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Isolation and characterization of a Korean porcine epidemic diarrhea virus strain KNU-141112

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ABSTRACT

Severe outbreaks of porcine epidemic diarrhea virus (PEDV) have re-emerged in Korea and rapidly swept across the country, causing tremendous economic losses to producers and customers. Despite the availability of PEDV vaccines in the domestic market, the disease continues to plague the Korean pork industry, raising issues regarding their protective efficacy and new vaccine development. Therefore, PEDV isolation in cell culture is urgently needed to develop efficacious vaccines and diagnostic assays and to conduct further studies on the virus biology. In the present study, one Korean PEDV strain, KOR/KNU-141112/2014, was successfully isolated and serially propagated in Vero cells for over 30 passages. The *in vitro* and *in vivo* characteristics of the Korean PEDV isolate were investigated. Virus production in cell culture was confirmed by cytopathology, immunofluorescence, and real-time RT-PCR. The infectious virus titers of the viruses during the first 30 passages ranged from $10^{5.1}$ to $10^{8.2}$ $TCID_{50}$ per ml. The inactivated KNU-141112 virus was found to mediate potent neutralizing antibody responses in immunized guinea pigs. Animal studies showed that KNU-141112 virus causes severe diarrhea and vomiting, fecal shedding, and acute atrophic enteritis, indicating that strain KNU-141112 is highly enteropathogenic in the natural host. In addition, the entire genomes or complete S genes of KNU-141112 viruses at selected cell culture passages were sequenced to assess the genetic stability and relatedness. Our genomic analyses indicated that the Korean isolate KNU-141112 is genetically stable during the first 30 passages in cell culture and is grouped within subgroup G2b together with the recent re-emergent Korean strains.

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1. Introduction

Porcine epidemic diarrhea (PED) is a devastating disease in pigs that is characterized by acute enteritis and lethal watery diarrhea followed by dehydration with high mortality in suckling piglets (Debouck and Pensaert, 1980; Saif et al., 2012; Pijpers et al., 1993). The disease was initially recognized in England in 1971 and has then spread to swine-producing European countries (Oldham, 1972; Pensaert et al., 1981). Since the 1990s, PED has become rare in Europe and is more often associated with post-weaning diarrhea in adult pigs (Saif et al., 2012). PED was first reported in Asia in 1982 and has since had a great economic impact on the Asian pork industry (Chen et al., 2008; Kweon et al., 1993; Li et al., 2012; Puranaveja et al., 2009; Takahashi et al., 1983). In May 2013, PED outbreaks suddenly appeared in the United States and have rapidly spread nationwide as well as to Canada and Mexico, causing high mortality in newborn piglets and significant financial concerns (Mole, 2013; Stevenson et al., 2013 Vlasova et al., 2014).

The etiological agent of PED, PED virus (PEDV), was identified as a coronavirus in 1978, which is a member of the genus *Alphacoronavirus* within the family Coronaviridae of the order Nidovirales (Lai et al., 2007; Pensaert and de Bouck, 1978; Saif et al., 2012). PEDV is a large, enveloped virus possessing a single-stranded positive-sense RNA genome of approximately 28 kb with a 5′ cap and a 3′ polyadenylated tail (Pensaert and de Bouck, 1978; Saif et al., 2012). The spike (S) protein of PEDV is the major envelope glycoprotein of the virion and plays pivotal roles in interacting with the cellular receptor for virus entry and mediating neutralizing antibodies in the natural host (Jackwood et al., 2001; Lai et al., 2007; Lee et al., 2010). Therefore, the PEDV S glycoprotein is known to be an appropriate viral gene for determining the genetic relatedness among PEDV isolates and for developing diagnostic assays and effective vaccines (Chen et al., 2014; Gerber et al., 2014; Lee et al., 2010; Lee and Lee, 2014; Oh et al., 2014).

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The first PED epizootic in Korea was confirmed in 1992 (Kweon et al., 1993). However, a retrospective study revealed that PEDV already existed as early as 1987 (Park and Lee, 1997). Since the emergence, PED outbreaks occurred every year, resulting in substantial economic losses to the Korean swine industry until early 2010. After severe outbreaks of foot-and-mouth disease (FMD) during 2010 to 2011, however, the prevalence of PEDV infections was occasional with only sporadic outbreaks in Korea. This epidemic situation probably resulted from the mass culling of more than one-third of the entire domestic pig population in Korea during the 2010–2011 FMD outbreaks. However, starting in November 2013, severe PED epidemics re-emerged in Korea and swept more than 40% of pig farms (Lee and Lee, 2014; Lee et al., 2014a,b). Although both modified live and killed vaccines against PED are commercially available in Korea, continuous PED epidemics indicate a low effectiveness of the domestic vaccines. This result appears to be due to genetic and antigenic differences between S proteins of vaccine and field strains (Lee et al., 2010; Oh et al., 2014; Lee and Lee, 2014). Thus, the lack of effective vaccines enhances the need for the development of next-generation vaccines to control PED.

PEDV isolation in cell culture is critical for developing effective vaccines for PED prevention as well as performing various PEDV research. However, the cell culture isolation of PEDV has shown to be difficult and even the isolated virus may be unable to maintain infectivity upon further passages in cell culture (Chen et al., 2014). To date, there have only been two reports in more than two decades on the cultivation of the Korean PEDV strain that is genetically divergent from field PEDVs (Kweon et al., 1999; Song et al., 2003), while a number of PEDV strains have been recently isolated in the US and successfully grown in cell culture for a year (Chen et al., 2014; Oka et al., 2014), In the present study, we attempted to isolate PEDV from various PEDV-positive samples using Vero cells. At this time, one highly virulent Korean strain KOR/KNU-141112/2014 has been successfully isolated and serially propagated in cell culture for over 30 passages. We aimed to characterize the growth and titer of the PEDV isolate KNU-141112 during the serial passages and the pathogenicity of the virus in suckling piglets. Our *in vivo* assessment demonstrated that KNU-141112 is highly entero-pathogenic in piglets, exhibiting severe clinical symptoms as well as macroscopic and microscopic lesions typical for PEDV infection. In addition, the complete genome or full-length S gene sequences of KNU-141112 were determined at selected passages to study the genetic stability and relationship. Our data indicated that KNU-141112 isolate is relatively stable during the first 30 passages in cell culture and is classified into subgroup G2b that includes PEDV strains responsible for recent severe outbreaks in Korea and the US.

2. Materials and methods

2.1. Cells, clinical samples, virus, and antibody

Vero cells (ATCC CCL-81) were cultured in alpha minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS; Invitrogen) and antibiotic-antimycotic solutions (100×; Invitrogen) and maintained at 37 °C in a humidified 5% CO_2 incubator. Seven small intestinal homogenates and 50 stool specimens that tested positive by RT-PCR using an *i*-TGE/PED Detection Kit (iNtRON Biotechnology, Seongnam, South Korea) were selected for virus isolation experiments. Intestinal homogenates were prepared to 10% (wt/vol) suspensions with phosphate-buffered saline (PBS) using a MagNA Lyser (Roche Diagnostics, Mannheim, Germany) by three repetitions of 15 s at a speed of 7000 rpm. Fecal samples were diluted with PBS to be 10% (wt/vol) suspensions. The suspensions were then vortexed and centrifuged for 10 min at 4500 × *g* (Hanil Centrifuge FLETA5, Incheon, South Korea). The supernatant was filtered through a 0.22-μm-pore-size syringe filter (Millipore, Billerica, MA) and stored at −80 °C as an inoculum for virus isolation until use. Virus isolation of PEDV was attempted on Vero cells as described previously with some modifications (Hofmann and Wyler, 1988). Briefly, prior to inoculation, inocula were prepared by adding trypsin (USB, Cleveland, OH) to intestinal or fecal suspensions prepared above to give a final concentration of 10 μg/ml. Confluent Vero cells grown in 6-well plates were washed with PBS and inoculated with 400 μl of each inoculum containing trypsin. After incubating at 37 °C for 1 h, 2 ml of virus growth medium [α-MEM supplemented with antibiotic-antimycotic solutions, 0.3% tryptose phosphate broth (TPB; Sigma, St. Louis, MO), 0.02% yeast extract (Difco, Detroit, MI), 10 mM HEPES (Invitrogen), and 5 μg/ml of trypsin] was added. The inoculated cells were maintained at 37 °C under 5% CO_2 and monitored daily for cytopathic effects (CPE). When 70% CPE appeared, inoculated cells were subjected to three rounds of freezing and thawing. The culture supernatants were then collected and centrifuged at 400 × *g* for 10 min. The clarified supernatants were aliquoted and stored at −80 °C as 'passage 1 (P1)' viral stocks until use. One hundred millimeter diameter tissue culture dishes were used for serial passages of the isolate. If no CPE was shown in inoculated cells for 7 days, the plates were frozen and thawed three times, and the supernatants were harvested by centrifugation and inoculated on fresh Vero cells for the next passage. If CPE and RT-PCR results were negative after 6 blind passages, the virus isolation was considered negative. The PEDV N protein-specific monoclonal antibody (MAb) was obtained from ChoogAng Vaccine Laboratory (CAVAC; Daejeon, South Korea).

2.2. Virus titration

Vero cells were infected with each passage KNU-141112 virus stock in the presence of trypsin as described above. The culture supernatants were collected at 24 or 48 h postinfection (hpi) at which a 70% CPE is commonly developed. For growth kinetics experiments, the supernatants were harvested from cells infected with each selected passage virus at different time points (6, 12, 24, 36, and 48 hpi) and stored at −80 °C. Virus titers were measured in 96-well plates by 10-fold serial dilution of the samples in triplicate per dilution to determine the quantity of viruses required to produce CPE in 50% of inoculated Vero cells and calculated as $TCID_{50}$ per ml using the Reed–Muench method (Reed and Muench, 1938). The PEDV titer was also determined by a plaque assay using Vero cells and quantified as plaque-forming units (PFU) per ml.

2.3. Immunofluorescence assay (IFA)

Vero cells grown on microscope coverslips placed in 6-well tissue culture plates were mock infected or infected with PEDV at a multiplicity of infection (MOI) of 0.1. The virus-infected cells were subsequently propagated until 24 hpi, fixed with 4% paraformaldehyde for 10 min at room temperature (RT) and permeabilized with 0.2% Triton X-100 in PBS at RT for 10 min. The cells were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at RT and then incubated with N-specific MAb for 2 h. After being washed five times in PBS, the cells were incubated for 1 h at RT with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Invitrogen), followed by counterstaining with 4′,6-diamidino-2-phenylindole (DAPI; Sigma). The coverslips were mounted on microscope glass slides in mounting buffer and cell staining was visualized using a fluorescence Leica DM IL LED microscope (Leica, Wetzlar, Germany).

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