Isolation and experimental inoculation of an S INDEL strain of porcine epidemic diarrhea virus in Japan

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ABSTRACT

In 2013, porcine epidemic diarrhea (PED) was reported in Japan for the first time in 7 years and caused significant economic losses. In the present study, we isolated PED virus (PEDV) circulating in Japan using Vero cell cultures and analyzed sequences of S1 genes of these PEDV isolates. Sequence analysis revealed that one of these strains contained distinct insertion and deletions in the S gene (i.e., S INDEL). Furthermore, inoculation of PEDV into 1-week-old pigs demonstrated that the S INDEL strain had a lower pathogenicity than the North American (NA) prototype strain. This is the first report comparing pathogenicity of an S INDEL strain with the NA prototype strain following experimental inoculation. Excretion of PEDV in the feces of S INDEL strain-inoculated pigs occurred later than in NA prototype strain-inoculated pigs. Thus, our findings suggested that the S INDEL strain had different viral dynamics than the NA prototype strain.

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Porcine epidemic diarrhea (PED) is an acute infection caused by porcine epidemic diarrhea virus (PEDV) (Debouck and Pensaert, 1980; Pijpers et al., 1993; Saif et al., 2012). In suckling piglets, PED has a high mortality rate approaching 100%. In Japan, PED was first recognized in 1982 (Takahashi et al., 1983). In October 2013, PED was reported for the first time in 7 years; in this outbreak, PED spread nationwide and caused severe economic losses.

PEDV, belonging to the family Coronaviridae, genus *Alphacoronavirus*, is an enveloped virus possessing a single-stranded, positive-sense RNA genome of approximately 28 kb (Pensaert and Debouck, 1978; Sun et al., 2012). The genome is composed of 5′ and 3′ untranslated regions (UTRs) and seven open reading frames, which encode four structural proteins (spike [S], envelope [E], membrane [M], and nucleocapsid [N]) and three nonstructural proteins (Duarte and Laude, 1994; Duarte et al., 1994; Kocherhans et al., 2001). The PEDV S protein is a type I glycoprotein composed of 1383–1386 amino acids (aa), depending on the strain (Oh et al., 2014). Although PEDV has an uncleaved S protein because of the lack of a furin cleavage site, the S protein can be divided into S1 and S2 domains based on its homology with S proteins of other coronaviruses (Sturman and Holmes, 1984; Duarte et al., 1994; Jackwood et al., 2001). Similar to that in other coronaviruses, the PEDV S protein, primarily S1 protein, plays an important role in regulating interactions with cellular receptor glycoproteins to mediate virus entry and contains neutralizing epitopes (Chang et al., 2002; Bosch et al., 2003; Lee et al., 2011; Oh et al., 2014). The PEDV S1 gene exhibits frequent variation between PEDV strains (Chen et al., 2012; Sun et al., 2012; Chen et al., 2014; Sun et al., 2014). Therefore, the S1 region of

the genome is considered to be useful for determining the genetic relationships of different PEDV isolates (Chen et al., 2014). In 2014, new variants of PEDV (OH851 and CH/HBQX/10) were reported in the USA and China (Zheng et al., 2013; Wang et al., 2014). These strains, called S INDEL strains, have three deletions and one insertion in comparison with the sequence of the initially reported prototype strain in the North America (NA prototype strain). S INDEL strains are thought to be associated with reduced severity of clinical disease in pigs under field conditions (Vlasova et al., 2014; Wang et al., 2014).

In the present study, we collected 11 fecal samples and 14 small intestinal tissue samples from four farms in Japan from March to May 2014. The samples were homogenized with the Eagle's minimum essential medium (EMEM) to make the 20% suspension. We attempted to isolate PEDV field strains in Vero cell cultures, as previously reported (Hofmann and Wyler, 1988; Kusanagi et al., 1992; Shibata et al., 2000), with a few modifications. At 7 days post inoculation (dpi), cytopathic effects (CPEs) were detected from the first to third passage in three samples inoculated with suspensions of small intestine tissue samples. In other samples, CPEs were not detected within five blind passages. The cells exhibiting CPEs were destroyed with three freeze-thaw cycles and centrifuged, and the supernatants were collected. Viral RNA was extracted from the supernatants using a QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed at 42 °C for 30 min, followed by predenaturation at 99 °C for 5 min with reaction mixtures containing 1 μL of sample RNA, 1 μL of a 10× PCR Gold Buffer, GeneAmp dNTP mix (0.8 mM of each dNTP), 2.5 mM of MgCl2 solution, 10 U RNase inhibitor, 25 U MuLV Reverse Transcriptase (Applied Biosystems, CT, USA), 2.5 μM of each specific sense and antisense primer, and up to 10 μL per sample of RNase-free water. The primers were designed based

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on the reported sequence of PEDV (Supplemental Table 1). The PCR conditions were as follows: 95 °C for 15 min; 40 cycles of 94 °C for 30 s, 42 °C for 30 s, and 72 °C for 2 min; and 72 °C for 10 min with reaction mixtures containing 10 μL of sample cDNA, 4 μL of a 10× PCR Buffer, 1 mM $MgCl_2$ solution, 1.25 U of Ampli Taq Gold (Applied Biosystems), and up to 50 μL per sample of RNase-free water. The supernatants collected from three CPE-positive samples were positive for PEDV by

RT-PCR. These three isolated PEDV strains were referred to as ZK-O, ZK-CTK, and ZK-CHR (accession numbers LC053343, LC053344, and LC053345, respectively).

In order to determine the sequences of the S1 genes of PEDV isolates, the purified RT-PCR products were sequenced by FASMAC Co., Ltd. (Kanagawa, Japan). S1 domain sequences were compared with genome sequences of several PEDV strains in GenBank. Sequence data were
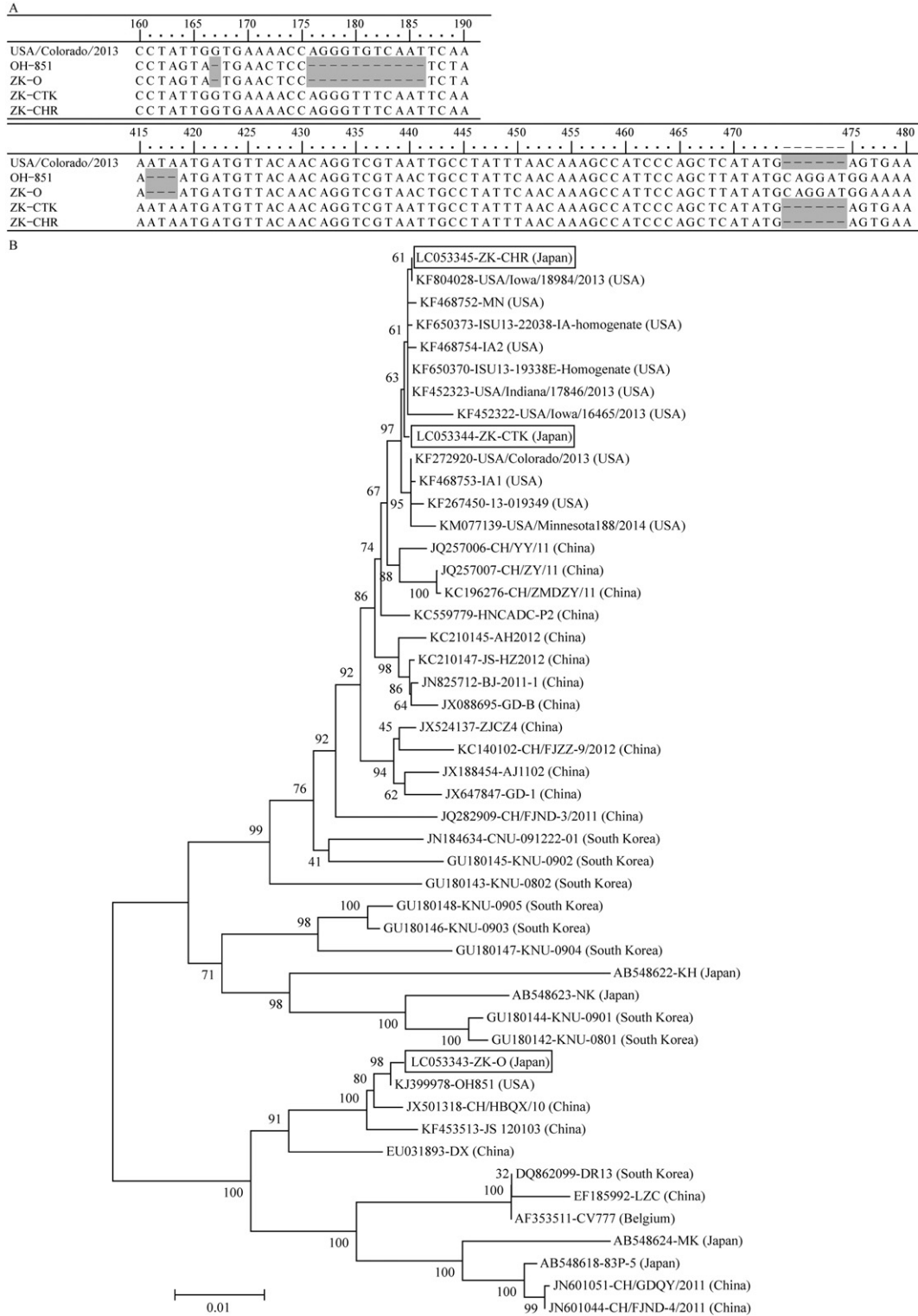
Fig. 1. (A) Alignment of partial S gene sequences of three PEDV isolates in this study and of the USA/Colorado/2013 strain (the NA prototype strain) and the OH-851 strain (S INDEL strain). (B) Phylogenetic trees of the S1 domain of PEDV isolates. The dendrograms were reconstructed by the neighbor-joining method using the MEGA 6 program. GenBank accession numbers are also shown.

aligned by the CLUSTAL W method using MEGA 6 software (Tamura et al., 2013). The trees were constructed by the neighbor-joining method with 1000 bootstrap replications using MEGA 6 software (Saitou and Nei, 1987). Genetic distances were calculated using Kimura's two-parameter correction at the nucleotide level (Kimura, 1980). Similar to OH-851 (an S INDEL strain), ZK-O had three deletions and one insertion in the S1 region as compared with the USA/Colorado/2013 strain (the NA prototype strain). ZK-CTK and ZK-CHR did not have these mutations (Fig. 1A). Thus, the ZK-O strain was classified as an S INDEL strain, whereas the other strains (ZK-CTK and ZK-CHR) were the NA prototype strains (Fig. 1B).

Next, we investigated the pathogenicity and viral dynamics of the two types of PEDVs isolated after inoculating piglets with the virus. Ten 1-week-old specific-pathogen-free (SPF) pigs were obtained from a PED-negative farm. The experimental protocol was approved by the Animal Care and Use Committee of the Zen-noh Institute of Animal Health. The pigs received care in accordance with the guide for Animal Experimentation of Zen-noh Institute of Animal Health. The pigs were divided into two groups (n = 5) and oral inoculation with 2 mL of isolated ZK-O (1.1 LogTCID$_{50}$/mL) and ZK-CHR (1.6 LogTCID$_{50}$/mL) virus stock was performed for pigs in each group. The virus stock contained 8.34×10^7 and 6.38×10^7 genome copies/mL, respectively, determined using real-time RT-PCR. Real-time RT-PCR was performed using an Ag Path-ID One-Step RT-PCR Kit (Life Technologies, MA, USA) according to the manufacturer's instructions. The sense and antisense primers (0.4 μM) and FAM-BHQ probe (0.2 μM) were used as previously reported (University of Minnesota, 2015). At 7 dpi, all pigs were sacrificed by exsanguination under anesthesia (0.03 mg/kg xylazine i.m., 0.5 mg/kg midazolam i.m., and 12.96 mg/kg pentobarbital sodium i.v.).

Clinical observations were performed twice a day. Four pigs from the group inoculated with ZK-CHR developed severe watery diarrhea, and one pig developed mild diarrhea. In contrast, in the group inoculated with ZK-O, two pigs did not develop diarrhea, while two pigs developed only mild diarrhea (Fig. 2A). Although all pigs in the group inoculated with ZK-CHR developed anorexia and exhibited lethargic behavior, no pigs inoculated with ZK-O exhibited these symptoms. Mortality rate is a useful measure of the pathogenicity of PEDV strains because suckling pigs exhibit high mortality rates in the field (Sun et al., 2012). In our study, no pigs from either group died within the experimental period. The pigs used in this experiment may have been too old to observe this effect. However, by assessing clinical symptoms, we confirmed that the S INDEL strain had a lower pathogenicity than the NA prototype strain, consistent with previous predictions.

Fecal samples were collected once a day and homogenized with PBS to make a 10% suspension. At 1 dpi, one pig inoculated with ZK-O and four pigs inoculated with ZK-CHR excreted PEDV in their feces. The next day, feces from two other pigs inoculated with ZK-O and one other pig inoculated with ZK-CHR were positive for PEDV in real-time RT-PCR. The other two pigs inoculated with ZK-O began to excrete PEDV at 3 dpi. All pigs excreted PEDV in feces by 7 dpi. The average quantity of virus excreted in the feces generally increased throughout the experimental period in the group inoculated with ZK-O, whereas it peaked at 3 dpi for the group inoculated with ZK-CHR (Fig. 2B, Supplemental Table 2). These results suggest that the viral dynamics of the S INDEL strain differed from that of the NA prototype strain.

At 7 dpi, there was no thinning of the walls of the intestine in all pigs. Some small intestine samples were homogenized with EMEM to generate a 10% suspension. The quantity of PEDV in the suspension was almost identical between the two groups. Significantly more PEDV was found in the ileum than in the jejunum in both groups (Fig. 2C). The remaining intestinal samples and samples from other organs were submitted for histopathological examination. In the small intestines of some pigs, mild villous atrophy was observed. The severities of the lesions were similar between the two groups. No other tissues showed abnormalities. Immunohistochemically, PEDV-positive cells were not
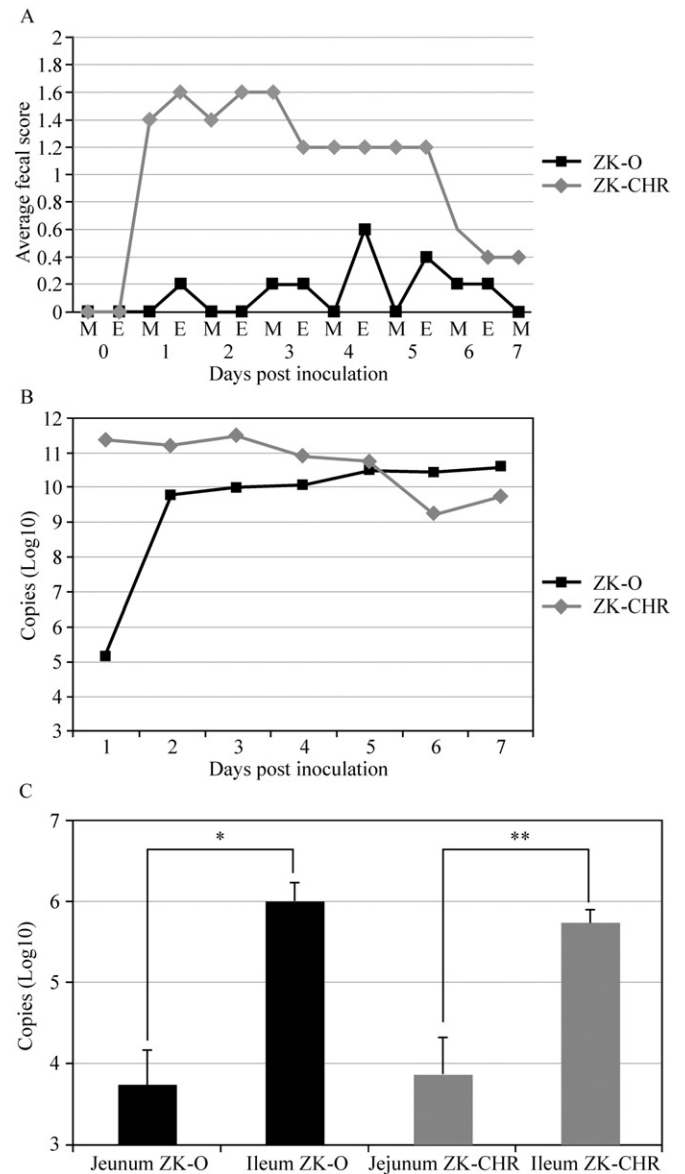
Fig. 2. (A) Average fecal score of inoculated pigs. (M: morning, E: evening, 0: normal, 1: loose, 2: watery). (B) Quantity of virus excreted into the feces, as determined by real-time RT-PCR. (C) Quantity of virus in suspensions of the small intestine, as determined by real-time RT-PCR. *: P < 0.05, **: P < 0.01 (Paired t-test).

detected in the small intestines of all pigs. These data suggested that almost all small intestine tissues reproduced within 7 dpi in the present experimental infection.

In summary, we isolated three PEDV strains, including an S INDEL strain, and confirmed that the S INDEL strain had lower pathogenicity than the NA prototype strain. This is the first report comparing the pathogenicity of an S INDEL strain with that of the NA prototype strain in an experimental infection model. Moreover, our findings suggested that the viral dynamics of the S INDEL strain differed from that of the NA prototype strain.

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Conflicts of interest

None.

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