Molecular Characterization of a Porcine Sapelovirus Strain Isolated in China

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Abstract

Background

Porcine sapelovirus (PSV) infections have been associated with a wide spectrum of symptoms ranging from asymptomatic infection to clinical signs including diarrhoea, pneumonia, reproductive disorders, and polioencephalomyelitis. Although it has a global distribution, studies on PSV in domestic have been relatively few so far which goes against for this pathogen's research and disease prevention.

Methods

In this study, we isolated a PSV strain, SHCM2019, from the swine faecal specimens with PK-15 cells. To investigate its molecular characteristics and pathogenicity, the genomic sequence of the SHCM2019 strain was analysed, and further the clinical manifestations and pathological changes exhibited following inoculation of the neonatal piglets were observed.

Results

The strain isolated with PK-15 cells, was identified as PSV by RT-PCR, IFA and TEM assays. Sequencing results showed that the full-length genome of the SHCM2019 strain was 7567 nucleotides including a 27-nucloetude poly (A) tail. Phylogenetic analysis demonstrated that the virus isolate belonged to PSV and was classified into the Chinese strain cluster. Virus recombination analysis indicated that there might be a recombination break point upstream of the 3D region in PSVs. Pathogenicity research demonstrated that the virus isolate could cause diarrhoea and pneumonia in piglets.

Conclusion

This study presented the isolation of a recombinant PSV strain, SHCM2019, and demonstrated the isolate was pathogenic. Our results may provide a reference for future researches of the pathogenic mechanism and evolutionary characteristics of PSV.

Introduction

The genus *Sapelovirus*, belonging to the family *Picornaviridae*, consists of three species: *Avian Sapelovirus*, *Sapelovirus A* (porcine sapelovirus), and *Sapelovirus B* (simian sapelovirus) [1]. Porcine sapelovirus (PSV) is a non-enveloped, positive-sense, single-stranded RNA virus, which was previously known as porcine enterovirus 8 (PEV-8) in the genus *Enterovirus* [2]. The genome of PSV measures approximately 7500 base pairs (bp) in length, consisting of 5' untranslated region (UTR), intermediate coding region, 3' untranslated region (UTR) and poly (A) tail. The intermediate coding region contains only a large open reading frame (ORF) that encodes a polyprotein precursor about 2,331 amino acids in length, which is subsequently cleaved by the self-coding protease into 12 mature functional proteins. The structure of the polyprotein is consistent with the L-4-3-4 structure of other picornaviruses, in which a

leading peptide L leads to four structural polypeptides (VP4, VP2, VP3, and VP1) and seven nonstructural polypeptides (2A, 2B, 2C, 3A, 3B, 3C, and 3D) [3–5].

Since PSV was first reported in the UK in 1958 [6], it has been identified in many countries, including Canada, Japan, Australia, Brazil, Spain and Korea, and China and other countries worldwide [5, 7–12]. PSV infection rates in pigs have been investigated globally, ranging from 7.1% in India to 71.0% in Hungary [13, 14]. PSV infections have been associated with a wide spectrum of symptoms ranging from asymptomatic infection to acute fatal encephalomyelitis, reproductive disorders, diarrhoea and pneumonia [12, 15–17]. Prodelalova et al reported that PSV infection rates were higher in asymptomatic pigs than in diarrhoeal pigs [15]. However, Zhang et al demonstrated there was a higher prevalence of PSV in diarrhoeal pigs by using metagenomic analysis of pig faeces [18]. Further, Kim et al reported that PSV could cause intestinal lesions and colonize the villous epithelial cells in the small intestine [19]. Like porcine epidemic diarrhea virus (PEDV), PSV can coinfect with porcine parvovirus, swine fever virus, porcine reproductive and respiratory disorder syndrome virus, porcine enterovirus and other viruses, leading to clinical atypical symptoms in sick pigs [20–22]. the symptoms of PSV are often covered up and overlooked due to the mixed infections [15], which poses a considerable threat to the pig industry.

In this study, a PSV strain was isolated using PK-15 cells. Based on the phylogenetic analysis, it is closely related to other Chinese isolates. The recombination analysis indicated a possible recombination break point in the 3D region. Animal regression experiments demonstrated that the isolated strain was pathogenic to piglets. The study laid a foundation on the pathogenic mechanism of PSV.

Materials And Methods

Cells and clinical samples

PK-15 cells were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, HyClone, USA) containing 10% heat-inactivated foetal bovine serum (FBS; Invitrogen, Australia) and 1% antibiotics (10,000 units/mL penicillin and 10,000 µg/mL streptomycin). The faecal specimens were collected from the diarrheic piglets from a pig farm in Shanghai, China, in 2019. Subsequently, these faecal samples were used for multiplex PCR detection of several potential pathogens including PSV, PEDV, porcine kobuvirus (PKV), porcine astrovirus (PAstV), porcine deltacoronavirus (PDCoV), porcine torovirus (PToV), porcine teschovirus (PTV), porcine sapovirus (PSaV), transmissible gastroenteritis virus (TGEV), porcine rotavirus (PoRV) and bovine viral diarrhoea virus (BVDV).

Virus isolation

PK-15 cells were inoculated with faecal specimens that were RNA-positive for PSV but RNA-negative for PEDV, TGEV, PKV, PAstV, PDCoV, PToV, PTV, PSaV, PoRV, and BVDV. In brief, the positive faecal samples were suspended in sterile phosphate-buffered saline (PBS) containing 1,000 units/mL penicillin and 1,000 µg/mL streptomycin and centrifuged for 10 min at 3,500×g. The supernatants were filtered through 0.22-µm pore membrane filters (Merck Millipore Ltd., USA), followed by inoculation in PK-15 cells for 1 h

at 37°C. Next, the inoculum was discarded, and the cells were washed three times with sterile PBS. Subsequently, DMEM supplemented with 0.5 μg/mL TPCK-trypsin (Gibco™, USA) was added, and cell culture was continued at 37°C in 5% CO_2, with daily observation for cytopathic effect (CPE). When more than 80% of cells appeared CPE, the supernatant and cells were harvested for continuous subculture.

RT-PCR

Total RNA of the first six generations was extracted from the culture supernatant of PSV inoculation using TRIzol Reagent (Takara, China) according to the manufacturer's instructions. The cDNAs were synthesized in a final volume of 20 μL containing 10 μL RNA, 4 μL 5×RT buffer, 1 μL dNTPs (10 mM), 2 μL random primer (25 μM), 0.5 μL Moloney murine leukaemia virus (M-MLV) reverse transcriptase (TaKaRa, China), 0.5 μL RNase inhibitor (TaKaRa, China) and 2 μL diethylpyrocarbonate (DEPC)-treated water. The reaction was incubated at 42°C for 1h followed by incubation at 70°C for 15 min. cDNA was used for PCR with the following primers, PSV-F:5'-GATGTGGCGCATGCTCTT-3', PSV-R:5'-TGCTGCCTCCTGTGTTGTTAT-3'. Amplified products were separated by electrophoresis in a 1.5% agarose gel and purified using a DNA gel extraction kit (Tiangen, China). The purified PCR products were subsequently cloned into the PEASY-Blunt Zero vector (TransGen, China) and sequenced (Biosune, China). Sequences were analysed using the BLAST search program (http://blast.ncbi.nlm.nih.gov/).

Transmission electron microscopy (TEM)

The PK-15 cells infected with strain SHCM2019 were frozen and thawed thrice when there were over 80% CPE, followed by centrifugation at 2,000×g for 30 min to remove cell debris. Crude virus was pelleted from the clarified supernatant by ultracentrifugation at 9,8900×g at 4°C for 3 h in a TYPE-70 Ti rotor (Beckman, USA). The resulting pellet was resuspended in a small amount of PBS and was subsequently layered onto a 20–60% (w/v) discontinuous iodixanol solution (OptiPrep™ Density GradientMedium, Sigma-Aldrich, USA) by centrifugation at 124,400×g at 4°C for 3 h in a SW-55 Ti rotor (Beckman, USA). The virus band at the interface was collected and placed onto a formvar grid (Electron Microscopy Sciences, USA) for 5 min, and excess liquid was subsequently removed by filter paper. The samples were observed using a transmission electron microscope (Hitachi, Japan) for the morphological identification of the virus.

Immunofluorescence assay (IFA)

To characterize the isolated strain, IFA was performed with a polyclonal antibody specific for the PSV-VP1 protein (prepared in our laboratory). Briefly, PK-15 cells in 6-well plates were mock infected or infected with the SHCM2019 strain, incubated for 24 h, fixed in 4% paraformaldehyde for 30 min at 4°C, and subsequently permeabilized with 0.3% Triton X-100 (Biodee, China) for 10 min at room temperature (RT). After the cells were washed three times with PBS, they were blocked with 5% bovine serum albumin (BSA; Sigma, USA) at RT for 1 h. Mouse polyclonal antibody against PSV and Alexa Fluor™ 488-conjugated rabbit anti-mouse IgG (Invitrogen, USA) were used as primary and secondary antibodies, respectively. The cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Beyotime, China) for 5 min at RT. After

the cells were washed with PBS, the stained cells were observed with a fluorescence microscope (Carl Zeiss, Germany).

Sequence and phylogenetic analysis

Third-generation viral RNA was extracted from PK-15 cells infected with the virus isolate using TRIzol Reagent. Reverse transcription and PCR were performed with M-MLV reverse transcriptase and TransStart FastPfu DNA polymerase (TransGen, China), respectively. The PCR products were cloned using the pEASY-Blunt Cloning Kit, and the resulting plasmids were sequenced. The 5'-UTR and 3'-UTR sequences of the isolated strain were obtained using a 5'-3' RACE kit (Takara, China) according to the manufacturer's instructions. The sequences fragments were assembled with DNAStar v. 7.1. Subsequently, the complete sequence was submitted to GenBank (GenBank ID: MN685785). All of the complete sequences of the polyprotein gene and reference sequences obtained from GenBank were utilized in sequence alignments and phylogenetic analyses. Phylogenetic trees were constructed using the neighbour-joining method with 1000 bootstrap replications in MEGA v. 7.0. Recombination analysis was performed using SimPlot v. 3.5.1 and Recombination Detection Program (RDP) v. 4.10.

Experimental infection of piglets

To assess the pathogenicity of the isolated SHCM2019 strain in piglets, four 5-day-old piglets from the same sow were used. Before inoculation, we observed for 3 days and collected faecal samples from all piglets to ensure that none of the piglets were infected with PSV. The four piglets were random divided into two groups (two piglets per-group). Each piglet in group 1 (L1 and L2) was given 5 mL PSV-SHCM ($10^{7.41}$ $TCID_{50}$/mL) by the oral route, and piglets in group 2 (C1 and C2) received 5 mL DMEM orally as a negative control. After inoculation, all piglets were monitored each day for clinical signs. Faecal samples of each piglet were collected for viral load detection by qRT-PCR at 0, 1, 2, 3, 4, 5 and 6 days post-inoculation (dpi), and serum samples were collected for seroconversion tests at 0, 1, 3 and 6 dpi. All piglets were artificial executed at 6 dpi, and one portion of the tissue samples were fixed with 4% paraformaldehyde solution for histological slides preparation and the other portion were collected for viral load detection by qRT-PCR.

Ethics statement

All animal experiments were performed under the guidance of the Institutional Animal Care and Use Committee at the Center for Disease Control and Prevention (CDC) and the Laboratory Animal Care International accredited facility.

Results

Virus isolation and identification

A PSV strain was isolated from diarrheal faecal samples originating from a farm in Shanghai, China. PK-15 cells infected with the isolate displayed a distinct CPE after three times of passages, which was

characterized by rounding and shrinking of the cells after 30 h and all cells fell off at 70 h (Fig. 1A). RT-PCR assays with a pair of primers specific to PSV amplifyed a 624 bp target fragment (Fig. 1B). Further IFA confirmed that the polyclonal antibody against PSV could be reacted with the SHCM2019 strain with the specific green fluorescences in cytoplasm (Fig. 1C). To determine its morphological characteristics, PSV particles of the SHCM2019 strain were purified and stained negatively, following imaged by transmission electronmicroscopy (TEM). It was revealed that the viral particles were nonenveloped, icosahedral, approximately 25–30 nm in diameter (Fig. 1D).

Full genome organization and phylogenetic analyses

To further explore the characteristics of the isolated virus, the complete genomic sequence of strain SHCM2019 was tested. The full-length genome of this virus strain was 7,567 nucleotides (nt) with only a single 6996-nt ORF flanked by a 465 nt 5'UTR and a106 nt 3' UTR. Although the genome of strain SHCM2019 in lengh is different from other PSV strains, its polyprotein gene is similar to most Chinese PSVs. Actually, the lengh of the polyprotein gene is varied in PSVs, however, it is relatively conserved in Chinese PSVs, exhibiting a consistent length of 6996 nt, except for the HuN4, HuN6, HuN21, and HuN32 strain with some extra nucleotides inserted in it.

The phylogenetic tree was constructed by the neighbour-joining method based on the complete polyprotein gene of the SHCM2019 strain and other representative picornaviruses from the NCBI database. The results indicated that SHCM2019 was most similar with the Chinese PSV strains, which were clustered in the same group (Fig. 2). Large genetic diversity was existed among the PSVs isolated from different countries. The Chinese PSV strains were closely related to the Korean PSV strains. In addition, the SHCM2019 strain showed high nucleotide (85.8–91.2%) and amino acid (71.5–79.8%) identities with the other PSV strains, but relatively low identities with the avian and simian sapelovirus strains (Supplemental Fig. 1).

Further the virus recombination analysis was carried out using SimPlot v. 3.5.1 and RDP v. 4.10 softwares. The SHCM2019 was used as separate queries to implement a standard similarity plot analysis. Next, we performed a bootscanning analysis to verify potential breakpoints in the recombinants. The SHCM2019 strain showed a high degree of nucleotide sequence similarity with HeB04 in the VP1,VP2 and VP3 regions, whereas the 3D regions were highly similar to those of ISU-SHIC, which suggested that there might also be a restructuring break point in 3D (Fig. 3A, B).

Experimental infection, necropsy, and histopathological examination

To test the virulence of the SHCM2019 strain, two 8-day-old healthy piglets were inoculated with SHCM2019. Inappetence and lethargy appeared in PSV-infected piglets at 2 dpi, and watery diarrhoea occurred at 5 dpi compared to the mock piglets (Fig. 4D). Necropsy results showed that lesions were mainly concentrated in the intestines and lungs. The intestines of PSV-infected piglets were thin and

translucent, and the intestinal contents were pulpy or watery (Fig. 4E). There was obvious congestion in the lungs compared to uninfected pigs (Fig. 4F). The piglets in the control group were clean around anus and had no obvious clinical symptoms (Fig. 4A). The intestines were thick and elastic, and the lungs were normal (Fig. 4B,C). Diseased tissues were also examined histopathologically. Histopathological results showed that villi in the small intestine were slightly atrophic, and submucosal oedema was obvious (Fig. 5C). The lung was damaged by thickened alveolar walls, serous exudation and inflammatory cell infiltration in the interalveolar septa (Fig. 5D). There was no significant change in the control group (Fig. 5A,B).

To explore the excretion and distribution of PSV, viral load detection of faecal samples collected daily and tissue samples obtained after necropsy of piglets were conducted by qRT-PCR. The results showed that there were high RNA copy numbers in faecal samples at 3–6 dpi (Fig. 6A). In addition, PSV was determined to be widely distributed in lung and intestinal tissues. However, PSV wasn't detected in the heart, liver, or kidney (Fig. 6C, D). The results of enzyme-linked immunosorbent assay (ELISA) showed that the IgG antibody levels of PSV-infected pigs were significantly higher than those of uninfected pigs, and antibody levels increased significantly at 6 dpi (Fig. 6B). These results confirmed the pathogenicity of SHCM2019 to piglets.

Discussion

PSV is widely popular among pig population in China. In this study, a PSV isolate from pig farms in Shanghai, SHCM2019, was identified and sequenced. The isolated strain has 7,567 nucleotides, consisting of a 5' UTR of 465 nucleotides, a 3' UTR of 106 nucleotides, and a single ORF of 6,996 nucleotides that encodes a polyprotein precursor of 2,331 amino acids. The lengh of the 5' UTR is varied in PSVs. According to previous research, the 5' terminal residues of the complete picornavirus should be UU [5], but the two 5' terminal residues were AC for strain SHCM2019, which was not accordance with those sequence features. The antigenic epitome of VP1 had high identity with other PSV strains, indicating the slow evolution, which will provide useful clues for disease prevention.

Phylogenetic analysis based on the polyprotein gene revealed that the PSV isolates from China exhibit genetic diversity, and are closely related to the South Korean strains, but are distantly related to the PSV isolates from Europe and America, suggesting that the prevalence of PSVs may has certain territoriality. Furthermore, we constructed an evolutionary tree of the VP1 gene (Supplementary Fig. 2). However, the topological structure and phylogenetic branching of the VP1 tree were slightly different from the ORF tree and the VP1 tree previously reported [23], which indicated that PSV could not be divided into genotypes at present. At present, many picornaviruses are genotyped by VP1 sequence alignment[24]. The classification criterion is that the VP1 sequence difference between isolates is greater than 25% [25]. Among PSVs, the nucleotide and amino acid sequence identities of the VP1 region were > 70.8% and > 75.1%, respectively, and these values were less multiple than those of porcine enteroviruses (> 53.9% and > 53.3%, respectively) [24]. This might be one of the reasons why the genotyping PSVs is considered to be hard.

The driving forces of viral evolution include gene mutation, natural selection, gene drift, and viral gene recombination. In which, mutation and recombination are two important mechanisms. The results of recombination analysis showed recombination existed among the PSV isolates, however, the recombination site was located in the 3D region, which was not consistent with the previous studies [23, 26–28]. The 3D protein is a virus-specific RNA polymerase[3], which has the ability to synthesize viral RNA, and its recombination plays an important role in viral replication. In view of the diversity of recombination sites reported to date [23, 26–28], we must accelerate the process of research on the molecular mechanism of PSV.

The SHCM2019 strain was found to replicate in the intestinal tracts of piglets and cause diarrhoea and a degree of pneumonia in pigs, suggesting that the SHCM2019 strain is pathogenic. In clinic, like porcine circovirus type 2 (PCV2), PSV infection rate was high in both domestic and wild pigs [9], but in most cases, the pigs infected with PSV did not exhibit clinical symptoms [8, 9, 14, 20], which suggests that PSV is not consistently pathogenic. This also led to PCV2 and PEDV being the main focus of most researchers when large-scale diarrhea occurred in pig populations, while, PSV, which could cause similar symptoms, didn't attract similar attention [29, 30]. Perhaps PSV require currently unidentified factors to show full pathogenicity in pigs, which warrant further investigation.

Conclusion

In this study, a swine sapelovirus strain was isolated and named SHCM2019. The isolated strain was confirmed as PSV by RT-PCR, IFAand TEM assays. The molecular characteristics and pathogenicity of the SHCM2019 strain were studied. Phylogenetic analyses showed that the virus isolate belongs to PSV and was classified into the China cluster. Recombination analysis indicated that there may be a recombination breakpoint upstream of the 3D region in PSVs. Pathogenicity research demonstrated that the SHCM2019 strain is pathogenic. To better assess the adverse effects of PSV on pig populations, we need to further explore the epidemiological characteristics and pathogenic mechanisms of PSV.

Abbreviations

PSV: Porcine sapelovirus; PEV-8: Porcineenterovirus 8; UTR: Untranslated region; ORF: Open reading frame; DMEM: Dulbecco's modified Eagle's medium; FBS: Foetal bovine serum; PKV: Porcine kobuvirus; PAstV: Porcine astrovirus; PDCoV: Porcine deltacoronavirus; PToV: Porcine torovirus; PTV: Porcine teschovirus; PSaV: Porcine sapovirus; PEDV: Porcine epidemic diarrhoea virus; TGEV: Transmissible gastroenteritis virus; PoRV: Porcine rotavirus; BVDV: Bovine viral diarrhoea virus; PCV2: porcine circovirus type 2; CPE: Cytopathic effect; PBS: Phosphate-buffered saline; M-MLV: Moloney murine leukaemia virus; DEPC: Diethylpyrocarbonate; TEM: Transmission electron microscopy; IFA: Immunofluorescence assay; RT: Room temperature; BSA: Bovine serum albumin; DAPI: Diamidino-phenylindole; RDP: Recombination Detection Program; ELISA: Enzyme-linked immunosorbent assay.

Declarations

Acknowledgements

Not applicable.

Author Contributions

HLL conceived and designed this study; NNL and BQL conducted experiments; JT, JHC and YS contributed their technical knowledge; NNL and HLL wrote this paper; BQL and TJ were involved in interpreting the results and critically reading the manuscript. All authors read and approved the final manuscript.

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Availabilty of data and materials

Data supporting the conclusions of this article are presented in the manuscript.

Ethics approval and consent to participate

All animal experiments were performed under the guidance of the Institutional Animal Care and Use Committee at the Center for Disease Control and Prevention (CDC) and the Laboratory Animal Care International accredited facility.

Consent to publication

Not applicable.

Competing interests

All authors declared that they have no competing interests.

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References

1. Adams MJ, Lefkowitz EJ, King AM, Bamford DH, Breitbart M, Davison AJ, Ghabrial SA, Gorbalenya AE, Knowles NJ, Krell P, Lavigne R, Prangishvili D, Sanfacon H, Siddell SG, Simmonds P, Carstens EB

(2015) Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses. Arch Virol 160:1837-1850.

2. Tseng CH, Tsai HJ (2007) Sequence analysis of a duck picornavirus isolate indicates that it together with porcine enterovirus type 8 and simian picornavirus type 2 should be assigned to a new picornavirus genus. Virus Res 129:104-114.
3. 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.
4. 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.
5. 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.
6. Lamont PH, Betts AO (1958) Enteroviruses in the pig. Nature 182:608-609.
7. Buitrago D, Cano-Gomez C, Aguero M, Fernandez-Pacheco P, Gomez-Tejedor C, Jimenez-Clavero MA (2010) A survey of porcine picornaviruses and adenoviruses in fecal samples in Spain. J Vet Diagn Invest 22:763-766.
8. L'Ecuyer C, Greig AS (1966) Serological and biological studies on porcine enteroviruses isolated in Canada. Can Vet J 7:148-154.
9. Morimoto T, Watanabe M (1964) Serological Identification of Porcine Enteroviruses Isolated in Japan. Natl Inst Anim Health Q 4:177-182.
10. Forman AJ, Pass DA, Connaughton ID (1982) The characterisation and pathogenicity of porcine enteroviruses isolated in Victoria. Aust Vet J 58:136-142.
11. 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.
12. 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.
13. 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.
14. Boros A, Laszlo Z, Pankovics P, Marosi A, Albert M, Csagola A, Biro H, Fahsbender E, Delwart E, Reuter G (2020) High prevalence, genetic diversity and a potentially novel genotype of Sapelovirus A (Picornaviridae) in enteric and respiratory samples in Hungarian swine farms. J Gen Virol 101:609-621.
15. Prodelalova 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.

16. Huang J, Gentry RF, Zarkower A (1980) Experimental infection of pregnant sows with porcine enteroviruses. Am J Vet Res 41:469-473.
17. 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.
18. Zhang B, Tang C, Yue H, Ren Y, Song Z (2014) Viral metagenomics analysis demonstrates the diversity of viral flora in piglet diarrhoeic faeces in China. J Gen Virol 95:1603-1611.
19. 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 SapelovirusA in piglets and chicks. J Gen Virol 97:2566-2574.
20. Shan T, Li L, Simmonds P, Wang C, Moeser A, Delwart E (2011) The fecal virome of pigs on a high-density farm. J Virol 85:11697-11708.
21. 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. J Virol 15:95.
22. Domingo E, Sheldon J, Perales C (2012) Viral quasispecies evolution. Microbiol Mol Biol Rev 76:159-216.
23. Sunaga F, Masuda T, Ito M, Akagami M, Naoi Y, Sano K, Katayama Y, Omatsu T, Oba M, Sakaguchi S, Furuya T, Yamasato H, Ouchi Y, Shirai J, Mizutani T, Nagai M (2019) Complete genomic analysis and molecular characterization of Japanese porcine sapeloviruses. Virus Genes 55:198-208.
24. 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.
25. 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.
26. Lukashev AN, Lashkevich VA, Ivanova OE, Koroleva GA, Hinkkanen AE, Ilonen J (2003) Recombination in circulating enteroviruses. J Virol 77:10423-10431.
27. Harima H, Kajihara M, Simulundu E, Bwalya E, Qiu Y, Isono M, Okuya K, Gonzalez G, Yamagishi J, Hang'ombe BM, Sawa H, Mweene AS, Takada A (2020) Genetic and Biological Diversity of Porcine Sapeloviruses Prevailing in Zambia. Viruses 12.
28. 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.
29. Li B, Ma J, Liu Y, Wen L, Yu Z, Ni Y, Zhang X, Zhou J, Guo R, Wang X, Feng Z, Hu Y, Lv L, He K (2012) Complete genome sequence of a highly prevalent porcine circovirus 2 isolated from piglet stool samples in China. J Virol 86:4716.
30. Sun RQ, Cai RJ, Chen YQ, Liang PS, Chen DK, Song CX (2012) Outbreak of porcine epidemic diarrhea in suckling piglets, China. Emerg Infect Dis 18:161-163.