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Complete genomic analysis and molecular characterization of Japanese porcine sapeloviruses

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Abstract

The *Porcine Sapelovirus* (PSV) is an enteric virus of pigs that can cause various disorders. However, there are few reports that describe the molecular characteristics of the PSV genome. In this study, almost the entire genomes of 23 PSVs detected in Japanese pigs were analyzed using bioinformatics. Analysis of the *cis*-active RNA elements showed that the predicted secondary structures of the internal ribosome entry site in the 5′ untranslated region (UTR) and a *cis*-replication element in the 2C coding region were conserved among PSVs. In contrast, those at the 3′ UTR were different for different PSVs; however, tertiary structures between domains were conserved across all PSVs. Phylogenetic analysis of nucleotide sequences of the complete VP1 region showed that PSVs exhibited sequence diversity; however, they could not be grouped into genotypes due to the low bootstrap support of clusters. The insertion and/or deletion patterns in the C-terminal VP1 region were not related to the topology of the VP1 tree. The 3CD phylogenetic tree was topologically different from the VP1 tree, and PSVs from the same country were clustered independently. Recombination analysis revealed that recombination events were found upstream of the P2 region and some recombination breakpoints involved insertions and/or deletions in the C-terminal VP1 region. These findings demonstrate that PSVs show genetic diversity and frequent recombination events, particularly in the region upstream of the P2 region; however, PSVs could currently not be classified into genotypes and conserved genetic structural features of the *cis*-active RNA elements are observed across all PSVs.

Keywords Complete genome analysis · Japan · Molecularly characterization · Porcine feces · *Sapelovirus A*

Introduction

The genus *Sapelovirus* belongs to the family *Picornaviridae*, which includes > 30 genera and > 75 species, including the species *Sapelovirus A* and *Sapelovirus B*, which were formerly known as *Porcine Sapelovirus* (PSV) and *Simian Sapelovirus*, respectively [55]. Sapeloviruses are

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phylogenetically classified into the picornavirus supergroup 3, together with *Enterovirus, Rabovirus*, and the proposed genus Antivirus, which is currently named *Avian sapelovirus*, and which belongs to the genus *Sapelovirus* (http://www.picornaviridae.com). Originally, PSV had been assigned to porcine enterovirus (PEV) 8 [5, 26]. After further genomic studies regarding PEV, PEV 1–7 and PEV 11–13 have been reclassified into the genus *Teschovirus*; PEV 9 and 10 were formally included in PEV-B, which has been renamed as *Enterovirus G*, and PEV 8, which was formally included in PEV-A, has been renamed as PSV [24, 27, 54, 55].

PSV can cause diseases with various symptoms, including polioencephalomyelitis, diarrhea, pneumonia, and reproductive disorders [3, 4, 17, 21, 22, 25, 29, 39, 41]; however, PSVs are also identified in the feces of healthy pigs [9, 38]. PSVs have been found in pigs in China, South Korea, the US, European countries, India, Brazil, and Japan [1, 4, 6, 10, 13–16, 29, 39, 41–43, 51, 52]. PSVs were spread by the fecal–oral transmission route among the pig population and are thought to be ubiquitous enteric viruses found in pigs [9, 38, 44].

PSV has an approximately 7.5-kb-long single-stranded RNA genome with a positive-sense polarity that contains a single, large open reading frame (ORF). The ORF encodes a single polyprotein flanked by the 5′ and 3′ untranslated regions (UTRs) and a poly-A tail at the end of the 3′ UTR. The single polyprotein is subsequently processed into a leader protein, four structural proteins (VP4, VP2, VP3, and VP1) derived from P1, and seven non-structural proteins (2A, 2B, and 2C derived from P2, and 3A, 3B, 3C, and 3D derived from P3) [55]. The 5′ UTR and 3′ UTR are known to contain important structural motifs. An internal ribosome entry site (IRES) located in the 5′ UTR is necessary for initiating translation from the uncapped viral genome [7, 12, 20, 37]. It is now known that there are five types of IRES, which are classified according to their nucleotide (nt) sequences and structural characteristics, and the mechanism of translation initiation is known [45, 53]. The 3′ UTR of picornaviruses contains secondary and tertiary structural elements and is required for efficiently carrying out viral RNA replication [8, 23, 32, 36]. In addition to the 5′ UTR and 3′ UTR structural elements, *cis*-acting replication elements (*CREs*), which are required for efficiently carrying out the initiation of a viral complementary RNA strand, have been identified within the coding region of several picornavirus genomes [2, 8, 11].

Enterovirus G, which belongs to the *Enterovirus*/*Sapelovirus* supergroup together with PSV, consists of 20 genotypes. These genotypes display more than 25% nt sequence divergence between each other in the VP1 gene region [46, 49]. On the other hand, although there have been reports regarding the antigenic diversity of PSV [18], there have been no reports describing the serotypes or genotypes of PSV.

In the present study, to characterize Japanese PSVs precisely and to compare them with PSVs from other countries, almost the entire genomes of 23 PSVs detected from the feces of pigs in Japan were analyzed using bioinformatics and phylogenetics, using PSV sequences that were obtained from the DDBJ/EMBL/GenBank database.

Materials and methods

Metagenomic and whole genome analysis of PSV

In a Japanese study regarding *Enterovirus G* that was conducted using 222 fecal samples of pigs with or without diarrhea in 2014–2016 [48], 21 sequence contigs, which were > 7000 bases in length and showed sequence homology with PSV, were identified. Two additional > 7000 base PSV sequence contigs were found from another 8 feces samples collected from healthy pigs during 2017–2018 in Japan, using the metagenomics approach. Metagenomic analyses were performed as described previously [33, 48]. Briefly, the total RNA was extracted directly from the supernatant of 10% fecal suspensions. cDNA libraries were prepared using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) from total RNA. Deep sequencing was performed using a MiSeq bench-top sequencer (Illumina, San Diego, CA, USA). FASTQ files created by the MiSeq Reporter (Illumina) were imported into the CLC Genomics Workbench 7.5.5 (CLC bio, Aarhus, Denmark). Contigs were created from trimmed reads using de novo assembly, with the use of default parameters in the CLC Genomics Workbench. To evaluate the confidence of generated contigs, mapping reads were conducted with regard to a reference using the CLC Genomics Workbench with the strictest parameter settings (mismatch cost, 2; insertion cost, 3; deletion cost, 3; length function, 0.9; and similarity function, 0.9), and 5′ and 3′ sequences with insufficient read depth (< 3) were omitted. To obtain the complete sequences at the 3′ end of the genome, commercial kits (3′-Full RACE Core Set, TaKaRa Bio, Otsu, Japan) were used to perform the rapid amplification of the cDNA end (RACE).

Genome analyses

Obtained contigs that were more than 7000 nts in length, for which there was sufficient read coverage, were aligned using ClustalW [47]. The secondary structures of the viral RNA were predicted using the Mfold program [56]. Phylogenetic analysis was performed based on nt sequence analysis using the maximum likelihood (ML) method with the best fit model (GTR + *G* + *I* for VP1, 3CD and P1) in

MEGA7 [28]. The trees were evaluated using bootstrap analysis with 1000 replicates [19]. Pairwise sequence identities were calculated using the CLC Genomics Workbench 7.5.5 (CLC bio). Recombination analysis was performed using the SimPlot software v. 3.5.1 [30] and Recombination Detection Program (RDP) v. 4.80 [31].

Results

Identification of nearly complete genome of PSVs

Using the metagenomics approach, 23 contigs that were > 7000 bases in length with sufficient coverage were identified from 10- to 80-day-old pigs (Supplementary Fig. 1). These contigs were confirmed to be sapelovirus sequences by BLAST analysis. Five of 23 sequences were found in diarrheal fecal samples from 10- to 80-day-old pigs, which also contained contigs that showed sequence similarity with *Porcine epidemic diarrhea virus* (1 sample), *Picobirnavirus* (3 samples), *Aichivirus C* (3 samples), *Teschovirus* (2 samples), *Enterovirus G* (1 sample), and *Rotavirus A* (1 sample). Eighteen sequences were found in fecal samples obtained from 15- to 60-day-old pigs without diarrhea and their contigs exhibited sequence similarity to those of *Teschovirus* (1 sample), *Porcine astrovirus* (14 samples), *Rotavirus C* (4 samples), *Enterovirus G* (5 samples), *Picobirnavirus* (6 samples), *Sapovirus* (5 samples), *Porcine torovirus* (1 sample), and *Posavirus* (1 sample). Information regarding Japanese PSV strains obtained during the course of this study is summarized in Table 1.

Analysis of the secondary RNA structure within the 5′ UTR, 2C coding region, and 3′ UTR

The secondary RNA structures of the 5′ UTR, the *CRE* within the 2C coding region, and the 3′ UTR were predicted using the Mfold program. *CRE* is located in the coding region for 2C in the PSV genome [42], and *CRE*s contain an essential AAACA motif [35]. The stem-loop structures with the AAACA motif (corresponding to genome position 4419–4478 of PSV V13/1957/GBR) were conserved; however, there were minor differences among PSVs (Fig. 1). All hairpin loops contained 16 nts; however, 42 PSVs, including 16 Japanese PSVs, included a single AAACA motif, while 29 PSVs, including 7 Japanese PSVs, had 2 copies of the AAACA motif (Fig. 1). To obtain complete 3′ UTR sequences, the RACE method was employed and the complete sequences of eight strains (Ishi-Ya8/2015, HgTa2-1, HgOg11/2018, MoI2-2/2015, Ishi-Im3/2015, Ishi-Miya3/2015, HkKa2-2/2015, and DeTk2-2/2015) were determined. The 3′ UTR of PSV was 82–86 nts long, excluding the poly-A tail. The longest 3′ UTR was found in HkKa2-2/2015 and DeTk2-2/2015 (Fig. 2a). Secondary structure analysis revealed that PSVs possessed 3 (domain X, Y, and Z) or 4 (domain X, Y-1, Y-2, and Z) stem-loop elements that could be divided into 10 patterns. Japanese PSVs were divided into seven patterns. Ishi-Im3, Ishi-Miya3/2015, and DeTk2-2/2015 possessed the small stem-loop domain Y-2, in addition to the common domains X, Y, and Z. Although the secondary structures of PSVs were varied, tertiary interactions between the X and Z domains were conserved. Seven nt interactions occurred between the loops of X and Z domains in the 3′ UTR of Jpsv1315/2009/JPN (Accession No. LC326555), Jpsv447/2009/JPN (Accession No. LC326556), and V13/1957/GBR; in contrast, the 3′ UTR of the other PSVs exhibited an interaction of 8 nts occurring between the loops of two constituent hairpins (Fig. 2b).

Sequences of PSV V13/1957/GBR (Accession No. NC_003987) at the nt positions 169–443 that corresponded to IRES stem-loop domains II and III [43] within the 5′ UTR of Japanese PSVs and PSVs from the DDBJ/EMBL/GenBank database were aligned and compared. Sequences of these regions, and particularly those in the domains IIId, IIIe, and IIIf were highly conserved (Supplementary Fig. 2a). The secondary structure analysis of these regions using the Mfold program showed that the stem-loop structure of domains II, IIIa, IIIb, IIId, IIIe, and IIIf were identical across all PSVs and that the domain IIIc was not present in any of the PSVs (Supplementary Fig. 2b).

Phylogenetic tree analysis, pairwise sequence comparison, similarity plot analysis, and recombination analysis

The phylogenetic tree was constructed by ML method using nt sequences from the complete VP1 region of 23 Japanese PSVs and 52 PSV strains from the DDBJ/EMBL/GenBank database. Japanese PSVs branched into at least 6 lineages consisting of only Japanese strains or PSVs from other countries such as China and the USA. PSVs displayed genetic diversity and formed several clusters; however, some clusters were not supported by ≥ 70% bootstrap values (Fig. 3a). Insertions and/or deletions were found in the 3′ terminal of the VP1 coding region and had different patterns; however, the patterns of the insertions and/or deletions were not correlated with the topology of the VP1 tree (Fig. 3b). The phylogenetic tree constructed using 3CD nt sequences of Japanese PSVs and PSVs from the DDBJ/EMBL/GenBank database using the ML method revealed that the tree topology was not consistent with that of the VP1 tree. Interestingly, PSVs formed clusters, which corresponded with the countries where each of the PSVs was isolated. Japanese PSVs alone formed two lineages (Fig. 3c). Pairwise sequence calculations were performed using the CLC Genomics Workbench.

Table 1 Summary of Japanese porcine sapeloviruses in this study

Strain names	Age of host (days)	Health status of host	Collection date	Genome length (excluding poly-A)	DDBJ accession nos.	Co-infection with other viruses
MoI2-2/2015	60	Without diarrhea	2015.7.2	7425	LC425394	*Teschovirus, Porcine astrovirus, Rotavirus C*
HgOg2-5/2015	60	Without diarrhea	2015.7.15	7480	LC425395	*Enterovirus G*
DeTk2-2/2015	60	Without diarrhea	2015.7.1	7428	LC425396	–
Iba26-489S/2014	10	Diarrhea	2014.3.21	7531	LC425397	*Porcine epidemic diarrhea virus, Picobirnavirus*
Ishi-Ka2/2015	21	Diarrhea	2015.11.5	7543	LC425398	*Aichivirus C, Teschovirus, Picobirnavirus*
Ishi-Miya3/2015	33	Diarrhea	2015.11.16	7508	LC425399	*Rotavirus A* (G9P[23), *Picobirnavirus*
HgTa2-1/2015	60	Without diarrhea	2015.7.15	7540	LC425401	*Rotavirus C, Porcine astrovirus, Enterovirus G*
HkKa2-2/2015	60	Without diarrhea	2015.7.1	7555	LC425402	–
HkKa2-3/2015	60	Without diarrhea	2015.7.1	7540	LC425403	*Porcine astrovirus*
HgTa2-2/2015	60	Without diarrhea	2015.7.15	7525	LC425404	*Enterovirus G, Porcine astrovirus, Picobirnavirus, Sapovirus*
HgYa2-1/2015	60	Without diarrhea	2015.7.15	7525	LC425405	*Rotavirus C, Porcine astrovirus, Porcine torovirus*
HgYa2-2/2015	60	Without diarrhea	2015.7.15	7515	LC425406	–
HgYa2-3/2015	60	Without diarrhea	2015.7.15	7535	LC425407	*Porcine astrovirus, Picobirnavirus*
HgYa2-4/2015	60	Without diarrhea	2015.7.15	7475	LC425408	*Porcine astrovirus, Picobirnavirus*
Ishi-Im1/2015	54	Without diarrhea	2015.11.6	7547	LC425409	*Sapovirus, Porcine astrovirus, Picobirnavirus, Posavirus*
Ishi-Im3/2015	41	Without diarrhea	2015.11.6	7554	LC425410	*Porcine astrovirus, Picobirnavirus*
Ishi-Ka1/2015	21	Diarrhea	2015.11.5	7545	LC425411	*Teschovirus, Aichivirus C*
Ishi-Ya8/2015	80	Diarrhea	2015.10.29	7528	LC425412	*Enterovirus G, Aichivirus C*
HgYa1/2016	60	Without diarrhea	2016.12.6	7526	LC425413	*Enterovirus G, Porcine astrovirus, Rotavirus C, Sapovirus*
MoI2/2016	60	Without diarrhea	2016.12.6	7514	LC425414	*Porcine astrovirus*
MoI3/2016	60	Without diarrhea	2016.12.6	7540	LC425415	*Porcine astrovirus, Picobirnavirus*
Ishi-Ka2/2017	15	Without diarrhea	2017.11.1	7534	LC425416	*Sapovirus, Porcine astrovirus*
HgOg11/2018	38	Without diarrhea	2018.6.27	7521	LC425417	*Porcine astrovirus, Enterovirus G, Sapovirus*

It was revealed that all Japanese PSVs shared high sequence identities with regard to their 3CD nts and amino acid (aa) sequences (nt: 88.8–100%, aa: 97.2–100%), but shared low nt and aa identities of 86.5–92.3% and 95.8–99.7%, respectively, with PSVs from other countries (Supplementary Tables 1, 2). Chinese PSVs also shared a higher sequence homology with regard to their 3CD region (nt: 91.0–100%, aa: 98.3–100%) than that observed for PSVs from other countries (nt: 86.8–92.3%, aa: 95.7–99.7%). In contrast, with regard to the VP1 region, Chinese strains shared ≥ 70.8% (nt) and ≥ 75.1% (aa) identities with each other, whereas Chinese strains showed ≥ 71.8% and ≥ 76.1% nt and aa sequence homologies, respectively, with strains from other countries. The lowest nt sequence identities were 70.8% for the VP1 (HuN9/2016/CHN vs. HuN16/2016/CHN) and 86.5% for the 3CD (Ishi-Miya3/2015/JPN vs. IVRI/PSV/SPF/C-6/2015/IND) sequences (Supplementary Table 1).

Since different topologies were observed between the VP1 tree and 3CD tree, recombination analyses were performed. Using SimPlot analysis, several crossover sites were identified. Ishi-Ka2/2015 had high nt sequence similarity with HgYa2-4/2015 in the 5′ UTR, L and VP4 coding regions, P2 and P3 coding regions, and 3′ UTR, whereas VP2, VP3, and VP1 coding regions were highly similar to

those of Ishi-Ka2/2017, suggesting a recombination event (Fig. 4a, b). HgYa-1/2016 displayed significant sequence identity with HgTa2-2 in the 5′ UTR, P1 region, and 3′ half of ORF, whereas HgYa-1/2016 showed higher sequence similarity with MoI3/2016 than HgTa2-2/2015 in 2A coding region, suggesting a recombination event (Fig. 4a, c). Almost all crossover sites were mapped in the 5′ UTR to 2B region. Bootstrap scanning analyses revealed that possible recombination breakpoints were identified in the 5′ UTR, VP4, VP2, VP1, 2A, and 2B regions (Fig. 4b, c; Supplementary Fig. 3). Among these, two possible recombination breakpoints were closely located at the nt insertion and/or deletion sites at the 3′ terminal of the VP1 coding region (Fig. 4b, c).

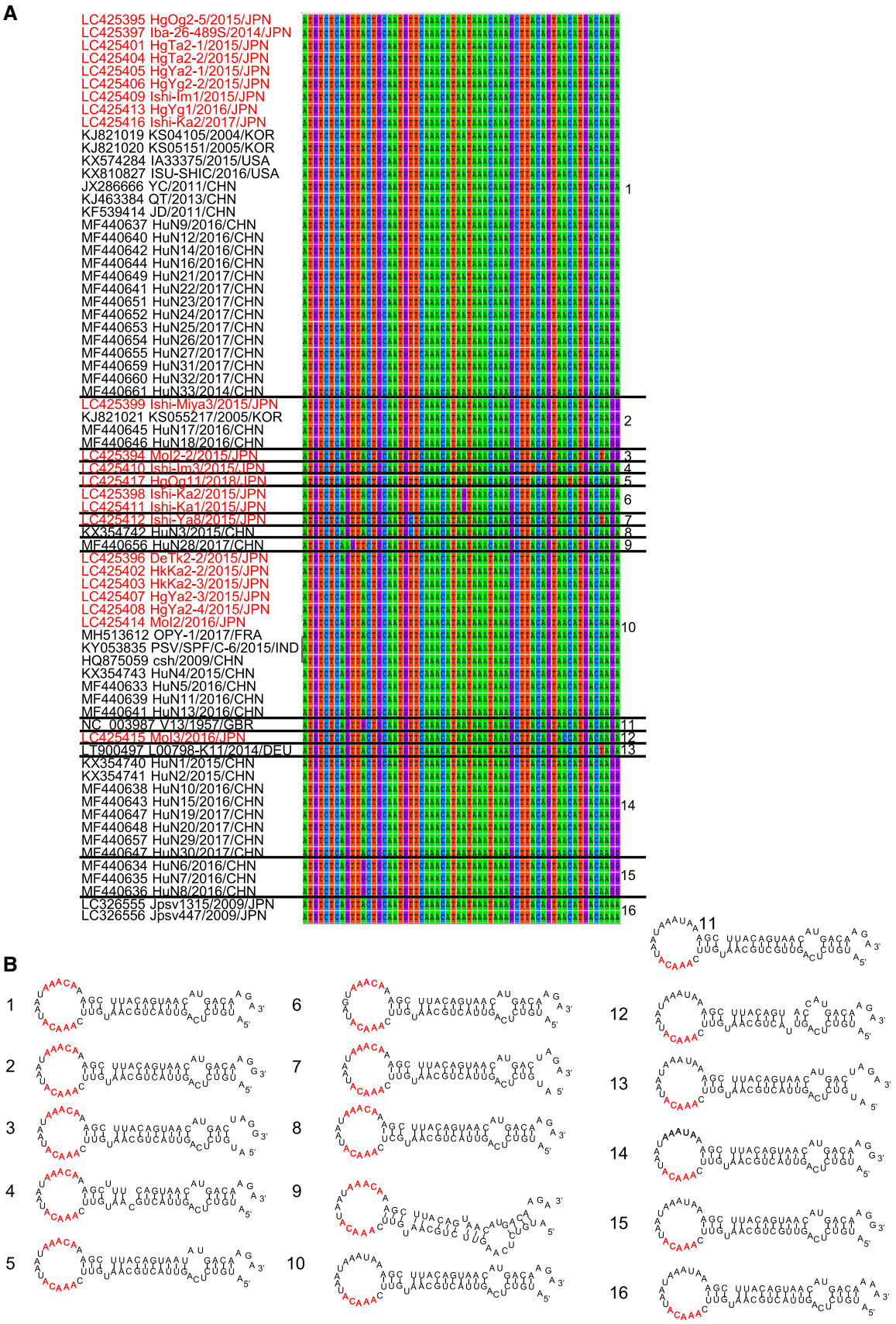
Discussion

Using the metagenomics approach, 23 nearly complete genomes of PSVs were identified from 230 fecal samples that were obtained from 10- to 80-day-old pigs in Japan. PSVs are believed to cause diarrhea [3, 21, 25, 29, 39]; on the other hand, PSVs are frequently found in apparently healthy pigs [9, 38]. Though this study did not aim to investigate PSV prevalence and the health status of detected pigs, we were able to identify > 7000 base contigs more frequently in healthy pigs (18/23; 78.3%) than in diarrheal pigs (5/23; 21.7%).

Picornaviruses possess several distinct *cis*-active RNA elements in their genomic RNA within the ORF or the 5′ or 3′ UTRs that are necessary for viral RNA replication [35]. However, there are few reports describing the *cis*-active RNA elements of PSV. Therefore, the *cis*-active RNA elements of PSVs were further analyzed in this study. Sequence comparison and secondary structural analysis of the 5′ UTR sequences corresponding to the IRES stem-loop domains II and III of 40 PSVs, including 23 Japanese PSVs detected in this study, showed that the sequences and structural features of the stem-loop domains II and III were highly conserved across all PSVs. Mfold analysis of the *CREs* located in the 2C regions of 23 Japanese PSVs and in 48 PSVs from other countries revealed that although the secondary structures of PSVs were slightly different, the stem-loop structures were well conserved. Within the predicted loop, an AAACA motif and two copies of the AAACA motif were found in the 2C region of 42 PSVs (including 16 Japanese PSVs) and 29 PSVs (including 7 Japanese PSVs), respectively. The *CRE* is an element that is important for positive-sense RNA synthesis in picornaviruses through VPg uridylylation, and it provides a template for the addition of uridine onto VPg [40, 43]. However, the effect of the difference in the copy number of the AAACA motif still needs to be elucidated. In contrast to the IRES and *CRE*, varied predicted secondary structures of 3′ UTRs were identified and could be classified into 10 patterns. A "kissing" interaction between domains X and Y of the 3′ UTR is thought to play an important role in viral negative-strand RNA synthesis [32]. Although an interaction of 7 or 8 nts between the loops of domains X and Z was observed, this tertiary structure was highly maintained among all PSVs. These data show that the genomic RNA of PSVs exhibits different nt sequences; however, the secondary or tertiary structures of *cis*-active RNA elements are conserved in their genomic RNA, within the 5′ UTR, 2C, and 3′ UTR.

Phylogenetic analysis was conducted using the VP1 coding region, and it revealed that Japanese PSVs exhibit genetic diversity and that they are either clustered with Japanese PSVs alone or with Chinese or US PSVs. Several clusters were found in the VP1 tree; however, many clusters were not supported by sufficient bootstrap values. We also constructed a phylogenetic tree using nt sequences of the complete P1 region (Supplementary Fig. 4). The topology and phylogenetic clustering of the P1 tree was slightly different from that of the VP1 tree and the P1 tree observed in the previous report [52], suggesting that PSVs could not be classified into genotypes at present. VP1 is one of the outer most viral protein, which induces host immunity, and VP1 sequence comparison has been adopted for the genotyping of several picornaviruses [34]. As for *Enterovirus G*, which belongs to the picornavirus supergroup 3 together with PSV, one of the criterions for genotype classification is > 25% nt sequence divergence in the complete VP1 region between isolates [49, 50]. The complete VP1 nt and aa sequences identities of PSVs were > 70.8% and > 75.1%, respectively, and these values were less diverse than those of porcine enteroviruses (> 53.9% and > 53.3%, respectively). This might be one of the reasons why the genotyping PSVs is known to be difficult. Further studies including reconstruction of phylogenetic tree after more sequence data accumulate for the PSV VP1 region and antigenicity tests by using antisera or sera isolated from seropositive pigs are needed to classify PSVs precisely.

The results of recombination analysis of PSVs revealed that the sites of possible RNA recombination events between Japanese PSVs and their possible recombination breakpoints were located upstream of the 2B region, which was consistent with the results of previously conducted studies [52, 53]. Some of the possible recombination breakpoints were located at the nt insertion and/or deletion sites in the C-terminal VP1 region. However, the insertions and/or deletion patterns did not correlate with the topology of the VP1 phylogenetic tree, suggesting that RNA recombination events do not attribute to the VP1 phylogeny. The topology of the 3CD phylogenetic tree was different from that of the VP1 tree,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LC425411 Ishi-Ka1/2015/JPN
LC425412 Ishi-Ya8/2015/JPN
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LC425414 Mol2/2016/JPN
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MF440633 HuN5/2016/CHN
MF440639 HuN11/2016/CHN
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MF440636 HuN8/2016/CHN
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◂**Fig. 1** Genome analysis of the putative *cis*-replication element (*CRE*) of Japanese PSVs using PSVs from DDBJ/EMBL/GenBank database. **a** Alignment of nt sequences of putative *CRE* in the 2C region of PSVs. **b** Secondary structure prediction of putative *CREs* within the 2C region of PSVs. The AAACA motifs are shown in red letters

i.e., PSV strains of each country were clustered together. This indicates that the VP1 and 3CD regions have evolved independently and that the 3CD region might be less affected by RNA recombination events, because the possible recombination breakpoints identified in this as well as previous studies were located upstream of the 2B region.

In summary, the results obtained in this study through phylogenetic analyses and pairwise sequence comparisons demonstrate that the PSVs are genetically diverse; however, PSVs could not be classified into genotypes at present. RNA recombination events were found to occur at sites upstream of the 2B region and some of them involve insertions and/or deletions in the C-terminal VP1 region. Genetic structural features are conserved among all PSVs, and this is important for the replication of the viral genome. These findings might provide evidence for carrying out the evaluation of the epidemiological basis of PSVs and uncover the mechanisms of evolution of PSVs.

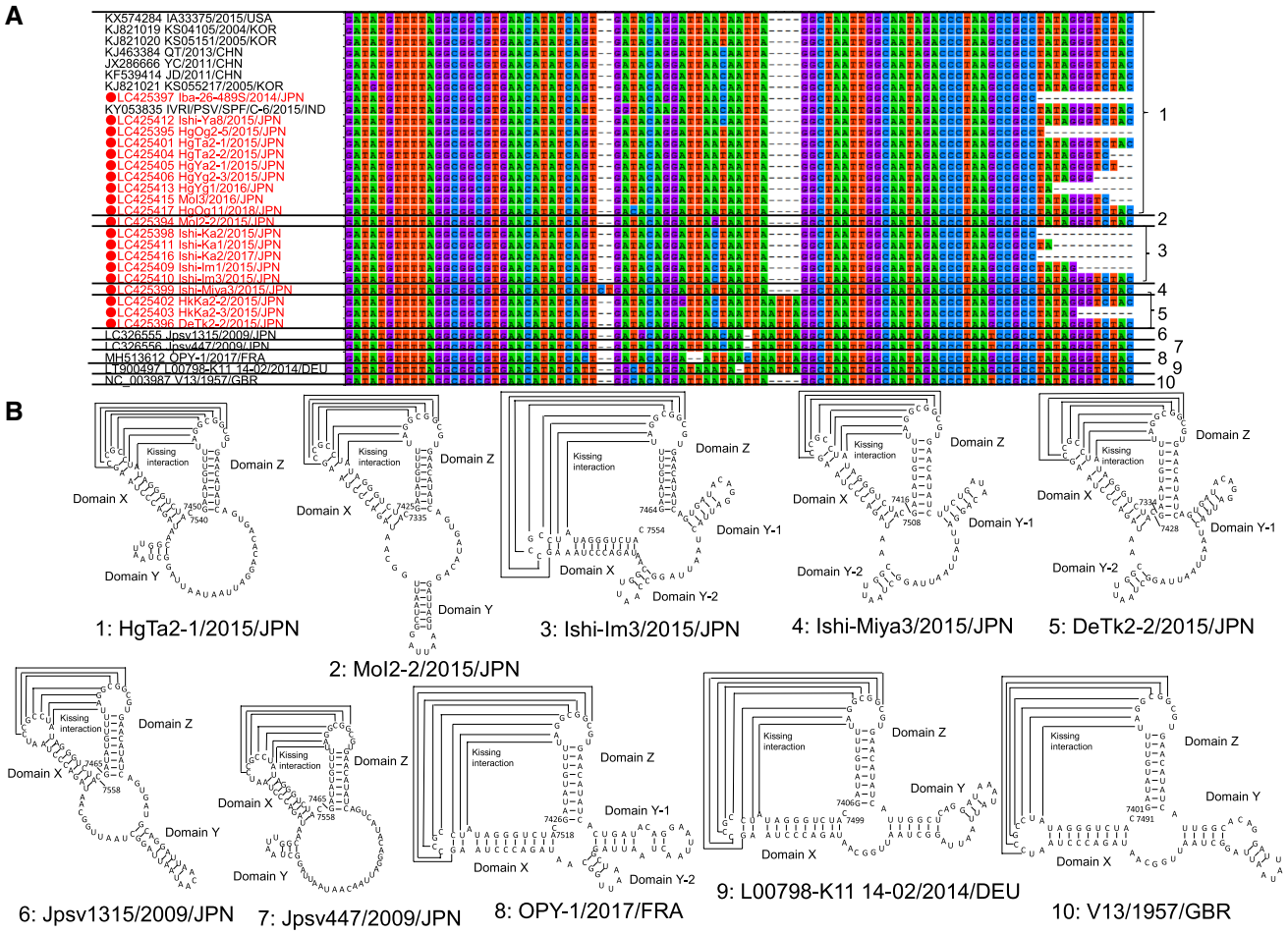

Fig. 2 Genome analysis of the 3′ UTR of Japanese PSVs using the PSVs from the DDBJ/EMBL/GenBank database. **a** Alignment of complete or almost complete nt sequences of PSVs. The extreme 3′ sequences of 12 strains (Iba-26-489S/2014, HgOg2-5/2015, HgTa2-2/2015, HgYa2-1/2015, HgYa2-3/2015, HgYa1/2016, MoI3/2016, Ishi-Ka2/2015, Ishi-Ka1/2015, Ishi-Ka2/2017Ishi-Im1/2015, and HkKa2-3/2015) are incomplete. **b** Secondary and tertiary structure prediction of the 3′ UTR. Predicted "kissing" interactions between the loops of X and Z domains are indicated by lines

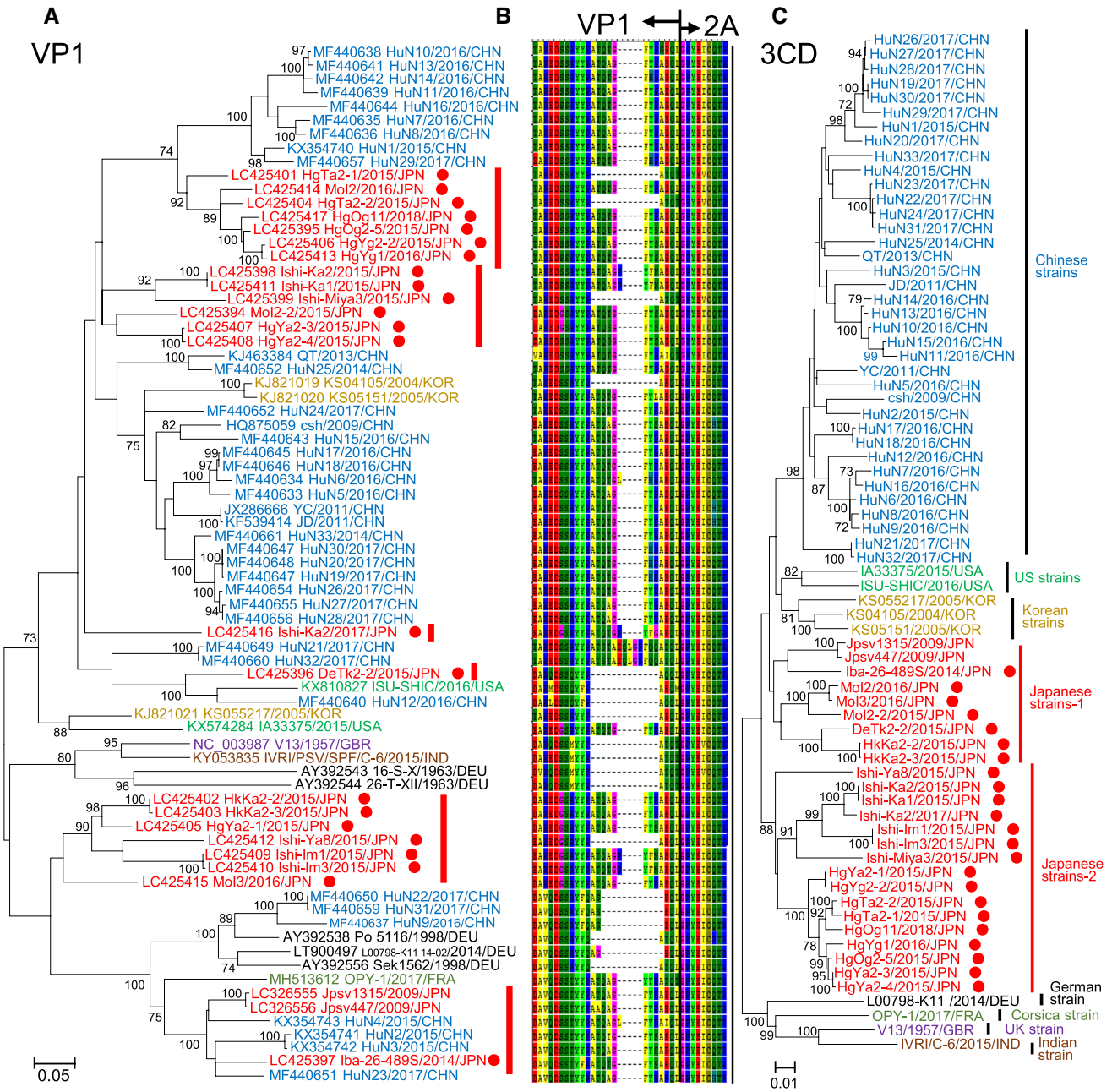

Fig. 3 **a** and **c** A phylogenetic tree is constructed based on complete nt sequences of VP1 (**a**) and the 3CD (**c**) region of 23 PSVs detected in this study, using PSVs from the DDBJ/EMBL/GenBank database. The phylogenetic tree was constructed using the maximum likelihood method in MEGA7, and bootstrap values (1000 replicates) above 70 are shown. The bar represents a corrected genetic distance. ● Denotes PSVs detected in the present study. PSVs detected in Japan, China, South Korea, the USA, German, France, the UK, and India are shown in red, blue, yellow, green, black, light green, purple, and brown, respectively. **b** Alignment of aa sequences of insertions and/or deletions in the C-terminal VP1 region of PSVs

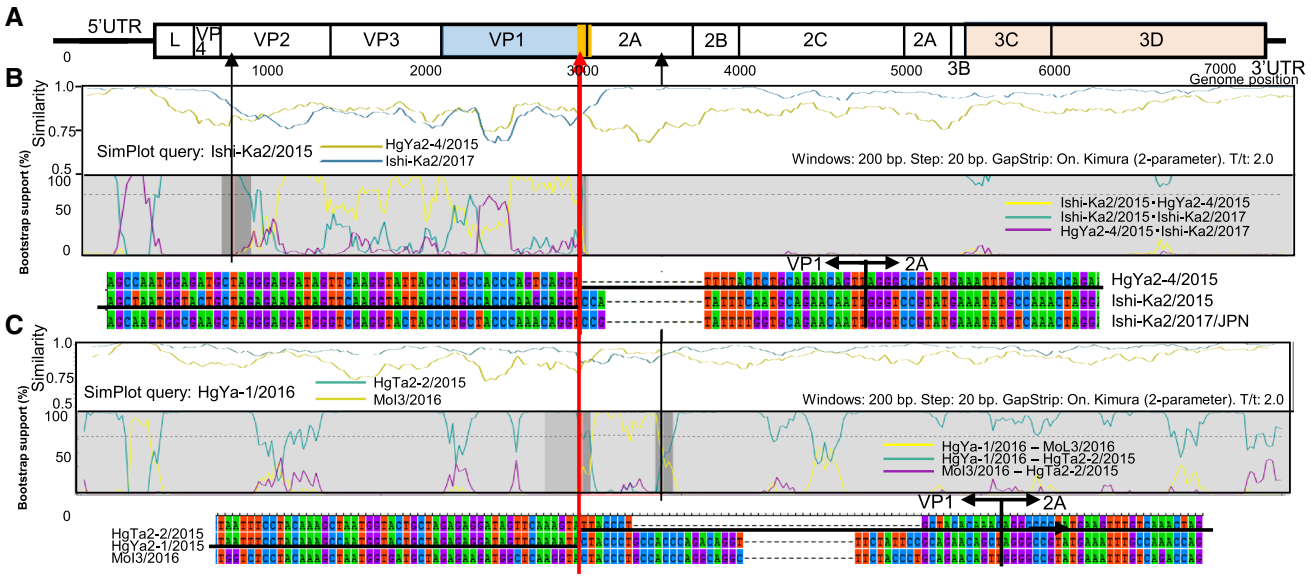

Fig. 4 **a** Genome structure of PSV. **b** and **c** Similarity plots of the entire genomes of HgYa2-4/2015 (yellow curve), Ishi-Ka2/2017 (blue curve), and Ishi-Ka2/2015 as query sequences (**b**), HgTa2-2/2015 (blue curve), MoI3/2016 (yellow curve), and HgYa-1 /2016 as query sequences (**c**), constructed using a sliding window of 200 nt and a moving step size of 20 nt (upper). Recombination breakpoint analysis of Ishi-Ka2/2015 versus HgYa2-4/2015 (yellow curve), Ishi-Ka2/2015 versus Ishi-Ka2/2017 (blue curve), and HgYa2-4/2015 versus Ishi-Ka2/2017 (purple curve) (**b**), HgYa-1/2016 versus MoI3/2016 (yellow curve), HgYa-1/2016 versus HgTa2-2/2015 (blue curve), and MoI3/2016 versus HgTa2-2/2015 (purple curve) (**c**) (lower)

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Author contributions FS, TM, MI, MA, YN, KS, YK, TO, MO, SS, TF, HY, YO, JS, TM, MN TM, and MN conceived of the study. FS, TF, JS, TM, and MN designed the study. TM, MI, MA, HY, and YO collected samples from pigs and carried out RT-PCR. YN, KS, YK, TO, MO, and SS analyzed data using bioinformatics. FS, KS, SS, TF, JS, TM, and MN wrote the paper. All authors approved the submitted manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals This study did not involve any human participants and animals.

Informed consent Not applicable.

References

1. Abe M, Ito N, Sakai K, Kaku Y, Oba M, Nishimura M, Kurane I, Saijo M, Morikawa S, Sugiyama M, Mizutani T (2011) A novel sapelovirus-like virus isolation from wild boar. Virus Genes 43:243–248
2. Agol VI, Paul AV, Wimmer E (1999) Paradoxes of the replication of picornaviral genomes. Virus Res 62:129–147
3. Alexanderson S, Knowles NJ, Dekker A, Belsham GJ, Zhang Z, Koenen F (2012) In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW (eds), Disease of swine, 10th edn. Blackwell Publishing, Inc., Oxford, pp 587–620
4. Arruda PH, Arruda BL, Schwartz KJ, Vannucci F, Resende T, Rovira A, Sundberg P, Nietfeld J, Hause BM (2017) Detection of a novel sapelovirus in central nervous tissue of pigs with polioencephalomyelitis in the USA. Transbound Emerg Dis 64:311–315
5. Auerbach J, Prager D, Neuhaus S, Loss U, Witte KH (1994) Grouping of porcine enteroviruses by indirect immunofluorescence and description of two new serotypes. Zent Vet B 41:277–282
6. Bai H, Liu J, Fang L, Kataoka M, Takeda N, Wakita T, Li TC (2018) Characterization of porcine sapelovirus isolated from Japanese swine with PLC/PRF/5 cells. Transbound Emerg Dis 65:727–734
7. Belsham GJ (2009) Divergent picornavirus IRES elements. Virus Res 139:183–192
8. Brown DM, Cornell CT, Tran GP, Nguyen JH, Semler BL (2005) An authentic 3′-noncoding region is necessary for efficient poliovirus replication. J Virol 79:11962–11973
9. Buitrago D, Cano-Gómez C, Agüero M, Fernandez-Pacheco P, Gómez-Tejedor C, Jiménez-Clavero MA (2010) A survey of porcine picornaviruses and adenoviruses in fecal samples in Spain. J Vet Diagn Investig 22:763–766
10. Cano-Gómez C, García-Casado MA, Soriguer R, Palero F, Jiménez-Clavero MA (2013) Teschoviruses and sapeloviruses in faecal samples from wild boar in Spain. Vet Microbiol 165:115–122
11. Cordey S, Gerlach D, Junier T, Zdobnov EM, Kaiser L, Tapparel C (2008) The *cis*-acting replication elements define human enterovirus and rhinovirus species. RNA 14:1568–1578
12. Chard LS, Kaku Y, Jones B, Nayak A, Belsham GJ (2006) Functional analyses of RNA structures shared between the internal ribosome entry sites of hepatitis C virus and the picornavirus porcine teschovirus 1 Talfan. J Virol 80:1271–1279

13. Chen J, Chen F, Zhou Q, Li W, Song Y, Pan Y, Zhang X, Xue C, Bi Y, Cao Y (2012) Complete genome sequence of a novel porcine Sapelovirus strain YC2011 isolated from piglets with diarrhea. J Virol 86:10898
14. Chen Q, Zheng Y, Guo B, Zhang J, Yoon KJ, Harmon KM, Main RG, Li G (2016) Complete genome sequence of porcine sapelovirus strain USA/IA33375/2015 identified in the United States. Genome Announc 4:e01055–e01016
15. Chen Q, Wang L, Zheng Y, Zhang J, Guo B, Yoon KJ, Gauger PC, Harmon KM, Main RG, Li G (2018) Metagenomic analysis of the RNA fraction of the fecal virome indicates high diversity in pigs infected by porcine endemic diarrhea virus in the United States. Virol J 15:95
16. Donin DG, de Arruda Leme R, Alfieri AF, Alberton GC, Alfieri AA (2014) First report of Porcine teschovirus (PTV), Porcine sapelovirus (PSV) and Enterovirus G (EV-G) in pig herds of Brazil. Trop Anim Health Prod 46:523–528
17. Dunne HW, Gobble JL, Hokanson JF, Kradel DC, Bubash GR (1965) Porcine reproductive failure associated with a newly identified "SMEDI" group of picorna viruses. Am J Vet Res 26:1284–1297
18. Dunne HW, Wang JT, Ammerman EH (1971) Classification of North American porcine enteroviruses: a comparison with European and Japanese strains. Infect Immun 4:619–631
19. Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
20. Fernández-Miragall O, López de Quinto S, Martínez-Salas E (2009) Relevance of RNA structure for the activity of picornavirus IRES elements. Virus Res 139:172–182
21. Honda E, Hattori I, Oohara Y, Taniguchi T, Ariyama K, Kimata A, Nagamine N, Kumagai T (1990) Sero- and CPE-types of porcine enteroviruses isolated from healthy and diarrheal pigs: possible association of CPE type II with diarrhea. Nihon Juigaku Zasshi 52:85–90
22. Huang J, Gentry RF, Zarkower A (1980) Experimental infection of pregnant sows with porcine enteroviruses. Am J Vet Res 41:469–473
23. Jacobson SJ, Konings DA, Sarnow P (1993) Biochemical and genetic evidence for a pseudoknot structure at the 3′-terminus of the poliovirus RNA genome and its role in viral RNA amplification. J Virol 67:2961–2971
24. Kaku Y, Sarai A, Murakami Y (2001) Genetic reclassification of porcine enteroviruses. J Gen Virol 82:417–424
25. Kim DS, Kang MI, Son KY, Bak GY, Park JG, Hosmillo M, Seo JY, Kim JY, Alfajaro MM, Soliman M, Baek YB, Cho EH, Lee JH, Kwon J, Choi JS, Goodfellow I, Cho KO (2016) Pathogenesis of Korean Sapelovirus A in piglets and chicks. J Gen Virol 97:2566–2574
26. Knowles NJ, Buckley LS, Pereira HG (1979) Classification of porcine enteroviruses by antigenic analysis and cytopathic effects in tissue culture: description of 3 new serotypes. Arch Virol 62:201–208
27. Krumbholz A, Dauber M, Henke A, Birch-Hirschfeld E, Knowles NJ, Stelzner A, Zell R (2002) Sequencing of porcine enterovirus groups II and III reveals unique features of both virus groups. J Virol 76:5813–5821
28. Kumar S, Stecher G, Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874
29. Lan D, Ji W, Yang S, Cui L, Yang Z, Yuan C, Hua X (2011) Isolation and characterization of the first Chinese porcine sapelovirus strain. Arch Virol 156:1567–1574
30. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC (1999) Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73:152–160
31. Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P (2010) RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics 26:2462–2463
32. Melchers WJ, Hoenderop JG, Bruins Slot HJ, Pleij CW, Pilipenko EV, Agol VI, Galama JM (1997) Kissing of the two predominant hairpin loops in the coxsackie B virus 3′-untranslated region is the essential structural feature of the origin of replication required for negative-strand RNA synthesis. J Virol 71:686–696
33. Nagai M, Omatsu T, Aoki H, Otomaru K, Uto T, Koizumi M, Minami-Fukuda F, Takai H, Murakami T, Masuda T, Yamasato H, Shiokawa M, Tsuchiaka S, Naoi Y, Sano K, Okazaki S, Katayama Y, Oba M, Furuya T, Shirai J, Mizutani T (2015) Full genome analysis of bovine astrovirus from fecal samples of cattle in Japan: identification of possible interspecies transmission of bovine astrovirus. Arch Virol 160:2491–2501
34. Oberste MS, Maher K, Kilpatrick DR, Pallansch MA (1999) Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. J Virol 73:1941–1948
35. Paul AV, Yin J, Mugavero J, Rieder E, Liu Y, Wimmer E (2003) A "slide-back" mechanism for the initiation of protein-primed RNA synthesis by the RNA polymerase of poliovirus. J Biol Chem 278:43951–43960
36. Pilipenko EV, Poperechny KV, Maslova SV, Melchers WJ, Slot HJ, Agol VI (1996) *Cis*-element, oriR, involved in the initiation of (−) strand poliovirus RNA: a quasi-globular multi-domain RNA structure maintained by tertiary ("kissing") interactions. EMBO J 15:5428–5436
37. Pisarev AV, Chard LS, Kaku Y, Johns HL, Shatsky IN, Belsham GJ (2003) Functional and structural similarities between the internal ribosome entry sites of hepatitis C virus and porcine teschovirus, a picornavirus. J Virol 78:4487–4497
38. Prodělalová J (2012) The survey of porcine teschoviruses, sapeloviruses and enteroviruses B infecting domestic pigs and wild boars in the Czech Republic between 2005 and 2011. Infect Genet Evol 12:1447–1451
39. Ray PK, Desingu PA, Kumari S, John JK, Sethi M, Sharma GK, Pattnaik B, Singh RK, Saikumar G (2018) Porcine sapelovirus among diarrhoeic piglets in India. Transbound Emerg Dis 65:261–263
40. Rieder E, Paul AV, Kim DW, van Boom JH, Wimmer E (2000) Genetic and biochemical studies of poliovirus *cis*-acting replication element cre in relation to VPg uridylylation. J Virol 74:10371–10380
41. Schock A, Gurrala R, Fuller H, Foyle L, Dauber M, Martelli F, Scholes S, Roberts L, Steinbach F, Dastjerdi A (2014) Investigation into an outbreak of encephalomyelitis caused by a neuroinvasive porcine sapelovirus in the United Kingdom. Vet Microbiol 172:381–389
42. Son KY, Kim DS, Kwon J, Choi JS, Kang MI, Belsham GJ, Cho KO (2014) Full-length genomic analysis of Korean porcine Sapelovirus strains. PLoS ONE 9:e107860
43. Son KY, Kim DS, Matthijnssens J, Kwon HJ, Park JG, Hosmillo M, Alfajaro MM, Ryu EH, Kim JY, Kang MI, Cho KO (2014) Molecular epidemiology of Korean porcine sapeloviruses. Arch Virol 159:1175–1180
44. Sozzi E, Barbieri I, Lavazza A, Lelli D, Moreno A, Canelli E, Bugnetti M, Cordioli P (2010) Molecular characterization and phylogenetic analysis of VP1 of porcine enteric picornaviruses isolates in Italy. Transbound Emerg Dis 57:434–442
45. Sweeney TR, Dhote V, Yu Y, Hellen CU (2011) A distinct class of internal ribosomal entry site in members of the Kobuvirus and proposed Salivirus and Paraturdivirus genera of the Picornaviridae. J Virol 86:1468–1486

46. Tapparel C, Siegrist F, Petty TJ, Kaiser L (2013) Picornavirus and enterovirus diversity with associated human diseases. Infect Genet Evol 14:282–293
47. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882
48. Tsuchiaka S, Naoi Y, Imai R, Masuda T, Ito M, Akagami M, Ouchi Y, Ishii K, Sakaguchi S, Omatsu T, Katayama Y, Oba M, Shirai J, Satani Y, Takashima Y, Taniguchi Y, Takasu M, Madarame H, Sunaga F, Aoki H, Makino S, Mizutani T, Nagai M (2018) Genetic diversity and recombination of enterovirus G strains in Japanese pigs: high prevalence of strains carrying a papain-like cysteine protease sequence in the enterovirus G population. PLoS ONE 11(1):e0190819 13(
49. Van Dung N, Anh PH, Van Cuong N, Hoa NT, Carrique-Mas J, Hien VB, Campbell J, Baker S, Farrar J, Woolhouse ME, Bryant JE, Simmonds P (2014) Prevalence, genetic diversity and recombination of species G enteroviruses infecting pigs in Vietnam. J Gen Virol 95:549–556
50. Van Dung N, Anh PH, Van Cuong N, Hoa NT, Carrique-Mas J, Hien VB, Sharp C, Rabaa M, Berto A, Campbell J, Baker S, Farrar J, Woolhouse ME, Bryant JE, Simmonds P (2016) Large-scale screening and characterization of enteroviruses and kobuviruses infecting pigs in Vietnam. J Gen Virol 97:378–388
51. Yang T, Li R, Peng W, Ge M, Luo B, Qu T, Yu X (2017) First isolation and genetic characteristics of porcine sapeloviruses in Hunan, China. Arch Virol 162:1589–1597
52. Yang T, Yu X, Yan M, Luo B, Li R, Qu T, Luo Z, Ge M, Zhao D (2017) Molecular characterization of Porcine sapelovirus in Hunan, China. J Gen Virol 98:2738–2747
53. Yu Y, Sweeney TR, Kafasla P, Jackson RJ, Pestova TV, Hellen CU (2011) The mechanism of translation initiation on Aichivirus RNA mediated by a novel type of picornavirus IRES. EMBO J 30:4423–4436
54. Zell R, Dauber M, Krumbholz A, Henke A, Birch-Hirschfeld E, Stelzner A, Prager D, Wurm R (2001) Porcine teschoviruses comprise at least eleven distinct serotypes: molecular and evolutionary aspects. J Virol 75:1620–1631
55. Zell R, Delwart E, Gorbalenya AE, Hovi T, King AMQ, Knowles NJ, Lindberg AM, Pallansch MA, Palmenberg AC, Reuter G, Simmonds P, Skern T, Stanway G, Yamashita T, ICTV Report Consortium (2017) ICTV virus taxonomy profile: Picornaviridae. J Gen Virol 98:2421–2422
56. Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406–3415