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Full-Length Genomic Analysis of Korean Porcine Sapelovirus Strains

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Abstract
Porcine sapelovirus (PSV), a species of the genus Sapelovirus within the family Picornaviridae, is associated with diarrhea, pneumonia, severe neurological disorders, and reproductive failure in pigs. However, the structural features of the complete PSV genome remain largely unknown. To analyze the structural features of PSV genomes, the full-length nucleotide sequences of three Korean PSV strains were determined and analyzed using bioinformatic techniques in comparison with other known PSV strains. The Korean PSV genomes ranged from 7,542 to 7,566 nucleotides excluding the 3′ poly(A) tail, and showed the typical picornavirus genome organization; 5′ untranslated region (UTR)-L-VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-3C-3D-3′ UTR. Three distinct cis-active RNA elements, the internal ribosome entry site (IRES) in the 5′ UTR, a cis-replication element (CRE) in the 2C coding region and 3′ UTR were identified and their structures were predicted. Interestingly, the structural features of the CRE and 3′ UTR were different between PSV strains. The availability of these first complete genome sequences for PSV strains will facilitate future investigations of the molecular pathogenesis and evolutionary characteristics of PSV.

Introduction
Picornaviruses are a family of positive-sense single stranded RNA viruses within the order Picornavirales [1]. They can cause intestinal, respiratory, neurological, cardiac, hepatic, mucocutaneous, and systemic diseases of varying severity in humans and animals [2]. Although different picornaviruses show various degrees of relatedness, all picornaviruses share a similar genomic organization, which consists of a covalently linked 5′ terminal protein called VPg (Viral Protein genome-linked), a 5′ untranslated region (UTR), a large open reading frame (ORF), a 3′ UTR and a poly(A) tail of variable length [2,3]. The genomic RNA of picornaviruses harbor several distinct cis-active RNA elements which are required for viral RNA replication; an internal ribosome entry site (IRES) in the 5′ UTR, a cis-replication element (CRE) within the ORF [3–6] or the 5′ UTR [7], the 3′ UTR, and the 3′ poly(A) tail [3]. Currently, five different types of IRES element [8] that direct cap-independent translation initiation on the viral RNA to produce the polyprotein have been identified from the primary sequence, secondary structure, location of the initiation codon and activity in different cell types [9,10]. In most picornaviruses, the polyprotein encoded by the ORF is cleaved into four structural viral particle proteins (VP4, VP2, VP3 and VP1) and seven non-structural proteins (2A, 2B, 2C, 3A, 3B, 3C and 3D). In addition, the members of the genera Cardioivirus, Aphthovirus, Erbovirus, Kobavirus, Teschovirus, Senecavirus and Sapelovirus possess a leader (L) protein at the N-terminus of polyprotein [11].

Although simian type 2 picornaviruses (SV-2-like viruses) and porcine enterovirus 8 (PEV-8) were once classified in the genus Enterovirus, SV-2-like viruses and PEV-8 along with duck picornavirus TW90A have an L protein at the N-terminus of the polyprotein that is lacking in the enteroviruses [12–14]. Moreover, those viruses contain distinct 2A proteins from those of the Enterovirus genus, and a highly divergent 5′ UTR with a type IV IRES [12,14–16]. Due to these particular genetic properties, these simian, avian, and porcine picornaviruses are now assigned as members of a new picornavirus genus, Sapelovirus [12–15,17,18].

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 [19–20]. Although PSVs can be important pathogens because of their wide distribution and high prevalence [21–25], the near-complete genomic sequences of only three PSV strains have been reported previously; one from the U.K. (V13
rocking, and EMEM containing 1% antibiotics and 1% NaHCO\textsubscript{3} plates. The suspensions were absorbed for 1 h with occasional tericin B) and 1% NaHCO\textsubscript{3}, and used to infect cells in 6-well diluted 10-times with Eagle’s minimal essential medium (EMEM) containing 1% antibiotics (Penicillin, Streptomycin, and Ampho-
diluted 10-times with Eagle’s minimal essential medium (EMEM) containing 1% antibiotics (Penicillin, Streptomycin, and Amphothericin B) and 1% NaHCO\textsubscript{3}, and used to infect cells in 6-well plates. The suspensions were absorbed for 1 h with occasional rocking, and EMEM containing 1% antibiotics and 1% NaHCO\textsubscript{3} was added. The cultures were incubated for 3 to 4 days at 37°C in a 5% CO\textsubscript{2} atmosphere and examined daily for cytopathic effects (CPE). Isolated PSVs were cloned by triple plaque purification. The PSV strains (KS04105, KS05151 and KS055217) were passaged eight times in LLC-PK cells, including isolation, adaptation, and triple plaque purification. The isolated viruses were confirmed as PSVs by an IFA, RT-PCR and transmission electron microscopy (TEM) assays, as described below.

Transmission electron microscopy (TEM)

LLC-PK cells infected with each of the above strains and showing over 70% CPE were frozen and thawed thrice, and centrifuged at 2,000 \( \times \) g for 30 min. To obtain purified virus, each supernatant was ultra-centrifuged at 200,000 \( \times \) g for 5 h at 4°C in a S30A-0015 rotor (Hitachi, Tokyo, Japan). The resulting pellets were resuspended in 40 \( \mu \)l of water and mixed with an equal volume of 2% (w/v) sodium phosphotungstic acid at pH 7.0. The samples were placed onto a formvar grid (Electron Microscopy Sciences, Hatfield, USA) for 5 min, and then excess liquid was removed by filter paper. The samples were examined using a High Resolution Transmission Electron Microscope (Hitachi) for the determination of purity of virus stock at Gwangju Center of Korea Basic Science Institute.

Immunofluorescence assay (IFA)

To characterize the PSV strains, the IFA was performed with a 153/5BS (IgG2a) monoclonal antibody specific for the PSV capsid protein (kindly provided by Dr. M Dauber, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany). Briefly, LLC-PK cells were infected with each strain, incubated for 18 h as above, fixed in 80% acetone for 5 min at 4°C, and allowed to air dry. Slides were washed thrice with PBS (pH 7.2), and incubated overnight at 4°C using a 1:40 dilution of monoclonal antibody specific for PSV capsid protein diluted in PBS (pH 7.2). Slides were washed thrice with PBS (pH 7.2), and incubated with FITC-conjugated goat anti-mouse IgG antibody (Santa Cruz biotechnology, Santa Cruz, USA) diluted 1:100 in PBS (pH 7.2) for 1 h at room temperature. After washing twice with PBS (pH 7.2), slides were stained with 4′-diamidino-2-phenylindole (DAPI) (Invitrogen, Lohne, Germany), and examined using a LSM confocal scanning microscope (Carl Zeiss, Jena, Germany).

RNA extraction and RT-PCR

Total RNA was extracted from the lysates of LLC-PK cells infected with each strain using the AccuPrep Viral RNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. To detect and amplify PSV RNA, RT-PCR with a primer pair specific for the PSV VPI coding sequence (Table S1 in File S1) was performed. To characterize the complete full-length genome sequences of each strain, ten primer sets (Table S1 in File S1) were designed to amplify the complete ORF sequences of each PSV strain based on the published genomic sequences of the PSV-V13 (GenBank ID: NC_003987), csh (GenBank ID: HQ875059) and YC2011 strains (GenBank ID: JX286666). Standard one-step RT-PCR assays were performed as previously described [25].

5′ and 3′ cDNA syntheses

cDNA of each strain was synthesized by the SMARTer Rapid Amplification of cDNA Ends (RACE) cDNA amplification kit (Clontech, Mountain View, USA) according to the manufacturer's instructions. For generating 3′ RACE-ready cDNA, 3.75 µl of the poly(A)\textsubscript{tail} tailing reaction mixture and 1 µl of 3′ cDNA Synthesis (CDS) Primer A were mixed and heated to 72°C for 3 min, followed by cooling to 42°C for 2 min using a thermo cycler. For generating 5′ RACE-ready cDNA, 3.75 µl of total RNA was mixed with 1 µl of 5′-CDS primer A, incubated at 72°C for 3 min, and cooled at 22°C for 2 min. The denatured RNA for each 3′ and 5′ cDNA generation was mixed with a reaction mixture composed of 2 µl 5′-First-Strand Buffer, 1 µl dithiothreitol (DTT) (20 mM), 1 µl dNTP mix (10 mM), 0.25 µl RNase inhibitor (40 U/µl), and 1 µl SMARTer Reverse Transcriptase (100 U). Samples were incubated at 42°C for 90 min and heated at 70°C for 10 min. The synthesized cDNAs were diluted with 7 µl of Tricine-EDTA buffer and used for RACE PCR.

RACE PCR, cloning and sequencing

For the generation of 3′ and 5′ RACE PCR reactions, Advantage 2 Polymerase Mix (Clontech) was used; 5 µl of 3′/5′-RACE-Ready cDNA, 32 µl of PCR-grade water, 5 µl of 10X Advantage 2 PCR Buffer, 1 µl dNTP Mix (10 mM), 5 µl Universal Primer Mix (10 X), 1 µl of 50 pmol/ml gene-specific primer (GSP) for 3′ and 5′ RACE (Table S1 in File S1), and 1 µl of 50X Advantage 2 Polymerase Mix. The reaction was performed with the following thermal cycling program: 5 cycles of 94°C or 94.5°C for 30 sec and 72°C for 2 min or 3 min; 3 three-step cycles of 94°C or 94.5°C for 30 sec, 65°C (applied melting temperature of GSP) for 30 sec, and 72°C for 2 min or 3 min; 25 three-step cycles of 94°C or 94.5°C for 30 sec, 60°C (applied lowered Tm values of GSP by 3°C to 5°C) for 30 sec, and 72°C for 2 min or 3 min.

The RACE PCR products were separated on a 1.5% (m/v) agarose gel; the bands were excised and purified using a PureLink Quick Gel Extraction kit (Invitrogen). The products were ligated into TA Vector Systems (Enzynomics, Daejeon, Korea) and...
introduced into DH5α competent cells. Individual colonies were
grown and plasmid was purified using Hybrid-Q Plasmid
(GeneAll, Seoul, Korea). Sequencing was performed using an
ABI System 3700 automated DNA sequencer (Applied Biosystems,
Foster, USA).

Full-length genomic characterization and secondary
structure prediction

To generate complete nucleotide sequences for each PSV strain,
both 5' and 3' end sequences of each strain were assembled with
the nucleotide sequences of the internal ORF sequences. The
full-length genomic and individual protein coding sequences
of three PSV strains were compared with those of the other
known PSV strains (Table S2 in File S1) using the DNA
Basic module (DNAsis MAX, Alameda, USA). Phylogenetic analyses
based on nucleotide and amino acid alignments were performed
using the neighbor-joining method with 1000 bootstrap
replicates and UPGMA Molecular Evolutionary Genetics Analysis
(MEGA version 5.2) employing pair-wise distance comparisons
[27]. Sequence identity calculations for the three PSV strains with
those of the other known PSV strains were performed using the
homology and distance matrices method of DNAMAN version 6.0
program (Lynnon, Vaudreuil, Canada). Secondary structure
elements in the PSV genomes were modeled using CLC Main
Workbench version 6.8.2 program (CLC bio, Katrinebjerg,
Denmark).

Ethics statement

No specific approval was needed since the fecal samples were
voluntarily submitted by the farms for pathogen screening in our
laboratory. No other specific permits were required for the
described field studies. The locations where we sampled are not
protected in any way. The field studies did not involve endangered
or protected animal species. Before beginning work on the study,
we contacted the farm owners and obtained their permission.

Results

Virus isolation and identification

Three Korean PSV strains were isolated from separate diarrhea
fecal samples originating from three different farms and plaque
purified. LLC-PK cells infected with each strain after 8 passages in
LLC-PK cells showed CPE at day 1 post-inoculation characterized
by shrinkage, rounding and detachment of cells (Figure 1A, 1B),
and displayed PSV-specific cytoplasmic fluorescence in the
indirect IFA using a monoclonal antibody against PSV capsid
protein (Figure 1A). RT-PCR assays with a primer pair specific for
the partial PSV VP1 coding region amplified a 636 bp amplicon
from alignments with other picornaviruses are shown in Table 1.
The complete genome sequence, excluding the poly(A) tail, and
the polyprotein sequences of three Korean strains were compared
with those of other known PSVs and representative picornavirus
strains available in the GenBank database. The Korean PSV
strains showed high nucleotide (84.7%–94.0%) and deduced
amino acid (92.9%–98.3%) identities with the other PSV strains
(Table 2), but showed relatively low nucleotide and polyprotein
sequence identities with the avian and simian sapelovirus strains
(Table 2).

Each of the major functional units in the genome, including the
5' and 3' UTRs, the capsid coding region (P1) and the regions
encoding the non-structural proteins (P2 and P3) of PSVs, were
phylogenetically analyzed (Figure 3). Representative simian and
avian sapelovirus reference strains were included in each of
the trees. The 5' UTR sequences of three Korean PSV strains were
in the same cluster and were more closely related to the English
strain V13 than to the Chinese strains csh and YC2011 (Figure 3A).
The 3' UTR sequences of five strains (KS04105, KS055217, KS05151,
V13, and YC2011) were 82 nucleotides long while that of Chinese
strain csh was 68 nucleotides in length, possibly due to incomplete sequencing (Table S3 in File S1). However, they were phylogenetically very close (Figure 3B).

The leader protein sequences of all PSV strains were 252
nucleotides (84 amino acids) in length (Table S3 in File S1) and
show high deduced amino acid identities (95.2–100%) (Table S4 in
File S1). The PSV leader polypeptide lacked the catalytic residue
motifs necessary for proteolytic activity and did not contain either
a zinc-finger motif [Cys2 His2-like fold group] in the leader amino
terminal region or a tyrosine-phosphorylation motif [KR]-x(2,3)-
[ED]-x(2,3)-[Y]. The nucleotide and deduced amino acid sequences of the PSV
capsid region varied in length from 2430 to 2454 nucleotides
(encoding 810 to 818 amino acids, Table S3 in File S1). To
investigate the genetic relationships between the PSV strains,
pairwise sequence identities were calculated for the deduced
complete capsid protein sequences of all 6 PSV strains and for the
sequences of each of the mature capsid proteins, VP1-VP4 (Table
S4 in File S1). All PSV strains showed 88.4% to 97.7% nucleotide
identities in the complete capsid coding sequences (Table S4 in

Genome organization

The complete nucleotide sequences of the whole genome of the
three Korean PSV strains were obtained and compared to the
previously determined PSV sequences. In picornavirus RNAs, the
two 5′ terminal UU residues are derived from the uridylation of
VPg to make VPg-pU-pU [28]. In the previously described PSV
sequences, however, the two 5′ terminal residues were AC for the
Chinese csh and English V13 strains, and UA for the Chinese
YC2011 strain, suggestive of incomplete sequences. To obtain the
correct 5′ terminal start residues, cDNA synthesis and then 5′
RACE PCR with 5′ RACE primer (Table S1 in File S1) were
performed. Using this approach, the 5′ terminal residues were UU
and the 5′ UTR length of three Korean strains was 25 nucleotides
longer than that of Chinese YC2011 strain (Table S3 in File S1)
[29]. In order to confirm this result, 5′ RACE PCR was performed
with another 5′ RACE PCR primer (Table S1 in File S1) and the
same 5′ terminal nucleotide residues, UU, were also observed.

The length of the complete genomes of Korean PSV strains,
excluding the poly(A) tail, was from 7,542 (KS04105 and
KS055217) to 7,566 nucleotides (KS05151) (Table S3 in File S1). These sequences contained a single large ORF whose lengths
were 6,966 nucleotides (strain V13; 2,322 amino acid polyprotein
precursor) to 6,993 nucleotides (strains csh, KS05151, YC2011;
2,331 amino acid polyprotein precursor) (Table S3 in File S1). The
predicted protease cleavage sites of the polyproteins, as determined
from alignments with other picornaviruses are shown in Table 1.
The polyprotein was predicted to be cleaved and processed into
twelve mature peptides: L-VP4-VP2-VP3-VP1-2A-2B-2C-3A-3B-
3CPro-3DPol (Figure 2). The ORF sequence in the three Korean
PSV strains was flanked by 5′ UTR which was 491 nucleotides
long and by a 3′ UTR which was 82 nucleotides long (Table S3 in
File S1).

Molecular and phylogenetic analyses

The complete genome sequence, excluding the poly(A) tail, and
the polyprotein sequences of three Korean strains were compared
with those of other known PSVs and representative picornavirus
strains available in the GenBank database. The Korean PSV
strains showed high nucleotide (84.7%–94.0%) and deduced
amino acid (92.9%–98.3%) identities with the other PSV strains
(Table 2), but showed relatively low nucleotide and polyprotein
sequence identities with the avian and simian sapelovirus strains
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The leader protein sequences of all PSV strains were 252
nucleotides (84 amino acids) in length (Table S3 in File S1) and
show high deduced amino acid identities (95.2–100%) (Table S4 in
File S1). The PSV leader polypeptide lacked the catalytic residue
motifs necessary for proteolytic activity and did not contain either
a zinc-finger motif [Cys2 His2-like fold group] in the leader amino
terminal region or a tyrosine-phosphorylation motif [KR]-x(2,3)-
[ED]-x(2,3)-[Y]. The nucleotide and deduced amino acid sequences of the PSV
capsid region varied in length from 2430 to 2454 nucleotides
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investigate the genetic relationships between the PSV strains,
pairwise sequence identities were calculated for the deduced
complete capsid protein sequences of all 6 PSV strains and for the
sequences of each of the mature capsid proteins, VP1-VP4 (Table
S4 in File S1). All PSV strains showed 88.4% to 97.7% nucleotide
identities in the complete capsid coding sequences (Table S4 in

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The phylogenetic relationships are shown in Figure 3C. The PSV strains had 85.6% to 98.2% nucleotide identities within the VP1 coding sequences (Table S4 in File S1). The phylogenetic analysis for the VP1 proteins among PSV strains is shown in Figure 3D. The VP1 sequences of the KS05151, csh, and YC2011 strains encoded an additional 8 amino acids in comparison with other strains (Table S3 in File S1).

The P2 region of all PSV strains was 1989 nucleotides (663 amino acids) in length and P3 was 2235–2238 nucleotides (745–746 amino acids) long (Table S3 in File S1). Pairwise deduced amino acid sequence identities of P2 and P3 regions were shown to be very high, ranging from 94.1% to 99.5% and 95.9% to 99.0%, respectively (Table S4 in File S1).

As described above, the use of 5’RACE allowed determination of the complete 5’UTR sequence of the Korean PSVs (some 491 nt in length); the 5’ UTR of these strains was 25 nucleotides longer than that of Chinese strain of PSV (YC2011). This allowed prediction of the secondary structure elements within the PSV 5’UTR (Figure 4). At the extreme 5’ terminus were two stem-loop structures, labelled domains Ia and Ib. The latter included two smaller stem loop structures, labelled Ic and Id. The 5’ UTR also contained two other domains, labelled domain II and domain III. These represent essential components of the IRES and are labelled in the same manner as other type IV IRES elements (related to that found in hepatitis C virus (HCV), the pestiviruses and certain picornaviruses, e.g. porcine teschovirus) [9,30,31].

### Table 1. Location of putative cleavage sites in the porcine sapelovirus polyprotein.

| Cleavage site | Amino acid sequence | Position of amino acid |
|---------------|---------------------|-----------------------|
| L/VP4         | GNKPQ/GAYNH         | 84/85                 |
| VP4/VP2       | GPSLK/APDKE         | 137/138               |
| VP2/VP3       | RQ/GFPVR            | 375/376               |
| VP3/VP1       | YQ/GD               | 609/610               |
| VP1/2A        | AEOQ” b (ATQTc)/GPYE | 902/903               |
| 2A/2B         | HDWQ/GLGQV          | 1128/1129             |
| 2B/2C         | EPHKQ/GPDSW         | 1233/1234             |
| 2C/3A         | DAIFQ/GPVQ          | 1565/1566             |
| 3A/3B         | KQ/GAY              | 1665/1666             |
| 3B/3C         | KAVVQ/GPDME         | 1687/1688             |
| 3C/3D         | FVNKO/GLITE         | 1869/1870             |
| 3D            | F/                  | 2331/                 |

Letters in bold represent conserved amino acid residues.

YC2011, KS05151 and csh strains.

KS055217 and KS04105 strains.

V13 strain.

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Figure 2. Genome organization of the porcine sapelovirus (PSV). The open reading frames are flanked on either side by UTRs. The numbers above or under each rectangle are the length of nucleotides or deduced amino acids. The length of VP1 and 3D regions are different among PSVs.

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Discussion

We report here the isolation of three Korean PSV strains, KS05151, KS04105 and KS055217 from porcine diarrhea specimens. The Korean PSV strains were identified as PSV by RT-PCR, IFA and TEM assays. The genome sequences of the Korean PSV strains were determined and proved to be the first complete genome sequences for PSVs. They have a genome organization typical for members of the genera Cardiovirus, Aplthovirus, Erbovirus, Kobavirus, Teschovirus and Senecavirus [11]. Moreover, these strains had distinct 2A proteins from those of the Enterovirus genus and a 5′ UTR with a type IV IRES [12,14,15,37].

The 5′ UTRs of picornaviruses are highly structured and contain an IRES that directs RNA translation by internal ribosome binding [8,9]. Picornavirus IRES are currently divided into five distinct types by the primary sequence, secondary structure, location of the initiation codon and activity in different cell types [9,10]. In a previous study [16], the IRES elements of PSV V13 strain and simian sapelovirus SV2 strain were found to be related functionally and structurally to the type IV IRES element from porcine teschovirus 1 and hepatitis C virus. Comparative sequence analysis of the Korean PSV strains with PSV V13 strain showed that the structural features of the IRES elements were well conserved in all PSV species including the Korean PSV strains but they lacked a domain IIIc [16]. At the 5′ UTR terminus, enteroviruses and rhinoviruses contain a cloverleaf structure which is involved in RNA replication [38]. In order to identify whether PSV species have a cloverleaf RNA structure at the 5′UTR, the complete 5′UTR needs to be known. However, the sequences of the known PSV strains including the English V13, and the Chinese csh and YC2011 strains lacked the 5′ terminal UU residues, which were necessary for picornavirus RNA replication [39]. Using 5′ RACE, an additional 25 nucleotides including 5′ terminal UU residues were identified compared to the recently sequenced Chinese YC2011 strain [29]. Unlike enteroviruses and rhinoviruses [30], the Korean PSV strains had no cloverleaf RNA structure at the 5′UTR. However, two conserved stem-loop motifs were present within the 5′-terminal 80 nucleotides (Figure 4). The role of these structures is not known but they may be expected to play a role in RNA replication analogous to the cloverleaf structure of the enteroviruses [38,39]. Overall the 5′UTRs of PSVs were quite short, for example the poliovirus 5′UTR is about 740 nucleotides in length while the foot-and-mouth disease virus (FMDV) 5′UTR is over 1300 nucleotides [40].

The picornaviruses that have a L protein preceding the capsid region are members of the genera Cardiovirus, Aplthovirus, Erbovirus, Kobavirus, Cardiovirus and Sapelovirus [41]. In aphthoviruses and erboviruses, the L proteins are papain-like cysteine proteases that are able to cleave at their own carboxy-terminus and also to induce the cleavage of the eukaryotic initiation factor (eIF) 4G, leading to the shut-off of host-cell protein synthesis [42,43]. The L protein of echymomyocarditis virus (a cardiovirus) binds zinc, is phosphorylated during viral infection, and has been reported to affect the efficiency of genome translation [41]. The properties of the sapelovirus L protein are not known; it has neither the catalytic dyad (Cys and His), conserved in a papain-like thiol protease found in FMDV L protein [44], nor a putative zinc-binding motif, Cys-His-Cys-Cys.
Table 2. Comparison of complete nucleotide and deduced amino acid sequences between the Korean porcine sapelovirus (PSV) strains and other known picornavirus strains.

| Strain1 | Genus     | Species | % nucleotide and deduced amino acid identities with strain: |
|---------|-----------|---------|-----------------------------------------------------------|
|         |           |         | KS04105 | KS05151 | KS055217 |
|         |           |         | nt2    | aa3    | nt      | aa      | nt      | aa      |
| KS04105 | Sapelovirus | PSV     | 94.0   | 98.3   | 90.6    | 97.0    |         |         |
| KS05151 | Sapelovirus | PSV     | 90.6   | 97.0   | 89.2    | 96.5    |         |         |
| KS055217| Sapelovirus | PSV     | 84.7   | 93.6   | 85.0    | 93.5    | 85.1    | 92.9    |
| V13     | Sapelovirus | PSV     | 88.0   | 97.1   | 88.8    | 97.4    | 87.5    | 95.6    |
| csb     | Sapelovirus | PSV     | 88.3   | 97.3   | 89.2    | 97.6    | 87.8    | 96.1    |
| YC2011  | Sapelovirus | PSV     | 56.3   | 55.0   | 56.2    | 54.8    | 56.7    | 54.9    |
| TW90A   | Sapelovirus | ASV4    | 44.2   | 43.3   | 44.1    | 43.4    | 43.8    | 43.8    |
| 2383    | Sapelovirus | SSV5    | 56.3   | 55.0   | 56.2    | 54.8    | 56.7    | 54.9    |
| F6S     | Teschovirus | PTV-18   | 39.0   | 24.3   | 39.4    | 24.5    | 39.2    | 24.5    |
| UKG/410/73 | Enterovirus | PEV-97 | 48.8   | 38.4   | 48.6    | 38.7    | 48.9    | 39.0    |
| Ruckert | Cardiovirus | EMCV8   | 37.0   | 24.8   | 36.9    | 24.5    | 36.8    | 24.8    |
| O’Tai   | Aphthovirus | FMDV9   | 32.3   | 24.3   | 32.1    | 24.3    | 32.4    | 24.1    |
| P1436/71| Erbovirus | EREBV-1 | 36.5   | 23.3   | 36.5    | 23.1    | 36.4    | 23.5    |
| Mahoney | Enterovirus | PV-111 | 48.7   | 38.2   | 48.9    | 38.0    | 48.8    | 37.9    |
| HM-175  | Hepatovirus | HAV13 | 35.7   | 18.6   | 35.8    | 18.6    | 36.1    | 18.6    |
| A8/46/88 | Kobavirus | AV13    | 32.7   | 19.3   | 32.7    | 19.4    | 32.6    | 19.4    |
| Gregory | Parechovirus | HPeV-14 | 35.5   | 16.1   | 35.1    | 16.2    | 35.3    | 16.2    |
| 89      | Rhinovirus | Human rhinovirus A | 47.1 | 38.0  | 47.0      | 38.3    | 47.3    | 38.1    |
| R85952  | Avihepatovirus | DHAV15 | 36.5   | 16.7   | 36.4    | 16.3    | 36.3    | 16.6    |
| SVV-001 | Senecavirus | SVV16  | 36.4   | 23.7   | 36.6    | 23.6    | 36.6    | 23.8    |
| Calnek  | Tremovirus | AEV17   | 37.3   | 19.1   | 37.5    | 19.2    | 37.3    | 19.1    |

1 GenBank accession numbers of strains used are in Table S2.
2 The full-length nucleotide sequence identities among PSVs and other picornaviruses.
3 The full-length deduced amino acid sequence identities among PSVs and other picornaviruses.
4 ASV: avian sapelovirus.
5 SSV: simian sapelovirus.
6 PTV-1: porcine teschovirus serotype 1.
7 PTV-9: porcine enterovirus serotype 9.
8 EMCV: encephalomyocarditis virus.
9 FMDV: foot-and-mouth disease virus type O.
10 ERBV-1: Equine rhinitis B virus serotype 1.
11 PV-1: Poliovirus serotype 1(Human enterovirus C serotype).
12 HAV: Hepatitis A virus.
13 AV: Aichi virus.
14 HPeV-1: Human parechovirus serotype 1.
15 DHAV: Duck hepatitis A virus.
16 SVV: Seneca Valley virus.
17 AEV: Avian encephalomyelitis virus.
18 doi:10.1371/journal.pone.0107860
found in encephalomyocarditis virus [45]. Further studies are required to address the function of PSV L protein.

In general, picornavirus 3’ UTRs vary in length between 40 and 165 nucleotides. The length of the 3’ UTR in PSV is 82 nucleotides (strain V13, YC2011, KS04105, KS05151 and KS055217) although a shorter sequence (68 nucleotides) was described for the csh strain [20]. In a previous report [12], PSV strain V13 was predicted to include three stem-loop structures in the region of the 3’ UTR using nine nucleotides of the terminal part of the 3D coding region. In the present study, the Korean viruses KS04105, KS05151 and KS055217 plus the Chinese YC2011 strain each appear to have these 3 stem-loop structures (X, Y and Z), but the csh strain showed only two stem-loop structures (X and Y) since the sequence was shorter. The domain Z, in which the stop codon (UGA) is located, is the most conserved region of the 3’ UTR within PSVs, whereas domains Y and X were considered more variable regions (Figure 5B, 5C), as they show heterogeneity in both length and nucleotide sequence. The differences between the 5 different PSV strains and the csh strain appear attributable to an incomplete 3’ terminal sequence for the csh 3’ UTR region. Moreover, five strains, except for the strain csh, were predicted to have an intramolecular kissing interaction between the X and Z domains. Due to the lack of 3’ terminal sequence of csh 3’ UTR region, no intramolecular kissing RNA interaction could be predicted (Fig. 5D). Interestingly, the V13 strain showed intramolecular interaction of 7 nucleotides, but the three Korean and one Chinese strains had intramolecular interaction of 8 nucleotides. The 3’ UTR plays an important role in picornavirus replication. Serial passage of mutant viruses in which such interactions were disrupted resulted in production of revertants in which the tertiary kissing interaction was restored, indicating the functional importance of the interaction in the enterovirus 3’ UTR [46–50]. Further study is required to determine whether these regions are important for PSV replication using modifications of the relevant PSV nucleotide sequences.

A CRE of picornaviruses has been identified in six genera of Picornaviridae family; Enterovirus [6,51], Rhinovirus [5], Cardiovirus [52], Aphthovirus [7], Parechoviruses [4], and Hepatovirus [53]. However, no putative CRE has yet been reported for viruses in the Sapelovirus, Kobuvirus, Teschovirus, Avihepatovirus, Senecavirus and Tremovirus genera. The location of the CRE in the genomic RNA varies between the picornavirus genera. It is located in the coding region for 2C in enteroviruses [6,36], for VP1 in species B rhinoviruses [32], for VP2 in species C rhinoviruses [38] and cardioviruses [32], for VP0 in parechoviruses [4] and for 3D of hepatoviruses [54]. In FMDV, the CRE is located just upstream of the IRES [7,55]. In the present study, a putative CRE was located in the 2C coding region of all PSV

Figure 3. Sequence comparisons and phylogenetic analysis of Korea porcine sapelovirus strains. The phylogenetic tree of 5’ untranslated region (UTR) sequence (A), 3’ UTR sequence (B), P1 nucleotide sequence (C), VP1 nucleotide sequence (D), P2 nucleotide sequence (E), and P3 nucleotide sequence (F) were constructed using the neighbor-joining method with 1,000 bootstrap replicates, and the branch length is indicated at each branch node. doi:10.1371/journal.pone.0107860.g003
strains. Generally, the AAACA motif is located in the loop of picornavirus CREs and the first and second A residues are involved in providing a template for the addition of uridine onto VPg [38,39,53,55,56]. In contrast to other known CREs, the PSV strains had two copies of the AAACA motif within the sequence CAAACAATAAATAACAA. This indicated that one or both of these AAACA motifs may be involved in being a template for the addition of uridine onto VPg. Future functional analyses are needed to identify whether one or both motifs are templates for the uridylylation of VPg to make VPg-pU-pUOH.

In this study, we characterized the structural features of three Korean PSV strains in comparison with the other known PSV strains. All PSV strains showed the typical picornavirus genome organization. We have identified putative RNA structures in the sequences and structural features of the porcine sapelovirus KS05151 strain. At the extreme 5' terminus are two stem-loop structures, labelled domains la and lb. Secondary stem-loops include two smaller stem loop structures labelled Ic and Id. A secondary structure model for the domains II and III of the type IV internal ribosome entry site element is shown (this model is based on previously published studies [9,29,30]).

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Figure 4. Sequences and structural features of 5’ untranslated region of the porcine sapelovirus KS05151 strain. At the extreme 5’ terminus are two stem-loop structures, labelled domains Ia and Ib. Secondary stem-loops include two smaller stem loop structures labelled Ic and Id. A secondary structure model for the domains II and III of the type IV internal ribosome entry site element is shown (this model is based on previously published studies [9,29,30]).

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Figure 5. Sequences and structural features of 3′ untranslated region (UTR) of the porcine sapeloviruses. (A) The nucleotide sequences of the 3′ UTR were compared using the Clustal W methods. (B–D) Secondary and tertiary structures of 3′ UTR of strains KS04105, KS05151, KS055217 and YC2011 (B), strain V13 (C), and strain csh (D) were predicted by the CLC program. Proposed tertiary interactions between the loops of X and Z domains are shown by lines.

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Figure 6. Sequences and structural feature of porcine sapelovirus cis-replication element (CRE). The first and second AAACA motifs are written in bold letter in the loop of the CRE.

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5’UTR and 3’ UTR plus a CRE in the 2C coding sequence. Interestingly, the structural features of the CRE in the 2C coding sequence and of the 3’UTR were different between the strains circulating in the recent and past decades. These first complete genome data for the PSV (for the Korean PSV strains KS05151, KS05152, and KS05217) will facilitate future investigations concerning the molecular pathogenesis and evolutionary characteristics of this virus.

Supporting Information

Figure S1 Phase contrast photomicrographs of control and infected LLC-PK1 cells, and RT-PCR assay for detecting porcine sapelovirus (PSV) VP1 coding region. (A) Mock-inoculated control cells. (B) Cells at 1 day after infection with Korean PSV strain KS05151. Note the shrinking and rounding up of the infected cells. Microscope settings Ocular: 10X. Scale bar, 200 μm. (C) RT-PCR with primers specific for part of the PSV VP1 coding region generated the expected 636 bp ampiclons. M: size marker. N: mock-infected LLC-PK cells. Lanes 1–3: KS04105, KS05151, and KS05217 strains. (TIF)

File S1 Supplementary Tables. Table S1. Oligonucleotide primers for amplifying and sequencing of porcine sapelovirus strains. Table S2. Strains of picornaviruses and their GenBank accession numbers used in this study. Table S3. The length of 5’ untranslated region, each part of the open reading frame, 3’ untranslated region and the complete genome excepting the poly(A) tail. Table S4. Comparison of nucleotide/deduced amino acid sequences between the porcine sapelovirus strains. (DOC)

Author Contributions

Conceived and designed the experiments: KOC KYS. Performed the experiments: KYS DSK JK JSC MIK GJB. Analyzed the data: KYS GJB KOC. Contributed reagents/materials/analysis tools: MIK KO C. Contributed to the writing of the manuscript: KYS DSK GJB KOC.

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