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ORIGINAL ARTICLE

Genetic characterization of porcine epidemic diarrhea virus in Korea from 1998 to 2013

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Abstract The number of porcine epidemic diarrhea (PED) cases has increased over the past 20 years in Korea, with a major outbreak in 2013. A total of 27 Korean strains from 1998 to 2013 were analyzed (excluding the noncoding regions) and divided into two groups for comparison of the spike (S), ORF3, envelope (E), membrane (M), and nucleocapsid (N) genes with those of reference strains, vaccine strains, and previously identified strains based on phylogenetic analysis. Analysis of the selection patterns of PEDV isolated in Korea indicated positive selection of nine nonsynonymous sites in the S and N proteins and negative selection at 97 sites for all of the proteins. Interestingly, eight nonsynonymous mutations in S showed no significant pattern change over the 15-year period, and one of eight mutation sites was found only in IC05TK, GN05DJ, and KNU0802 in the epidemic years 2005 and 2008. These eight mutations were also present during the epidemic years in China. Furthermore, of the signs of positive selection in the S protein, the conservative substitutions were more frequent than radical substitutions in PEDVs, suggesting that the evolution of Korean strains has been slow. Serological cross-reactivity was detected between three field PEDVs and two vaccine strains, with different serum neutralization titers. In conclusion, although Korean PEDVs have been evolving slowly, their diverse antigenicity and genetics imply that multilateral efforts to prevent future PED outbreaks are required.

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Introduction

Porcine epidemic diarrhea (PED) is a viral diarrheal disease that affects swine of all ages, often leading to high piglet mortality rates (>90 % in severe cases) [19]. Porcine epidemic diarrhea virus (PEDV) is transmitted mainly via direct or indirect fecal-oral routes. PEDV is a member of the family *Coronaviridae* and is an enveloped, single-strand, positive-sense RNA virus with a genome of ~30 kb in length encoding a set of seven nested mRNAs for production of four major structural proteins (spike [S] protein, envelope [E] protein, membrane glycoprotein [M], and nucleocapsid [N] protein), and three non-structural proteins (replicases 1a and 1b, and 3) [6, 11, 13]. The S protein, a glycoprotein peplomer on the viral surface, plays an important role in induction of neutralizing antibodies and is cleaved by host-derived proteases into two subunits – namely, S1 (binds to the receptor) and S2 (responsible for fusion activity) [20]. The other structural genes of coronaviruses – namely, E, M, and N, play important roles during viral replication, modulation of cell signaling pathways and the interferon response [5, 14, 16, 28].

Outbreaks of PED have been reported in many swine-raising countries, notably in Europe and Asia [19]. PED has resulted in economic losses for swine farms in Korea since the reported outbreak in 1993 [12]. In addition, sporadic and epidemic outbreaks of PED have been observed year round, not just during the winter season. Recently, PED cases were found for the first time in the USA in 2013; the origin of this infection, which spread rapidly through swine farms, is unclear, but the viruses were closely related to those of Chinese origin [3, 9]. At present, no specific treatment is available, but live, oral, and inactivated PED vaccines have been used for 10 years for protection against PED in Korea as well as in parts of Asia in where PED has occurred [7]. However, although some farms have reported effective protection from PED by these vaccines, PED outbreaks have occurred in Korea. This may be due to several factors, such as incorrect vaccination, high-density management systems, or the environmental conditions on individual farms. However, before discussing the reasons for these continuous outbreaks, it is necessary to determine the prevalence of PEDV in Korea.

For molecular and phylogenetic characterization, the full S, ORF3, E, M, and N nucleotide (nt) and amino acid (aa) sequences of PEDV were analyzed. Previous molecular studies of Korean PEDVs were limited to analysis of single genes or small numbers of strains collected over short periods. Therefore, we collected piglet samples from several provinces in Korea from 1998 to 2013 and characterized the viruses by sequencing each gene and comparing the sequences with those of previously reported Korean, other Asian, US, and European PEDVs in terms of their selection patterns. Furthermore, the antigenic properties of the viruses were determined by serum neutralization (SN) assays with pig sera raised against field and vaccine strains.

Materials and methods

Sample collection and preparation

A total of 19 samples of diarrhea and 8 samples of intestinal tissue from piglets from 1998 to 2013 were collected from farms in eight provinces of Korea. PEDV was detected in the 27 samples by real-time reverse transcription polymerase chain reaction (RT-PCR) amplification of a portion of the N gene sequence [10]. Each sample from farms was designated by a capital letter representing the province, a number that identified the sampling year, and the farm name. To extract viral RNA, 10 % suspensions of diarrhea samples in phosphate-buffered saline (PBS; 0.1 M, pH 7.2) were clarified by centrifugation for 10 min at 8,000*g* to eliminate fecal debris. The supernatant was then stored at −70 °C until use. In the case of intestinal samples, 1 g of intestinal tissue was diluted with 5 ml of α-minimal essential medium (α-MEM), ground, and clarified as described above for diarrhea samples, followed by storage at −70 °C until use.

Virus isolation

Eight intestine samples from dead piglets were used for PEDV isolation. Supernatants in α-MEM were passed through 0.2-μm syringe filters and then inoculated onto confluent Vero cells grown in 25-cm^2 flasks. After adsorption at 37 °C for 1 h, the cells were incubated in α-MEM supplemented with antibiotic/antimycotic solution and 2 μg of crystal trypsin. PEDV strains were identified by real-time RT-PCR and indirect fluorescence assay (IFA). For IFA, Vero cells were fixed with 80 % cold acetone and incubated with and anti-PEDV S-specific mouse monoclonal antibody 2C10 for 30 min, followed by a fluorescein-labeled goat anti-mouse IgG. Cell staining was examined under a fluorescence microscope.

RT-PCR and sequencing

Viral RNA was extracted from 300 μl of the diluted sample and eluted in 30 μl of RNase/DNase-free water using an RNeasy® Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription was performed with extracted RNA, oligo (dT), and a PrimeScript First-Strand cDNA Synthesis Kit (Takara, Kyoto, Japan), according to the manual provided by the manufacturer. PCR was conducted to amplify PEDV S, E, ORF3, M, and N cDNA fragments from the RT product using an Advantage® 2 PCR Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's protocol under the following conditions: denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 45 s, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Primers for S, ORF3, E, M, and N were designed based on the alignment of published PEDV genome sequences available in the NCBI GenBank database. The sequences of the primers used in this study are presented in Table S1. The amplified PCR products in 1 % agarose gels were purified using a QIAquick Gel Extraction Kit (QIAGEN) and cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA). The sequencing reaction for all samples of each gene was performed in triplicate, and sequences were confirmed in both directions using M13 forward and reverse primers.

Sequence analysis

The nucleotide sequence of each gene was trimmed and aligned with other sequences using the ClustalW [25] and

BioEdit (version 7.2.5) [8] software packages. Unrooted phylogenetic trees were constructed using MEGA 5 (5.0) with the distance-based neighbor-joining algorithm and bootstrap analysis with 1000 replicates [23]. The multiple alignment results were used as input for ADAPTSITE [22] to estimate natural selection. The natural selection calculation requires the ratio of non-synonymous and synonymous mutations, for which all gaps were removed, and sequences of the same length were used to achieve the ideal fit for the statistical selection model.

Antigenic characterization

Two 5-week-old pigs were inoculated with commercial vaccines commonly used in Korea; i.e., SM98 Korean PED live/inactivated vaccine (Jungang Product Co., Ltd. Daejeon, Korea) and P-5V Japanese PED Live Vaccine (Nisseiken Co, Ltd., Tokyo, Japan), according to the manufacturer's instructions. Briefly, P-5V vaccine was injected intramuscularly (2 ml) twice at two-week intervals, for a total of four weeks. For the SM98 vaccine, pigs were injected intramuscularly with 1 ml of live SM98 and then 2 ml of inactivated SM98 twice at two-week intervals for a total of six weeks. Two-day-old piglets from PEDV-negative sows were inoculated orally with 2 ml of supernatant from ground intestinal tissue containing one of the three field viruses. Blood was collected every week for a total of six weeks. Furthermore, field sera were collected from pigs infected with KDGN13_295BG (which has over 99.0 % homology to the US strain) on a pig farm. The antigenic properties of the virus were determined by SN assay [18] with pig antisera raised against those Korean field PEDVs and vaccines 4 weeks after infection and 3 weeks after the last vaccination, respectively.

Results

Virus isolation

We collected 27 diarrhea and intestine samples between 1998 and 2013, which were used for comparison of PEDV sequences (Table 1). The SM98, CJ98, and IC05TK strains were isolated from eight intestine samples, using Vero cells, and their identity was confirmed by IFA (data not shown). The SM98 strain underwent 116 serial passages in Vero cells (accession no. GU937797).

Phylogenetic and sequence analysis of S, ORF3, E, M, and N sequences

The full S sequences of a total of 27 PEDV strains and 48 reference strains, including vaccine strains used in Korea, previous Korean PEDV strains, and strains from China, Japan, Europe, and the USA, were compared. Based on the phylogenetic trees prepared using the neighbor-joining method, the Korean strains could be divided into two groups (G1 and G2) at the nt and aa levels; G2 was further divided into G2-1 and G2-2 as shown in Fig. 1A. G1 consisted of vaccine strains, European strains, Japanese strains, and a few Korean and Chinese strains. Fourteen of the S genes of G2-1 sequenced in this study were grouped with only Korean field strains, with the exception of KH from Japan, and G2-2 consisted mostly of Chinese, several Korean, and US strains. Interestingly, three strains (KDGG13_2DJ, KDGG14_6IC, and KDGN13_295BG) in G2-2 were highly similar to the US strains that spread in 2013, with 99.2–99.8 % and 98.8–99.7 % identity at the nt and aa level, respectively. Of the 39 sequences of Korean PEDVs, all were 4,161 nt in length, encoding 1,386 aa residues, with the exception of two strains (KDJN10NJ and KDCN12KH) with 4,158 nt (1,386 aa), four strains (Chinju99, DR13, KDGN13DJ, and KDJN13_1003SW) with 4,152 nt (1,384 aa), and one strain, SM98-5P, with 4,131 nt (1,377 aa). The most frequent insertion was at aa positions 56–59 of the S protein in 34 Korean viruses, followed by one additional inserted D or N residue at aa position 140 in 31 strains. Furthermore, six viruses also had an insertion of two aa residues between residues 160 and 161 of the S protein (DG/DK/DR). The S genes used in this study, including those of previously reported strains, were not homologous, with nt and aa similarities of 89.7–99.8 %; the nt and aa sequence identity of the Korean PEDV S genes of group G1, into which most vaccine strains were grouped, was 93.9–98.1 % and 93.0–97.5 %, respectively, within the group, and they showed 91.4–95.7 % nt and 89.7–94.2 % aa sequence identity with members of group G2. The G2 group showed 92.8–99.9 % nt and 90.7–99.8 % aa sequence identity within the group. Furthermore, vaccine strains used in Korea showed 93.5–99.3 % nt and 92.6–98.9 % aa sequence identity to members of group G1 and 91.8–94.2 % nt and 90.0–92.7 % aa identity to members of G2. Finally, Korean S genes showed 92.4–99.6 % nt and 91.3–99.2 % aa sequence identity to strains from other countries, including China. Previous studies of the S protein of PEDV have identified the core neutralizing epitope (COE) region (aa position 748–755), SS2 (aa position 748–755) and SS6 (aa position 764–771) epitopes in the S1 domain and the 2C10 epitope (aa position 1368–374) in the cytoplasmic domain [1, 4, 21]. We found partial mutations in the COE region and SS6, whereas the SS2 and 2C10 neutralizing epitopes had very low mutation rates (1–2 aa) in all Korean strains used in this study (data not shown).

The complete 675-nt and 225-aa sequences of ORF3 of a total of 56 Korean strains, including Chinju99, virulent

Table 1 Porcine epidemic diarrhea virus strains used in this study

No.	Virus designation	Province	Sample origin	GenBank accession number				
				S	ORF3	E	M	N
1	SM98-5P	Gyeongnam	Intestine	KJ857455	KJ857428	KJ857352	KJ857378	KJ857402
2	CJ98	Jeju	Intestine	KJ857456	KJ857429	KJ857353	KJ857379	KJ857403
3	IC05TK05	Incheon	Intestine	KJ857457	KJ857430	KJ857354	KJ857380	KJ857404
4	JB0536	Jeonbuk	Intestine	KJ857458	KJ857431	KJ857355	KJ857381	KJ857405
5	GN05DJ	Gyeongnam	Diarrhea	KJ857459	KJ857432	KJ857356	KJ857382	KJ857406
6	GB06SY	Gyeongbuk	Intestine	KJ857460	KJ857433	-[a]	KJ857383	-
7	GW06CHC	Gwangwon	Diarrhea	KJ857461	KJ857434	KJ857357	KJ857384	KJ857407
8	KDGG10AN	Gyeonggi	Diarrhea	KJ857462	KJ857435	KJ857358	-	KJ857408
9	KDGG10DA	Gyeonggi	Diarrhea	KJ857463	KJ857436	KJ857359	KJ857385	KJ857409
10	KDGG10HA	Gyeonggi	Diarrhea	KJ857464	KJ857437	KJ857360	KJ857386	KJ857410
11	KDGG10KA	Gyeonggi	Diarrhea	KJ857465	KJ857438	KJ857361	KJ857387	KJ857411
12	KDGG10YO	Gyeonggi	Intestine	KJ857466	KJ857439	KJ857362	KJ857388	KJ857412
13	KDJN10NJ	Jeonnam	Diarrhea	KJ857467	KJ857440	KJ857363	KJ857389	KJ857413
14	KDGN10HOR	Gyeongnam	Diarrhea	KJ857468	KJ857441	KJ857364	-	KJ857414
15	KDGN10WC	Gyeongnam	Diarrhea	KJ857469	KJ857442	KJ857365	-	KJ857415
16	KDGG12KAN	Gyeonggi	Diarrhea	KJ857470	KJ857443	KJ857366	KJ857390	KJ857416
17	KDCN12KH	Chungnam	Diarrhea	KJ857471	KJ857444	KJ857367	KJ857391	KJ857417
18	KDJN12JNJ	Jeonnam	Diarrhea	KJ857472	KJ857445	KJ857368	KJ857392	KJ857418
19	KDJN12GAM	Jeonnam	Diarrhea	KJ857473	KJ857446	KJ857369	KJ857393	KJ857419
20	KDJN12JU	Jeonnam	Diarrhea	KJ857474	KJ857447	KJ857370	KJ857394	KJ857420
21	KDJN12YG	Jeonnam	Diarrhea	KJ857475	KJ857448	KJ857371	KJ857395	KJ857421
22	KDGG13HWN	Gyeonggi	Intestine	KJ857476	KJ857449	KJ857372	KJ857396	KJ857422
23	KDGN13DJ	Gyeongnam	Intestine	KJ857477	KJ857450	KJ857373	KJ857397	KJ857423
24	KDJN13_1003SW	Jeonnam	Diarrhea	KJ857478	KJ857451	KJ857374	KJ857398	KJ857424
25	KDCN13_2DJ	Chungnam	Diarrhea	KJ857479	KJ857452	KJ857375	KJ857399	KJ857425
26	KDGG13_6IC	Gyeonggi	Diarrhea	KJ857480	KJ857453	KJ857376	KJ857400	KJ857426
27	KDGN13_295BG	Gyeongnam	Diarrhea	KJ857481	KJ857454	KJ857377	KJ857401	KJ857427

[a] Not detected by RT-PCR

DR13, attenuated DR13, and attenuated SM98, as well as 13 reference strains from other countries were compared. Multiple alignment data showed a large nucleotide deletion in some of the PEDVs used in this study. SM98P-5P (passage 5) and SM98 (cell-attenuated strain, passage 116) had a 30-nt deletion from positions 1 to 30 of the 675-nt sequence and a 52-nt deletion from nt 22 at the end of the spike to nt 30 at the start of ORF3. All of the P-5V and DR13 cell-attenuated strains except the DBI865 strain had a 49-nt deletion from nt 245 to nt 293. Furthermore, four Chinese strains, JS2008, CH3, CH4, and SD-M, used as reference strains in PubMed, showed the same 49-nt deletion from nt 245 to 293 that was present in the P-5V and DR13 cell-attenuated strains. The ORF3 sequences of Korean strains were divided into G1 and G2, and G2 was further divided into G2-1 and G2-2 (Fig. 1B). Most Korean strains were grouped into G2-2 and showed 96.1–100 % and 95.9–100 % identity among Korean strains (with the exception of DBI865) at the nt and aa level, respectively.

The E gene in all Korean PEDVs—with the exception of the attenuated DR13 strain—was 231 nt in length and encoded a protein of 76 aa. The E sequences of Korean strains were divided into two groups, G1 and G2, and G2 was further divided into G2-1 and G2-2 (Fig. 1C). Most Korean PEDV strains belonged to G2-2 and showed 95.6–100 % nt and 96.0–100 % aa sequence identity among Korean strains. These strains showed 93.9–99.5 % nt and 94.7–100 % aa sequence identity to the Chinese strains used in this study. The E gene of vaccine strains used in Korea showed 87.4–99.5 % nt and 86.8–98.6 % aa sequence identity to members of group G1 and 86.5–97.8 % nt and 85.5–97.3 % aa sequence similarity to members of group G2.

A total of 45 Korean strains and 17 reference and vaccine strains were compared in terms of genetic diversity

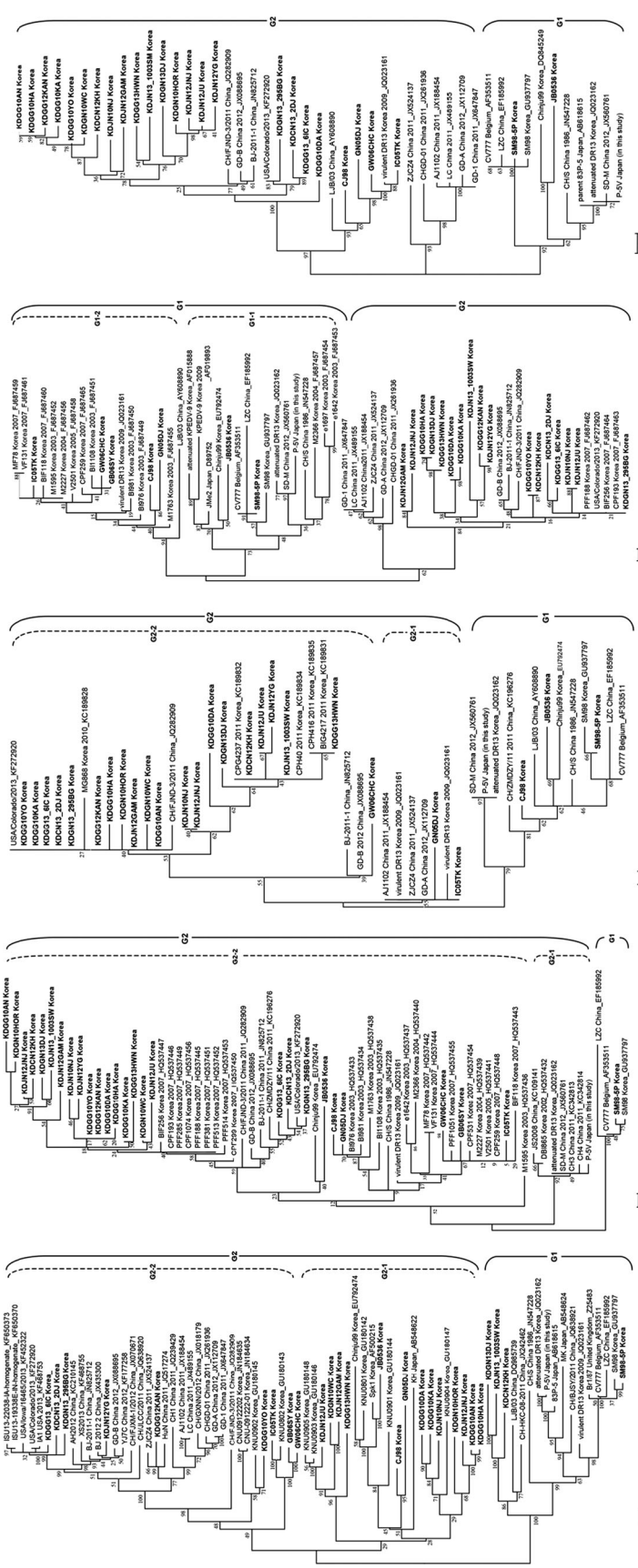

Fig. 1 Phylogenetic tree generated based on the nucleotide sequences of complete S (A), ORF3(B), E (C), M (D), and N (E) genes of Korean PEDV strains with reference PEDV strains using the neighbor-joining method with MEGA 5.2 software. The numbers at branches are bootstrap values (%) from 1,000 replications

and similarity by sequencing the complete 681 nt of the M gene and deducing the 226-aa sequence of the encoded protein. The M gene consisted of 681 nt in all strains, except the cell-attenuated SM98 strain, which had an insertion of 12 nt (ATGCTAGTACTT) between nt 36 and 37, encoding 4 aa residues (MLVL). This could be utilized as a genetic marker for differentiating other field PEDVs. This insertion may have arisen during serial passage under cell culture conditions. The B-cell epitope [27], WAFYVR, located at positions 195–200, was conserved in all 62 strains. As shown Fig. 1D, these sequences were divided into two groups (G1 and G2); G1 was further divided into two subgroups (G1-1 and G1-2). Furthermore, they were grouped according to year of detection; most of the 1998–2007 Korean PEDVs were grouped into G1, and those from subsequent years belonged to G2. The levels of nt and aa sequence identity in G1 were 97.0–99.5 % and 96.9–99.1 %, respectively, and they showed 96.7–100 % nt and 96.9–100 % aa sequence identity to members of group G2. Isolates in group G2 showed 98.0–100 % nt and 96.9–100 % aa sequence identity to each other. Furthermore, vaccine strains used in Korea showed 95.9–99.1 % nt and 95.2–99.1 % amino acid sequence identity to members of group G1 and 95.2–97.9 % nt and 94.7–97.7 % aa sequence identity to members of group G2. Korean M genes showed 96.0–99.8 % nt 95.1–100 % aa sequence identity to the Chinese strains used in this analysis.

The complete 1,326 nt and 442 aa of the N gene and protein from a total of 28 Korean field strains and 19 reference and vaccine strains—with the exception of the GW06CHC strain—were analyzed. Based on the phylogenetic tree, the Korean strains were divided into two groups, G1 and G2 (Fig. 1E). G1 consisted mostly of vaccine strains and CV777. The G2 Korean PEDV N genes showed 95.0–99.9 % nt and 94.5–100 % aa sequence identity to each other and 93.2–96.3 % nt and 92.5–96.8 % aa sequence identity to members of group G1. Finally, Korean N genes showed 96.0–99.8 % nt and 95.1–100 % aa sequence identity to the Chinese strains used in this study.

Natural selection analysis of S, ORF3, E, M, and N genes

In the present study, the selection patterns of PEDV strains from neighboring countries were explored by examining the sequences in GenBank and those from epidemic years in Korea using the ADAPSITE tool. As selection analysis is highly sensitive to quality factors, such as differences in sequence length and insertions/deletions, sequences that did not meet the quality criteria were removed during ADAPSITE analysis. Due to the size limit of spreadsheets in Microsoft Excel, multiple alignment of the S sequence was divided into five sites. Sites 5 and 1 in the S and N genes in the multiple alignments using ClustalW were used to analyze the mutation sites in PEDV strains from neighboring countries (data not shown).

In statistical analysis, one-tailed and two-tailed tests are alternative methods of computing the statistical significance of a dataset, depending on whether only one direction was considered extreme (and unlikely) or both directions were considered equally likely. Parsimony-based detection of positively and negatively selected sites by ADAPTSITE was performed; the significance of positive selection was determined by one-tailed test, while that of negative selection was determined by two-tailed test. The cutoffs for significance of codon usage bias patterns were set at $P < 0.05$. Selection analysis revealed no significant positive selection for sites in ORF3, M, and E, while the S and N proteins showed positive selection for eight sites and one site, respectively (Tables S2A and S2B). As shown in Table 2, the S protein showed 8 non-synonymous (SN) sites, 16 conservative sites (SC), 3 radical sites (SR), and 3 radical substitutions (CR) in nodes in the phylogenetic tree; the N protein showed 1 SN, 2 SC, and 2 CR; the others showed no positive selection. The results indicated that 106 of the total variations could be attributed to mutational pressure, while 8.5 % (9/106) and 91.5 % (97/106) of the total variations could be explained by positive (Dn/Ds > 1) and negative (Dn/Ds < 1) selection for PEDVs, respectively. Of the PEDV proteins, only S and N showed both positive and negative selection (9 and 93, respectively), while the others (E, M, and ORF3) showed negative selection (0 and 4). In addition, amino acid substitutions could be classified as conservative or radical according to whether the properties of the substituted amino acid were retained. Interestingly, of the signs for positive selection in

Table 2 Summary of natural selection for PEDV proteins

Proteins	Selection	SN[a]	SC	SR	CR
E	Positive	0	0	0	0
	Negative	2	2	0	0
M	Positive	0	0	0	2
	Negative	1	1	0	0
N	Positive	1	2	0	2
	Negative	53	32	31	3
ORF3	Positive	0	0	0	0
	Negative	1	1	0	0
S	Positive	8	16	3	3
	Negative	40	21	10	13

[a] SN, SC, SR and CR represent average numbers of sites with non-synonymous, conservative, and radical substitutions and the total number of radical substitutions, respectively

Table 3 Positively selected mutations in the PEDV spike protein according to epidemic year

Sample strain	Codon position								Year
	36	303	375	399	422	459	504	515	
CV777 Belgium,	TCA	AAT	TCA	TTA	CCT	ACT	CTC	CGG	
CJ98 Korea	TCA	AAT	TCA	TTA	CCT	ACT	CTC	CGG	1998
IC05TK Korea	TCA[c]	A[a]TT	TCA	TTA	CCT	ACT	CTC	CGG	
GN05DJ Korea	TTA	A[a]TT	TCA	TTA	CCT	ACT	CTC	TGG	2005
JB0536 Korea	TCA	AAT	TCA	TTA	CCT	ACT	TTC	CGG	
GB06SY Korea	TCA	AAT	TCA	TCA	CCT	ACT	CCC	CGG	2006
GW06CHC Korea	TCA	AAT	TCA	TCA	CCT	ACT	CCC	CGG	
GU180143 Korea	TCA	A[a]TT	TCA	TCA	CCT	ACT	CTC	CGG	2008
GU180142 Korea	TTA[c]	AAT	TCA	TTA	CCT	ACT	TTC	CGG	
GU180144 Korea	TTA	AAT	TCA	TTA	CCT	ACT	TTC	CGG	
GU180147 Korea	TTA	ACT	TCA	TTA	CCT	ACT	CTC	CGG	
KC879277 Korea	TTA	ACT	TCA	TTA	CCT	ACT	CTC	CGG	
JN184635 Korea	TCA	AAT	TTA	TCA	CTT	ACT	CTC	CGG	2009
JN184634 Korea	TCA	AAT	TTA	TCA	CTT	ACT	CTC	CGG	
GU180145 Korea	TCA	ACT	TTA	TCA	CTT	ACT	CTC	CGG	
GU180148 Korea	TCA	ACT	TTA	TCA	CCT	ACT	CTC	CGG	
GU180146 Korea	TCA	ACT	TTA	TCA	CCT	ACT	CTC	CGG	
KDGN10HOR Korea	TCA	ACT	TCA	TCA	CTT	ACT	TTC	CGG	
KDGG10YO Korea	TCA	ACT	TTA	TCA	CTT	ACT	CTC	CGG	
KDGN10WC Korea	TCA	ACT	TCA	TTA	CCT	ACT	CTC	CGG	2010
KDGG10DA Korea	TTA	ACT	TCA	TTA	CCT	ACT	TTC	CGG	
KDGG10KA Korea	TTA	AAT	TCA	TTA	CCT	ACT	TTC	CGG	
KC879275 Korea	TTA	ACT	TCA	TTA	CCT	ACT	CTC	CGG	
KC879278 Korea	TCA	ACT	TCA	TCA	CCT	ACT	CTC	CGG	2011
KC879280 Korea	TCA	AAT	TCA	TCA	CCT	ACT	CTC	CGG	
KC879282 Korea	TCA	AAT	TCA	TCA	CCT	ACT	CTC	CGG	
KC879281 Korea	TCA	AAT	TCA	TCA	CCT	ACT	CTC	CGG	
KC879276 Korea	TTA	ACT	TCA	TTA	CCT	ACT	CTC	CGG	
KDJN12YG Korea	TTA	AAT	TCA	TCA	CCT	ACT	CTC	CGG	2012
KDJN12GAM Korea	TCA	ACT	TCA	TTA	CCT	ACT	CTC	CGG	
KDJN12JU Korea	TCA	ACT	TTA	TCA	CCT	ACT	CTC	CGG	
KDGG12KAN Korea	TCA	ACT	TTA	TCA	CTT	ACT	CTC	CGG	
KDGG13HWN Korea	TCA	ACT	TTA	TCA	CTT	ACT	CTC	CGG	
KDGG13_6IC Korea	TTA	AAT	TCA	TCA	CCT	GCT	CTC	CGG	2013
KDCN13_2DJ Korea	TTA	AAT	TCA	TCA	CCT	GCT	CTC	CGG	
KDGN13_295BG Korea	TTA	AAT	TCA	TCA	CCT	ACT	CTC	CGG	
China	**-Y[b]-**	**-Y-**	**-Y-**	**-Y-**	**-Y-**	**Y--**	**YY-**	**Y--**	
Thailand	**-N[b]-**	**-N-**	**-Y-**	**-Y-**	**-Y-**	**Y--**	**YN-**	**N--**	
USA	**-Y-**	**-N-**	**-N-**	**-Y-**	**-N-**	**Y--**	**NN-**	**N--**	

[a] Found only in Korea

[b] Y and N represent the same mutation found and not found, respectively, in the indicated countries

[c] Red squares indicate mutated sites in isolates from non-consecutive years, and black indicate those from consecutive years

the S protein, the numbers of SC in S protein were relatively high compared to the numbers of CR in PEDVs.

The numbers of PEDVs from Korea, China, Japan, Thailand, and the USA used in this selection study were 27, 86, 1, 0, and 10, respectively, for N, and 35, 92, 0, 6, and 10, respectively, for S, as shown in Tables S2C and S2D. Analysis of codon usage bias patterns of PEDV strains during the period 1998 to 2013 indicated that only eight codons (aa positions 36, 303, 375, 399, 422, 459, 504, and 515) in the S protein and one codon at aa position 412 in the N protein showed at least one positive selection, but no significant pattern changes were detected during this period. In addition, comparative analysis of strains from China, Thailand, and the USA indicated that the eight mutation sites found in China were frequently detected in Korea (Tables 2, S2C, and S2D). Among Korean PEDVs, SM98-5P, Chinju99, KDGG13DJ, KDJN13_1003SW, and virulent DR13 were not addressed in this study because of the

Table 4 Cross-reactivity of vaccine strains with antisera to vaccine and field viruses

Vaccine strain	SN titer					
	P-5V	SM98	KDGG10YO	KDGG12HWN	KDGN12DJ	KDGN13_295BG
P-5V	**16**[a]	256	8	4	4	16
SM98	32	**512**	32	16	8	32

[a] Boldface, SN titers for homologous PEDV strains

quality factors in ADAPSITE analysis. As shown in Table 3, the amino acid substitutions in eight codons, which resulted in 10 translation products, were TCA > TTA (S > L) for residue 36, AAT > ATT (N > I) and AAT > ACT (N > T) for residue 303, TCA > TTA (S > L) for residue 375, TTA > TCA (L > S) for residue 399, CCT > CTT (P > L) for residue 422, ACT > GCT (T > A) for residue 459, CTC > TTC (L > F), and CTC > CCC (L > P) for residue 504, and CGG > TGG (R > W) for residue 515. The amino acid mutation at position 303 (N > T) in IC05TK, GN05DJ, and KNU0802 (accession no. GU180143) during the epidemic years 2005 and 2008 was unique and was not found in neighboring countries. Of the 15 mutation sites indicated in Table 3 by squares, seven mutations (red) were found in non-consecutive years, and eight (black) were found in consecutive years. In particular, six of the seven mutations in isolated years appeared during 2005, 2006, and 2008.

Characterization of antigenic properties

PED antisera from pigs were produced against three field viruses (KDGG10YO, KDGG12HWN, and KDGN12DJ) and two vaccine viruses (SM98 and P-5V). Furthermore, field sera from pigs at a farm infected with KDGN13_295BG were used for the SN assay. The selected field viruses were not passaged in Vero cells, and the vaccine viruses were subjected to SN assays using each antiserum. Variable serological cross-reactivity was observed between SM98 and field viruses, with SN titers of 8–32. However, limited cross-reactivity was detected between P-5V and field viruses, with a reduction in the SN titer (Table 4).

Discussion

PEDV causes acute enteritis in piglets, with severe damage, and is detected continually in pigs on Korean pig farms. A nationwide PED outbreak occurred in 2013, and the disease remains prevalent on Korean pig farms. PEDVs detected in 2013 were placed into various groups based on the results of phylogenetic analysis. Among them, three strains (KDGG13_2DJ, KDGG14_6IC, and KDGN13_295BG) showed high levels of sequence identity with strains detected in the USA in 2013. One possible cause of the transmission of PEDVs similar to the US strain is the importation of pigs for breeding into Korea from the USA. Furthermore, transmission may also arise due to importation of goods from China through unknown paths, as the US strains are genetically similar to Chinese viruses [3]. However, we cannot exclude the possibility that preexisting Korean PEDVs caused the 2013 PED outbreak. KDJN12YG (detected in 2012) showed 99.1–100 % nt and 98.4–100 % aa sequence identity in its structural genes to US strains.

An epitope in the S glycoprotein gene can serve as a target for analysis of genetic variation of PEDV strains in the field. Many complete sequences of S genes have been deposited in the GenBank database, representing a variety of genetic groups and subgroups from China, Japan, Thailand, and Korea [7, 15, 17, 24, 26]. Based on analysis of complete S-gene nucleotide sequences, the strains were divided into two distinct groups, G1 and G2. Vaccine strains, reference strains, and several of the Korean and Chinese strains were found to belong to G1, whereas all Korean field strains clustered together within G2. The data obtained from the present phylogenetic analyses were similar to those reported previously [15]. However G2 was further divided into G2-1 and G2-2 in this study: G2-1 consisted only of field Korean strains, with the exception of one Japanese strain (KH), and was closely related to the old Korean strains Spk1 and Chinju99. G2-2 consisted mostly of Chinese strains and six of the Korean field strains that were more closely related to Chinese strains than other Korean viruses. These findings indicated that the prevalent PEDV strains in Korea are genetically diverse and can be divided into three groups according to their putative origin: Chinese-like (G2-2), Korean-like (G2-1), and vaccine-like (G1) viruses.

Neighbor-joining trees based on different genes showed limited similarity to each other in their topology. Neighbor-joining trees constructed using S, ORF3, E, M, and N genes showed that the strains could be divided into two groups, G1 and G2. G1 consisted mostly of vaccine viruses, and G2 comprised field strains, regardless of year. When S and M phylogenetic trees were compared, some strains that were associated with G1 in the S tree were relocated into G2 in the M tree. This phylogenetic incongruity may be explained by a higher degree of conservation of the M gene

than the S gene; alignments among Korean strains indicated differences of 0.1–8.4 % (0.2–10.3 %) in S, 0.1–6.8 % (0–7.5 %) in N, 0–5.3 % (0–6.5 %) in E, 0–3.9 % (0–4.1 %) in ORF3, and 0–4 % (0–4 %) in M at the nt (aa) level. Further studies may be required to confirm recombination events of the different groups of PEDVs.

Similar to the homology analysis, natural selection analysis also showed significant patterns in S and N proteins. In addition, long-term analysis of PEDV indicated continuous accumulation of mutations. In particular, one of eight nonsynonymous sites in three Korean strains showed unique mutations. Six mutation sites appeared during 2005, 2006, and 2008, suggesting the occurrence of environmental events during this period. Overall, all strains from Korea appeared in China at the same time, but this was less true in Thailand, suggesting independent evolution of Korean strains to be less likely. However, there has been no official importation of Chinese goods, including pigs or vaccines to Korea. The results showed a high level of similarity (92.3–99.3 %) between the S genes of Korean strains and those isolated in China during epidemic years [2], suggesting that variations may arise independently in each country as a result of similar evolutionary processes.

Of the PEDV proteins—E, M, N, ORF3, and S—96 % of all selected mutations were found in N and S, while the remainder (E, M, and ORF3) showed a rate of only 4 %. Of these, 91.5 % showed negative selection. In natural selection, negative selection is the selective removal of deleterious alleles. Purging of deleterious alleles can be achieved at the population genetics level, with as little as a single point mutation being the unit of selection. The N and S proteins of PEDV are subjected to greater mutational pressure than the E, M, and ORF3 proteins. Thus, carriers of harmful point mutations have fewer offspring in each generation, reducing the frequency of the mutation in the gene pool. In the case of strong negative selection at a locus, the purging of deleterious variants will result in the occasional removal of linked variation, producing a decrease in the level of variation surrounding the locus under selection. Positive selection is a process by which new advantageous genetic variants sweep through a population. Interestingly, in our study, the positive selection rate in the S and N proteins was only 8.5 %, and their mutations with positive selection were even more conservative, with few radical changes, suggesting that PEDV strains in Korean undergo a slow evolutionary process.

The S gene sequences of Korean field strains exhibited 0.1–8.4 % and 0.2–10.3 % differences at the nt and aa level, respectively, and vaccine strains commonly used in Korea showed a maximum 10 % difference at the aa level compared with field viruses. Thus, cross-reactivity was examined by SN assay using vaccine viruses and pig antisera against vaccine viruses and field strains. The SM98 vaccine strain exhibited greater variability in serological cross-reactivity with these antisera than did the P-5V vaccine strain; thus, the SM98 vaccine was inferred to confer protection against field PEDVs despite the 10 % difference in S gene sequences. Conversely, the two viruses showed limited genetic variation in the epitope region. The variable serological cross-reactivity of the SM98 vaccine may be due to the presence of as-yet-unidentified neutralizing epitopes in the SM98 strain or its ability to induce high titers of neutralizing antibodies compared to the P5-V vaccine.

With nonperiodic sampling over a $\sim$15-year period, the strains from outbreaks or sporadic episodes in Korea between 1993 and 2013 showed a group mean divergence of $\sim$10 %, with placement in two groups/subgroups. This genetic relationship suggested that, besides evolving independently, transborder movement might be a cause of disease dissemination. This would complicate efforts to understand the origins of these viruses. PED no longer occurs only in Asia and part of Europe but has also been identified in the USA and Canada, and so is now classified as a worldwide disease. The appropriate use of vaccines and biosecurity measures is necessary to control the disease. The results presented here will contribute to an understanding of the prevalence of current PEDVs in Korea and facilitate evaluation of PED vaccines.

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