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Heterogeneity in spike protein genes of porcine epidemic diarrhea viruses isolated in Korea

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Porcine epidemic diarrhea virus (PEDV) has plagued the domestic swine industry in Korea causing significant economic impacts on pig production nationwide. In the present study, we determined the complete nucleotide sequences of the spike (S) glycoprotein genes of seven Korean PEDV isolates. The entire S genes of all isolates were found to be nine nucleotides longer in length than other PEDV reference strains. This size difference was due to the combined presence of notable 15 bp insertion and 6 bp deletion within the N-terminal region of the S1 domain of the Korean isolates. In addition, the largest number of amino acid variations was accumulated in the S1 N-terminal region, leading to the presence of hypervariability in the sequences. Sequence comparisons at the peptide level of the S proteins revealed that all seven Korean isolates shared diverse similarities ranging from a 93.6% to 99.6% identity with each other but exhibited a 92.2% to 93.7% identity with other reference strains. Collectively, the sequence analysis data indicate the diversity of the PEDV isolates currently prevalent in Korea that represents a heterogeneous group. Phylogenetic analyses showed two separate clusters, in which all Korean field isolates were grouped together in the second cluster (group 2). The results indicate that prevailing isolates in Korea are phylogenetically more closely related to each other rather than other reference strains. Interestingly, the tree topology based on the nucleotide sequences representing the S1 domain or the S1 N-terminal region most nearly resembled the full S gene-based phylogenetic tree. Therefore, our data implicate a potential usefulness of the partial S protein gene including the N-terminal region in unveiling genetic relatedness of PEDV isolates.

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1. Introduction

Porcine epidemic diarrhea (PED) is a devastating swine disease that is characterized by acute enteritis and lethal watery diarrhea followed by dehydration leading to death with a high mortality rate in piglets (Debouck and Pensaert, 1980; Pensaert and Yeo, 2006; Pijpers et al., 1993). The disease was first recognized in England in 1971 (Oldham, 1972), and since then, outbreaks have been reported in Europe and Asia (Chen et al., 2008; Pensaert et al., 1981; Puranaveja et al., 2009; Takahashi et al., 1983). The causative agent of this disease, the PED virus (PEDV), was first described in 1978 (Pensaert and Debouck, 1978). Thereafter, a suitable cell culture system for PEDV propagation was developed and successfully used for virus isolation (Hofmann and Wyler, 1988). In Korea, PEDV was first recognized in 1992 (Kweon et al., 1993); however, a retrospective study indicated that the virus had already existed as early as 1987 (Park and Lee, 1997). Since the emergence, a periodic vaccination strategy has been applied nationwide to control the disease on pig farms but PEDV outbreaks have continuously occurred causing tremendous economical losses to the Korean swine industry.

PEDV, a member of the family Coronaviridae, is an enveloped virus possessing a single-stranded positive-sense approximately 28 kb RNA genome with a 5′ cap and a 3′ polyadenylated tail (Pensaert and Debouck, 1978; Pensaert and Yeo, 2006). The PEDV genome is composed of the 5′ untranslated region (UTR), at least 7 open reading frames (ORF1a, ORF1b, and ORF2–6), and the 3′ UTR (Kocherhans et al., 2001). The two large ORF1a and 1b cover the 5′ two-third of the genome and encode the non-structural replicase genes. The remaining ORFs in the 3′ terminal region code for four major structural proteins, the 150–220 kDa glycosylated spike (S) protein, 20–30 kDa membrane (M) protein, 7 kDa envelope (E) protein, and 58 kDa nucleoprotein (N) protein (Duarte and Laude, 1994; Pensaert and Yeo, 2006).

The S protein of PEDV is a type I membrane glycoprotein composed of 1383 amino acids (aa), which contains a signal peptide (1–24 aa), a large extracellular region, a single transmembrane domain (1334–1356 aa), and a short cytoplasmic tail. In addition,
the S protein can be divided into S1 (1–735 aa) and S2 (736–1383 aa) domains based on its homology with S proteins of other coronaviruses (Jackwood et al., 2001; Sturman and Holmes, 1984). As with other coronavirus S proteins, the PEDV S glycoprotein is known to play pivotal roles in interacting with the cellular receptor to mediate viral entry and inducing neutralizing antibodies in the natural host (Bosch et al., 2003; Chang et al., 2002; Godet et al., 1994). Thus, the S glycoprotein would be a primary target for the development of effective vaccines against PEDV. It is also the major envelope glycoprotein found in the virion, which serves as an important viral component in the study to understand genetic relationships among PEDV isolates and the epidemiological status of PEDV in the field (Park et al., 2007b; Puranaveja et al., 2009; Spaan et al., 1988). In the present study, therefore, we sought to determine the full-length sequences of the S glycoproteins of PEDV field isolates in order to investigate genetic relatedness and to understand the diversity and prevalence of PEDV isolates in Korea.

2. Materials and methods

2.1. Sample collection

Small intestine or stool specimens were taken from piglets showing acute enteritis and watery diarrhea on seven different swine farms in the Gyeongbuk province in 2008–2009. Intestinal samples were prepared into 10% suspensions through homogenization with phosphate-buffered saline (PBS). Fecal samples were diluted with PBS to be 10% suspensions. The suspensions were then vortexed and centrifuged for 10 min at 1700 × g (Hanil Centrifuge FLETA 5, South Korea). The clarified supernatants were initially subjected to RT-PCR with PEDV N gene-specific primers N-Fwd and N-Rev (Table 1), and nucleotide sequencing in order to confirm PEDV infection. The PEDV-positive supernatants were stored at –80°C as the field isolate until use.

2.2. RT-PCR, DNA cloning and sequence analysis

In order to determine the full-length S glycoprotein gene sequences of the Korean PEDV isolates, primers were first selected based on published known sequences of reference PEDV strains (Table 1). Two overlapping cDNA fragments spanning the entire S gene were RT-PCR amplified by using gene-specific primer sets. Briefly, viral RNA was extracted from the PBS-diluted or homogenized samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Reverse transcription (RT) was performed by using 1 μg of viral RNA, specific reverse primers, and SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to the manual provided by the manufacturer. PCR was conducted to amplify each cDNA fragment from the RT product by using Takara Ex Taq DNA polymerase (Takara, Japan) according to the manufacturer’s protocol under the following conditions: denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. 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. General procedures for DNA manipulation and cloning were performed according to standard procedures (Sambrook and Russell, 2001). The complete S glycoprotein gene sequences of the Korean PEDV isolates were deposited to the GenBank database under their accession numbers (Table 2).

2.3. Multiple alignments and phylogenetic analyses

The sequences of 17 fully and 8 partially sequenced S genes of PEDV isolates were independently used in sequence alignments and phylogenetic analyses (Table 2). The multiple-sequencing alignments were generated with ClustalX 2.0 program (Thompson et al., 1997), and percentage nucleotide sequence divergences were further assessed by using the same software application. Phylogenetic trees were constructed from the aligned nucleotide 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 by the Mega 4.0 software (Kumar et al., 2004). A similarity plot was generated by Simplot ver. 3.5.1 (Lole et al., 1999) to compare the full S genes of the PEDV isolates with those of the published reference strains.

3. Results

3.1. Nucleotide and amino acid sequence analyses

The full-length nucleotide sequences of S glycoprotein genes of PEDV isolates were determined to investigate their genetic characterization. The sequence data found that the S genes of seven Korean PEDV isolates (KNU-0801, KNU-0802, and KNU-0901–KNU-0905) consisted of 4161 nucleotides (nt) in length, which encoded 1386 amino acid residues. The complete nucleotide sequences of the Korean PEDV isolates were further compared to those of reference PEDV strains described in Table 2. The full-length S genes of the Korean PEDV isolates were determined to be 9 nt (3 amino acids) longer in size than that of other PEDV strains. This consequence was due to the presence of the number of inserted or deleted nucleotides that was accumulated in the N-terminus of the S1 domain (Fig. 1). Remarkably, the first notable insertion existed at amino acid positions 56–59 of the S protein for all seven Korean PEDV isolates followed by the presence of one additional inserted aspartic acid residue at position 140. Interestingly, the unique deletion was also located between amino acids 156 and 157 of the PEDV S protein, which is commonly present in reference PEDV strains. The S proteins of seven Korean PEDV isolates contained 25–27 potential N-linked glycosylation sites (identified by the sequence NXS/T, where X is any amino acid except proline). Deletion or modification of N-glycosylation sites was found predominantly in the N-terminus of the S1 domain. Furthermore, the largest number of amino acid differences was also clustered in the N-terminal region of the S1 domain. In addition, the genetic distances between the query strain CV777 and the Korean field isolates were plotted against the nucleotide position. Likewise, the N-terminal region of the S1 domain (1–800 bp) showed the lowest similarity with the PEDV reference strain (Fig. 2). Taken together, our data indicate that the S1 domain including the peptide signal sequences of the Korean field isolates contain regions that exhibited sequence hypervariability.

The similarity between the S protein genes was measured, and the sequence homology results are described in Table 3. The comparison of nucleotide sequences showed variable similarities among the seven Korean PEDV isolates, ranging from 94.8% to 99.7% identity. The sequence analysis was further extended to the deduced amino acid level and exhibited 93.6–99.6% identity with each other. In addition, all isolates shared diverse identities with the first Korean field isolate Chinju99, showing 93.8–97.7% homology at the nucleotide sequence level and accordingly, the percent identity ranged between 91.8% and 96.8%. However,
when the seven isolates were compared to other published PEDV reference strains, the similarity was relatively low but consistently ranged from 93.0% to 94.6% and 92.2% to 93.7% identities at the nucleotide and amino acid sequence levels, respectively. Thus, sequence homology results suggest that the diversity in the S protein is present in the group of seven Korean field isolates.

### 3.2. Phylogenetic analyses

For studies to establish genetic relationships of the fully sequenced S proteins, phylogenetic analyses were performed. The previous phylogeny based on the partial S glycoprotein (495–697 aa) indicated that the Korean PEDV isolates are divided into three groups (G1–G3) with three subgroups (G1-1–3), suggest-
ing that many isolates are closely related to the G1-1 subgroup (Park et al., 2007b). To confirm the previous conclusions, the phylogeny was initially performed by using the nucleotide sequences of the corresponding C-terminal region of the S1 domain of Korean isolates and reference strains (Fig. 3A). The phylogenetic tree topology was shown to form three distinct groups, which was consistent with the previous studies (Park et al., 2007b; Puranaveja et al., 2009). Likewise, most field isolates (5/7) were found to be grouped into the G1-1 subgroup, whereas the remaining two isolates (KNU-0801 and KNU-0901) were clustered in the G2 group with Spk1.

To conduct more precise evolutionary studies, phylogenetic analyses were further expanded to the full-length nucleotide sequences of the S protein genes in the Korean field isolates and published PEDV reference strains (Fig. 3B). In addition, the partial nucleotide sequences corresponding the S1 domain (1–735 aa), S1 N-terminal

Fig. 1. Amino acid sequence alignment of the S glycoprotein genes of the Korean PEDV isolates and PEDV reference strains. The dashes (−) indicate the deleted sequences. Potential N-glycosylation sites predicted by GlycoMod Tool (http://www.expasy.ch/tools/glycomod/) are shown in boldface type. Insertions and deletions in PEDV isolates are shaded. Putative signal sequences (dotted line box) and amino acids representing potential hypervariable domains (solid boxes) are indicated.
Fig. 1. (Continued).

Fig. 2. Similarity plot of the full-length nucleotide sequences of seven PEDV isolates with other reference strains. The similarity plot was constructed using the two-parameter (Kimura) distance model with a sliding window of 200 bp and step size of 20 bp. The vertical and horizontal axes indicate the percent nucleotide similarity and nucleotide position (bp) in the graph, respectively.

region (1–494 aa), and S2 domain (736–1383 aa) of the S glycoprotein genes, respectively, were independently examined in the phylogenetic study (Fig. 3C to E). Unlike the result obtained above, the full S gene-based phylogenetic tree revealed that all PEDV strains and field isolates used in the present study were defined into two separate clusters (Fig. 3B). The first cluster (group 1; G1) included the PEDV reference strains, Korean vaccine strains (SM98P and DR13) and all Chinese strains. In contrast, the second cluster (group 2; G2) contained all Korean field isolates, including previously identified Korean field isolates Spk1 and Chinju99 that were reported formerly to belong to G2 and G3 in the S1 C-terminal based phylogenetic tree, respectively (Park et al., 2007b). These data indicated that the Korean field isolates were more closely related to each other rather than other reference strains. More importantly, the phylogenetic trees based on the nucleotides representing either the S1 domain or the S1 N-terminal part were most closely similar to the tree based on the full-length S gene in that they also exhibit two distinct phylogenetic groups (Fig. 3C and D, compared to Fig. 3B). This result suggests that only the N-terminal region of the S1 domain would be sufficient for revealing genetic relatedness among the different PEDV isolates. Subsequent phylogenetic analyses on the basis of the amino acid sequences further showed the
Fig. 3. Phylogenetic analyses based on the nucleotide sequences corresponding to the S1 C-terminal region (495–697 aa) (A), full-length S gene (B), S1 domain (C), S1 N-terminal region (1–494 aa) (D), and S2 domain (736–1383 aa) (E). Putative similar regions of spike proteins of other distantly related coronaviruses, transmissible gastroenteritis virus (TGEV) and human coronavirus HCoV-229E, were also included in this study. Multiple-sequencing alignments were performed using ClustalX program and the phylogenetic tree was constructed from the aligned nucleotide sequences by using the neighbor-joining method. The numbers at each branch represent bootstrap values greater than 50% of 1000 replicates. The scale bars indicate the number of 0.1 inferred substitutions per site.

4. Discussion

The S glycoprotein of the coronavirus is a type 1 glycoprotein associated with the viral envelope. For viral entry, the S protein recognizes the cellular receptor via specific interactions and in the natural host, it is responsible for mediating viral neutralizing antibodies (Bosch et al., 2003; Chang et al., 2002). In addition, the S protein gene is considered the most useful in revealing the genetic diversity of coronavirus isolates (Kim et al., 2003; Paton and Lowings, 1997; Puranaveja et al., 2009; Sánchez et al., 1992). Indeed, sequence analysis based on the S protein genes of PEDV isolates has been recently reported to show the diversity of Korean PEDV isolates (Park et al., 2007b). However, this previous study assessed only partial S protein genes including the epitope region (Chang et al., 2002), thereby implying that the data might not represent the results expected from the full S gene-based analysis. A more precise study with the full S genes will be needed to better understand the genetic relationships among prevailing PEDVs in Korea. In the current study, therefore, the complete nucleotide and deduced peptide sequences of the S protein genes of seven Korean PEDV isolates were determined and compared with those of published PEDV reference strains. As consistent with the previous study, we demonstrated that Korean PEDV isolates undergo the genetic diversity in their S glycoprotein genes, and notably distinct results were obtained in our study.

The first unique characteristic of the field isolates indicated in the present study was the presence of the insertion and deletion same results as found in the nucleotide-based phylogeny (data not shown).
in the S protein genes of all seven Korean PEDV isolates. These nucleotide variations were found to exist within the N-terminus of the S1 domain that was excluded in the previous study. The similarity plot showed that most of the diversity between the G1 and G2 clusters is located within the S' most 800 nt of the S gene. Although further studies would be needed to investigate this phenomenon, it might be explained by a possible recombination event with an unknown PEDV strain or other porcine coronaviruses in the field. In addition, the S1 N-terminus showed the high occurrence of amino acid mutations that led to the creation or elimination of potential N-glycosylation sites. Thus, this N-terminal part of the S1 domain contains several potential hypervariable regions which will be useful in differentiating specific PEDV isolates and in studying genetic relatedness among field isolates.

Our findings showed that the S protein genes of the Korean PEDV isolates exhibited low levels of sequence conservation compared to other reference strains. More interestingly, sequence homology analysis of the S proteins suggests that Korean isolates are genetically diverse among themselves, showing various identities with each other. These results further indicate heterogeneity among prevailing PEDV isolates in Korea. Although the mechanism for the origin of the diversity is unclear, one possibility is that the virus might have evolved independently since PEDV introduction into Korea in the early 1990s.

The data obtained from the present phylogenetic analyses were divided into three separate groups based on the partial S gene including the C-terminal region of S1 domain. In that study, most Korean isolates were found to belong to the group 1 and to be highly homologous to reference strains that include CV777, Br1/87, Korean vaccine strains, and the Chinese strain JS-2004-2. It was further reported that the Korean isolates were distantly related to other previously identified Korean field isolates Spk1 and Chinhu99. These results were reproducible in the current analysis of the same region with Korean isolates, reference strains, and Thailand isolates. However, the full S gene-based phylogenetic analysis in the present study resulted in two distinct clusters. PEDV reference strains including Korean vaccine strains and Chinese isolates were determined to belong to the group 1, whereas all Korean field isolates including Spk1 and Chinhu99 were clustered together within the group 2. These data demonstrate that prevalent PEDV isolates in Korea are more closely related to each other and to other previously identified Korean isolates Spk 1 and Chinhu99 rather than reference strains (CV777 and Br1/87), Korean vaccine strains (DR13 and SM98P), and all Chinese strains (DX, JS-2004-2, LBJ/03, and LZC). The reason for the contradictory result is likely due to the difference of the target region analyzed between the previous and present studies, which led to the contrary interpretation of the sequence phylogeny. Nevertheless, our study indicates that the genetic basis of the C-terminal part of the S1 domain, including the epitope region, does not represent the entire S gene-based analysis, thereby displaying inconsistent results in the present study. It is further noteworthy that the phylogenetic tree topologies based on the S1 domain and the S1 N-terminus correspond well to the full S gene-based tree. This indicates that the N-terminal region of the S1 domain will be required for analyzing the relationships and diversity of PEDV isolates in future studies.

In conclusion, this study provided the complete nucleotide sequences of the Korean PEDV isolates and their phylogenetic relatedness. The results presented here indicate that PEDV isolates prevalent in Korea represent a heterogeneous group but all isolates are phylogenetically more closely related to each other. Our data further suggest that the N-terminal region of the S1 domain may be an ideal marker for monitoring the genetic variation and for developing differential diagnostic assays. The current study should lead to a better understanding of the genetic diversity among PEDV isolates and contribute to the development of more effective vaccines.

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