopathological examinations. Fresh and formalin-fixed samples were collected during necropsy. Representative tissue samples were collected, fixed in 10% neutral-buffered formalin, processed routinely, sectioned at a thickness of 5 μm, stained with hematoxylin and eosin stain (H&E stain), and then analyzed for histopathological changes.

For the morphometric analysis of intestines, three intestinal mucosa samples containing full-length villi and intact crypts from each of the three sections of the small intestine (duodenum, jejunum, and ileum) were assessed using a computerized image system. The ratio of villous height to crypt depth (VH:CD) was calculated as previously suggested (Jung et al., 2014; Madson et al., 2014). The histopathological examination was recorded and assessed blindly by one veterinary pathologist.

Immunohistochemistry (IHC)

To detect the distribution of PEDV antigens, 4-μm-thick paraffin-embedded tissue sections were cut and heated at 60 °C for an h. The tissue sections were deparaffinized with xylene, rehydrated with decreasing concentrations of ethanol, and rinsed with TBST (Tris-buffered saline, 0.1% Tween 20). Heat-induced epitope retrieval was carried out in EDTA-based retrieval buffer (Trilogy™; Cell Marque, CA, USA) at 95 °C for 10 min. Endogenous peroxidase activity was blocked by immersion in 3% hydrogen peroxide (Merck, Darmstadt, Germany) for 15 min. To block non-specific binding sites, the slides were incubated with 2.5% normal goat serum (Dako, CA, USA) diluted in phosphate-buffered saline (PBS; pH 7.4) at room temperature (RT) for 30 min. The slides were then stained with the monoclonal antibody DE-1 (diluted 1:1000 in 2.5% normal goat serum) against the nucleocapsid protein of PEDV suspended in 2.5% normal goat serum for 1 h. The sections were then treated with Dako Real Envision-HRP (rabbit/mouse) (Dako Denmark A/S, Glostrup, Denmark) for 60 min at RT. After exposure to aminoethylcarbazole (AEC) substrate chromogen (Dako Denmark A/S, Glostrup, Denmark) or 2% diaminobenzidine (Real DAB+ chromogen, Dako Denmark A/S, Glostrup, Denmark.) for 3 min, the slides were counterstained with Mayer's hematoxylin solution (MUTO, Tokyo, Japan) and mounted in Entellan (Merck, Darmstadt, Germany). As a negative control, a duplicate section was stained simultaneously without adding the primary antibody. Tissue sections were considered IHC-positive when there was cytoplasmic dark brown staining in the intralesional enterocytes. The IHC scores used were a modified version of those in a previous study (Madson et al., 2014; Lin et al., 2015). The IHC signal was scored 0–3 according to the following criteria: 0 = no positive signal, 1 = less than 10% of the villous enterocytes with a positive signal, 2 = 10–50% of the villous enterocytes with a positive signal, and 3 = more than 50% of the villous enterocytes with a positive signal.

Statistical analysis

All values were expressed as the mean ± standard deviation (SD). The results of ratio of villous height to crypt depth (VH:CD) were analyzed using statistical software GraphPad Prism (GraphPad Prism Inc.). Variables were compared using the non-parametrical Kruskal–Wallis test with $P < 0.05$ considered significant.

Results

Full-length genome sequence analysis and phylogenetic analysis of the Taiwan PEDV-PT strain

The 28,038 base pairs (bp)-long Taiwan PEDV-PT genome sequence (GenBank accession number KY929405), excluding the polyadenosine tail, was aligned with the complete genome sequences of 25 PEDV strains. Phylogenetic analysis of the full-length genome of the Taiwan PEDV-PT strain, shown in Appendix: Supplementary Fig. 1, indicated that it belongs to the subgroup G2b. The full-length genome sequence of this strain shared the highest nucleotide identity (99.8%) with the USA/Iowa/18984/2013