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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 $10^{8.2}$ TCID₅₀ 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 \times ; Invitrogen) and maintained at 37 °C in a humidified 5% CO₂ 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 \times g (Hanil Centrifuge FLETA5, Incheon, South

Korea). The supernatant was filtered through a 0.22- μ m-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 μ g/ml. Confluent Vero cells grown in 6-well plates were washed with PBS and inoculated with 400 μ l 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 μ g/ml of trypsin] was added. The inoculated cells were maintained at 37 °C under 5% CO₂ 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 \times 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 TCID₅₀ 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).

2.4. Quantitative real-time RT-PCR

Viral RNA was extracted from virus isolates or fecal samples prepared as described above using an *i*-TGE/PED Detection Kit according to the manufacturer's protocol. Quantitative real-time RT-PCR was performed using a One Step SYBR PrimeScript RT-PCR Kit (TaKaRa, Otsu, Japan) as described elsewhere (Kim et al., 2007; Sagong and Lee, 2011). The reaction took place using a Thermal Cycler Dice Real Time System (TaKaRa) and the results were analyzed by the system software as described previously (Sagong and Lee, 2011).

2.5. Immunization of guinea pigs

Eight 3–4 month-old guinea pigs (weighing 300–350 g) were randomly allocated into inoculated ($n=6$) and control ($n=2$) groups. Six guinea pigs were immunized subcutaneously with 0.5 ml of binary ethylenimine (BEI)-inactivated KNU-141112-P10 virus in the presence of Freund's complete adjuvant (Sigma) and boosted once with a freshly prepared emulsion of the inactivated virus and Freund's incomplete adjuvant (Sigma) at a 2-week interval. Two sham-inoculated guinea pigs were administered and boosted with cell culture media in the presence of the respective adjuvant. Pre-immune sera were collected before starting the immunization and antisera were collected at 2 weeks after the final boost.

2.6. Serum neutralization

The presence of PEDV-specific neutralizing antibodies in serum samples collected from guinea pigs in all groups was determined using a serum neutralization (SN) test in 96-well microtiter plates using PEDV isolate KNU-141112 or vaccine strain SM98-1 as previously described (Oh et al., 2014) with minor modifications. Briefly, Vero cells were grown at 2×10^4 /well in 96-well tissue culture plates for 1 day. The KNU-141112-P10 virus stock was diluted in serum-free α -MEM to make 200 TCID in a 50 μ l volume. The diluted virus was then mixed with 50 μ l of 2-fold serial dilutions of individual inactivated sera in 96-well plates and incubated at 37 °C for 1 h. The mixture was inoculated into Vero cells and incubated at 37 °C for 1 h. After removing the mixture, the cells were thoroughly rinsed 5 times with PBS and maintained in virus growth medium at 37 °C in a 5% CO₂ incubator for 2 days. For the SN test using the vaccine strain, PEDV strain SM98-1 propagated in the absence of trypsin was diluted in serum-free α -MEM to make 200 PFU in a 50 μ l volume. The diluted virus was then mixed with each serum sample in 96-well plates as described above and incubated at 37 °C for 1 h. Subsequently, approximately 1×10^4 Vero cells in 100 μ l of α -MEM containing 5% FBS were added to each well and the mixture was maintained at 37 °C in a 5% CO₂ incubator for 3 days. The neutralization titer was calculated as the reciprocal of the highest dilution of serum that inhibited virus-specific CPE in all of the duplicate wells.

2.7. Pig infection experiments

The *in vivo* swine studies described here were performed at the ImproAH Animal Facility under the guidelines established by its Institutional Animal Care and Use Committee. A total of 8 suckling piglets of 7 days of age were obtained from a commercial pig farm with no known prior PED outbreak or vaccination with PEDV. All animals were determined to be free of antibodies to PEDV as well as to transmissible gastroenteritis virus (TGEV) and porcine reproductive and respiratory syndrome virus. Pigs were randomly assigned to 3 experimental groups housed in 2 separated rooms: PEDV-inoculated ($n=4$) and contact control ($n=2$) in room 1 and

sham-inoculated control ($n=2$) group in room 2. Following a 1 day acclimation period, only piglets in the PEDV-inoculated group orally received a 1 ml dose of 10^3 TCID₅₀/ml of KNU-141112-P10 virus. Two piglets were exposed to the virus by direct contact with inoculated piglets in the same room. The sham-inoculated pigs were administered with cell culture media as a placebo. Clinical signs of diarrhea and mortality were monitored daily for the duration of the study. Stool samples from all groups were collected prior to inoculation and daily with 16 inch, cotton-tipped swabs and subjected to RT-PCR using an *i*-TGE/PED Detection Kit and real-time RT-PCR as described above. PEDV-inoculated piglets were necropsied daily (days 1, 2, 3, and 4) after challenge for post-mortem examinations throughout the study, whereas all pigs from the contact and control groups were euthanized at 4 days post-challenge for post-mortem examinations. Small intestinal tissue specimens (<3 mm thick) collected from each piglet were fixed with 10% formalin for 24 h at RT and embedded in paraffin according to standard laboratory procedures. The formalin-fixed paraffin-embedded tissues were cut at 5–8 μ m thick on a microtome (Leica), floated on a 40 °C water bath containing distilled water, and transferred onto glass slides. The tissues were then deparaffinized in xylene for 5 min and washed in decreasing concentrations of ethanol (100%, 95%, 90%, 80%, and 70%) for 3 min each. The deparaffinized intestinal tissue sections were stained with hematoxylin and eosin (Sigma) for histopathology or subjected to immunofluorescence assay using PEDV N-specific MAb as described above.

2.8. Immunohistochemistry

The paraffin-embedded tissue sections were deparaffinized, treated with 0.01 M citrate buffer (pH 6.0) in a microwave oven for 5 min, chilled at RT for 20 min, and then incubated with 0.3% hydrogen peroxide in DW for 20 min to block endogenous peroxidase. After being washed three times in PBS, the sections were blocked with normal horse serum (VECTASTAIN ABC Kit; Vector Laboratories, Burlingame, CA) and incubated 1 h at RT with N-specific MAb. After rinsing in PBS, the samples were reacted for 45 min at RT with a horse anti-mouse secondary antibody (VECTASTAIN ABC Kit), incubated with avidin-biotin peroxidase complex for 45 min (VECTASTAIN ABC Kit), and developed using the DAB substrate Kit (Vector Laboratories) according to the manufacturer's instructions. The slides were then counterstained with hematoxylin, dehydrated, cleared with xylene, and mounted on microscope glass slides in mounting buffer and tissue staining was visualized using a microscope.

2.9. Nucleotide sequence analysis

The complete genomic sequences of the original fecal sample as well as those of the P5 and P10 isolates were determined by next-generation sequencing (NGS) technology. Total RNA was extracted from the feces as well as P5 and P10 isolates using a RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and used as a template to amplify cDNA fragments as described elsewhere (Lee and Lee, 2014; Lee et al., 2014b). Ten overlapping cDNA fragments were generated to encompass the entire genome of each sample, 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; Lee et al., 2014b; Rothberg et al., 2011). The single-nucleotide variants (SNVs) were analyzed using the CLC Genomic Workbench version 7.0 (CLC Bio, Cambridge, MA) and the individual NGS reads were assembled using the complete genome of PEDV reference strain KOR/KNU-1305/2013 (Genbank accession no. KJ662670). The 5' and 3' ends of the genomes of

the original feces and the P5 and P10 isolates were determined by rapid amplification of cDNA ends (RACE) as described previously (Lee and Lee, 2013). The full-length genomic nucleotide sequences of the KNU-141112-feces, KNU-141112-P5, and KNU-141112-P10 were deposited in GenBank under accession numbers KR873431, KR873434, and KR873435, respectively. The S glycoprotein gene sequences of the virus isolates were also determined by the traditional Sanger method. Two overlapping cDNA fragments spanning the entire S gene of each isolate were RT-PCR amplified as described previously (Lee et al., 2010). The individual cDNA amplicons were gel-purified, cloned into pGEM-T easy (Promega, Madison, WI), and sequenced in both directions using two commercial vector-specific T7 and SP6 primers and the S gene-specific primers. In addition, the complete structural gene sequences of the virus isolate at selected passages (KNU-141112-P3, KNU-141112-P4, KNU-141112-P20, and KNU-141112-P30) were determined by the Sanger method as described above and deposited to the GenBank database under their accession numbers shown in Fig. 5.

2.10. Phylogenetic analysis

The sequences of 42 fully sequenced S genes and 25 complete genomes of PEDV isolates were independently used in sequence alignments and phylogenetic analyses. The multiple-sequence alignments were generated with the ClustalX 2.0 program (Thompson et al., 1997) and the percentages of nucleotide sequence divergences were further assessed with the same software program. Phylogenetic trees were constructed from the aligned nucleotide or amino acid sequences by using the neighbor-joining method and subsequently subjected to bootstrap analysis with 1000 replicates in order to determine percentage reliability values on each internal node of the tree (Saitou and Nei, 1987). All tree figures were produced using Mega 4.0 software (Tamura et al., 2007).

2.11. Statistical analysis

Student's *t* test was used for all statistical analyses, and *p*-values of less than 0.05 were considered statistically significant.

3. Results

3.1. Virus isolation and in vitro characterization

We attempted to isolate PEDV from PCR-positive clinical samples, including 50 feces and 7 intestinal homogenates on Vero cells. One PEDV isolate designated KNU-141112 was successfully isolated from the feces of a naturally infected piglet from a commercial farm located in Kyungpook Province obtained on September 29, 2014. The KNU-141112 virus was capable of producing distinct CPEs typical for PEDV infection, such as cell fusion, syncytium, and detachment, in infected Vero cells from passage 3 (P3). We then investigated whether the isolate can be efficiently cultivated and maintained in cell culture. Thus, the isolated PEDV strain KNU-141112 was further serially passaged in Vero cells for a total of 30 passages (P1 to P30). The time of CPE onset was at 24 hpi and, accordingly, prominent CPE was observed within 48 hpi in the first 2 productive passages (P3 and P4). In the later passages, visible CPE appeared at 12 hpi and became predominant by 24 hpi. Virus propagation was confirmed by detecting PEDV antigens by IFA using a PEDV N protein-specific MAb. The distinct staining was distributed in the cytoplasm of typical syncytial cells. In contrast, no CPE and N-specific staining was evident in mock-inoculated Vero cells. Examples of CPE and corresponding IFA images in selected passages are shown in Fig. 1. The level of viral genome in each selected passage was further assessed by real-time RT-PCR and the

mean cycle threshold (Ct) value was determined to be 16.7, ranging from 15.3 (P10) to 18.7 (P5). The infectious titer of the isolate ranged from $10^{5.1}$ to $10^{6.1}$ TCID₅₀/ml up to P5, whereas it was determined to be approximately 10^7 TCID₅₀/ml in the later passages. The peak viral titer reached $10^{7.8}$ TCID₅₀/ml (equivalent to $10^{7.5}$ PFU/ml) or more since passage 10 (Fig. 2A). The growth kinetics study further showed that KNU-141112 replicated rapidly and efficiently in Vero cells, reaching a maximum titer $>10^7$ TCID₅₀/ml by 24 dpi (Fig. 2B).

3.2. Antibody response and pathogenicity of KNU-141112

Guinea pig antisera were collected before immunization (pre-immune) and at 2 weeks after the final boost and tested for their neutralizing activity against the isolate KNU-141112 or vaccine strain. As shown in Fig. 3, the guinea pig antisera were highly effective in inhibiting KNU-141112 infection with mean neutralizing antibody (NA) titers of 1:112, whereas the antisera at relatively low dilution fully protect Vero cells from SM98-1 infection with mean NA titers of 1:37. In contrast, none of the pre-immune and non-immunized sera showed neutralizing activity against either strain. Taken together, our data indicated that the isolate KNU-141112 elicits potent antibody responses in immunized animals. However, the antisera strongly recognized the homologous field isolate, but inefficiently the heterologous PEDV vaccine strain, suggesting the antigenic variations between the vaccine strain and field PEDVs.

Four piglets were challenged orally with the KNU-141112 virus, while 2 control animals were inoculated with cell culture media. Two piglets were housed together with inoculated piglets in the same room for direct contact exposure. During the acclimation period, all piglets were active and had normal fecal consistency. PEDV-challenged piglets exhibited clinical signs including lethargy and diarrhetic feces by 1 day post-inoculation (DPI) and experienced severe watery diarrhea with vomiting thereafter. PEDV-associated mortality occurred in one of the inoculated pigs at 1 DPI. Contact piglets housed with the inoculated group exhibited diarrhetic feces with vomiting by 2 DPI and the progression of clinical signs was similar to that of the inoculated animals. Furthermore, all inoculated and contact piglets were positive for PEDV, as determined by RT-PCR, by 1 or 2 DPI and shed PEDV in feces with mean Ct values of 18.7 (range 16.4–21.0) and 20.1 (range 15.4–23.1), respectively. Negative control pigs remained active with normal feces and continued to be undetectable for fecal shedding of PEDV throughout the study period.

One piglet died from PEDV and was subsequently necropsied at 1 DPI and the remaining inoculated piglets were randomly selected for necropsy thereafter. All contact and control animals were euthanized at the end of the study for postmortem assessments. All inoculated and naïve contact pigs macroscopically displayed typical PED-like lesions. The small intestine was dilated and accumulated with yellow fluid and its wall was thin and transparent, due to the villous atrophy (Fig. 4, panel A). The stomach was distended and filled with curdled and undigested milk. In contrast, the other intestinal organs appeared grossly normal. Microscopic intestinal observations consistent with viral enteritis were developed in all inoculated and contact piglets, which included vacuolation of small intestinal enterocytes and shortening and fusion of small intestinal villi (Fig. 4, panels B and C). Similar microscopic lesions were continuously present in the challenged and contact pigs through 4 DPI. Furthermore, both IFA and IHC staining revealed that the viral antigen was predominantly detected in the cytoplasm of epithelial cells on atrophied villi in all segments of the small intestines (Fig. 4, panels D–F). These findings are comparable to those in pigs naturally or experimentally infected with virulent strains of PEDV (Debouck et al., 1981; Jung et al., 2014; Madson et al., 2014; Stevenson et al.,

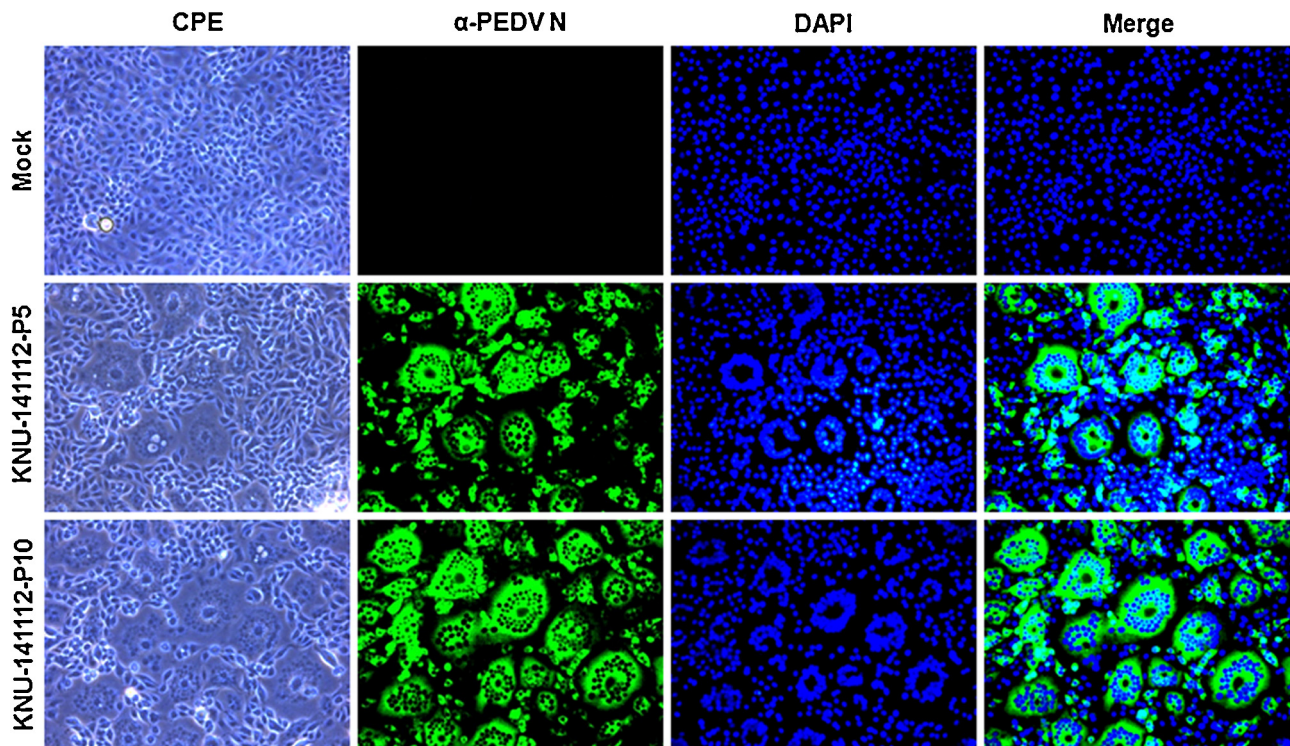


Fig. 1. Cytopathology and IFA of a PEDV isolate KNU-141112 in infected Vero cells. Vero cells were mock infected or infected with PEDV KNU-141112-P5 and KNU-141112-P10 isolates. PEDV-specific CPEs were observed daily and were photographed at 24 hpi using an inverted microscope at a magnification of 200 \times (first panels). For immunostaining, infected cells were fixed at 24 hpi and incubated with MAb against the N protein, followed by Alexa green-conjugated goat anti-mouse secondary antibody (second panels). The cells were then counterstained with DAPI (third panels) and examined using a fluorescence microscope at 200 \times magnification. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

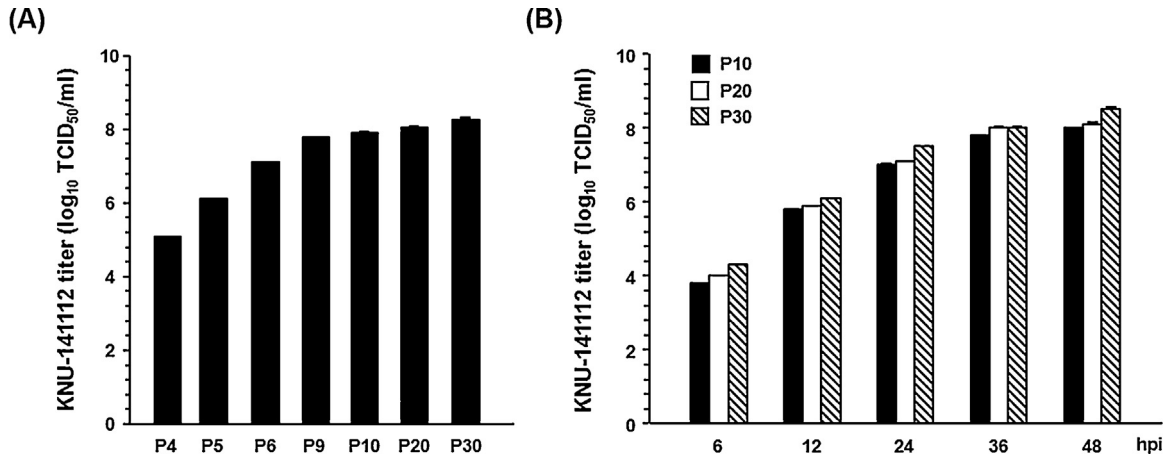


Fig. 2. Growth kinetics of KNU-141112 in Vero cells. (A) Virus titers at selected passages. Vero cells were infected with PEDV KNU-141112 harvested at indicated passage numbers. At 24 or 48 hpi, the virus supernatants were collected and virus titers were determined. (B) One-step growth curves for KNU-141112 strains. Vero cells were independently infected with PEDV KNU-141112-P10, -P20, and -P30. At the indicated time points postinfection, culture supernatants were harvested and virus titers were determined. The 50% tissue culture infectious dose (TCID₅₀) was calculated. Results are expressed as the mean values from triplicate wells and error bars represent standard deviations.

2013). Neither macroscopic nor microscopic intestinal lesions were observed in the negative control piglets during the experiment.

3.3. Complete genomic characterization of KNU-141112

Entire genome sequence data of the original fecal sample (KNU-141112-feces) and cell culture-passaged KNU-141112-P5 and -P10 viruses were determined using the NGS technology. The full-length genome of KOR/KNU-1305/2013 PEDV was used as the initial reference for each NGS read and the individual complete genome

sequences were successfully obtained by the assembly of respective NGS reads. The 5' and 3' ends of their genomes were also sequenced by RACE. All three genomes were 28,038 nucleotides (nt) in length, excluding the 3' poly(A) tail and exhibited the genomic organization typical of all previously sequenced PEDV strains, consisting of the 292-nt 5' UTR, the 20,345-nt ORF1a/1b (nt 293 to 12,601 for 1a and nt 12,601 to 20,637 for 1b), the 4161-nt S gene (nt 20,634 to 24,794), the 675-nt ORF3 (nt 24,794 to 25,468), the 231-nt E gene (nt 25,449 to 25,679), the 681-nt membrane (M) gene (nt 25,687 to 26,367), the 1326-nt N gene (nt 26,379 to 27,704), and the 334-

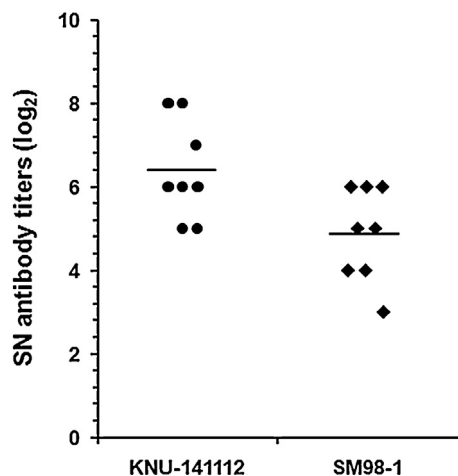


Fig. 3. Virus-neutralizing antibody titers in the sera of guinea pigs inoculated with a PEDV KNU-141112 isolate. Guinea pigs were immunized twice with inactivated KNU-141112-P10 virus by subcutaneous injection. Blood samples were collected prior to immunization and at 2 weeks after the second immunization and subjected to the virus neutralization assay using homologous (KNU-141112; solid circles) and heterologous (SM98-1; solid diamonds) strains. Neutralizing antibody titers for individual infected animals were spotted as a log₂ scale. Values are representative of the mean from three independent experiments in duplicate and error bars denote standard deviations.

nt 3' UTR. The slippery heptamer sequence, TTAAAC, followed by a stem-loop structure was present at the 3' end of ORF1a in the PEDV genome, which is a potential signal for a ribosomal frame shift during translation to generate the pp1ab.

The complete genome sequences of all three viruses were compared, and the results are summarized in Table 1. Compared to

the KNU-141112-feces, KNU-141112-P5 showed one different nt at position 21,756 resulting in one amino acid (aa) change (Leu to Phe) in the S protein, while KNU-141112-P10 gained two additional nt changes at positions 21,448 and 24,492 causing two aa mutations located in the S protein. The full-length S genes of the original feces and KNU-141112 viruses at selected passages (P3, P4, P5, P10, P20, and P30) were also sequenced using the traditional sequencing method. The S protein sequences of KNU-141112-feces, -P5, and -P10 were completely identical to those determined by NGS. One nt change present in the S gene of KNU-141112-P5 had been acquired since passage 3 (KNU-141112-P3). A total of three nt mutations, identified at passage 10 compared to the feces, were sustained through passage 30 (KNU-141112-30). In addition, compared to KNU-141112-P10, we were able to detect two independent mutations at positions 24,869 and 25,656, resulting in amino acid changes in ORF3 (Asp to Tyr) and E (Pro to Ser) of KNU-141112-P20, respectively, which were maintained at passage 30. Altogether, our results revealed that the PEDV isolate KNU-141112 is genetically stable during serial passages in cell culture.

The entire genome sequences of PEDVs determined in this study were further compared to those of 3 other Korean and 8 non-Korean PEDV strains available in GenBank, and the nucleotide homology and difference data are described in Table 2. KNU-141112 isolates (feces, P5, and P10) had the highest nucleotide identity (99.9%) with the Korean re-emergent strain KOR-KNU-1305 and US strains, IN17846 and MN, showing 8 to 46 different nt at the genomic level. All three viruses were genetically distinct from the Korean vaccine strains, SM98-1 and DR-13, and the prototype CV777 strain and exhibited relatively low nucleotide identity ranging from 96.3% to 96.8%. By alignment of the global PEDV strains, a single-nucleotide insertion between nucleotides 20,204 and 20,205 has been previously identified in one Chinese AH2012 and three US strains,

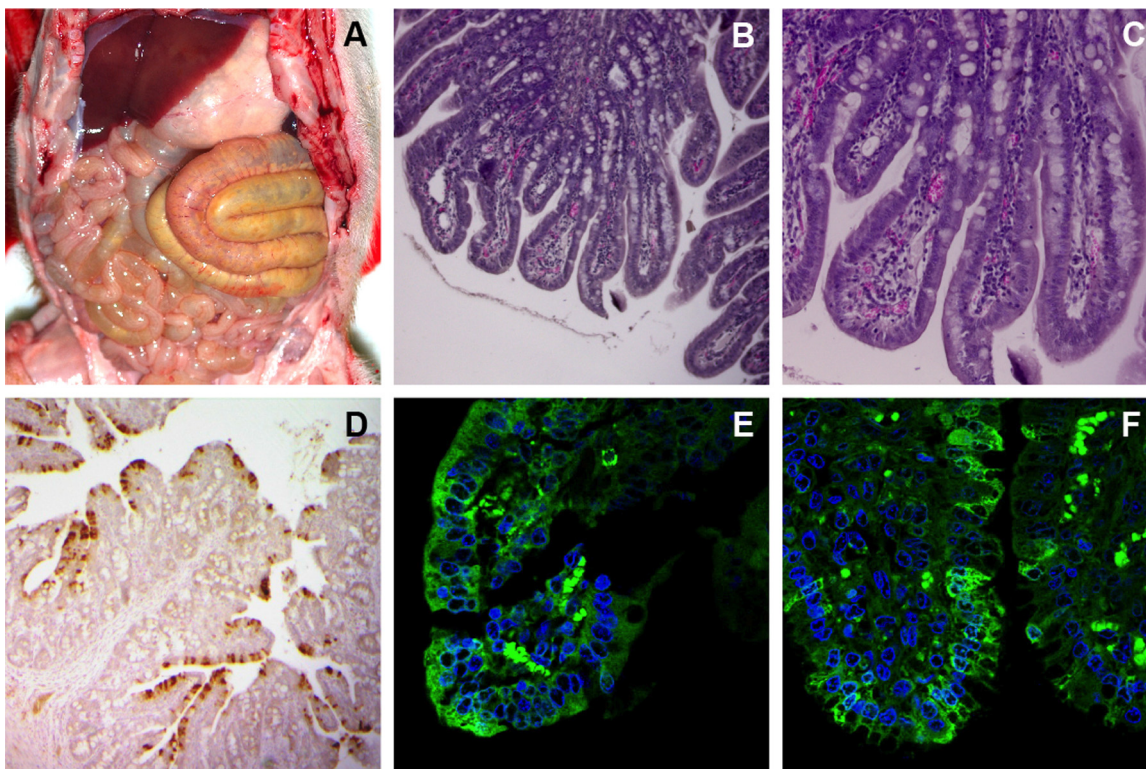


Fig. 4. Macroscopic and microscopic examinations of the intestine of piglets inoculated with the Korean PEDV strain KNU-141112. (A) Small intestines of an inoculated pig at 2 DPI showing thin and transparent intestinal walls and extended stomach filled with curdled milk. (B and C) Hematoxylin and eosin-stained jejunum of a KNU-141112-inoculated pig at 3 DPI (100 \times and 200 \times magnifications, respectively). (D) IHC analysis of jejunum tissue sections from a pig at 3 DPI (100 \times magnification). (E and F) Immunofluorescence staining in small intestine sections from pigs inoculated with KNU-141112 at 3 or 4 DPI. The sections were counterstained with DAPI and examined using a fluorescence microscope at 400 \times magnification. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

Table 1
Nucleotide and amino acid changes of KNU-141112 during serial passages in cell culture.

Genome region (nucleotide length)	Nucleotide								Amino acid							
	Position	feces	P3	P4	P5	P10	P20	P30	Position	feces	P3	P4	P5	P10	P20	P30
5' UTR (292)	– ^a	–	ND ^b	ND	–	–	ND	ND	–	–	ND	ND	–	–	ND	ND
ORF1a (12,354)	–	–	ND	ND	–	–	ND	ND	–	–	ND	ND	–	–	ND	ND
ORF1ab (20,345)	–	–	ND	ND	–	–	ND	ND	–	–	ND	ND	–	–	ND	ND
S	21448	A	A	A	A	C	C	C	272	K	K	K	K	T	T	T
(4,161)	21756	C	T	T	T	T	T	T	375	L	F	F	F	F	F	F
	24492	G	G	G	G	C	C	C	1287	E	E	E	E	Q	Q	Q
ORF3 (675)	24869	G	G	G	G	G	T	T	26	D	D	D	D	D	Y	Y
E (231)	25656	C	C	C	C	C	T	T	70	P	P	P	P	P	S	S
M (681)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
N (1,326)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
3'UTR (334)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

^a No nucleotide change found.

^b Not determined (ND).

resulting in the shorter pp1ab protein in length (Chen et al., 2014). However, none of the genomes of the three PEDVs included such an insertion, and this result was further confirmed by the traditional Sanger sequencing method (data not shown).

In agreement with previous results (Lee et al., 2010; Lee and Lee, 2014; Lee et al., 2014a,b), the full-length S gene-based phylogenetic tree revealed that the global PEDV strains were clearly defined into 2 separate clusters, designated genogroup 1 (G1; classical) and genogroup 2 (G2; field epidemic). Each genogroup can be further divided into subgroups 1a, 1b, 2a, and 2b (Fig. 5A). All original feces and passaged PEDV viruses through passages 30 were classified into subgroup 2b along with the recent Korean field isolates, which were most closely clustered together with the emergent US strains in an adjacent clade with the same subgroup. Subsequent phylogenetic analysis of the S1 region showed the same grouping structure as the S gene-based tree (data not shown). In addition, phylogenetic analysis based on the entire genome sequences demonstrated that strain KNU-141112 is grouped within the same cluster with the recent Korean and US strains (Fig. 5B).

4. Discussion

In Korea, three PEDV strains, SM98-1, DR-13, and Chinju99, were initially isolated almost two decades ago in Korea. Genetic and phylogenetic analyses revealed that SM98-1 and DR-13 belong to the classical group 1, whereas Chinju99 is classified into the field epidemic group 2. Of these, only SM98-1 and DR-13 isolates can be grown in cell culture and, accordingly, have been used as commercial vaccine seeds. Since 2008, we have been monitoring the genetic diversity of PEDV prevalent in the field based on the S gene. Our data demonstrated the existence of antigenic and genetic variations between vaccine and field strains, exhibiting a more than 10% amino acid difference in the S1 domain (Lee et al., 2010; Lee and Lee, 2014; Oh et al., 2014). This may be one of the reasons for the incomplete efficacy of current vaccines in Korea, suggesting that the isolation of field PEDV is required for the development of a next-generation vaccine. Although virus isolation in cell culture from clinical samples of naturally or experimentally infected pigs is fastidious, recent studies reported the successful isolation and propagation of several US original PEDV strains using Vero cells (Chen et al., 2014; Oka et al., 2014). In this study, we initially sought to isolate PEDV efficiently propagated *in vitro* from 7 intestine homogenates and 50 fecal samples of naturally infected piglets (field cases) and were able to obtain only 1 isolate from feces in Vero cell culture. The virus isolation rate, in the present study, was less than 2% and was relatively lower than that in recent US studies ranging from 5 to 10% (Chen et al., 2014; Oka et al., 2014). In previous studies, all isolated PEDV strains originated from intestinal

contents of naturally infected or experimentally inoculated pigs, suggesting that intestine samples may be a better source for virus isolation (Chen et al., 2014; Oka et al., 2014). However, we failed to isolate PEDV from intestine homogenates, which may be due to the number of intestinal contents ($n=7$) included in our study and be responsible for the low isolation rate. Although PEDV isolation might be affected by multiple factors, it appears to depend on the number of samples with good quality rather than the type of samples (intestinal contents or feces). Further studies are needed to improve the isolation methodology or to determine the contributing factor(s) to enhance the success rate of PEDV isolation in cell culture. The PEDV isolate, KNU-141112, was cytopathogenic in Vero cells from passage 3 and after passage 5, exhibited more severe and rapid CPE characterized by fusion of infected cells (syncytium or polykaryon formation). The initial viral infectious titers ranged from approximately 5 to 6 \log_{10} TCID₅₀/ml and increased after several more passages reaching to 8 \log_{10} TCID₅₀/ml or more. These growth characteristics, including cytopathology, infectious titer, and growth kinetics were unchanged or even more efficient throughout the experiment (30 passages), indicating that the isolate KNU-141112 is phenotypically stable during serial passages in Vero cells.

Since the antibody response is a critical indicator to prove the cause of viral infection, we immunized guinea pigs twice with the inactivated isolate (KNU-141112-P10) and determined whether the animals developed humoral immunity using an SN test. The guinea pig sera raised against the isolate contained high levels of NA (6.4 \log_2), demonstrating the ability of KNU-141112 to elicit immune responses. On the other hand, the antisera showed a relatively weak neutralizing response (an almost 2- \log_2 reduction), when the heterologous vaccine strain SM98-1 was used for an SN test. This weak neutralizing activity of the anti-KNU-141112 guinea pig sera against SM98-1 was somewhat expected because of a high degree of genetic diversity between the S proteins of the vaccine strain and field isolates (Lee et al., 2010; Lee and Lee, 2014; Oh et al., 2014).

Experimental oral inoculation of nursery pigs with PEDV isolate KNU-141112 induced severe clinical disease typical of acute enteritis throughout the study, demonstrating that the isolate was highly enteropathogenic in neonatal piglets. Onset of clinical signs including lethargy and watery diarrhea in inoculated pigs was found as early as 1 DPI. The PEDV genome was detected in feces in 100% of challenged pigs on DPI 1 and virus quantities from fecal shedding were maintained thereafter in all challenged pigs throughout the study period, leading to the source for direct transmission of virus to other animals. This result is inconsistent with a previous study using a US PEDV strain that demonstrated virus fecal shedding on DPI 1 in only one-fourth of the challenged pigs (Madson

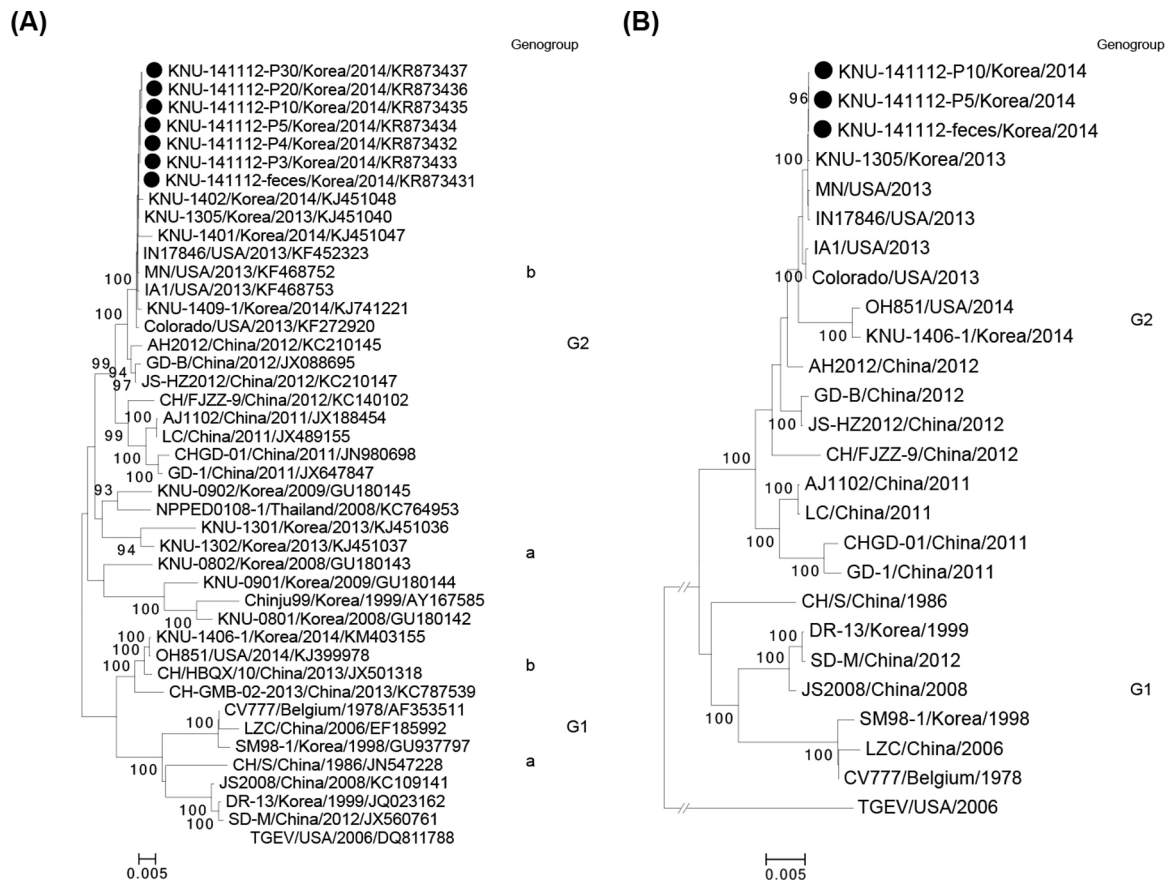


Fig. 5. Phylogenetic analyses based on the nucleotide sequences of the spike genes (A) and the full-length genomes (B) of PEDV strains. A putative similar region of the spike protein and the complete genome sequence of TGEV was included as an outgroup in each panel. Multiple-sequence alignments were performed using ClustalX program and the phylogenetic tree was constructed from the aligned nucleotide sequences by using the neighbor-joining method. Numbers at each branch represent bootstrap values greater than 50% of 1000 replicates. Names of the strains, countries and years of isolation, GenBank accession numbers, and genogroups and subgroups proposed in this study are shown. The PEDV isolates identified in this study are indicated by solid circles. Scale bars indicate nucleotide substitutions per site.

et al., 2014). The difference between the current and previous studies is the age of the pigs: 1-week-old neonatal pigs and 3-week-old weaned pigs used in the present and previous experiments, respectively. Therefore, younger piglets in this study were more sensitive to PEDV infection shedding virus in feces earlier than older pigs, probably due to an age-dependent disease severity as previously described (Shibata et al., 2000). IHC and IFA revealed that viral antigen in villous enterocytes were observed at 1 DPI in all segments of the small intestine of inoculated piglets. The onset of clinical signs and viral fecal shedding and the detection time of viral antigen in the target tissue were similar to recent independent reports using different US PEDV strains (Madson et al., 2014; Jung et al., 2014). Two non-challenged piglets were included in this study for direct contact to inoculated piglets in the same space. All contact piglets displayed PED-like symptoms within 24 h after the onset of clinical signs in inoculated piglets. The presence of PEDV in feces and infected tissues was further verified in contact piglets, showing 100% morbidity in our study. Mortality averages 50% in suckling piglets up to 1 week of age, often approaching 100% in 1- to 3-day-old piglets, and decreases to 10% thereafter (Saif et al., 2012). In our study, mortality was observed only in one out of four inoculated piglets, resulting in 25% mortality in the current study involving 8-day-old piglets. For reproducible challenge studies *in vivo* using the isolate in the future, a neonatal swine bioassay will be needed to determine either the median pig diarrhea dose or lethal dose as a standardized dose and this aspect is currently under investigation.

Whole-genome sequences of 3 Korean PEDV strains (KNU-141112-feces, P5, and P10) were determined using NGS approaches

coupled with RACE experiments. Regions covering the structural genes were also sequenced at the selected passages by the Sanger method for confirmation. Compared to the original feces (KNU-141112-feces), only one nt difference at position 21,756 was identified for the first 5 passages (KNU-141112-P5) in cell culture, which led to a non-synonymous mutation at the corresponding position 375 of the S protein. This nt change was initially found at passage 3 and further maintained through passage 30. However, we were unable to investigate whether this mutation had been acquired at the beginning of the Vero cell culture since infectious virus was not obtained during the first two passages. Interestingly, the identical C21756T mutation at the whole-genome level (L375F at the aa level of S) has been previously reported in a US PEDV isolate ISU-19338E during cell culture passage (Chen et al., 2014), suggesting its potential importance for adaption of the field virus to growth *in vitro*. At passage 10, two more nt differences at positions 21,448 and 24,492 of the genome (A21448C and G24492C) were detected when compared to KNU-141112-feces. All of the changes acquired in KNU-141112-P10 led to non-synonymous mutations at the respective positions 272 and 1287 of the S protein (K272T and E1287Q). These findings were similar to recent data reported by Chen et al. (2014) that two US isolates individually gained the 4 mutations located in ORF1b, S, and E through passage 9 in cell culture. However, no nucleotide changes were identified in ORF1b and E for the first 10 passages in Vero cells. The 3 mutations in the S protein were persistent for 30 passages in cell culture. Cell-adapted PEDV vaccine strains, SM98-1 and DR-13, are known to contain a 52-nt deletion spanned from the end of S to the start of

Table 2
Nucleotide comparison of the full-length genomes of Korean isolates to other PEDV strains.

Strain	Nucleotide identity (%) (No. of nucleotide differences)													
	KNU-141112 -P5	KNU-141112 -P10	KNU-141112 -feces	KNU-1305/Korea/2013	Co/13/USA/2013	IA1/USA/2013	MN/USA/2013	INI7846/USA/2013	AH2012/China/2012	GD-B/China/2012	JS-HZ2012/China/2012	CV777/Belgium/1978	SM98-1/Korea/1998	DR-13/Korea/1999
KNU-141112-feces	99.9 (1)	99.9 (3)	99.9 (8)	99.9 (8)	99.8 (38)	99.8 (43)	99.9 (12)	99.9 (13)	99.5 (117)	99.2 (200)	99.3 (180)	96.7 (905)	96.3 (1034)	96.8 (882)
KNU-141112-P5		99.9 (2)	99.9 (9)	99.9 (9)	99.8 (39)	99.8 (44)	99.9 (13)	99.9 (14)	99.5 (118)	99.2 (201)	99.3 (181)	96.7 (906)	96.3 (1035)	96.8 (883)
KNU-141112-P10			99.9 (11)	99.9 (11)	99.8 (41)	99.8 (46)	99.9 (15)	99.9 (16)	99.5 (120)	99.2 (203)	99.3 (183)	96.7 (908)	96.3 (1037)	96.8 (885)
KNU-1305/Korea/2013					99.8 (44)	99.8 (49)	99.9 (18)	99.9 (19)	99.5 (119)	99.2 (202)	99.3 (182)	96.7 (905)	96.3 (1034)	96.8 (884)
Co/13/USA/2013						99.9 (11)	99.8 (40)	99.8 (43)	99.5 (139)	99.3 (192)	99.3 (172)	96.7 (908)	96.3 (1037)	96.8 (890)
IA1/USA/2013							99.8 (45)	99.8 (48)	99.4 (144)	99.2 (197)	99.3 (177)	96.7 (913)	96.2 (1042)	96.8 (895)
MN/USA/2013								99.9 (13)	99.5 (119)	99.2 (202)	99.3 (182)	96.7 (906)	96.3 (1035)	96.8 (885)
INI7846/USA/2013									99.5 (120)	99.2 (205)	99.3 (185)	96.7 (910)	96.2 (1039)	96.8 (887)
AH2012/China/2012										99.4 (164)	99.4 (147)	96.8 (896)	96.3 (1028)	96.8 (880)
GD-B/China/2012											99.8 (32)	96.8 (897)	96.3 (1027)	96.8 (875)
JS-HZ2012/China/2012												96.8 (879)	96.4 (1009)	96.9 (857)
CV777/Belgium/1978													99.4 (148)	97.2 (651)
SM98-1/Korea/1998														97.6 (782)
DR-13/Korea/1999														

ORF3 and a 49-nt deletion in the middle of ORF3. Thus, we further sequenced structural genes of KNU-141112-P20 and -P30 and identified two additional nucleotide changes (G24869T and C25656T) acquiring non-synonymous amino acid mutations in ORF3 and E, respectively. Except for those nucleotide substitutions, the isolate had no extra change, including such large deletions in structural genes through passage 30, indicating the genetic stability of KNU-141112 during serial passages in Vero cells. Sequence comparisons with other PEDV strains showed that KNU-141112 isolates are genetically most similar to recent Korean and US epidemic strains with 99.8–99.9% identities, but most distinctly related to classical strains, CV777, SM98-1, and DR-13, with 96.3–96.8% identities at the genomic level. All phylogenetic analyses based on the complete genome, the full-length S gene, and the S1 portion formed the similar tree structure, revealing that the Korean KNU-141112 strains are clustered together with the re-emergent Korean strains in subgroup 2b that also includes 2013 emergent US strains and 2011–2012 field epidemic Chinese strains.

In conclusion, we isolated and serially propagated PEDV in cell culture that is phenotypically and genotypically identical to field strains responsible for the recent severe PED outbreaks in Korea. To our knowledge, this is the first report describing the isolation and *in vitro* and *in vivo* characterization of Korean PEDV associated with the field epidemic. With the availability of the Korean isolate, we are now able to spur the development of new effective and safe vaccines for PED prevention. Indeed, our PEDV isolate has been used for the development of an inactivated vaccine that is currently being evaluated under experimental and field conditions. Furthermore, we are continuing to passage the isolate in Vero cells to develop a live attenuated vaccine that generally prove to provide a more efficient protective immunity than killed viral vaccines. For this purpose, pathogenic and molecular characterization of the isolates at selected passages will be assessed to determine their phenotypes in pigs and to identify the genetic changes involved in PEDV attenuation.

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