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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.

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

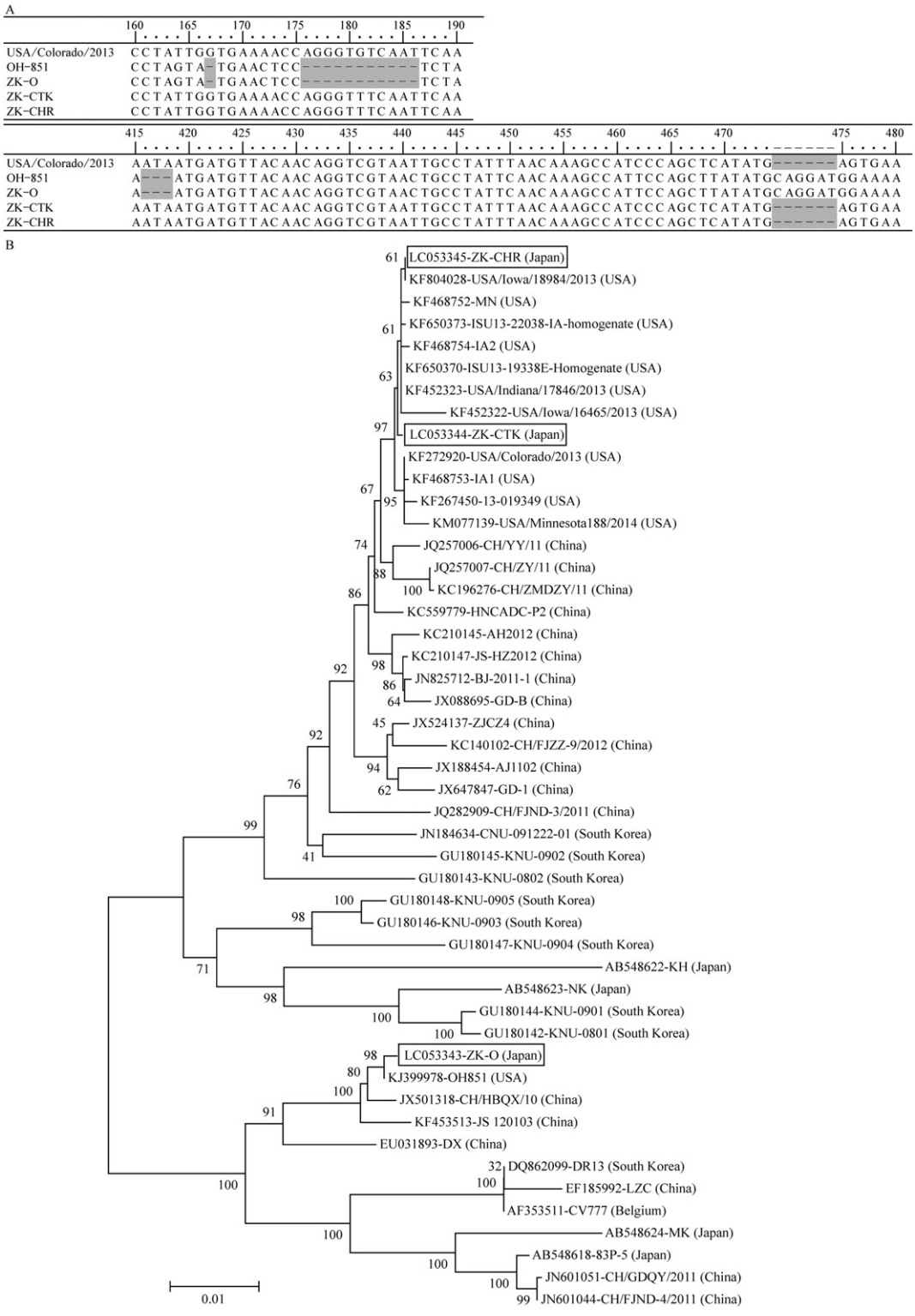

Fig. 1. (A) Alignment of partial S gene sequences of three PEDV isolates in this study and of the USA/Colorado/2013 strain (the NA prototype strain) and the OH-851 strain (S INDEL strain). (B) Phylogenetic trees of the S1 domain of PEDV isolates. The dendrograms were reconstructed by the neighbor-joining method using the MEGA 6 program. GenBank accession numbers are also shown.