

New porcine epidemic diarrhoea virus variant with a large deletion in the spike gene identified in domestic pigs

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Abstract From October 2013 to date, approximately 1,000 outbreaks of porcine epidemic diarrhoea virus (PEDV) have occurred in Japan. Porcine epidemic diarrhoea with non-lethal effects in piglets was identified in Tottori prefecture in October 2014. Complete genome analysis revealed that the causative pathogen, Tottori2, is a new PEDV variant with a large (582 nt) deletion in the spike gene. Phylogenetic analysis indicated that the Tottori2 PEDV strain might have been derived from the current PEDV strains circulating in domestic pigs. Moreover, the Tottori2 PEDV strain was successfully isolated in Vero cells by serial passage.

Porcine epidemic diarrhoea (PED) was first discovered in the United Kingdom in 1971 [1], and outbreaks of this disease were reported in European and Asian countries during the 1970s and 2000s [2–4]. In April 2013, the first case of PED in the United States was identified, and the disease then rapidly became widespread in Canada,

Mexico, Korea, Taiwan, and Germany [5–10]. The disease is caused by the porcine epidemic diarrhoea virus (PEDV), an enveloped, single-stranded positive-sense RNA virus belonging to the family *Coronaviridae*. PED is characterised by severe watery diarrhoea, leading to dehydration and a high mortality rate in piglets [4, 7]. In October 2013, outbreaks of PED re-emerged in Japan after an absence of 7 years. From October 2013 to date, PED has occurred at about 1,000 farms throughout Japan, causing the deaths of about 440,000 pigs according to a report by the Ministry of Agriculture, Forestry and Fisheries (MAFF) (<http://www.maff.go.jp>). Phylogenetic analysis of the full-length spike (S) gene from several PEDV strains detected in Japan between 2013 and 2014 showed that some strains were classified as highly virulent types, including recent global strains from the US, Canada, China, Korea, and Taiwan, while other strains were grouped into the S INDEL type, including the global strains from the US, China, and Germany (data not shown).

In October 2014, the second PED case was identified in Tottori prefecture, Japan, following the first PED case detected in March 2014. First, suckling pigs (7 days old) from several sows showed mild diarrhoea and vomiting in a breed-to-wean farm (a total of 500 sows). Although the disease had spread to 7 sows and 120 piglets, all affected pigs recovered within a week, and no piglets died from the diarrhoea. The sows on this farm had never been vaccinated with modified live PEDV, because the farm had a high level of biosecurity. In addition, there was no prevalence of PEDV antibody in the sera of gilts introduced from other prefectures at 2-month intervals for the last 6 months prior to the outbreak.

Small-intestine specimens were sampled from eight piglets with watery diarrhoea. The intestine samples were prepared as 10 % suspensions in phosphate-buffered saline

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Colorado/USA/2013	1:MKSLTYFWLFLPVLSTLSLPQDVTRCSANTNFRFFSKFNVQAPAVVVLGGYLPIGENQG	60
Tottori2/JPN/2014	1:MKSLTYFWLFLPVLSTLSLPQD-----	22
PC177/USA/2013	1:MKSLTYFWLFLPVLSTLSLPQDVTRCSANTNFR-----	33

Colorado/USA/2013	61:VNSTWYCAGQHPTASGVHGFVSHIRGGHGFEGISQEPDPSGYQLYLHKATNGNTNAT	120
Tottori2/JPN/2014	22:-----	22
PC177/USA/2013	33:-----	33
Colorado/USA/2013	121:ARLRICQFPSIKTLGPTANNDVTGRNCLFNKAIPAHMSEHSVVGITWDNDRVTVFSDKI	180
Tottori2/JPN/2014	22:-----	22
PC177/USA/2013	33:-----	33
Colorado/USA/2013	181:YFYFKNDWSRVATKCYNSGGCAMQYVYEPTYMLNVTSAGEDGISYQPCTANCIGYAAN	240
Tottori2/JPN/2014	23:-----VTSAGEDGISYQPCTANCIGYAAN	46
PC177/USA/2013	34:-----TANCIGYAAN	43

Colorado/USA/2013	241:VFATEPNGHIPEGFSFNNWFLLSNDSTLVHGKVVSNQPLLNVNCLLAIPKIYGLGQFFSFN	300
Tottori2/JPN/2014	47:VFATEPNGHIPEGFSFNNWFLLSNDSTLVHGKVVSNQPLLNVNCLLAIPKIYGLGQFFSFN	106
PC177/USA/2013	44:VFATEPNGHIPEGFSFNNWFLLSNDSTLVHGKVVSNQPLLNVNCLLAIPKIYGLGQFFSFN	103

Colorado/USA/2013	301:QTIDGVCNGAAVQRAPEALRFNINDTSVILAEGSIVLHTALGTNFSFVCSNSSNPHLATF	360
Tottori2/JPN/2014	107:QTIDGVCNGAAVQRAPEALRFNINDTSVILAEGSIVLHTALGTNFSFVCSNSSNPHLATF	166
PC177/USA/2013	104:QTIDGVCNGAAVQRAPEALRFNINDTSVILAEGSIVLHTALGTNFSFVCSNSSNPHLATF	163

Colorado/USA/2013	361:AIPLGATQVPYYCFLKVDTYNSTVYKFLAVLPPTVREIVITKYGDVYVNGFGYLHLGLLD	420
Tottori2/JPN/2014	167:AIPLGATQVPYYCFLKVDTYNSTVYKFLAVLPPTVREIVITKYGDVYVNGFGYLHLGLLD	226
PC177/USA/2013	164:AIPLGATQVPYYCFLKVDTYNSTVYKFLAVLPPTVREIVITKYGDVYVNGFGYLHLGLLD	223

Fig. 1 Amino acid sequence alignments of partial S proteins (aa 1–420) from the Tottori2 PEDV strain and two reference US PEDV strains, Colorado/USA/2013 (highly virulent type of PEDV) and

PC177/USA/2013 (197-aa deletion). Deletions are labelled as '-'. The conserved amino acid residues are labelled as '*'

(PBS) following centrifugation ($3,000 \times g$ for 10 min at 4 °C) and filtration. Viral RNA was extracted from the 10 % suspensions using a QIAamp Viral RNA Mini Kit (QIAGEN, Venlo, Limburg, the Netherlands) and subjected to amplification by reverse transcription PCR (RT-PCR) with a set of primers (S1-20320-F, 5'-AACACGT CATCGTCAGAGGC-3'; S1-21816R, 5'-CGGTTGGAGG TAAACAGC-3'; S2-U, 5'-CTGATTCTGGACAGTTG TTA-3'; S2-1L, 5'-TTGGACAGCATCCAAAGACA-3') for the PEDV S1 (5'-terminal portion of the S gene) and S2 regions (3'-terminal portion of the S gene) as described previously [11, 12]. The PCR product of the S1 region from this field strain, designated as Tottori2, is approximately 600 nucleotides (nt) shorter than the expected size (1,500 nt). To determine the full-length S nucleotide sequence from the Tottori2 PEDV strain, the products of the PEDV S gene were cloned into a TA cloning vector and sequenced by cycle sequencing using an automated sequencer (ABI PRISM 3130; Life Technologies, Carlsbad, CA, USA). In addition, the cDNA of the Tottori2 PEDV strain was synthesised using a random hexamer primer. The entire genome (except for 48 nt at the 5' end

and 66 nt at the 3' end) with reference to the US PEDV strain USA/Colorado/2013, consisting of eight overlapping amplicons (approximately 5 kb in length), was generated from the cDNA using sets of primers originally designed with reference to 74 US PEDV strains described previously [7, 13]. Eight amplicons were pooled in equal amounts and analysed using a next-generation sequencer (Ion Torrent PGM; Life Technologies). The consensus full-genome sequence of the Tottori2 PEDV strain was determined with reference to the complete genome of the US PEDV strain published in GenBank. Phylogenetic analysis for the S gene and the entire genome were performed using the maximum-likelihood method with the general time-reversible nucleotide substitution model and bootstrap tests of 1,000 replicates in the MEGA 6 program [14].

Virus isolation was attempted from 10 % PCR-positive suspensions of Vero cells. After the suspensions were inoculated onto the cells at 37 °C for 1 h, the inoculum was removed, and Eagle's minimal essential medium containing 10 µg of trypsin per ml was added. Until the isolated virus was efficiently propagated in Vero cells, and clear cytopathic effect (CPE)—characterized by cell fusion and

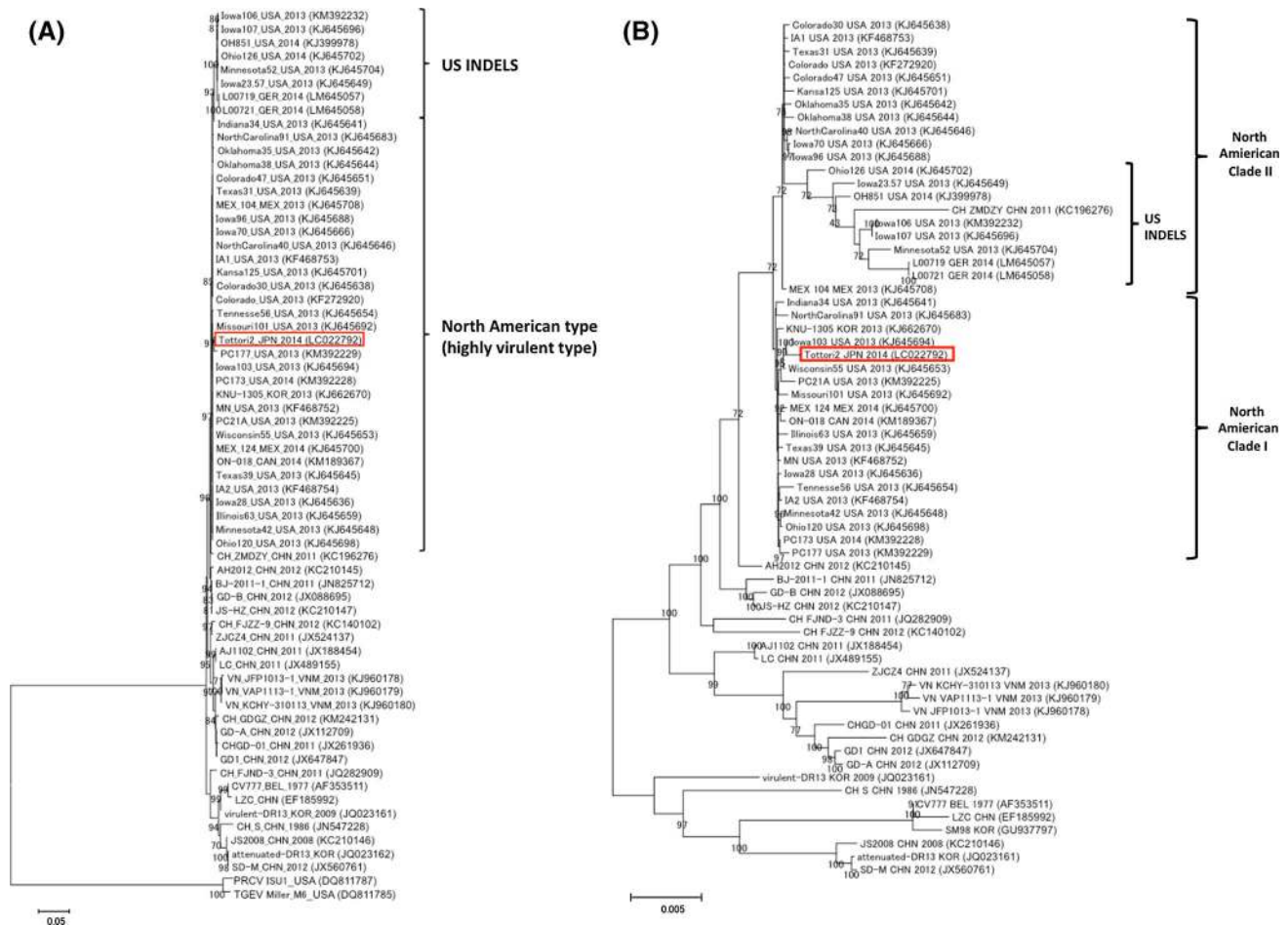


Fig. 2 Phylogenetic trees based on full-length nucleotide sequences of the spike gene (A) and complete genome sequences (B) of pig epidemic diarrhoea virus (PEDV) strains, including recent PEDV strains identified worldwide. TGEV and PRCV were used as outgroup controls. Dendrograms were constructed by using the maximum-likelihood method in the MEGA 6 program. A bootstrap test was

performed with 1,000 replicates; bootstrap values >70 % are indicated on each branch. Reference PEDV strains were obtained from GenBank: common names of strains, country and year of collection, and accession number are shown in parentheses. Types of PEDV strains are represented on the right. Scale bars indicate nucleotide substitutions per site

syncytium formation—was observed, serial passages were conducted at 7-day intervals.

We determined the full-length S nucleotide sequence from the Tottori2 PEDV strain by TA cloning. Comparison of the S1 sequences to the reference US PEDV strain showed that the S1 region of the Tottori2 PEDV strain was shorter than that of the USA/Colorado/2013 PEDV strain [13]. This demonstrates that the Tottori2 PEDV strain has a large deletion of 582 nt (194 aa) in the S1 region (Fig. 1). Phylogenetic analysis of S genes demonstrated that the Tottori2 PEDV strain belonged to a cluster including the reference PEDV strains and showed the closest genetic relationship to the US PEDV strain TC-PC177 (97.89 % identity at the nucleotide sequence level), which was derived from cell adaptation, with a large, 197-aa deletion (Fig. 2A). In addition, there was a difference in the pattern of deletion of the S1 amino acid sequences between

Tottori2 and the TC-PC177 PEDV strains (Fig. 1). The entire genome of the Tottori2 PEDV strain was finally determined to be 27342 nt in length, with no changes other than the large deletion in the S gene and the unidentified regions at the 5' and 3' termini. Comparative sequence analysis showed that the complete genome of the Tottori2 PEDV strain had 99.73 %–99.87 %, 99.64 %–99.69 %, and 98.90 %–99.45 % nucleotide sequence identity with those of North American clades I and II and US S INDEL PEDV strains used in this analysis, respectively. A phylogenetic dendrogram constructed using the sequence data without considering gaps indicated that the Tottori2 PEDV strain was included in a cluster in North American clade I and was most closely related to US PEDV strain Iowa103 (Fig. 2B). Hence, the Tottori2 PEDV strain detected in the present study might have spontaneously originated from the current PEDV strains in the field, and the deletion in the

S gene identified in this strain might be closely associated with its pathogenicity and be the reason for the non-lethal effect in affected pigs. In future studies, the pathogenicity of this strain needs to be confirmed by experimental infection of gnotobiotic pigs.

We succeeded in propagating the Tottori2 PEDV strain from 10 % intestinal suspensions in Vero cells. Despite the large deletion in the S protein, the Tottori2 isolate cultivated in Vero cells demonstrated a CPE similar to that of the reference PEDV isolate CV777 (Supplementary Fig. 1). Moreover, no clear difference in morphology was observed between the Tottori2 and CV777 PEDV strains.

In this study, we identified and characterized a new PEDV variant, Tottori2, with a 194-aa deletion in the N-terminal portion of the S protein, which might have arisen spontaneously from recent field PEDV strains. In coronaviruses, mutations in the S gene are known to be strongly involved in pathogenicity and tissue tropism [15, 16]. In fact, in porcine respiratory coronavirus (PRCV), a mutant that might have been naturally derived from transmissible gastroenteritis virus (TGEV) has low pathogenicity and tropism switching owing to a 224-aa deletion in the N-terminal portion of the S gene. In this study, the Tottori2 PEDV strain could grow in a cell culture system as successfully as other PEDV strains, but its virulence was much lower than that of other PEDV strains, as evidenced by the survival of affected piglets. In addition, the presence of PEDV in respiratory tracts and lungs collected from dead piglets were not detected by immunohistochemistry (data not shown). Therefore, our findings demonstrate that the deletions in the S gene identified in this strain would affect its virulence. Although it remains unknown how the PEDV variant has evolved, our data suggest that the S gene of PEDV might also be closely associated with its pathogenicity, similar to what has been observed for PRCV and TGEV. Further accumulation of extensive genomic information for novel PEDV variants and understanding their biological properties such as pathogenicity, tissue tropism, and transmissibility would contribute to the prevention and control of this disease, including the development of effective PEDV vaccines in the future.

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