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Short communication

Phylogenetic and antigenic characterization of newly isolated porcine epidemic diarrhea viruses in Japan

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ABSTRACT

To evaluate the mechanism by which a large outbreak of porcine epidemic diarrhea (PED) occurred in Japan, where the majority of sows are vaccinated, we isolated two new strains of PED virus (PEDV) from the intestines of piglets and found that they showed greater similarity to US isolates (group II PEDV) than to the Japanese vaccine strain (group I PEDV). We compared the antigenicity of the vaccine type strain and newly isolated strains by means of a neutralization test using sera from a number of pigs from various farms; the results revealed that they are antigenically similar. This is the first report of the similarity of group I and II viruses using sera from individual pigs vaccinated with group I virus. These data suggest that the large outbreak of PED in Japan cannot be attributed to inefficient vaccination but may be due to the extremely high virulence of the newly appearing viruses.

Porcine epidemic diarrhea virus (PEDV) is classified into the genus *Alphacoronavirus* in the family *Coronaviridae* of the order *Nidovirales*. It is an enveloped virus with a single-stranded, positive-sense genomic RNA of ca. 28 kb (Lee, 2015; Sung et al., 2015; Oka et al., 2014; Chen et al., 2014; Masters, 2006). PEDV infects the epithelial cells lining the small intestine of pigs and causes severe diarrhea, resulting in fatal dehydration in piglets (Lee, 2015; Liu et al., 2015; Song and Park, 2012).

PED was initially reported in Europe, and the causative virus, the PEDV CV777 strain, was first isolated in 1971 in Belgium (Chasey and Cartwright, 1978). Thereafter, PED spread to Asian countries, in which viruses were isolated from diseased pigs (Kweon et al., 1993; Cheng, 1992; Xuan et al., 1984; Takahashi et al., 1983). In the US, PEDV was first detected in May 2013 (Stevenson et al., 2013), and a huge outbreak of PED occurred in the US thereafter. By the end of April 2014, the outbreak had spread to 30 US states, causing the death of ca. 8 million pigs, most of which were piglets. The infection subsequently spread throughout North America, including Canada and Mexico (Vlasova et al., 2014). In October 2013, an outbreak of PED occurred in Japan after a 7-year absence. PED has affected more than 1000 farms throughout Japan, causing the deaths of ~440,000 piglets (Masuda et al., 2015), despite vaccination of pig herds nationwide.

Vaccination of sows is the principal strategy to control and eradicate epidemic or endemic PED outbreaks. Even though PED first emerged in Europe, PED outbreaks have become more serious issue in Asian countries, and therefore different kinds of PEDV vaccines have been developed in Asia. In China, CV777-attenuated or −inactivated vaccines have been regularly used. Inactivated, bivalent TGEV and PEDV vaccine (Ma et al., 1995) and attenuated, bivalent TGEV and PEDV vaccine (Tong et al., 1999) are available. Two South Korean virulent PEDV strains were also attenuated by the cell-culture adaptation and used as live or killed vaccine (Lee, 2015; Kweon et al., 1999). In Japan two different live vaccines are available, both of which belong to group I virus. These vaccines were made by attenuation of the virulence by serial passages through Vero cells (Lee, 2015; Sato et al., 2011). In spite of the vaccination, PED is prevalent in many Asian countries.

The present study was performed to determine why the large outbreak occurred in Japan, where PED vaccination has been performed. We first isolated two PEDV strains from infected

Abbreviation: aa, amino acid; cDNA, complementary deoxyribonucleic acid; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HR, heptad-repeat; NT, neutralization test; PEDV, porcine epidemic diarrhea virus; PBS, phosphate-buffered saline; RT-PCR, reverse transcription polymerase chain reaction; TCID, tissue culture infective dose; TPB, tryptose phosphate broth; US, United States.

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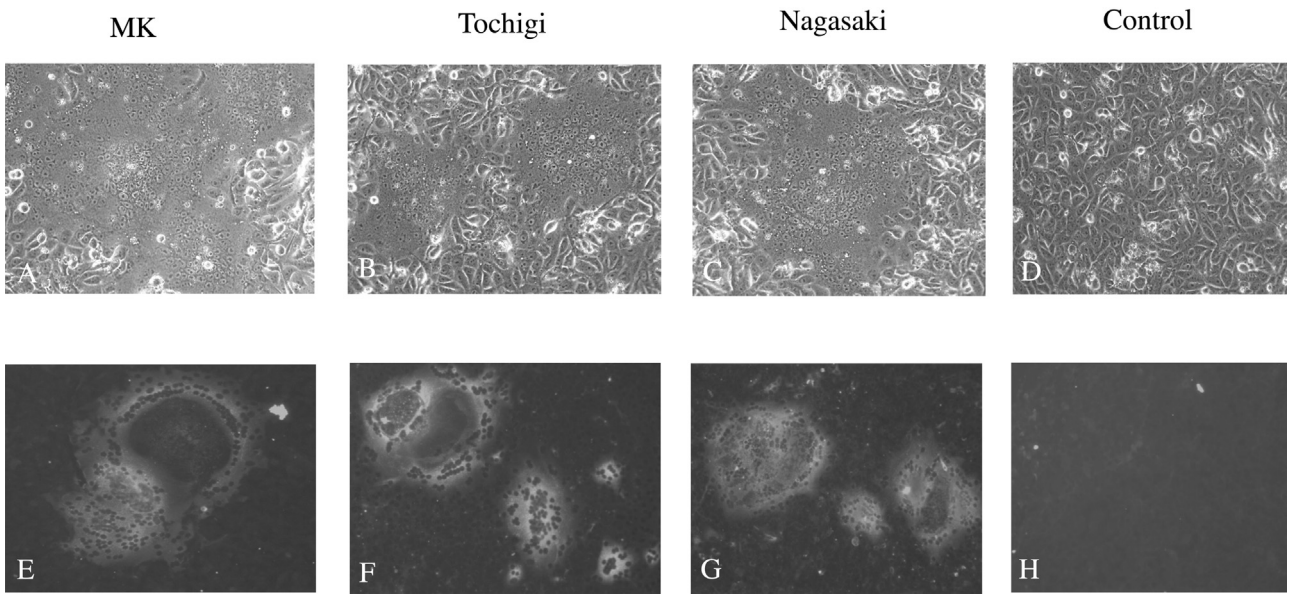

Fig. 1. Cytopathic effects and detection of viral antigen by immunofluorescence in Vero cells infected with the MK, Tochigi and Nagasaki strains. At 48 h postinfection, cytopathic effects were observed by phase-contrast microscopy (A–D). At the same time, PEDV-infected Vero cells were fixed with acetone-methanol, and the presence of virus-specific antigen was determined by immunofluorescence assay using anti-PEDV pig serum collected from pig vaccinated by group I vaccine but not infected by newly appearing PEDV and fluorescence isothiocyanate-conjugated goat anti-swine IgG (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) (E–H).

piglet intestines, which were designated Tochigi (LC144542) and Nagasaki (LC144543). These viruses were found to be phylogenetically similar to newly isolated US strains (group II) and different from CV777 and other strains classified in group I. A neutralization test (NT) showed no significant difference in antigenicity between our new isolates and a group I strain using a number of sera samples collected from pigs vaccinated with group I virus. Our results suggest that the PED outbreak in Japan was not caused by inefficient vaccination, but by the high virulence of newly circulating viruses.

We obtained specimens (feces and small intestines) from piglets infected by PEDV in 2014 from several prefectures in Japan and used 14 specimens PCR-positive for the PEDV N and S genes (data not shown). Seven samples were diarrheic feces, and the other seven were small intestines from piglets. We failed to isolate the virus from feces but successfully isolated two viruses from intestines obtained from Tochigi and Nagasaki prefectures. To isolate the virus, we used Vero-KY5 cells (Vero) (Suzuki et al., 2015), which were kindly provided by the National Institute of Animal Health (NIAH), Japan. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sigma-Aldrich, St. Louis, MO, USA), penicillin (100 IU/ml), and streptomycin (100 μg/ml) and 0.3% tryptose phosphate broth (TPB, Difco, Detroit, MI, USA). The transparent parts of the small intestine, indicating infection with PEDV, 10–15 cm in length, were filled with 5–10 ml phosphate-buffered saline (PBS), pH 7.2, and massaged gently to detach infected epithelial cells. PBS was collected, homogenized using a glass homogenizer and centrifuged at 5000 rpm for 10 min at 4 °C to remove cell debris. The supernatants were filtered through a 450 nm pore-size filter to remove contaminating bacteria. Diarrheic feces were diluted 10-fold with PBS and treated in the same manner as the intestines, described above. These samples were inoculated onto Vero cells prepared in 24-well plates by spinoculation, as described previously (Watanabe et al., 2006). Briefly, Vero cells seeded at a concentration of 2×10^5 per well in a 24-well culture plate (Falcon, Franklin Lakes, NJ, USA) were inoculated with 50 μl filtered samples and 400 μl DMEM per well. The plates were centrifuged at 3000 rpm for 1 h at 4 °C and then at 3000 rpm for 1 h at 24 °C. Then, cells were incubated with 0.5 ml DMEM supplemented with 5% TPB and trypsin (10 μg/ml), and cytopathic effects were monitored during incubation at 37 °C for 2–4 days. We also tried to isolate PEDV by conventional method, i.e., we inoculated specimens onto Vero cells and let the virus adsorb cells for 1 h at 37 °C and cultured the inoculated cells for 4–5 days in the presence of trypsin, Two PEDVs were isolated by spinoculation but not by conventional method, one from Tochigi and the other from Nagasaki. Both viruses produced syncytia in Vero cells, although the syncytia were smaller than those generated by the vaccine type MK strain, which is adapted to Vero cells (Kusanagi et al., 1992) (Fig. 1A–C). Syncytial cells contained viral-specific antigen, as revealed by immunofluorescence (Fig. 1E–G). The initial homogenate of the specimens, from which the virus was isolated successfully, were used to isolate viral RNA using an RNA isolation kit (Direct-Zol™ RNA Miniprep, Zymo Research, CA, USA). cDNA was then generated from the total RNA using the PrimeScript II First-strand cDNA Synthesis kit (Takara Bio Inc., Shiga, Japan). From the cDNA, three fragments covering the entire S gene were amplified using primers F1 and R1, F2 and R2 as well as F3 and R3 (Table 1). The PCR products were purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) and then sequenced using the primers shown in Table 1. Sequence analysis was performed by Eurofins Genomics, Tokyo, Japan, and amino acid (aa) sequences of the S proteins were deduced from the nucleotide sequences.

An aa alignment of the S protein of the two novel isolates, MK and three US PEDV strains (MN-KF468752, IA1-KF468753, and IA2-KF468754), is shown in Fig. 2. All of the US strains and Nagasaki and Tochigi were closely related genetically (99.42–99.97% aa identity) and differed from each other by 1–8 aa throughout the entire S protein. However, the new isolates and the US strains were genetically different from the vaccine type MK strain, ranging from 92.84–93.15% similarity (95–99 aa differences between MK versus Tochigi, Nagasaki and the other US strain). Alignment of the aa sequence of those strains identified two regions of aa deletions in the MK strain (aa 59–62 and 140) compared with the other strains. There was also one region of aa insertion in MK (aa 160 and 161) compared with the other strains (Fig. 2).

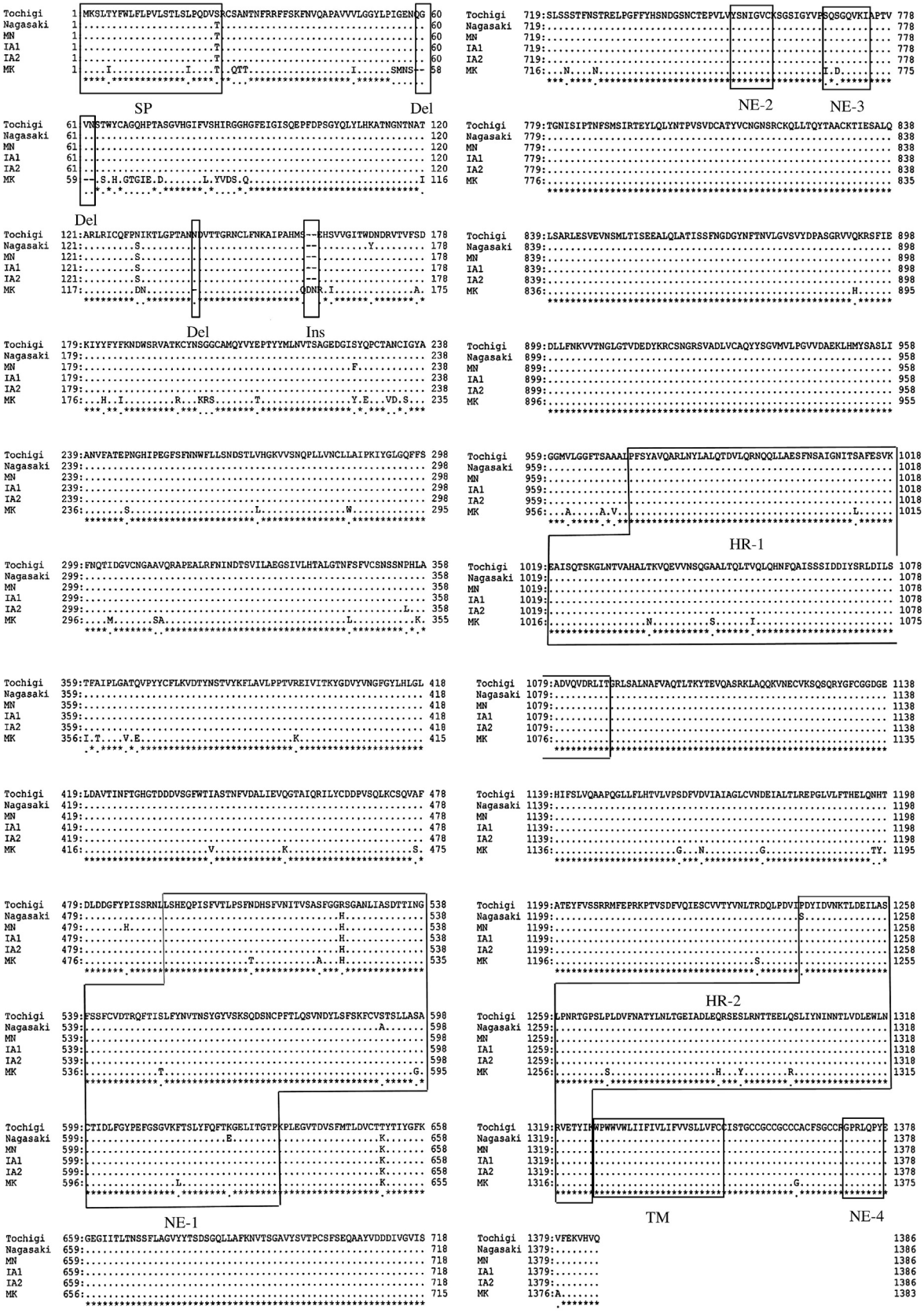

Fig. 2. Alignment of the S-protein amino acid sequences of six PEDV strains (Tochigi, Nagasaki and MK are described in the text, and MN, IA1 and IA2 are US isolates). Signal peptide (SP), deletion (Del), insertion (Ins), neutralizing epitope 1 to 4 (NE-1 to NE-4), heptad-repeat 1 and 2 (HR-1 and HR-2) and transmembrane domain (TM) are indicated by boxes.

Table 1
List of primers used for sequencing of S gene of PEDV.

Fragments of S gene	Primer name	Primer sequence (5′–3′)	Position in PEDV genome
S1	F1	gaatggtaagttgctagtgcg	20562–20582
	R1	cctgagaacacttgagttggc	22039–22059
	F-4	gggccccactgctaataatg	21035–21054
	R-4	ctcatatgagctgggatggc	21090–21109
	F-5	ggagctgctgtgcagcgtgc	21558–21577
	R-5	ccttcagcaagaatgacagagg	21610–21631
S2	F2	cagggaaattgtcatcacc	21818–21836
	R2	ccaccgatgagagacgcac	23494–23512
	F-6	ggtgccaaccttattgcatc	22209–22228
	R-6	gggcaattactgtcctgtg	22333–22351
	F-7	ggtgagggtatcattaccc	22608–22626
	R-7	ggctaacaactgtccagaatc	22668–22688
	F-8	gcacccacggttactggg	22956–22973
	R-8	gcacaatcaacactaacaggcg	23038–23059
S3	F3	cgctctgtggcagatctagtc	23403–23423
	R3	cattgagctccaactcttgg	24877–24896
	F-9	ccgtacagctgcaacacaac	23791–23810
	R-9	gctgataatctgccggtgatg	23891–23911
	F-10	gttgtgtggtcacctatgtc	24307–24326
	R-10	cgatgtaatctgggattac	24354–24372

A total of 45 PEDV viruses (2 isolated in this study, 3 US strains, the MK strain, 5 S-INDEL PEDV strains and 34 other globally endemic PEDV strains) were used for phylogenetic analysis based on the S protein (Fig. 3), which demonstrated that the new Japanese isolates were closely related to the new US isolates. Forty five PEDV strains clustered into group I or II. The above-mentioned five strains, but not the MK strain and seven Taiwan PEDV isolates as well as 5 S-INDEL PEDV strains, were clustered within group II together with 13 strains detected in China from 2011 to 2012. On the other hand, the European CV777-based vaccine strain, MK strain, Korean DR13 strain, and JS2008 were clustered into group I (Fig. 3). These results indicated that the PEDV currently circulating in Japan is classified into group II, as are US isolates, but in contrast to viruses previously isolated in Japan, which are classified in group I (Fig. 3). These data are in agreement with previous reports (Masuda et al., 2015; Suzuki et al., 2015) that the currently prevalent PEDV in Japan is similar to US isolates (group II), but different from Japanese vaccine type viruses (group I).

We next compared the antigenic properties of the new isolates with those of the vaccine-type MK strain by NT. The MK strain induces syncytium formation in the presence of trypsin (Shirato et al., 2010; Kusanagi et al., 1992), as did the new isolates Tochigi and Nagasaki, while the vaccine strain commercially available in Japan induces cell rounding and detachment without trypsin treatment. We therefore used the MK strain rather than the vaccine strain to perform NT, because the MK, Tochigi and Nagasaki strains can be examined under identical conditions. Moreover, there was no antigenic difference between Tochigi and Nagasaki in NT (data not shown). Two types of PEDV antisera were used. One type comprised 18 sera collected from herds of pigs that were vaccinated but not recently infected by PEDV, and the other comprised 17 sera from herds of pigs that were vaccinated and infected with a new PEDV strain. NT was performed as follows. Two hundred μl of serum were serially diluted in twofold steps with DMEM and then mixed with an equal volume of viral suspension containing 100 $TCID_{50}$/100 μl and incubated at 37 °C for 60 min. Then, 50 μl of each dilution were inoculated into each well (4 wells per dilution) of a 96-well plate containing confluent Vero cells and incubated at 37 °C for 1 h. Then, the mixture was removed, and cells were washed twice with DMEM and, finally, incubated at 37 °C with 50 μl DMEM containing 5% TPB and trypsin (10 μg/ml). Neutralization antibody titers were expressed as the reciprocal of the highest serum dilution that inhibited syncytium formation in at least two of four wells. As shown in Fig. 4A, all sera from pigs vaccinated but not infected by the new PEDV strain, namely pig farms vaccinated without recent PED incidence, showed almost identical neutralization titers against the MK strain and the new isolate. In some cases, the titer was twofold higher for group I virus compared with group II virus. These data indicate no significant difference in the antigenicity of viruses in terms of neutralization activity. A twofold difference in titer between group I and II viruses has also been reported by others (Wang et al., 2016). Similar results were obtained using sera from pigs vaccinated and infected with the new PEDV strain; namely pig farms vaccinated and recent PED incidence, (Fig. 4B). No pig serum showed a higher titer to the new isolates than to MK, suggesting that those pigs were not infected even if the farm was affected by the new PEDV strain. Alternatively, it could indicate that antiserum against group I vaccine also protected against infection by group II viruses, and thus no antibodies were elicited by group II viruses. Collectively, these data indicated that neutralizing antigenicity does not differ between MK (group I) and the new isolates (group II). Our NT showed for the first time that group I and II viruses have similar neutralizing antigenicity using a number of sera from individual pigs vaccinated with group I virus.

In the present study, we isolated two new PEDVs from piglets sacrificed during the PED outbreak in Japan. Although we firstly have tried to isolate new PEDV from more than 10 samples by conventional method, we failed isolation. Then we applied spinoculation and successfully isolated 2 viruses from 7 intestinal specimens but not from 7 feces, though isolation was not successful by conventional method. Feces were cytotoxic in spinoculation and conventional isolation. These results show that spinoculation is more efficient for isolation of PEDV from specimens than conventional isolation method and feces were not appropriate for isolation. Successful isolation of PEDV by spinoculation could be attributed to 10–100 fold enhanced attachment of coronaviruses to cells by spinoculation as previously reported (Watanabe et al., 2006).

As anticipated, the viruses that caused the 2013 outbreak in Japan, which started 6 months after the initial detection of PED in the US, were highly similar to the US isolates and to the viruses circulating in China since 2010 (Chen et al., 2014; Huang et al., 2013). New cases of PED caused by group II viruses had not been reported prior to the large PED outbreak in September 2013 in Japan (Suzuki et al., 2015). The new PEDV currently prevalent in Japan could have

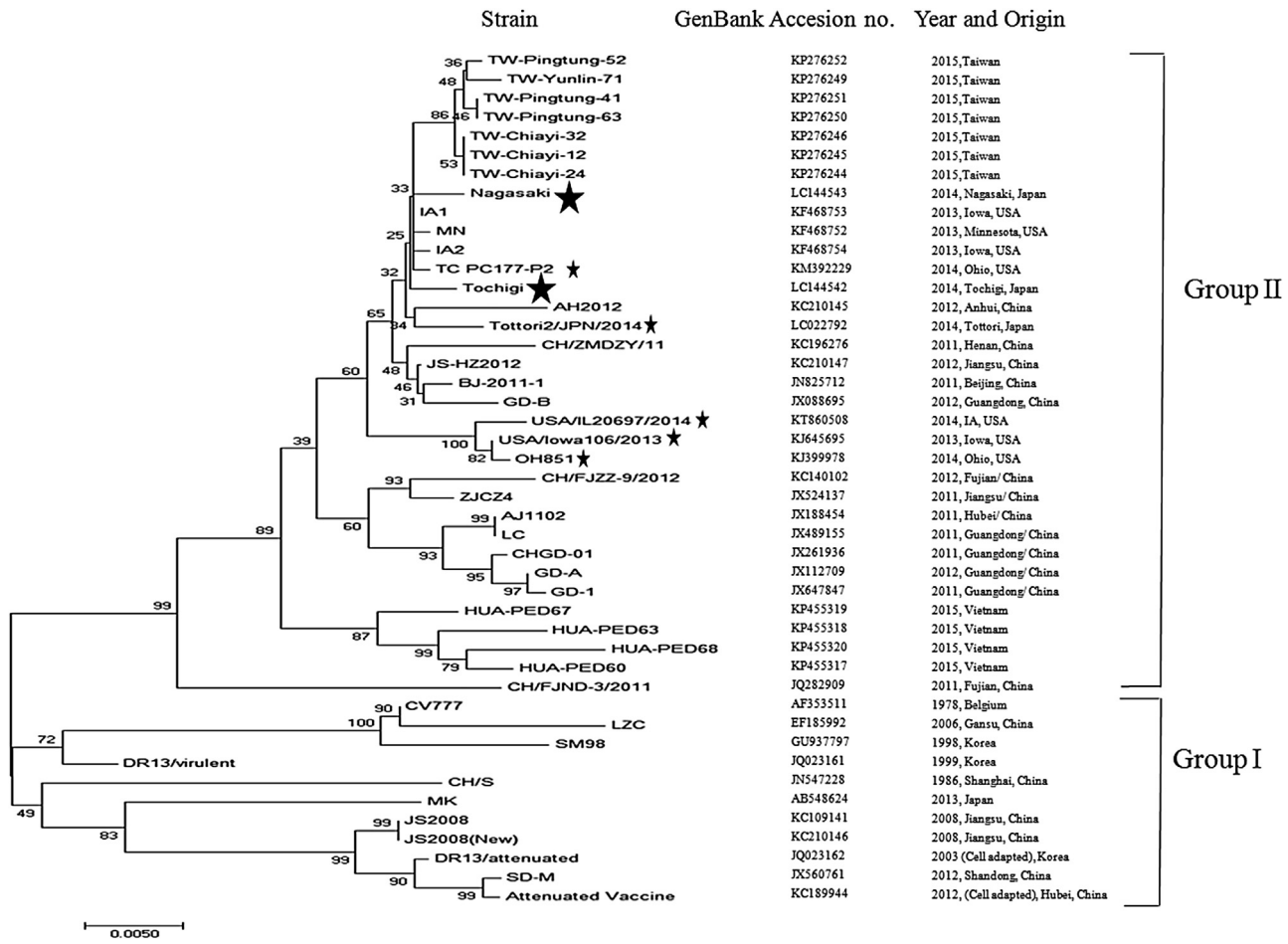

Fig. 3. Phylogenetic analysis of the entire amino acid sequence of the S proteins of two new PEDV isolates (Tochigi and Nagasaki) and those of 43 previously published PEDVs. The trees were constructed using the distance-based neighbor-joining method of the MEGA 6.06 software. Bootstrap analysis was carried out on 1000 replicate datasets, and values are indicated adjacent to the branching points. The viruses identified in this study are indicated by large asterisks. S-INDEL PEDV strains are indicated by small asterisks. The scale represents the number of amino acid substitutions per site.

originated from countries in which similar viruses are prevalent, but its origin and mechanism of spread nationwide are unknown.

We showed in this study that new Japanese isolates classified in group II do not differ antigenically from the group I vaccine type MK strain, as reported for US isolates (Lin et al., 2015). Our result is not in disagreement with previous reports that CV777 (group I) and a new strain isolated in China (group II) differed in antigenicity by twofold (Wang et al., 2016). These reports are in accordance with the findings that the neutralizing epitopes of the S protein of new isolates are highly conserved and similar to those of the CV777 vaccine strain (Sun et al., 2015; Hao et al., 2014; Chen et al., 2013; Sun et al., 2008).

In the US, PED spread rapidly nationwide from the initial case in Ohio, as well as to Canada and Mexico. The huge outbreak in the US may have been caused by the fact that no pigs were vaccinated. However, the spread of PED was similar in Japan, where pregnant sows are usually vaccinated with a virus that elicits production of antibodies that can also neutralize PEDVs of other groups. The vaccine currently used in Japan is effective against group I viruses, as evidenced by the lack of PED for 7 years in Japan prior to invasion by group II viruses. It was reported that the vaccine against group I viruses increased the survival rate of piglets challenged with virulent wild-type PEDV, although it failed to reduce morbidity (Lee, 2015). In agreement with those reports, our findings suggested that group II viruses are neutralized in pigs by the antibodies elicited by a vaccine comprising a group I virus. Collectively, these findings suggest that outbreaks in countries where vaccines are available are not due to ineffective vaccination but rather are attributed to another, as-yet-unknown, cause.

It was also reported that the antisera against the S-INDEL virus neutralized non-S-INDEL virus (Chen et al., 2016) and sows inoculated with S-INDEL could reduce mortality rate and fecal viral shedding in newborn piglets challenged with a US-virulent non-S-INDEL virus (Goede et al., 2015). The relationship between S-INDEL and non-S-INDEL may be similar to that between group 1 and group II PEDVs.

Newly appearing PEDVs are reported to be more virulent than classic group I strains (Liu et al., 2015; Authie et al., 2014); infection and disease in piglets resulted from less than 1 infectious unit (Liu et al., 2015). This suggests that large outbreaks are due to the high pathogenicity of the viruses; escape of even a small number of viruses from antibody neutralization causes devastating diseases in piglets. As commercially available vaccines can neutralize new viruses and protect piglets via antibodies contained in colostrum and ordinary milk, factors—such as sanitation, hygiene and health conditions of lactating sows—must also be important for preventing outbreaks of highly pathogenic viruses (Lee, 2015). Moreover, PEDV-infected sows failed to endow a full amount of milk with neutralizing antibodies to their piglets, which could also have led to the high mortality rate in the present PED outbreak (Lee, 2015; Park and Lee, 2009).

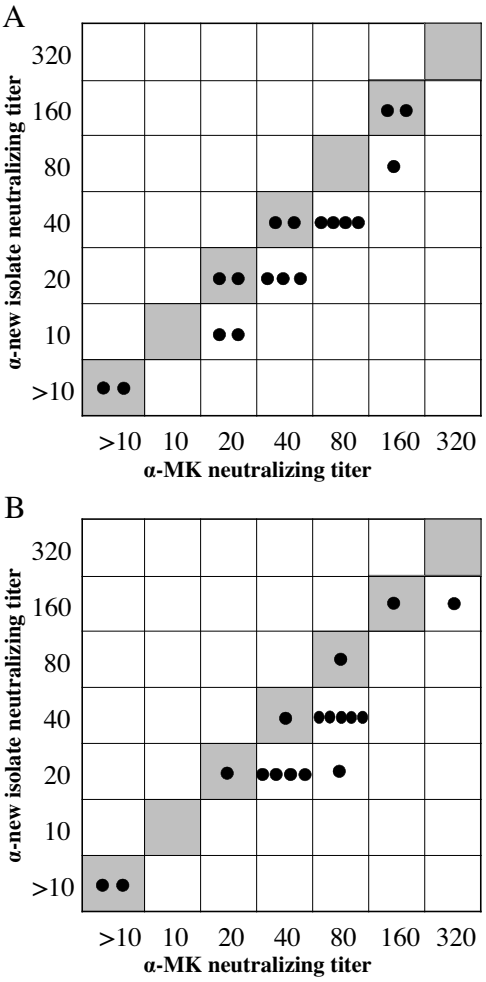

Fig. 4. Neutralizing titers of various pig sera against the new isolates (Tochigi and Nagasaki) and the MK vaccine strain. Two types of antisera against PEDV were used. One group comprised 18 sera collected from herds of pigs that were vaccinated but not recently affected by PEDV (A), and the other group comprised 17 sera from herds of pigs that were vaccinated and affected by the new PEDV (B). Sera were collected from different pig farms. • Individual pig serum.

The large outbreak that occurred recently in the US was due mainly to poor biosecurity (Lee, 2015). Namely, the virus was transmitted by movement of contaminated vehicles from the farm where the PEDV outbreak occurred to PEDV-free farms (Lowe et al., 2014). Also, transmission by contaminated food was suspected (Dee et al., 2014). To prevent large outbreaks, a high level of biosecurity and highly effective vaccines is required.

Conclusively, to prevent infection by highly pathogenic group II viruses, establishment of rigid biosecurity, in which PEDV infection is contained at an early phase, is critical. Also, vaccination with group II virus strains could assist to protect pigs from large outbreaks of PEDV.

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