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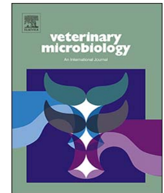

Genetic characteristics, pathogenicity, and immunogenicity associated with cell adaptation of a virulent genotype 2b porcine epidemic diarrhea virus

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ABSTRACT

Porcine epidemic diarrhea virus (PEDV) has emerged or re-emerged worldwide, posing a significant financial threat to major pig-producing countries. In the present study, a virulent Korean pandemic PEDV strain, KNU-141112, was serially propagated in Vero cells for up to 100 passages. Through cell culture adaptation, we obtained four distinct deletion (DEL) mutants by plaque purification followed by nucleotide sequencing of the spike (S)/ORF3 gene-coding region, which were designated KNU-141112-S DEL2, −S DEL5, −S DEL2/ORF3, and −S DEL5/ORF3. Further whole genome sequencing identified 12 or 14 amino acid changes in the cell-adapted DEL strains. Animal inoculation studies revealed that the virulence of both S DEL2/ORF3 and S DEL5/ORF3 viruses with a large 46-nt deletion in the intergenic portion of S and ORF3 was remarkably diminished, indicating viral attenuation in the natural host. Furthermore, these cell-adapted strains elicited potent neutralizing antibody responses in immunized pigs. Taken together, our data indicate that the cell-attenuated S DEL2/ORF3 and S DEL5/ORF3 strains are promising candidates for the development of a safe and effective live PEDV vaccine.

1. Introduction

Porcine epidemic diarrhea (PED) is a deadly and highly contagious enteric pig disease that is characterized by acute watery diarrhea/vomiting, and dehydration, resulting in high mortality in newborn piglets (Lee, 2015; Saif et al., 2012). PED virus (PEDV), the etiological agent of PED, is a large, enveloped, single-stranded, positive-sense RNA virus that belongs to the genus *Alphacoronavirus* in the family *Coronaviridae* of the order *Nidovirales* (Pensaert and Debouck, 1978; Lee, 2015). The PEDV genome is approximately 28 kb in length with a 5′ cap and a 3′ polyadenylated tail, and is composed of a 5′-untranslated region (UTR), at least seven open reading frames (ORFs) designated ORF1a, ORF1b, and ORFs 2 through 6, and a 3′-UTR (Pensaert and Debouck, 1978; Kocherhans et al., 2001; Saif et al., 2012). The first two large ORFs, ORF1a and 1b, encode replicase polyproteins, pp la and pp 1ab, which undergo autoproteolysis by viral proteases to eventually produce 16 processing nonstructural proteins (nsp1–16). The remaining ORFs code for the four canonical structural spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins of coronaviruses and a single accessory gene, ORF3 (Lai et al., 2007; Lee, 2015). On the basis of phylogenetic analysis of the S gene, PEDV can be divided into two genotypes, designated genogroup 1 (G1; classical or recombinant and low-pathogenic) and genogroup 2 (G2; field epidemic or pandemic and high-pathogenic), which are genetically and antigenically distinct. Each genogroup is composed of two subgroups, 1a and 1b, and 2a and 2b, respectively (Lee, 2015; Lee et al., 2010; Lee and Lee, 2014; Oh et al., 2014).

Although PED outbreaks have been reported in Europe and Asia, the veterinary health impact and related economic losses have been most devastating in Asian swine-producing nations in the past two decades. Despite its notorious reputation in Asia, PED was not well-recognized worldwide until the disease hit the United States (US) in 2013. Since its emergence in the US, PEDV has spread quickly throughout most of the states and to adjacent countries, sustaining a tremendous threat in the North American pork business (Mole, 2013; Stevenson et al., 2013; Vlasova et al., 2014). Subsequently, large, severe PED outbreaks recurred almost simultaneously in South Korea, Japan, and Taiwan, and US prototype-like G2b PEDV strains were found to be accountable for these recent epizootics (Lee and Lee, 2014; Lin et al., 2014; Suzuki et al., 2015). Furthermore, recombinant G1b or pandemic G2b PEDVs re-emerged throughout western and central Europe (Boniotti et al., 2016; Dastjerdi et al., 2015; Grasland et al., 2015; Hanke et al., 2015;

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Mesquita et al., 2015; Steinrigl et al., 2015; Theuns et al., 2015). Therefore, PED has become a globally emerging and re-emerging viral swine disease that causes enormous financial damage throughout the world and is considered one of the most economically important diseases in countries with intensive swine industries.

A PED epizootic in South Korea was first reported in 1992 (Kweon et al., 1993). Since that time, PED has remained rampant, devastating the domestic hog industry. More recently, the 2013–2014 PED epidemic swept through mainland South Korea, followed by Jeju Island, and killed hundreds of thousands of piglets in domestic herds (Lee et al., 2014a; Lee and Lee, 2014). Currently, a limited number of PEDV vaccines, either modified live or inactivated/killed, are commercially available in South Korea. These vaccines contain a single G1a classical strain (Korean SM98-1 or DR-13 strains, or Japanese 83P-5) and, in many cases, are not fully protective against genetically divergent field strains. The incomplete efficacies of current PEDV vaccines might be ascribed to antigenic or genetic differences between the major S glycoprotein of the vaccine and field epidemic strains (Kim et al., 2015; Lee, 2015; Lee et al., 2010, 2014a; Lee and Lee, 2014; Oh et al., 2014). Considering these issues, there is a strong demand for a new vaccine to be developed against G2b epizootic or related strains prevalent in the field. In general, attenuation of the virulence of a wild-type virus can be achieved by sequential passage in non-host cell lines. The attenuated virus can be explored to prepare a modified live virus (MLV) vaccine. Indeed, current live PEDV vaccines were created by continuous passages of classical G1a strains in African green monkey kidney-derived Vero cells (Kweon et al., 1999; Sato et al., 2011; Song et al., 2007). In the present study, a highly virulent Korean G2b strain KOR/KNU-141112/2014 was serially propagated in Vero cells for up to 100 passages for attenuation so that it could be further used for the development of an MLV vaccine.

2. Materials and methods

2.1. Cells, 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. Virulent Korean PEDV strain KOR/KNU-141112/2014 was isolated and propagated in Vero cells as described previously (Lee et al., 2015). A viral stock was prepared from the 5th passage in cell culture (KNU-141112-P5) and used as the parental virus in this study (GenBank accession no. KR873434). PEDV N protein-specific monoclonal antibody (MAb) was obtained from ChoogAng Vaccine Laboratory (CAVAC; Daejeon, South Korea).

2.2. Serial passage of the virus

PEDV strain KNU-141112-P5 was plaque purified twice in Vero cells, and the purified virus was continuously passaged on Vero cells as described previously with some modifications (Lee et al., 2015). Confluent Vero cells grown in 100-mm diameter tissue culture dishes were washed with PBS and inoculated with 1 ml of 10-fold diluted PEDV KNU-141112 with trypsin (USB, Cleveland, OH). After incubation at 37 °C for 1 h, 7 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 effect (CPE). When ~70% of cells showed CPE, the inoculated cells were subjected to three rounds of freezing and thawing. The culture supernatants were then centrifuged for 10 min at 400 × *g* (Hanil Centrifuge FLETA5, Incheon, South Korea) and filtered through a 0.45-μm pore-size filter (Millipore, Billerica, MA). The clarified supernatants were aliquoted and stored at −80 °C as the viral stock for the next passage. In the same manner, 100 subsequent passages were performed in Vero cells. Beginning at the 70th passage in cell culture, the virus was plaque cloned every 10 passages. Single plaques were chosen and subjected to nucleotide (nt) sequencing to identify mutations in the S gene, and the selected viruses were further passaged.

2.3. Immunofluorescence assay (IFA)

Vero cells grown on microscope coverslips placed in 6-well tissue culture plates were infected with PEDV at a multiplicity of infection (MOI) of 0.1. The virus-infected cells were subsequently cultured 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 glass microscope slides in mounting buffer, and cell staining was visualized using a fluorescence Leica DM IL LED microscope (Leica, Wetzlar, Germany).

2.4. Virus titration

Vero cells were infected with each KNU-141112 virus stock in the presence of trypsin as described above. The culture supernatants were collected at 24 or 48 h postinfection (hpi), when 70% CPE commonly developed. For the growth kinetics experiments, supernatants were harvested from cells infected with each virus strain at various time points (6, 12, 24, 36, and 48 hpi) and stored at −80 °C. Virus titers were measured in duplicate by plaque assay using Vero cells and defined as plaque-forming units (PFU) per ml. In brief, Vero cells grown in 6-well plates were inoculated with 200 μl/well of 10-fold serially diluted virus suspensions containing trypsin and adsorbed for 1 h at 37 °C. The inoculated cells were overlaid with 2 ml of premixed virus growth medium and 1.5% Bacto Agar (Difco) and incubated for 2 days at 37 °C. Plaque morphologies were assessed by staining with 1% crystal violet in ethanol at 48 hpi.

2.5. Nucleotide sequence analysis

The full-length genomic sequences of cell adapted KNU-141112 DEL strains were determined by next-generation sequencing (NGS) technology. Total RNA was extracted from each virus stock serially passaged in Vero cells using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and was used as a template to amplify cDNA fragments as described elsewhere (Lee and Lee, 2014; Lee et al., 2014b, 2015). Ten overlapping cDNA fragments encompassing the entire genome of each virus strain were generated, pooled in equimolar amounts, and subjected to NGS using the Ion Torrent Personal Genome Machine (PGM) Sequencer System (Life Technologies, Carlsbad, CA) and a 316 v2 sequencing chip (Life Technologies) as described previously (Lee and Lee, 2014; Rothberg et al., 2011). The single-nucleotide variants (SNVs) were analyzed using CLC Genomic Workbench version 7.0 (CLC Bio, Cambridge, MA), and the individual NGS reads were assembled using the complete genome of PEDV strain KNU-141112-P5 (GenBank accession no. KR873434) as a reference. The 5′ and 3′ ends of the genomes of cell-adapted KNU-141112 strains were determined by rapid amplification of cDNA ends (RACE) as described previously (Lee and Lee, 2013). The full-length genomic nt sequences of high passage KNU-141112 DEL derivatives (KNU-141112-S DEL2, –S DEL2/ORF3, –S DEL5, and –S DEL5/ORF3) were deposited in the GenBank database under accession numbers KY825240 to KY825243, respectively. In addition, the unique nt deletions identified in S and/or

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