

Molecular epidemiology of Korean porcine sapeloviruses

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Abstract To evaluate the prevalence and genetic diversity of porcine sapeloviruses (PSVs) in Korea, a total of 100 diarrhea fecal samples from pigs were analyzed by RT-PCR and nested PCR assays with primer pairs specific for the VP1 gene. Overall, 34 % of the diarrhea samples tested positive for PSV, and a high proportion of infections occurred along with a variety of other enteric viruses and bacteria. Genomic and phylogenetic analysis of the VP1 genes revealed pronounced genetic diversities between PSVs from Korean and elsewhere. Our results indicate that PSV infections are very common in Korean pigs with diarrhea. The infecting strains are genetically diverse.

Keywords Porcine · Sapelovirus · Epidemiology · Genetic diversity

Picornaviruses are small, non-enveloped viruses with single-stranded, positive-sense genomic RNA that are found in humans and a wide variety of other animals, and which can be the cause of respiratory, cardiac, hepatic, neurological, mucocutaneous, and systemic diseases of varying severity [1]. Porcine sapelovirus (PSV) is a member of a new genus, *Sapelovirus*, together with simian sapelovirus and avian sapelovirus [2–6]. Similar to all members of the family *Picornaviridae*, sapeloviruses contain 7.5- to 8.3-kb single-stranded RNA genomes of positive polarity with a genome-linked protein (VPg) at the 5' terminus, an internal ribosome entry site, polyprotein regions, and a 3'-poly A tail [7].

PSV infections have been associated with a wide spectrum of symptoms ranging from asymptomatic infection to clinical signs including diarrhea, pneumonia, polioencephalomyelitis, and reproductive disorders [8, 9]. A recent experimental study reported PSV-mediated acute diarrhea, respiratory distress, and polioencephalomyelitis in pigs in China [9]. However, precise data about the prevalence of PSV infections and the genetic diversity of this virus in pigs and wild boars have been reported only in a limited number of countries [10, 11]. Moreover, the exact status of their association with co-infections with other enteric pathogens remains largely unknown.

The prevalence and genetic diversity of PSVs in South Korea have never been studied. Therefore, the molecular epidemiology of PSV infections in South Korea is needed to determine the prevalence as well as the extent of genetic diversity in the circulating strains to develop vaccination programs and to establish a surveillance system for PSV

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infections. The aim of this study was to determine the exact prevalence of PSV infections in Korean pigs with gastroenteritis using PCR-based assays and to obtain information on the genetic heterogeneity of PSVs circulating in South Korea.

To determine the prevalence of PSV infections and the genetic diversity of this virus in Korean pigs, a total of 100 diarrheic fecal samples from 45 farms were selected from the archives of the Fecal Registry of the Laboratory of Veterinary Pathology, College of Veterinary Medicine, Chonnam National University (Supplementary Table 1). These fecal samples were collected during 2004 to 2007 across four Korean provinces (Chonnam, Chonbuk, Jeju, and Chungnam). For the extraction of viral RNA, fecal suspensions of each sample were prepared by diluting the feces 1:10 in 0.01 M phosphate-buffered saline (PBS), pH 7.2. The suspensions were vortexed for 30 s, and centrifuged ($1,200 \times g$ for 20 min), and the supernatants were collected and stored at -80°C for further testing.

Two sets of primers for detecting and sequencing PSVs were designed based on the sequences of the VP1 genes of the csh (GenBank accession number; HQ875059) and V13 (GenBank accession number; NC_003987) strains (Supplementary Table 2). Primer pairs for the detection of other enteric viruses (porcine kobuvirus [PKV], porcine enterovirus [PEV], porcine teschovirus [PTV], group A-C rotaviruses [RVA-C], porcine sapovirus [PSaV], porcine norovirus [PNoV], transmissible gastroenteritis coronavirus [TGEV], and porcine epidemic diarrhea coronavirus [PEDV]) were designed in this study or are described in other studies (Supplementary Table 2). Fecal samples were examined for common bacterial enteric pathogens including *Escherichia coli* and *Salmonella* spp. using specific agar media [12, 13]. *Lawsonia intracellularis*, *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, *Brachyspira intermedia* and *Clostridium perfringens* were detected by PCR assays with specific primers [14–16].

RNA was extracted from a 200- μl starting volume of each centrifuged 10 % fecal suspension using TRIzol-LS (Gibco-BRL, Grand Island, NY). The total RNA that was recovered was suspended in 40 μl of RNase-free water and used directly for RT-PCR. RT-PCR assays with different primer sets (Supplementary Table 2) for the detection of PSVs and other enteric viruses were performed using a standard one-step RT-PCR as described elsewhere (see Supplementary Table 2). To increase the sensitivity and specificity for RT-PCR, nested PCR assays with primer pairs specific to each gene (Supplementary Table 2) were performed as described previously [17]. The amplification products were analyzed by 1.5 % or 2 % agarose gel electrophoresis and visualized by ultraviolet illumination after ethidium bromide staining.

The nested PCR products for the partial VP1 (542 bp) genes (Supplementary Table 2) were all selected from different test reactions and sequenced to verify the specificity of each reaction and to obtain genomic data. The nested PCR products were purified using a GENECLAN II Kit (BIO 101, LaJolla, CA) according to the manufacturer's instructions. DNA sequencing was carried out using an automated DNA sequencer (ABI system 3700; Applied Biosystem, Foster City, CA).

Using the DNA Basic module (DNAsis MAX, Alameda, CA), the partial nucleotide sequences of VP1 (500 bp; lacking primer sequences) genes were compared with those of other known PSV sequences (Supplementary Table 3). Phylogenetic analysis based on nucleotide and deduced amino acid alignments was conducted using the neighbor-joining method and the unweighted pair group method with the arithmetic mean (UPGMA) method of Molecular Evolutionary Genetics Analysis (MEGA version 5.1, DNAMAN version 5.2.2) [18] with a pairwise distance.

Eleven (11 %) out of 100 fecal samples (11/45 farms; 24.4 %) tested positive using a one-step RT-PCR assay, targeting a 636-bp fragment of the VP1 gene of PSV. Nested PCR, targeting a 542-bp fragment of the VP1 gene, detected 34 (34 %) positive fecal samples from 24 farms (53.3 %) (Table 1). These data indicate that PSV infections are epidemic in Korean pigs with diarrhea. Of the 34 PSV-positive diarrheic fecal specimens, two (2 %) tested positive for PSV alone, while the other 32 samples (32 %) also tested positive for other enteric pathogens, including PEV, PTV, PKV, PRVA, PRVC, PSaV, *Escherichia coli*, and *Salmonella* (Table 1). Interestingly, PEVs were most prevalently co-infected with PSVs; 30 of 34 PSV-positive fecal samples also tested positive for PEV (Table 1). In addition, the 64 fecal specimens (64 %) that were negative for PSV also tested positive for other enteric pathogens (Table 1). Among these PSV-negative fecal samples, 56 samples were also positive for PEVs (Table 1). These results indicate that PSVs and other enteric pathogens, particularly PEVs, commonly co-infect Korean pigs.

To confirm the results of the PCR-based assays and to obtain genomic data, all of the amplicons generated by nested PCR assays were sequenced. Comparison of the nucleotide sequences of partial VP1 genes of 34 Korean PSVs revealed high genetic diversity, with 76.2–100 % nucleotide and 81.9–100 % deduced amino acid identity (Supplementary Table 4). The high genetic diversity in the VP1 gene was also observed between the Korean and other known PSVs, showing 73–85.6 % nucleotide and 77.1–88.6 % deduced amino acid sequence identity to European PSVs and 74.6–85.4 % nucleotide and 81.9–98.8 % deduced amino acid sequence identity to Chinese PSVs. All PSVs as a group are monophyletic with respect to PSV species in VP1, but they make 11 different clusters (Fig. 1).

Table 1 Summary of enteric pathogens present in the samples obtained from pigs with diarrhea

Enteric pathogens present ^a	No. of farms (%) ^b	No. of samples (%) ^c	Enteric pathogens present	No. of farms (%)	No. of samples (%)
PSV alone	2 (4.4)	2 (2)	PRVA	1 (2.2)	1 (1)
PSV plus one pathogen			PRVC	1 (2.2)	1 (1)
PEV	5 (11.1)	7 (7)	PKV	1 (2.2)	1 (1)
<i>E. coli</i>	1 (2.2)	1 (1)	Two pathogens		
PSV plus two pathogens			PEV, PTV	7 (15.6)	9 (9)
PEV, PTV	4 (8.9)	4 (4)	PEV, PKV	3 (6.7)	5 (5)
PEV, PKV	1 (2.2)	1 (1)	PEV, PRVC	3 (6.7)	4 (4)
PEV, PRVA	2 (4.4)	2 (2)	PEV, PSaV	2 (4.4)	2 (2)
PEV, PRVC	3 (6.7)	3 (3)	PEV, <i>E. coli</i>	2 (4.4)	2 (2)
PEV, PSaV	1 (2.2)	1 (1)	PRVA, <i>E. coli</i>	1 (2.2)	1 (1)
PEV, <i>E. coli</i>	3 (6.7)	3 (3)	Three pathogens		
PTV, PKV	1 (2.2)	1 (1)	PEV, PRVA, <i>E. coli</i>	3 (6.7)	5 (5)
PEV, <i>Salmonella</i> spp.	1 (2.2)	1 (1)	PEV, PRVA, PRVC	1 (2.2)	3 (3)
PSV plus three pathogens			PEV, PSaV, PRVC	2 (4.4)	2 (2)
PEV, PRVA, <i>E. coli</i>	3 (6.7)	3 (3)	PEV, PKV, <i>E. coli</i>	2 (4.4)	2 (2)
PEV, PSaV, <i>E. coli</i>	2 (4.4)	2 (2)	PEV, PSaV, PRVA	1 (2.2)	1 (1)
PEV, PTV, PKV	1 (2.2)	1 (1)	PEV, PSaV, <i>E. coli</i>	1 (2.2)	1 (1)
PEV, <i>E. coli</i> , <i>Salmonella</i> spp.	1 (2.2)	1 (1)	PEV, PKV, PTV	1 (2.2)	1 (1)
PSV plus four pathogens			PEV, PKV, PRVA	1 (2.2)	1 (1)
PEV, PKV, PRVA, <i>E. coli</i>	1 (2.2)	1 (1)	PEV, PRVC, <i>Salmonella</i> spp.	1 (2.2)	1 (1)
SUBTOTAL	24 (53.3)	34 (34)	PKV, PRVA, <i>E. coli</i>	1 (2.2)	1 (1)
Other enteric pathogens detected	31 (68.9)	64 (64)	Four pathogens		
One pathogen			PKV, PRVA, <i>E. coli</i> , <i>Salmonella</i> spp.	1 (2.2)	1 (1)
PEV	12 (26.7)	17 (17)	No enteric pathogens detected	2 (4.4)	2 (2)
<i>E. coli</i>	2 (4.4)	2 (2)	Total	45 (100)	100 (100)

^a PSV, porcine sapelovirus; PTV, porcine teschovirus; PEV, porcine enterovirus; PKV, porcine kobuvirus; PRVA-C, porcine group A, B and C rotaviruses; PSaV, porcine sapovirus

^b Number of positive farms

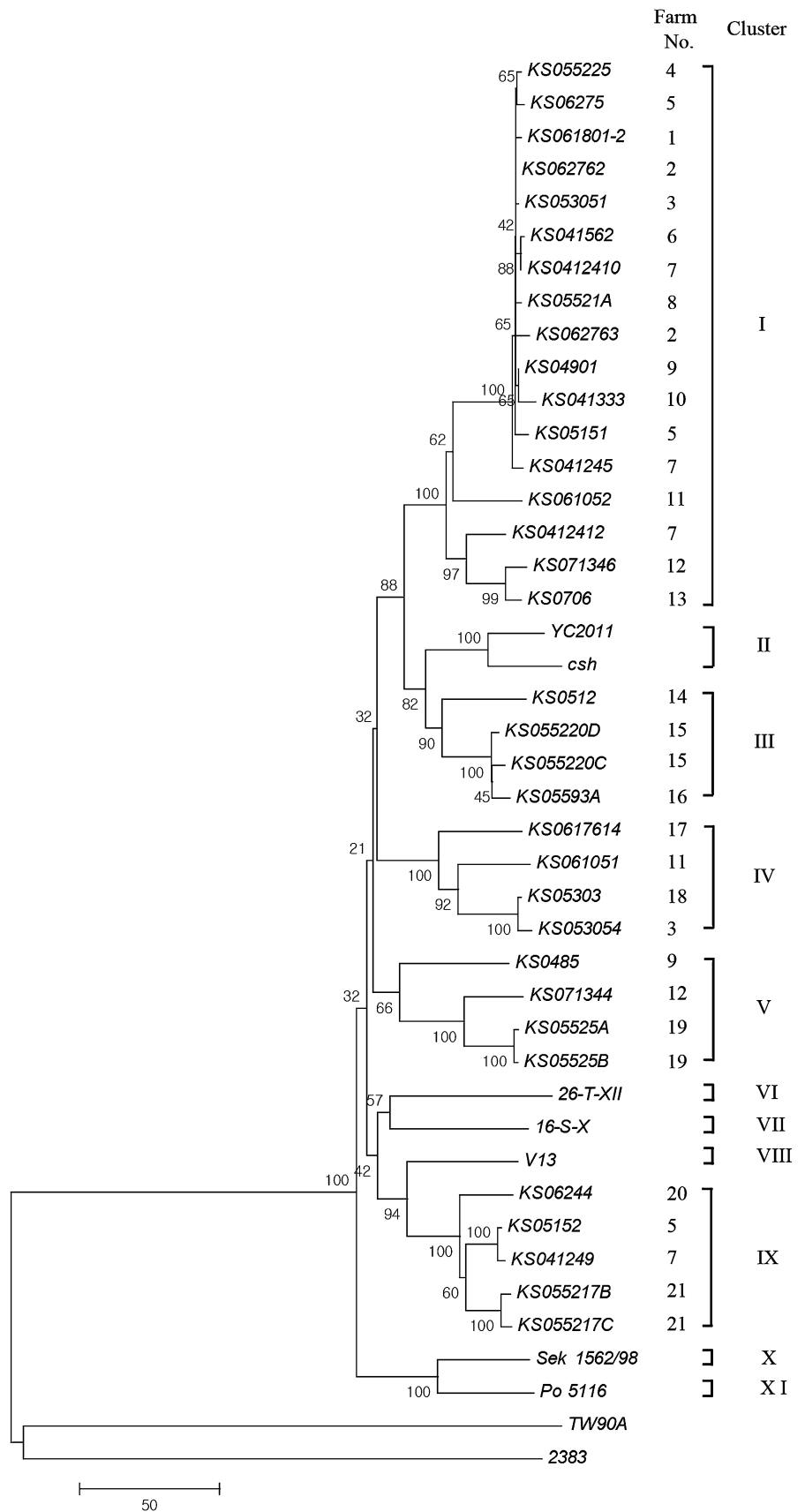
^c Number of positive fecal samples

The 34 Korean PSVs were placed in five different branches (clusters I, III, IV, V, and IX), distinct from those of German (clusters VI, VII, X, and XI), English (cluster VIII) and Chinese PSVs (cluster II). Interestingly, PSVs sequenced from the same Korean farm often belonged to different clusters (example: PSVs KS06275 and KS05152 from farm number 5 belonged to clusters I and IX, respectively), indicating the presence of genetic diversity in the same farm (Fig. 1). The inter-cluster distances among the 11 cluster were analyzed by the UPGMA method and are shown in Supplementary Table 5. The intra-cluster distances in each clusters were 0-0.193, whereas the inter-cluster distances ranged from 0.157-0.209 between clusters I (17 Korean strains) and III (4 Korean strains) to 0.266-0.346 between clusters II (Chinese csh and YC2011 strains) and X I (German AY392538 strain). These data indicate that there is genetic diversity not only among the Korean PSVs, even on the same farm,

but also between the Korean and other known PSVs, resulting in the formation of 11 different clusters.

Detection and classification of PSV strains or isolates had usually been done using a cross-neutralization test [10, 19, 20]. Recently, PCR-based assays including RT-PCR, nested PCR, and real-time PCR have been applied to detect PSVs in field fecal samples from pigs and to investigate their genetic diversity [11, 21]. The fecal prevalence of PSV infections in diarrheic pigs has varied from 36.6 % in the Czech Republic [11] to 69.2 % in Japan [10]. In the present study, two primer sets for RT-PCR and nested PCR assays, targeting the VP1 gene of PSV, were designed to determine the prevalence rate more precisely. Based on the nested PCR assay, the PSVs were detected in 34 % of the fecal samples examined in this study. This suggests that PSV infections are epidemic in pigs with diarrhea in South Korea in a similar manner to the prevalence of PSV infections in the Czech Republic [11].

Fig. 1 Phylogenetic tree based on the nucleotide sequences of partial VP1 genes of porcine sapeloviruses. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates, using MEGA version 5.1 with pairwise distance. The scale bar indicates nucleotide substitution per site. GenBank accession numbers for each strain used in this tree are in Supplementary Table 3



Interestingly, two diarrheic fecal samples among the 34 PSV positive samples obtained from the archives of the Fecal Registry tested positive for PSV alone. These data may support the idea that PSV plays a role in pig diarrhea as a causative agent of gastroenteritis [9]. The remaining 32 diarrheic fecal samples were positive for not only PSV but also other enteric pathogens. It is possible that the PSV infections may augment the severity of diarrhea, interacting with other enteric pathogens, including PSaV, RVA-C, PKV, PEV, PTV, and PNoV. This suggests that a number of enteric pathogens, either singly or in combination, can influence the clinical course of PSV infections. In addition, PSVs have been isolated or detected in healthy pigs and wild boars [10, 21–23]. Since we analyzed only diarrheic fecal samples from pigs, future studies should use many more fecal samples from pigs with and without gastroenteritis across different age groups. Interestingly, PEVs were the most frequently detected pathogens associated with PSV infections and in PSV-negative fecal samples. The high prevalence of PEV infections (88 %) in Korean pigs is very similar to that (80 %) in the US [24]. Although PEVs are detected in porcine fecal samples, its precise role as an enteric pathogen remains largely unknown [8]. Moreover, PEVs are detected in both diarrheic and clinically healthy pigs [24–26]. Therefore, further studies should address the enteropathogenicity of PEVs.

A comparison of the nucleotide sequences of partial VP1 genes of PSVs showed the largest genomic diversity. Since VP1 is the most external and immunodominant of the picornavirus capsid proteins, it contains a number of major neutralization sites of many picornaviruses [27], explaining why certain regions of the VP1 gene are hypervariable. Interestingly, a high degree of genomic diversity was observed between PSV strains circulating on the same Korean farm. Moreover, VP1 genes of Korean PSVs were distinct from those of the other known PSVs detected in China, the UK and Germany. These data suggest that the Korean PSVs had distinct genetic properties, resulting from a distinct evolutionary pathway.

Due to the properties of VP1 protein, Oberste and colleagues suggested that VP1 sequence comparisons may be valuable to determine genetic relationship among picornaviruses [28]. Although the species *Porcine sapelovirus* includes only one serotype, phylogenetic analysis of VP1 genes showed genetic diversity, resulting in 11 clusters. Depending on the country, moreover, PSVs made distinctly different clusters. Like the other RNA viruses, PSVs show broad genomic sequence diversity between the circulating strains, which is partially due to the poor template fidelity of their RNA polymerases. Taken together, it is assumed that a distinct evolutionary pathway of PSVs occurs throughout the world. It is expected that more genetic diversity will be identified among PSVs in the future when more sequence data for PSVs become available.

In summary, this study demonstrates that PSV infections are epidemic in pigs with diarrhea in South Korea. The infecting PSV strains are genetically diverse. Our data are useful for determining the importance of different PSV strains and may enable us to select representative strains for future biological or pathological studies, for the evaluation of diagnostic methods, and possibly for the study of candidate strains for vaccine production.

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