Short Communication

Pig epidemic diarrhoea virus S gene variant with a large deletion non-lethal to colostrum-deprived newborn piglets

Tohru Suzuki,1 Tomoyuki Shibahara,2 Ryosaku Yamaguchi,3 Keisuke Nakade,4 Takehisa Yamamoto,1 Ayako Miyazaki1 and Seiichi Ohashi1

Correspondence
Tohru Suzuki
tohru_suzuki@affrc.go.jp

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1Viral Disease and Epidemiology Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), Tsukuba, Japan
2Pathology and Pathophysiology Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), Tsukuba, Japan
3Institute of Animal Health, National Federation of Agricultural Co-operative Associations, Sakura, Japan
4Oita Livestock Hygiene Service Center, Oita, Japan

We previously identified a third porcine epidemic diarrhoea virus (PEDV) S variant with a large deletion of 582 nucleotides in the 5‘ terminal region of the S gene, in addition to the North American type and the S INDELs type. To investigate the pathogenicity of this variant, TTR-2/JPN/2014, we performed experimental infection using colostrum-deprived piglets and compared the results with those from the North American type PEDV, OKN-1/JPN/2013. Fifteen newborn piglets were divided into two groups of 7–8 piglets each and inoculated orally with one of PEDV isolates maintained at the eighth passage in Vero cell culture. Although all PEDV-inoculated piglets showed acute watery diarrhoea, lethality clearly differed between both PEDV-inoculated groups. Moreover, there were differences in virus distribution and lesions on the intestines between the two PEDV-inoculated groups. Therefore, our data suggest that the OKN-1/JPN/2013 PEDV isolate is virulent, whereas the TTR-2/JPN/2014 PEDV isolate is avirulent.

Porcine epidemic diarrhoea (PED) is caused by porcine epidemic diarrhoea virus (PEDV), which belongs to the family Coronaviridae, genus Alphacoronavirus. PEDV is an enveolped, positive-sense, single-stranded RNA virus that was first discovered in the UK in 1971 (Pensaert & de Bouck, 1978). From the 1970s–1990s, PED became widespread in multiple swine-producing countries in Europe and Asia (Chasey & Cartwright, 1978; Kweon et al., 1993; Nagy et al., 1996; Takahashi et al., 1983; Van Reeth & Pensaert, 1994). Since late 2010, severe PED outbreaks with considerable morbidity and high mortality among suckling pigs were reported in China (Chen et al., 2012; Li et al., 2012a, b). The first case of PED in the USA occurred in April 2013, and PED spread rapidly to over 30 states in the first year (Huang et al., 2013; Stevenson et al., 2013). Two main types of PEDVs have been identified in the USA based on genetic analysis of the spike (S) gene: original highly virulent USA PEDV strains (defined as the North American type) and S INDELs PEDV strains, which contain insertions and deletions in the 5‘ terminus of the S gene (Wang et al., 2014; Vlasova et al., 2014). After this initial outbreak, USA-like PED epidemics have occurred in North America, Asia and Europe (Vlasova et al., 2014; Pasick et al., 2014; Lee & Lee, 2014; Chiu et al., 2014; Hanke et al., 2015).

In Japan, PED was first reported in 1982 (Takahashi et al., 1983). In 1996, PED outbreaks occurred in 80 000 pigs in 102 farms in nine prefectures, and approximately half of the affected pigs died. Thereafter, PED was listed as a notifiable infectious disease in Japan, resulting in the establishment of immunohistochemical methods for detecting PEDV (Tsuda, 1997). In October 2013, an outbreak of PED re-emerged in Japan after a period of seven years without a reported case. Over 1000 outbreaks of PED in nearly all (39/47) prefectures of Japan have occurred from 2013 to 2015, as reported by the Ministry of Agriculture, Forestry and Fisheries (http://www.maff.go.jp). Phylogenetic analysis based on the S gene showed that several Japanese PEDV
strains obtained from these outbreaks were predominantly classified into two PEDV types: the North American type, including a novel PEDV S variant (defined as the S large-DEL type) with a large deletion of 582 nucleotides in the 5' terminal region of the S gene, and the S INDELs type (Masuda et al., 2015; Murakami et al., 2015; Suzuki et al., 2015). Additionally, Japanese PEDV strains were more closely related to global PEDV strains recently detected worldwide than to classical PEDV strains detected in Japan in prior decades. Moreover, a phylogenetic dendrogram of the complete genome sequences indicated that the S large-DEL variant may have been spontaneously derived from the current PEDV strains circulating in domestic pigs.

In this study, we investigated and characterized the symptoms, virulence, and viral shedding in the faeces, sera, and various tissues in highly susceptible colostrum-deprived neonates experimentally inoculated with the S large-DEL type PEDV isolate, TTR-2/JPN/2014, compared with those in piglets inoculated with the North American type PEDV isolate, OKN-1/JPN/2013.

PEDV isolates, originally obtained from intestinal samples of PEDV-affected piglets with severe and moderate diarrhoea detected in Okinawa prefecture in September 2013 and in Tottori prefecture in October 2014, were maintained and stocked at the eighth passage in Vero cell culture as described in our previous study (Suzuki et al., 2015). Each virus stock was serially diluted 10-fold with minimum essential medium supplemented with 5 µg ml⁻¹ trypsin 1:250 and inoculated into Vero cells grown in 96-well plates (100 µl per well) with duplicate wells per dilution. The plates were incubated at 37°C with 5% CO₂ for 5 days. Viral cytopathic effects were monitored daily, and virus titres, expressed as TCID₅₀ ml⁻¹, were determined as described by Reed & Muench (1938). Whole-genome sequences of the OKN-1/JPN/2013 and TTR-2/JPN/2014 (GenBank accession numbers LC063836 and L022792, respectively) PEDV isolates were determined using a next-generation sequencer (Ion PGM; Thermo Fisher Scientific) as described previously (Murakami et al., 2015; Suzuki et al., 2015). Comparative sequence analysis showed that the genome sequences of the TTR-2/JPN/2014 PEDV isolate had high nucleotide identities (99.6–100%) with those of the OKN-1/JPN/2013 PEDV isolate in six remaining genes except the S gene (85.7%).

Colostrum-deprived newborn piglets were obtained from two specific-pathogen-free sows in our institute according to the guidelines for proper conduct of animal experiments. Nineteen piglets (6 days old) were randomly separated into three groups: group 1 (piglets inoculated with the OKN-1/JPN/2013; n=8), group 2 (piglets inoculated with TTR-2/JPN/2014; n=7), and group 3 (negative control; n=4). Individuals from groups 1 and 2 were inoculated orally with 10¹⁴ TCID₅₀ per head and 10¹⁴ TCID₅₀ per head, respectively. Rectal swab samples were collected from each piglet every two days from 0–8 days p.i. and every 3–4 days after 8 days p.i. Four piglets from each group were euthanized for pathological examination at 2 days p.i. The remaining piglets from groups 1 and 2 were monitored for clinical signs and viral shedding until 25 days p.i. Rectal swab samples were prepared as 20% suspensions diluted in PBS and were subjected to centrifugation at 3000 g for 10 min at 4°C to remove debris. Viral RNA was extracted from 20% suspensions, sera, and 10% tissue homogenates using the QIAamp viral RNA mini kit (Qiagen) according to the manufacturer’s instructions. Titres of virus shed in the faeces, sera and various tissues were determined using real-time RT-PCR; viral standards with known titres were used for quantification. Real-time RT-PCR was performed using a Takara Onestep PrimeScript RT-PCR kit (Takara Bio), with PEDV N gene-specific primers (forward: 5'-GAA TTCCCAAGGGCGAAAT-3', and reverse: 5'-TTTTTCGAAATTCGCACTTC3') and FAM-labeled probe (5'-G TACGACGGTCTGCTCAGCCA-3'). For the PCR, an ABI 7500 (Thermo Fisher Scientific) was used with the following conditions: one cycle of 42°C for 5 min, one cycle of 95°C for 10 s, and 45 cycles of 95°C for 5 s and 60°C for 35 s.

Small and large intestines and other organs (lung, heart, liver, spleen, kidney, trachea, tonsil, muscle, stomach, and mesenteric lymph nodes) were collected from PEDV-infected and control piglets at 2 days p.i., fixed in 10% formalin, embedded in paraffin, sectioned, and mounted on glass slides. The tissues were stained with haematoxylin and eosin and PEDV-specific rabbit antisera as previously described (Matsubayashi et al., 2016). Five representative villi and crypts with integrated longitudinal sections were randomly selected from stained duodenum, jejunum and ileum samples. Villus heights and crypt depths in various small intestine tissues were measured blindly by two veterinary pathologists using a computerized image system. Antigen detection by immunohistochemistry (IHC) was semiquantitatively scored based on the percentage of villous enterocytes in sections showing positive staining signals.

Statistical analysis was performed with linear mixed models. Villus length and crypt depths were independently treated as fixed and random effects, respectively. Coefficients for PEDV-inoculated groups were diagonally treated as fixed and random effects, respectively. Pedigrees for PEDV-inoculated groups were diagnosed as statistically significant if P values were <0.05.

All pigs recovered and survived within one week, although multiple sows and piglets exhibited moderate diarrhoea at the farm where the S large-DEL variant had been detected (Masuda et al., 2015). To evaluate the true pathogenicity of this variant, colostrum-deprived piglets, who are highly susceptible to causative pathogens, were experimentally infected as described previously (Jung et al., 2015). Piglets inoculated with the TTR-2/JPN/2014 PEDV isolate exhibited acute watery diarrhoea from 1–14 days p.i., and all piglets recovered to mild diarrhoea and survived until 25 days p.i. In contrast, all piglets inoculated with the OKN-
1/JPN/2013 PEDV isolate, at 1/1000 the amount of the TTR-2/JPN/2014 PEDV isolate, showed lethargy, loss of body-weight, and severe dehydration from 3–4 days p.i., and all piglets died by 4 days p.i. These findings indicate that the OKN-1/JPN/2013 PEDV isolate is lethal, while the TTR-2/JPN/2014 PEDV isolate is non-lethal in sensitive piglets. This phenomenon has been reported in transmissible gastroenteritis virus (TGEV), a member of the genus Alphacoronavirus. In fact, porcine respiratory coronavirus (PRCV), a variant potentially naturally derived from TGEV, showed low pathogenicity and tropism switching because of a deletion of 224 amino acids in the N-terminus of the S protein (Pensaert et al., 1986; Gallagher et al., 2001). Our data suggest that the PEDV S gene is genetically similar to that of TGEV. Thus, in Alphacoronaviruses, variations in the S gene may be closely related to virus pathogenicity and tissue tropism.

All piglets inoculated with the OKN-1/JPN/2013 PEDV isolate were positive in PEDV-specific PCR from 1 day p.i. in faecal samples and showed peaks of virus shedding for $10^{11}$–$10^{13}$ genome equivalents (GE) ml$^{-1}$ at 1–2 days p.i.; these virus shedding titres were sustained until death (Fig. 1a). All TTR-2/JPN/2014 PEDV-inoculated piglets exhibited peaks of virus shedding of $10^{12}$ GE ml$^{-1}$ at 2 days p.i., and virus shedding was high in the faeces for approximately one week and then gradually decreased, reaching undetectable levels by PCR at 24 days p.i. Viral RNAs with a range of $10^{4}$–$10^{6}$ GE ml$^{-1}$ were also detected in the sera of OKN-1/JPN/2013-inoculated piglets at 2 and 4 days p.i. (Fig. 1b). Virus shedding in the sera of piglets inoculated with the TTR-2/JPN/2014 PEDV isolate peaked at 2 days p.i. for $10^{6}$–$10^{8}$ GE ml$^{-1}$, and decreased gradually thereafter. We detected large amounts of faecal virus shedding and lower amounts of virus shedding in the serum of piglets inoculated with the two types of PEDVs, consistent with a previous report (Madson et al., 2016). Moreover, nearly all piglets inoculated with the OKN-1/JPN/2013 PEDV isolate showed peak virus shedding in faeces at 1 day p.i., and all piglets inoculated with the TTR-2/JPN/2014 PEDV isolate exhibited peak virus shedding in faeces at 2 days p.i., despite the marked difference in inoculum amounts between the two groups. These results suggested that there were differences in the rate of viral replication between the two PEDVs. However, we could not confirm that the growth rate of the OKN-1/JPN/2013 PEDV isolate differed from that of the TTR-2/JPN/2014 PEDV isolate in Vero cell culture (Masuda et al., 2015). Virus distributions in various tissues from both PEDV-inoculated groups were examined at 2 days p.i. using PEDV-specific PCR (Fig. S1, available in the online Supplementary Material). In

![Fig. 1. Virus shedding in rectal swabs (a) and serum samples (b) from OKN-1/JPN/2013- and TTR-2/JPN/2014-inoculated piglets. Individual virus shedding (shown in different colours) was detected by a quantitative PEDV N specific real-time RT-PCR at each time point.](http://jgv.microbiologyresearch.org)
the OKN-1/JPN/2013 PEDV-inoculated group, viruses were mainly detected in intestinal tissues from the proximal jejunum to the colon and in the mesenteric lymph nodes, with a range of $10^6$–$10^{10}$ GE ml$^{-1}$. In TTR-2/JPN/2014 PEDV-inoculated piglets, viruses were widely distributed in the small and large intestines and in the mesenteric lymph nodes at high levels ($10^6$–$10^{12}$ GE ml$^{-1}$). In addition, virus antigens were detected only in the intestines from both PEDV-inoculated groups by IHC staining. The detection of virus antigen indicated that virus replication was active in the examined tissues; thus, PEDV may not replicate in non-enteric tissues, where low levels of PEDV genomic RNA were detected, as reported previously (Madson et al., 2016).

All faecal, serum and tissue samples from piglets in the negative-control group were negative by PEDV-specific PCR.

Severe villus blunting and atrophy were observed in the small intestine from the duodenum to the ileum in all OKN-1/JPN/2013 PEDV-inoculated piglets at 2 days p.i. (Fig. S2). In contrast, lesions with moderate or severe villus atrophy were observed in the distal jejum to the ileum, but not in the duodenum to the middle jejum in the TTR-2/JPN/2014 PEDV-inoculated group. No significant microscopic lesions were observed in small intestinal tissues in negative-control piglets at 2 days p.i. Villus heights and crypt depths in small intestinal samples at 2 days p.i. were measured using a computerized image system and compared between the two PEDV-inoculated and negative-control groups (Fig. 2). The OKN-1/JPN/2013 PEDV-inoculated piglets showed significantly decreased average villus heights in various small intestinal tissues from the duodenum to the ileum compared with the negative controls. Average villus heights of the TTR-2/JPN/2014 PEDV-inoculated piglets did not significantly differ from those of the negative-control piglets in the duodenum and proximal and middle jejunums, but were significantly shorter than those of controls in the distal jejum and ileum. Moreover, villus lengths of the TTR-2/JPN/2014 PEDV-inoculated group were significantly different from those of the OKN-1/JPN/2013 PEDV-inoculated group in the three parts of the jejunum. There were no significant differences in the average crypt depths in small intestinal tissues among the three groups. PEDV antigens were detected by IHC in the cytoplasm of villus enterocytes from the duodenum to the colon in the two PEDV-inoculated groups, but not in the negative-control group at 2 days p.i. (Fig. S3). The patterns of positive signals by IHC staining differed between the two PEDV-inoculated groups (Table 1). OKN-1/JPN/2013 PEDV-inoculated piglets showed increased IHC scores in the proximal and middle jejum and decreased IHC scores from the distal jejum to the colon. In contrast, IHC scores in TTR-2/JPN/2014 PEDV-inoculated piglets were low in the proximal and middle jejum, high in the distal jejum to the colon, and negative in the duodenum. No positive staining was observed in various tissues other than the intestines in both PEDV-inoculated groups. Our findings suggest that there were differences in tissue tropism between the two types of PEDVs, which contain variations in the S gene. Recently, Deng et al. (2016) reported that the C-terminal domain (CTD) of the PEDV S1 domain is responsible for binding to porcine aminopeptidase N as its major receptor, and the N-terminal domain (NTD) of the PEDV S1 domain is responsible for binding to sugar, which acts as a co-receptor. Additionally, the NTD of the TGEV S1 domain is responsible for enteric tropism; PRCV, which lacks this domain, does not show enteric tropism (Sanchez et al., 1992). The TTR-2/JPN/2014 PEDV isolate has large deletions in the NTD of PEDV S1 domain, which is

### Table 1. Immunohistochemical (IHC) scores of various intestinal tissues of two types of PEDV-inoculated piglets and negative-control piglets at 2 days post-infection

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<tr>
<th>Group</th>
<th>ID</th>
<th>Duodenum</th>
<th>Proximal jejum</th>
<th>Middle jejum</th>
<th>Distal jejum</th>
<th>Ileum</th>
<th>Cecum</th>
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Criteria of IHC scores: –, no staining; +, 1–10 %; ++, 11–24 %; ++++, 25–50 %; ++++, 51–100 % enterocytes with positive staining; ND, not determined.
different from that of PRCV. Therefore, the TTR-2/JPN/2014 PEDV isolate may be distributed from the distal jejunum to the colon widely possessing enteric tropism but not respiratory tropism.

In conclusion, we demonstrated that the novel PEDV variant TTR-2/JPN/2014, which harbours a large deletion in the S gene, was non-lethal in highly susceptible neonatal piglets. Moreover, there was an important region within the deletion of 582 nucleotides in the S gene that was responsible for tissue invasion, directivity, and virulence in newborn piglets. We will further examine the function of the S gene and the important regions regulating these features using reverse genetic technology.

Acknowledgements


References


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