RAPID COMMUNICATION

Genomic and antigenic characterization of porcine epidemic diarrhoea virus strains isolated from South Korea, 2017

Sunhee Lee | Changhee Lee

Animal Virology Laboratory, School of Life Sciences, BK21 Plus KNU Creative BioResearch Group, Kyungpook National University, Daegu, Korea

Correspondence
Changhee Lee, School of Life Sciences, College of Natural Sciences, Kyungpook National University, Daegu 41566, Korea. Email: changhee@knu.ac.kr

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Summary
Porcine epidemic diarrhoea virus (PEDV) is a globally emerging and re-emerging enteric coronavirus in pigs causing serious economic threats to the world swine industry. Since the re-emergence of massive PEDV outbreaks in South Korea in 2013–2014, domestic pig farms have continued to experience PED epidemics or endemics. This study represents the molecular characterization of PEDV isolates identified in diarrhoeic animals collected across the country in 2017. Initial sequencing analysis of the full-length S genes revealed that 70% of the 2017 isolates (7/10) belong to the G2b subgroup, while the remaining isolates were classified as G1b. The data indicated that both variant G1b and global epidemic G2b strains were responsible for current PED outbreaks in South Korea. The 2017 G1b and G2b isolates shared 98.7%–99.4% and 98.1%–99.2% amino acid sequence identity at the S gene level and 99.3% and 99.0%–99.6% nucleotide sequence homology at the genome level compared to the corresponding Korean prototype G1b and G2b strains, respectively. In an interesting manner, one G2b-like KNU-1705 strain was found to possess a large 39-nucleotide deletion in the ORF1a region theoretically encoding nonstructural protein 3. Phylogenetic analysis based on the entire genome and spike protein sequences indicated that the 2017 isolates were most closely related to other global G1b or G2b strains but formed different branches within the same genogroup. These results indicate that PEDVs undergo continuous evolution in the field. In addition, one 2017 PEDV strain, KOR/KNU-1705/2017, was successfully isolated and propagated in Vero cells. The antisera raised against the Korean prototype 2014 G2b strain efficiently neutralized KNU-1705 virus infection, suggesting antigenic homology between the 2014 and 2017 PEDV strains. Our data advance the understanding of the molecular epidemiology and antigenicity of PEDV circulating in South Korea.

KEYWORDS
antigenicity, full-length genome, phylogenetic analysis, porcine epidemic diarrhoea virus, prevalence

1 | INTRODUCTION

Porcine epidemic diarrhoea (PED) is a highly contagious, deadly enteric viral disease in pigs with economically effects on pig production. The disease causes severe watery diarrhoea, followed by fatal dehydration, and a high mortality rate in neonatal piglets (Lee, Kim, & Lee, 2015). The causative agent, PED virus (PEDV), is a large, enveloped, single-stranded, positive-sense RNA virus belonging to the genus Alphacoronavirus within the family Coronaviridae of the order Nidovirales (Cavanagh, 1997; Gorbalenya, Enjuanes, Ziebuhr, & Snijder, 2006; Pensaert & Debouck, 1978; Saif, Pensaert, Sestack, Yeo, & Jung, 2012). The PEDV genome is approximately 28 kb in length with a 5’ cap and 3’...
Porcine epidemic diarrhoea was first observed in feeder and fattening pigs in England in 1971 (Oldham, 1972) and caused widespread epidemics in multiple swine-producing countries in Europe during the 1970s (Opriessnig, 2016). A marked decrease in acute PED epizootics occurred in Europe in the 1980s and 1990s, and only sporadic outbreaks have occurred in recent years (Opriessnig, 2016). In Asia, PED was first reported in 1982, and unlike in Europe, it has since posed a huge economic threat to the Asian pork industry (Chen et al., 2008; Kweon et al., 1993; Li et al., 2012; Puranaveja et al., 2009; Takahashi, Okada, & Ohshima, 1983). In May 2013, PED outbreaks suddenly appeared in the United States and swiftly spread across the nation, as well as to adjacent countries. This outbreak caused the death of more than 8 million newborn piglets in the United States alone during a 1-year-epidemic period, leading to annual losses in the range of $900 million to $1.8 billion (Langel, Paim, Lager, Vlasova, & Saif, 2016). The US emergent strain-like viruses further reached East Asian countries, resulting in nationwide PED disasters (Lee, 2015; Lee & Lee, 2014; Lin et al., 2014; MAFF, 2018). During the 2013–2014 pandemics, PED rapidly swept across mainland South Korea and Jeju Island, killing hundreds of thousands of piglets in domestic herds (Lee & Lee, 2014, 2017; Lee & Lee, 2014). Since then, PED epizootics or enzootics have regionally occurred through provinces in South Korea with intensive swine industries. To investigate the diversity of PEDVs responsible for the ongoing outbreaks in South Korea, in this study, we determined the full-length sequences of the S proteins of field isolates and complete genome sequences of representative strains identified throughout 2017. In addition, we isolated and serially propagated a KOR/KNU-1705/2017 strain and assessed the antigenic cross-reactivity between 2014 and 2017 PEDV field isolates.

2 | MATERIALS AND METHODS

2.1 | Clinical sample collection

The small intestine or stool specimens were collected from piglets showing acute watery diarrhoea at various swine farms located in eight different provinces from March through December 2017. Intestinal homogenates were prepared as 10% (wt/vol) suspensions in phosphate-buffered saline (PBS) using a MagNA Lyser (Roche Diagnostics, Mannheim, Germany) by three repetitions of 15 s at a speed of 8,000 g. Faecal samples were also diluted with PBS to 10% (wt/vol) suspensions. The suspensions were then vortexed and centrifuged for 10 min at 4,500 × g (Hanil Centrifuge FLETAS, Inchon, South Korea). The clarified supernatants were initially subjected to RT-PCR using a TGE/PED Detection Kit (InnRION Biotechnology, Seongnam, South Korea) according to the manufacturer’s instructions. PEDV-positive samples were filtered through a 0.22-μm-pore-size syringe filter (Millipore, Billerica, MA) and stored at −80°C until subsequent sequencing analysis and virus isolation.

2.2 | Nucleotide sequence analysis

The S glycoprotein gene sequences of the virus isolates were determined by the traditional Sanger method. Two overlapping cDNA fragments spanning the entire S gene of each isolate were amplified by RT-PCR as described previously (Lee, Park, Kim, & Lee, 2010). The individual cDNA amplicons were gel-purified, cloned into a pGEM-T Easy Vector System (Promega, Madison, WI) and sequenced in both directions using two commercial vector-specific T7 and SP6 primers and gene-specific primers. The full-length S sequences of 10 PEDV, designated KNU-1701 to -1710, were deposited in the GenBank database under the accession numbers shown in Figure 1a. In addition, the complete genomes of representative PEDV field strains were sequenced by the traditional Sanger method. Ten overlapping cDNA fragments spanning the entire genome of each virus strain were RT-PCR-amplified as described previously (Lee & Lee, 2014; Lee et al., 2015, 2017), and each PCR product was sequenced as described above. The 5’ and 3’ ends of the genomes of individual isolates were determined by rapid amplification of cDNA ends (RACE) as described previously (Lee & Lee, 2013). General procedures for DNA manipulation and cloning were performed according to standard procedures (Sambrook & Russell, 2001). The complete genomic sequences of the 2017 viruses were deposited in the GenBank database under the accession numbers shown in Figure 1b.

2.3 | Multiple alignments and phylogenetic analyses

The sequences of the 48 fully sequenced S genes and 31 complete genomes of global PEDV isolates were independently used in sequence alignments and phylogenetic analyses. Multiple sequence alignments were generated with the ClustalX 2.0 program (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997), and the percentages of nucleotide sequence divergences were further assessed using the same software program. Phylogenetic trees were constructed from the aligned nucleotide or amino acid sequences using the neighbour-joining method and subsequently subjected to bootstrap analysis with 1,000 replicates to determine the percentage reliability values of each internal node of the tree (Saitou & Nei, 1987). All figures involving phylogenetic trees were generated using Mega 4.0 software (Tamura, Dudley, Nei, & Kumar, 2007).
2.4 Virus isolation and serum neutralization

PEDV isolation was conducted from faecal suspensions on Vero cells in the presence of trypsin (USB, Cleveland, OH) as described previously (Lee et al., 2015). Virus isolation was confirmed by cytopathic effect (CPE) observation, immunofluorescence assay (IFA) and nucleotide sequencing as described previously (Lee et al., 2015). The isolated PEDV strain was propagated for serial passages in Vero cells, and virus titres were determined as described previously (Lee et al., 2015). The cross-reactivity of antisera collected from sows inoculated with a Korean pandemic G2b strain KNU-141112 isolated in 2014 (Baek et al., 2016) was evaluated by a serum neutralization (SN) test in 96-well microtiter plates against the past 2014 and present 2017 isolates as previously described (Lee et al., 2015; Oh, Lee, Choi, & Lee, 2014). The neutralization titre was calculated as the reciprocal of the highest dilution of serum that inhibited virus-specific CPE in all duplicate wells.

3 RESULTS

3.1 Genetic and phylogenetic characterizations of 2017 Korean PEDV strains

The PEDV S glycoprotein is a suitable viral gene for investigating genetic relatedness among isolates and the molecular epidemiology of PEDV (Chen et al., 2014; Gerber et al., 2014; Lee, 2015; Lee & Lee, 2014; Lee et al., 2010; Oh et al., 2014). Based on the S gene sequences, therefore, PEDV can be genetically separated into two genogroup clusters, genogroup 1 (G1, classical and recombinant: low-pathogenic) and genogroup 2 (G2, field epizootic or panzootic: high-pathogenic), which are further divided into subgroups 1a and 1b.
The number of nt/aa differences and percent identity shared between the 2017 isolates and genogroup representative strains is summarized in Table S2.

To establish the genetic relationships involved, phylogenetic analyses were carried out using the nucleotide sequences of the S gene and full-length genome of the 2017 isolates, which were determined in this study and are available from GenBank (Figure 1). Consistent with previous studies (Lee, 2015; Lee & Lee, 2014; Lee et al., 2015), phylogenetic analysis based on the PEDV S genes revealed clear separation among the G1a, G1b, G2a and G2b subgroups. All G2b strains identified in 2017 were grouped within the G2b clade; however, they were in different branches from the emergent US strains and past re-emergent Korean field isolates (Figure 1a). The 2017 G1b isolates were most closely clustered together, forming an independent branch within the G1b subgroup. Furthermore, a phylogenetic tree subsequently reconstructed from the complete genome showed the same grouping structure as the S gene-based tree (Figure 1b). As shown previously (Lee, 2015; Lee et al., 2015), the entire genome-based phylogenetic tree revealed that the G1b strains including KNU-1702 were grouped within the G2 clade because of the similarity between the G1b and G2b genomes, except for the N-terminal one-third of the S gene. These phylogenetic data suggest that the contemporary G1b and G2b epidemic strains detected in 2017 differ from the emergent 2013–2014 pandemic strains in South Korea.

3.2 | Virus isolation and neutralizing activity

We attempted to isolate PEDV from PCR-positive clinical suspensions on Vero cells. One PEDV isolate designated as KNU-1705 was successfully isolated from the faeces of a naturally infected piglet from a commercial farm located in Chungnam Province obtained on December 19, 2017. KNU-1705 produced apparent CPEs typical of PEDV infection, such as cell fusion, syncytium and detachment, in infected Vero cells from passage 3 (P3) (Figure 2a). Virus propagation was confirmed by detecting PEDV antigens by IFA using a PEDV N protein-specific MAb (CAVAC, Daejeon, South Korea). As shown in Figure 2a, distinct staining was distributed in the cytoplasm of typical syncytial cells. In addition, S glycoprotein gene sequencing revealed that the P10 isolate (KNU-180117-P10) contains one nucleotide change, resulting in a nonsynonymous mutation (Ser to Leu) at amino acid position 1,708, compared to the original faecal sample KNU-1705 faeces. Growth kinetics analysis further indicated that KNU-1705 replicated efficiently in Vero cells, reaching a maximum titre of \(>10^5\) TCID\(_{50}\)/ml by 24 hr postinfection (hpi) (Figure 2b). Next, we tested sow antisera previously elicited by a PEDV G2b KNU-141112 strain (Baek et al., 2016; Lee et al., 2015) to determine their cross-neutralization ability against the contemporary KNU-1705 isolate (Figure 2c). In agreement with previous studies, the antisera were greatly effective in inhibiting KNU-141112 infection with mean neutralizing antibody (NA) titres of 1:131. Similar to that, highly diluted antisera efficiently protect Vero cells infected with the recent KNU-1705 isolate with mean NA titres of 1:80, respectively.
TABLE 1  Pairwise comparisons of the nucleotide and protein sequences of the S protein genes of the 2017 isolates and genogroup representative porcine epidemic diarrhoea virus strains

| Strain name (Genogroup) | CV777 | SM98-1 | DR-13 | OH851 | KNU-1406 | KNU-1701 | KNU-1702 | KNU-1707 | GD-B | Co/13 | KNU-141112 | KNU-1703 | KNU-1704 | KNU-1705 | KNU-1706 | KNU-1708 | KNU-1709 | KNU-1710 |
|-------------------------|-------|--------|--------|-------|---------|---------|---------|---------|-------|-------|---------|---------|---------|---------|---------|---------|---------|---------|
| CV777 (G1a)             | 98.8  | 96.8   | 95.7   | 95.7  | 95.5    | 95.4    | 95.3    | 93.7    | 93.6  | 93.6  | 93.6    | 93.6    | 93.6    | 93.6    | 93.2    | 93.4    | 93.2    | 93.2    |
| SM98-1 (G1a)            | 98.3  | 95.7   | 94.6   | 94.6  | 94.4    | 94.3    | 94.2    | 92.6    | 92.6  | 92.5  | 92.5    | 92.1    | 92.1    | 92.1    | 92.1    | 92.1    | 92.1    | 92.1    |
| DR-13 (G1a)             | 96.0  | 94.5   | 95.9   | 95.9  | 95.7    | 95.6    | 95.4    | 93.6    | 93.5  | 93.5  | 93.5    | 93.0    | 93.0    | 93.0    | 93.0    | 93.0    | 93.0    | 93.1    |
| OH851 (G1b)             | 96.1  | 94.6   | 96.0   | 99.9  | 99.6    | 99.5    | 99.4    | 95.8    | 96.5  | 96.3  | 96.3    | 95.9    | 96.3    | 96.2    | 96.2    | 96.4    | 95.7    | 95.9    |
| KNU-1406 (G1b)          | 96.2  | 94.7   | 96.0   | 99.7  | 99.6    | 99.5    | 99.4    | 95.8    | 96.4  | 96.3  | 96.3    | 96.0    | 96.3    | 96.2    | 96.2    | 96.4    | 95.7    | 95.9    |
| KNU-1701 (G1b)          | 96.0  | 94.5   | 95.9   | 99.4  | 99.4    | 99.7    | 99.6    | 95.6    | 96.3  | 96.2  | 96.1    | 96.1    | 96.1    | 96.4    | 96.4    | 96.2    | 95.5    | 95.7    |
| KNU-1702 (G1b)          | 95.7  | 94.2   | 95.8   | 99.2  | 99.1    | 99.7    | 99.5    | 95.5    | 96.1  | 96.0  | 96.0    | 96.1    | 96.0    | 96.4    | 96.4    | 96.1    | 95.4    | 95.7    |
| KNU-1707 (G1b)          | 95.5  | 94.0   | 95.5   | 98.8  | 98.7    | 99.3    | 99.0    | 95.4    | 96.1  | 96.0  | 95.9    | 96.0    | 95.9    | 96.3    | 96.3    | 96.0    | 95.4    | 95.6    |
| GD-B (G2b)              | 93.1  | 91.6   | 92.5   | 95.4  | 95.3    | 95.2    | 94.9    | 95.0    | 99.2  | 99.2  | 99.2    | 98.3    | 98.8    | 98.4    | 98.3    | 98.8    | 98.4    | 98.5    |
| Co/13 (G2b)             | 93.2  | 91.8   | 92.8   | 95.8  | 95.8    | 95.6    | 95.3    | 95.3    | 99.4  | 99.8  | 99.8    | 98.9    | 99.4    | 99.1    | 99.0    | 99.5    | 99.0    | 99.1    |
| KNU-1305 (G2b)          | 93.1  | 91.6   | 92.7   | 95.7  | 95.6    | 95.6    | 95.3    | 95.2    | 99.2  | 99.8  | 99.9    | 98.8    | 99.3    | 99.0    | 98.9    | 99.4    | 99.1    | 99.2    |
| KNU-141112 (G2b)        | 93.0  | 91.5   | 92.6   | 95.6  | 95.6    | 95.5    | 95.2    | 95.1    | 99.2  | 99.7  | 99.7    | 98.8    | 99.3    | 98.9    | 99.4    | 99.1    | 99.2    | 99.2    |
| KNU-1703 (G2b)          | 92.5  | 91.0   | 92.0   | 94.5  | 94.6    | 94.9    | 94.9    | 94.5    | 97.9  | 98.2  | 98.1    | 98.1    | 98.7    | 99.3    | 99.2    | 98.8    | 98.2    | 98.3    |
| KNU-1704 (G2b)          | 93.0  | 91.5   | 92.6   | 95.6  | 95.6    | 95.4    | 95.1    | 95.1    | 98.9  | 99.4  | 99.2    | 99.2    | 97.9    | 98.8    | 98.7    | 99.8    | 98.7    | 98.7    |
| KNU-1705 (G2b)          | 92.5  | 91.0   | 92.5   | 95.2  | 95.1    | 95.6    | 95.6    | 95.3    | 98.0  | 98.5  | 98.4    | 98.4    | 98.4    | 98.1    | 99.6    | 98.9    | 98.2    | 98.4    |
| KNU-1706 (G2b)          | 92.7  | 91.3   | 92.6   | 95.5  | 95.4    | 95.8    | 95.8    | 95.5    | 98.1  | 98.6  | 98.3    | 98.6    | 98.3    | 99.4    | 98.8    | 98.1    | 98.3    | 98.3    |
| KNU-1708 (G2b)          | 93.2  | 91.7   | 92.7   | 95.8  | 95.7    | 95.6    | 95.3    | 95.1    | 98.9  | 99.4  | 99.2    | 99.2    | 97.9    | 98.1    | 98.3    | 98.7    | 98.7    | 98.7    |
| KNU-1709 (G2b)          | 92.4  | 90.9   | 91.8   | 94.6  | 94.5    | 94.5    | 94.2    | 94.2    | 98.0  | 98.5  | 98.4    | 98.3    | 97.1    | 98.1    | 97.3    | 97.4    | 98.1    | 98.6    |
| KNU-1710 (G2b)          | 92.9  | 91.5   | 92.3   | 95.2  | 95.1    | 95.0    | 94.8    | 94.7    | 98.2  | 98.7  | 98.6    | 97.1    | 98.1    | 97.4    | 97.5    | 98.1    | 97.6    |

The percent nucleotide identity was shown in the upper right and the percent amino acid identity was presented in the lower left.
which were less than 1-log₂ lower but not significantly different compared to those against KNU-141112. Taken together, our data indicate that the antisera cross-reacted well between the homologous G2b field isolates, suggesting antigenic similarity between the 2014 and 2017 PEDV strains.

4 | DISCUSSION

PEDV has emerged or re-emerged as one of the deadliest and most contagious viral pathogens in swine, leading to large financial losses in the global swine industry. Along with strict biosecurity, vaccination is a fundamental tool for managing and eradicating PEDV during epidemic or endemic outbreaks. Although G1a-based vaccines against PEDV were developed and used to combat this disease in South Korea over the past decade, their efficacy in the field, as well as the advantages and disadvantages of their use, is continuously debated. Furthermore, a growing body of evidence suggests that their incomplete effectiveness may result from antigenic, genetic (>10% aa variation between respective S proteins) and phylogenetic (G1 versus G2) differences between vaccine and field epidemic strains (Lee et al., 2010; Lee & Lee, 2014; Oh et al., 2014; Kim et al., 2015; Lee et al., 2015; Lee, 2015). The advent of the 2013–2014 PEDV pandemic led to a breakthrough in the development of G2b-based vaccines phenotypically and genotypically homologous to field strains responsible for global PED epidemics, and these G2b vaccines are currently applied to prevent PEDV in South Korea. Another important policy for controlling PEDV is to operate a monitoring and surveillance system (MOSS) to monitor genetic diversity among field isolates and surveil the emergence of novel variants in the field, which will contribute to preventing future outbreaks. To provide insight into the understanding of the current epidemiological status of PEDV in South Korea, the present study aimed to investigate the genetic, phylogenetic and antigenic characteristics of PEDVs responsible for regional outbreaks in South Korea in 2017.

Nucleotide sequencing analysis revealed that two different PEDV genotypes, low-pathogenic G1b and high-pathogenic G2b, caused regional outbreaks in South Korea, with the latter genotype more prevalent and associated with more serious and fatal clinical outcomes on domestic pig farms. The 2017 isolates exhibited less than

![FIGURE 2](image-url)
1% nucleotide sequence variations at the genome level with the 2013–2014 pandemic strains. However, field G2b isolates with nearly 2% amino acid sequence divergence compared to previous G2b strains at the S gene level were identified in the present study. Furthermore, mutations within the S protein were randomly and extensively distributed in the S1 and S2 regions among the 2017 isolates (Figure S1). This finding may warn the emergence of new genotypes or variant if broad S mutations are incidentally accumulated in a specific virus in the natural host that is predominant in the field. Moreover, the 2017 G1b and G2b isolates were found to be phylogenetically different from former respective Korean strains, indicating that continuous and independent evolution in the natural host occurs under environmental pressures. In an interesting manner, a large 13-aa DEL in the region of ORF1a encoding nsp3 was identified in G2b KNU-1705 virus, which is the first report of a novel nsp3-DEL variant. Nsp3 critically serves as a PLPro that posttranslationally trims replicase polyproteins into functional nsps during virus replication. However, this unique DEL is in the Glu-rich acidic region, which does not affect the authentic roles of nsp3 and thus is nonessential for coronavirus replication (Lei, Kusov, & Hilgenfeld, 2018). Although the virus can tolerate the large nsp3-DEL which is dispensable for PEDV replication as shown in Figure 2, the pathogenicity of the KNU-1705 virus remains unclear. We have obtained a KNU-1705 isolate that can grow efficiently in cell culture and are currently investigating its biological properties. Based on the high degree of cross-neutralization between the 2014 and 2017 PEDV strains, the antigenicity may be maintained for at least 4 years. This finding indicates the effectiveness of 2013–2014 pandemic strain (KNU-141112)-derived vaccines currently available in South Korea against existing PEDV epidemics. However, consistent with small-scale genetic changes in the 2017 isolates within the identical G2b genotype, antigenic variations appeared to be ongoing under various field conditions. Therefore, the timeline of this situation is unclear and it is unknown whether antigenic differences among PEDV epidemic strains will contribute to the failure of current G2b vaccines. To counteract the prospective scenario, further studies are critical for securing culturable PEDV epidemic strains that are genetically, phenotypically and antigenically characterized in the laboratory.

In summary, genetic and phylogenetic analyses indicated that the 2017 epidemic-related isolates are closely related with corresponding global G1b or G2b strains identified in previous outbreaks and that the virus continues to evolve in its host environment. Despite their genetic diversity, antigenicity currently seems to remain unchanged among G2b strains, indirectly confirming the efficacy of G2b-based vaccines against homologous G2b PEDVs responsible for current epidemics. Because the virus is assumed to undergo an evolutionary process to accumulate mutations to ensure viral fitness in the field, new genotypes or variants of PEDV, against which the current G2b vaccine may provide partial protection, will eventually emerge. Furthermore, this circumstance may advent earlier than expected if PEDV outbreaks fade from our attention following sporadic or endemic outbreaks without serious economic problems. Therefore, it is important to execute mandatory notification of PED-like outbreaks essentially followed by activating an MOSS, including early diagnosis, to survey forthcoming PEDV strains that may emerge locally or globally through genetic drift (e.g., nonsilent point mutations) or genetic shift (e.g., recombination events) and obtain and characterize epidemic field isolates to predict and prepare for future epizootics or panzootics.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ORCID

Changhee Lee http://orcid.org/0000-0002-5930-5461

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