Virus Research 222 (2016) 113–119

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

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

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ARTICLE INFO

Article history:
Received 10 April 2016
Received in revised form 5 June 2016
Accepted 8 June 2016
Available online 9 June 2016

Keywords:
Porcine epidemic diarrhea virus (PEDV)
Phylogenetic analysis
Neutralization test

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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http://dx.doi.org/10.1016/j.virusres.2016.06.006
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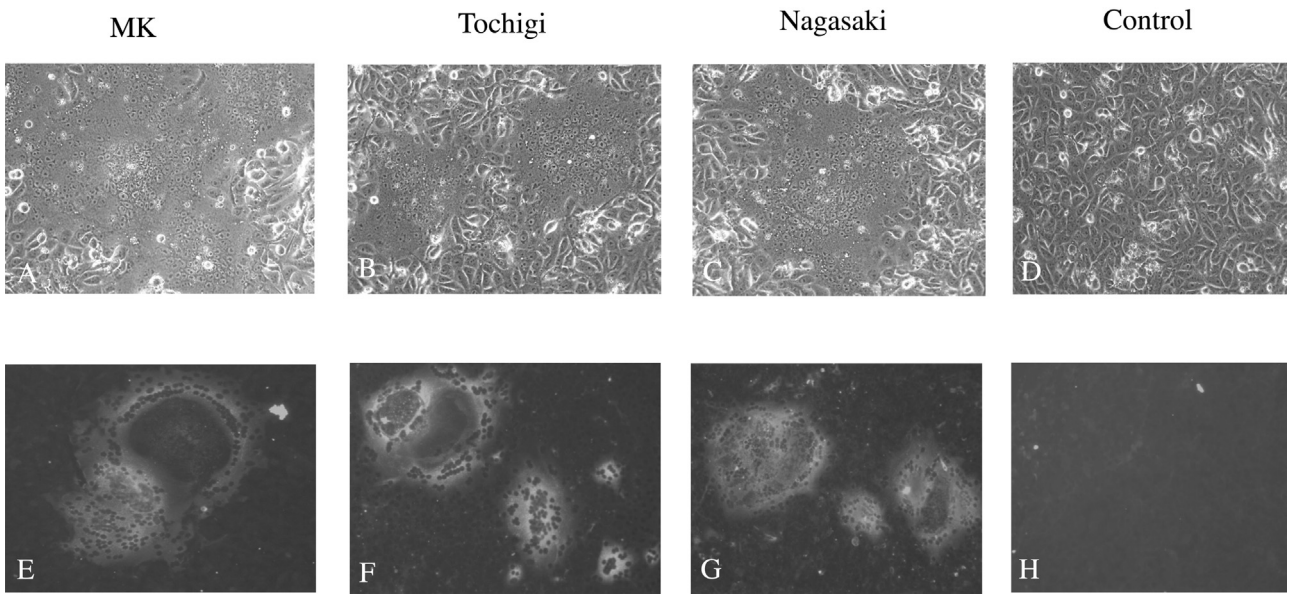

Fig. 1. Cytopathic effects and detection of viral antigen by immunofluorescence in Vero cells infected with the MK, Tochigi and Nagasaki strains. At 48 h postinfection, cytopathic effects were observed by phase-contrast microscopy (A–D). At the same time, PEDV-infected Vero cells were fixed with acetone-methanol, 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).