Received: 10 February 2021 | Revised: 25 March 2021 | Accepted: 26 March 2021

DOI: 10.1111/tbed.14087

ORIGINAL ARTICLE

Transboundary and Emerging Diseases WILEY

Genetic characterization of porcine sapoviruses identified from pigs during a diarrhoea outbreak in Iowa, 2019

Huigang Shen[1] | Jianfeng Zhang[1,2] | Phillip C. Gauger[1] | Eric R Burrough[1] | Jianqiang Zhang[1] | Karen Harmon | Leyi Wang[3] | Ying Zheng[1] | Thomas Petznick[4] | Ganwu Li[1]

[1]Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA

[2]Key Laboratory of Livestock Disease Prevention of Guangdong Province, Scientific Observation and Experiment Station of Veterinary Drugs and Diagnostic Techniques of Guangdong Province, Institute of Animal Health, Guangdong Academy of Agricultural Sciences, Guangzhou, China

[3]Department of Veterinary Clinical Medicine and the Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Illinois, Urbana, IL, USA

[4]Arkcare, Fairbury, NE, USA

Correspondence
Ganwu Li, Department of Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, USA.
Email: liganwu@iastate.edu

Funding information
Swine Health Information Center, Grant/Award Number: SHIC#19-220

Abstract

Porcine sapovirus (SaV) was first identified by electron microscopy in the United States in 1980 and has since been reported from both asymptomatic and diarrhoeic pigs usually in mixed infection with other enteric pathogens. SaV as the sole aetiological agent of diarrhoea in naturally infected pigs has not previously been reported in the United States. Here, we used four independent lines of evidence including metagenomics analysis, real-time RT-PCR (rRT-PCR), histopathology, and in situ hybridization to confirm porcine SaV genogroup III (GIII) as the sole cause of enteritis and diarrhoea in pigs. A highly sensitive and specific rRT-PCR was established to detect porcine SaV GIII. Examination of 184 faecal samples from an outbreak of diarrhoea on a pig farm showed that pigs with clinical diarrhoea had significantly lower C_t values (15.9 ± 0.59) compared to clinically unaffected pigs (35.8 ± 0.71). Further survey of 336 faecal samples from different states in the United States demonstrated that samples from pigs with clinical diarrhoea had a comparable positive rate (45.3%) with those from asymptomatic pigs (43.1%). However, the SaV-positive pigs with clinical diarrhoea had significantly higher viral loads (C_t = 26.0 ± 0.5) than the SAV-positive but clinically healthy pigs (C_t = 33.2 ± 0.9). Phylogenetic analysis of 20 field SaVs revealed that all belonged to SaV GIII and recombination analysis indicated that intragenogroup recombination had occurred within the field isolates of SaV GIII. These results suggest that porcine SaV GIII plays an important aetiologic role in swine enteritis and diarrhoea and rRT-PCR is a reliable method to detect porcine SaV. Our findings provide significant insights to better understand the epidemiology and pathogenicity of porcine SaV infection.

KEYWORDS

diarrhoea, phylogenetic analysis, porcine sapovirus, real-time PCR, recombination

Huigang Shen and Jianfeng Zhang contributed to this work equally.

1 | INTRODUCTION

Sapovirus (SaV) is a member of the genus *Sapovirus* within the family *Caliciviridae*. It is a positive sense, single-stranded RNA virus with a genome of approximately 7.1–7.7 kb in length and a poly A tail at the 3′ end (Alhatlani et al., 2015; Oka et al., 2016). The SaV genome contains two open reading frames (ORFs). The ORF1 encodes a large polyprotein and the major capsid protein VP1 and the ORF2 encodes the minor structural protein VP2 (Oka et al., 2016). The ORF1-encoded polyprotein undergoes protease processing to produce non-structural (NS) proteins including NS1, NS2, NS3 (putative NTPase), NS4, NS5 (VPg) and NS6-NS7 (protease and RNA dependent RNA polymerase, RdRp, respectively) (Oka et al., 2015).

Sapoviruses are classified into different genogroups based on the VP1 sequences (Farkas et al., 2004; Scheuer et al., 2013). A pairwise identity cut-off value of VP1 amino acid sequence (<57%) was used to define a different SaV genogroup (Oka et al., 2016; Yinda et al., 2017), and we followed the proposal described by Oka et al. (2016). At present, SaVs are classified into 19 genogroups and at least 52 genotypes based on complete VP1 sequences using a pairwise distance cut-off value of ≤0.488 to distinguish different genogroups and ≤0.169 to distinguish different genotypes (Nagai et al., 2020; Oka et al., 2015). Among them, 17 genogroups (GII, GIII and GV–GXIX) have been identified in animals and four (GI, GII, GIV and GV) in humans (Scheuer et al., 2013) with genogroups II (GII) and V (GV) detected in both animals and humans (Oka et al., 2015). Thus far, at least eight genogroups (GIII, GV, GVI, GVII, GVIII, GIX, GX, and GXI) of SaVs have been associated with pigs (Nagai et al., 2020; Oka et al., 2016; Scheuer et al., 2013). Our recent genomic characterization study reveals that highly divergent porcine SaV strains co-circulate in the field in the United States (Wang et al., 2020). In addition to nucleotide mutations, genome insertions and deletions, and recombination events might contribute to virus evolution (Wang et al., 2020).

Sapoviruses can cause gastroenteritis in humans and animals (Oka et al., 2015). Porcine SaV was first identified by electron microscopy in the United States in 1980 in a mixed infection with other viruses (Saif et al., 1980) and since then, SaVs have been reported from both diarrhoeic and asymptomatic pigs (das Merces Hernandez et al., 2014; Wang et al., 2020; Wang et al., 2006). Experimental infection of cell culture-adapted SaVs in gnotobiotic piglets successfully induced enteritis and diarrhoea (Flynn et al., 1988; Guo et al., 2001). However, in the field, SaVs are often identified as a mixed infection with other diarrhoeic pathogens (Dufkova et al., 2013; Mijovski et al., 2010; Scheuer et al., 2013), while SaV as the sole aetiological agent of diarrhoea in naturally infected pigs has, until this time, never been reported in the United States.

2 | MATERIALS AND METHODS

2.1 | Clinical samples and history

In February of 2019, the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) received a diagnostic submission from a farm experiencing an ongoing problem with piglet diarrhoea in the lactation phase for more than 2 years (Case #1 in Table 1). The pigs reportedly exhibited a pasty to occasional semiliquid diarrhoea starting at around 10 days of age. The diarrhoea was generally described as self-limiting, but the pigs would typically lose 1–2 lbs of expected weaning weight. Faecal and intestinal tissue samples were received at the ISU VDL as part of this diagnostic submission. Histopathological examination of intestinal samples from five clinically affected piglets revealed that 5/5 small intestines had moderate villous atrophy with lymphocytic infiltration in the lamina propria suggestive of an enteric viral infection (Figure 1a-d). Porcine epidemic diarrhoea virus (PEDV), porcine deltacoronavirus (PDCoV), transmissible gastroenteritis virus (TGEV) and rotaviruses (groups/species A-C) were not detected in the intestinal contents using real-time RT-PCR (rRT-PCR) assays. Additionally, there was no significant growth of common bacterial pathogens from the affected small intestines.

As part of a follow-up investigation, a total of 184 faecal swab samples were collected from pigs with clinical diarrhoea and healthy pigs of varying ages. These swabs included 34 samples from pigs between 8 and 12 days of age, 100 samples from two-week-old pigs, and 50 samples from pigs between 12 and 16 weeks of age. All samples were collected from the original submitting farm and were subjected to SaV GIII rRT-PCR.

In addition, 234 faecal samples from swine with diarrhoea and 102 from clinically healthy pigs that were submitted to the ISU VDL for routine diagnostics by unrelated farms from different states across the United States were also tested by SaV GIII rRT-PCR.

2.2 | Next generation sequencing

Total nucleic acid of clinical samples was extracted using MagMAX Pathogen RNA/DNA Kit with KingFisher™ Flex System (Thermo Fisher Scientific) (Zhang et al., 2017). Double stranded cDNA was synthesized using NEXTflex™ Rapid RNA-Seq Kit (Bioo Scientific Corp). Sequencing library was prepared using Nextera XT DNA

TABLE 1 Presence of viral pathogens in faecal samples of two NGS cases

Case No.	Sequence ID	Age	SaV GIII	PEDV[a]	PDCoV	TGEV	Rota A	Rota B	Rota C
1	MW316743_NE9550B	2 weeks	(+)	(–)	(–)	(–)	(–)	(–)	(–)
2	MW316759_IA0365	8–10 days	(+)	(–)	(–)	(–)	(–)	(–)	(–)

[a]All viruses other than SaV GIII were tested by real-time PCR; (+), positive; (–), negative, that is $C_t \geq 36$.

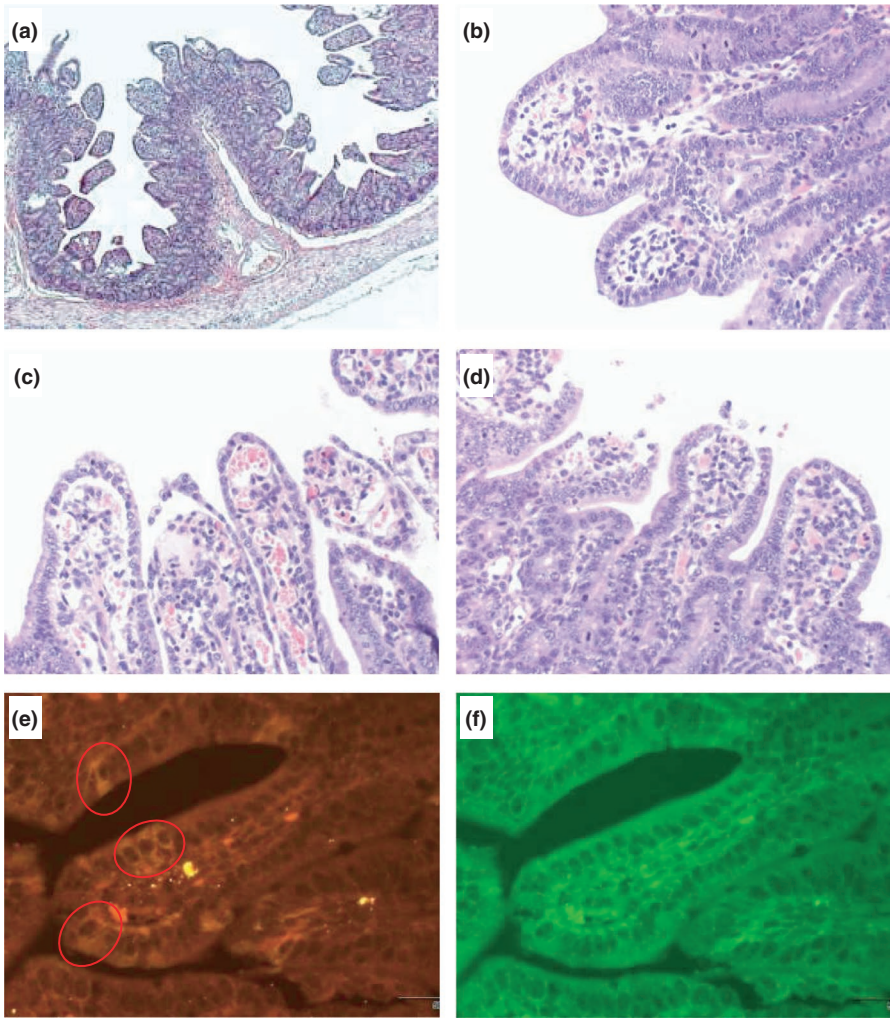

FIGURE 1 Microscopic lesions and fluorescent in situ hybridization (FISH) in the small intestine of pigs naturally infected with porcine sapovirus GIII. (a) There is marked villous atrophy and rare fusion. HE. 40×. (b) Mild lymphocytic infiltration in the lamina propria. HE. 200×. (c) Variable villous vascular congestion. HE. 200×. (d) Mildly vacuolated villus enterocytes that are often detached and exposing the lamina propria. HE. 200×. (e) Scattered FISH-positive villus enterocytes (in the red circle) are observed within sections with villus attenuation; FISH was performed with Alexa555-labelled sapovirus probe; observed with CY3 Filter. 200×. (f) No fluorescence was observed in the FISH-positive cells when observed with a FITC Filter. 200×

library preparation kit (Illumina) with dual indexing. The pooled libraries were sequenced on an Illumina MiSeq platform at the next generation sequencing (NGS) unit in the ISU VDL, with 500-Cycle v2 Reagent Kit (Illumina) to generate 250 base-pair paired-end reads by following standard Illumina protocols. Raw reads of each sample were demultiplexed automatically on the MiSeq platform with the default settings.

2.3 | Bioinformatics analysis

Raw sequencing reads were pre-processed using Trimmomatic v0.36 to remove adapters and trim low quality ends (Bolger et al., 2014). Raw reads and pre-processed reads were subjected to sequencing quality analysis with FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to ensure the efficiency of cleaning. Cleaned reads were fed to a comprehensive reference-assisted virus genome assembly pipeline (Chen et al., 2018; Zhang et al., 2017) with modifications. The cleaned reads were aligned to host reference genome using Burrow-Wheeler Alignment (BWA) (Li & Durbin, 2009). The non-host reads were classified using Kraken v1.0 (Wood & Salzberg, 2014) and the unclassified reads were further classified using Kaiju v1.6.2 (Menzel et al., 2016). KronaTools-2.7 (Ondov et al., 2011) was used to generate the interactive html charts for hierarchical classification results. Reads of interest were extracted and used for assembly by using ABySS v1.3.9 (Simpson et al., 2009). The resulting contigs were manually curated to remove contaminated contigs and trim chimeric contigs in SeqMan Pro (DNASTAR® Lasergene 11 Core Suite) and finally refined to obtain the genome sequence.

2.4 | Histopathology and Fluorescent in situ hybridization (FISH)

Sections of duodenum, jejunum and ileum were fixed in 10% neutral buffered formalin and processed by standard paraffin embedding technique and then stained with haematoxylin and eosin (HE). Histopathological evaluation was performed by diagnostic pathologists at the ISU VDL. Sections from selected paraffin blocks with lesions of atrophic enteritis were then used for FISH development with an Alexa555-labeled probe Psap5122 (Table 2) and an in-house hybridization buffer as previously described (Burrough et al., 2013) with slight modification (20 mM of Tris, 0.9 M of NaCl,

TABLE 2 Primers, probe and oligo sequences used in this study

Name	Sequence (5′to 3′)	Position	Accession no.	Used for
PSapV-F	AACGCRGTGGCAACGTACAA	5050–5069	MK965904	rRT-PCR
PSapV-R	GCCTCCATCACGAACACTTC	5128–5147	MK965904	rRT-PCR
PSapV-P	FAM-TGGCTCYTCATCTTCATTGGTGGGAGC-TAMSp	5101–5127	MK965904	rRT-PCR
Sap-GV-115	AATTACGAGCAGGCCACTGCCTGTTACAACAGCTGGTTTATTGGCGGGAGCCAACCTGAGGTGCCCACTA CCAGTGAAGGCTATGGGCTATTAGTGTTTGAGATGGAGGGCAATC	5103–5217	AB521771	Specificity assay
Sap-GVI-112	GATTACACAGAGGCATTTGACGTCATGCGCAACTTGTCATCTACCCAACCCGATGGCGGGGCTGTTGTGTA CACAATGGAGGGGCCCAAGCCCTCTCGTGCGTCGAATGGGT	4897–5008	LC215886	Specificity assay
Sap-GVII-107	GAATACTCTGAGGCCATCGAGGTGATAACCAACATTTCATCAGGCACGCCCGAGGGCGAGGCAATTGTGTA TAAAATGGAGGGTCCTGAGGGGCCAAAGCCCACAGA	4897–5003	AB221130	Specificity assay
Sap-GVIII-116	CACCAATTATGCTCAAGCTGCAGCCACCTACAATGCGTGGTATATTGGTGGCGTGGAACCACAATTGGGGAG CCTCGCCAGTGAAGGTTCAGCTCAAGTAGTGTTTGAGATGGAGG	4083–4198	KC309417	Specificity assay
Sap-GIX-117	CTGGTGTGGAATACTCAGAGGCAATTAACGTGCTAACATCTATTTCATCCAGACCGCCTGAGGGTGAGGCAAT AGTGTATGTGATGGAGGGTCCAAACGGCCCTAAGGGCGCTCAGC	1436–1552	KC309418	Specificity assay
Sap-GX-116	CTGGTGTGGAATACTCAGAGGCAATACACGTATTGACATCAATTTCATCCAAACCGCCCGAGGGCGAGGCAA TAGTGTATGTGATGGAGGGCCCAAAGGGTCCCGTTGAGGGCGAG	4889–5004	LC215897	Specificity assay
Sap-GXI-116	TACGCTGGGACAAAATACTCTGAGGCCATCGAGTTGATAACCAACATTTCATCAATCCCG CCCGATGGCGAG GCTATTGTGTATGTCATGGAGGGCCCAGAGGGCTCCATGCAGAA	2136–2251	LC215902	Specificity assay
Psap5122probe	Alex555N-GCCTCCATCACGAACACTTCTGGCTC	5122–5147	MK965904	FISH
Sap-T7-F1	TAATACGACTCACTATAGGGAACGCAGTGGCAACGTACAA	5050-5069[a]	MK965904	Create RNA standards
Sap5193R	TTGAGTACCCTCTGGGTTGCT	5173–5193	MK965904	Create RNA standards

[a]Only show the position corresponding to SaV sequence.

0.1% sodium dodecyl sulphate [SDS] buffer, 20% formamide, 10% dextran sulphate [pH 7.2]). In situ hybridization was performed using an automated system (Discovery Ultra; Roche Diagnostics) with the manufacturer's standard protocol and commercial reagents with the exception of the hybridization buffer as noted above. Hybridization was carried out at 52°C for 12 hr.

2.5 | Development of real-time RT-PCR (rRT-PCR) for porcine SaV GIII

An rRT-PCR assay specific for porcine SaV GIII was developed with the primers PSapV-F and PSapV-R, and probe PSapV-P (Table 2), which were commercially synthesized (Integrated DNA Technologies). The real-time RT-PCR was performed in a reaction mixture of 25 μl containing 12.5 μl 2× AgPath-ID RT-PCR Buffer (Applied Biosystems), 1 μl 25× RT-PCR Enzyme Mix, 1 μl (final concentration 0.4 μM) of each of the primers, 0.5 μl (final concentration 0.2 μM) of probe, 4 μl nuclease-free water and 5 μl extracted RNA. The amplification was performed at 48°C for 10 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 55°C for 20 s and 60°C for 45 s. For each amplification plot, a cycle threshold (C_t) value was calculated representing the highest cycle number at which the reporter signal was above threshold.

The sensitivity of the real-time PCR assay was evaluated by using serial 10-fold dilutions of the in vitro transcribed RNA, which was produced by Transcript Aid T7 High Yield Transcription Kit (Thermo Fisher Scientific, Waltham, MA) with primers Sap-T7-F1 and Sap5193R (Table 2).

The specificity of SaV GIII rRT-PCR was evaluated using oligo DNA fragments with length between 107 and 117 nt designed for SaV GV, GVI, GVII, GVIII, GIX, GX and GXI according to their corresponding sequences at the same region that SaV GIII rRT-PCR targeted (Table 2). All oligo DNAs were synthesized in the DNA Facility at Iowa State University. In addition, the specificity was evaluated with other viral pathogens related to swine diarrhoea including virus isolates of PEDV, TGEV, PDCoV and rotavirus A, and faecal samples positive for rotavirus B and C, and bacterial pathogens *Salmonella* spp. and *E. coli*.

2.6 | Sequence comparison and analysis

Sequence comparison was performed with MegAlign (DNASTAR® Lasergene 11 Core Suite). Sequences representative of different SaV genogroups were obtained from GenBank and aligned with the 20 SaV genome sequences in the present study. Phylogenetic trees were constructed using the neighbour-joining method in MEGA 7.0 (Kumar et al., 2016) with p-distance as the substitution model. Bootstrap analysis was carried out with 1,000 replicates. Recombination analysis was performed in the region from ORF1 55 nt to ORF2 522 nt using SimPlot software v. 3.5.1 (Lole et al., 1999).

3 | RESULTS

3.1 | Next-generation sequencing

Next-generation sequencing and bioinformatics analysis were performed on the faecal and tissue samples from the outbreak farm (case 1 in Table 1). The hierarchical classification results were shown as an html pie-chart by Kraken and Krona Tools. Surprisingly, the classification results showed that 100% of the viral reads belong to SaV. The complete genome sequence of porcine SaV was then assembled and designated as NE9550B. No other viral pathogens were identified in the classification; these results were consistent with the real-time PCR results in which all other common viruses related to porcine enteric disease were negative (Table 1). NGS analysis also identified SaV as the sole viral pathogen from faecal samples of 8 to 10-day-old diarrhoeic piglets (case # 2) (Table 1).

3.2 | Histopathology and fluorescent in situ hybridization (FISH)

Histopathological examination revealed lesions consistent with viral infection in the small intestine. Specifically, there was moderate segmental villous blunting, atrophy and contraction (Figure 1a,b) with marked, locally extensive villous vascular congestion (Figure 1c). Low to moderate numbers of lymphocytes, macrophages and fewer neutrophils were observed in the lamina propria (Figure 1b,c) and multifocal crypts contained low to moderate numbers of neutrophils and cellular debris. Apical enterocytes over affected villi ranged from vacuolated to flattened or attenuated (Figure 1c) and some villi lacked apical enterocytes with exposed lamina propria (Figure 1d). In the colon, multifocal glands contained low to moderate numbers of neutrophils admixed with cellular debris and low numbers of lymphocytes and macrophages were identified in the lamina propria (data not shown).

For slides with histopathological lesions that also originated from SaV rRT-PCR positive intestine tissue samples, additional sections of small intestine were further subjected to FISH. Fluorescently-labelled enterocytes were identified along the attenuated villi indicating that the lesion was caused by SaV infection (Figure 1e,f).

3.3 | Development of real-time RT-PCR (rRT-PCR)

Standard curves were established using the 10-fold serially diluted RNA control in vitro transcribed from a DNA fragment amplified by SaV GIII primers Sap-T7-F1 and Sap5193R (Table 2). The concentration (ng/μl) of the RNA control was determined by a NanoDrop (Thermo Fisher Scientific), and the copy numbers were calculated with the mass and molecular weight using an online calculator (http://www.scienceprimer.com/copy-number-calculator-for-realtime-pcr). RNA control was then serially diluted from 2.4×10^1 to 2.4×10^9 copies per reaction (in 5 μl) and amplified in triplicate. The threshold cycle (C_t) values were plotted against the copy numbers. The SaV GIII

rRT-PCR was able to detect the RNA control over a linear range. The standard curve obtained showed a slope of −3.80, an intercept of 43.33, and a regression coefficient (R^2) of 0.9,998. The detection limit of the assay using RNA control as template was five copy numbers per reaction. No PCR amplification signals were detected in the SaV GV, GVI, GVII, GVIII, GIX, GX and GXI oligos, PEDV, TGEV, PDCoV and rotavirus A isolates, rotavirus B and C faecal samples, or the *Salmonella* spp. and *E. coli* cultures by the SaV GIII rRT-PCR ($C_t \geq 40$).

3.4 | rRT-PCR examination of porcine SaV in clinical and non-clinical pigs from the outbreak farm

The intestine and faecal samples of the original case from the outbreak farm were analysed using the SaV rRT-PCR assay. High amount of SaV was detected in both intestine and faecal samples, with C_t values of 18.6 in the tissue sample and 15.4 in the faecal sample, respectively.

A follow-up investigation was performed with samples from the outbreak farm. In total, 34 faecal samples from pigs 8 to 12 days old with clinical signs were examined and 85.5% of samples were found positive with an average C_t value of 21.5 ± 3.38 (Figure 2). Another 100 faecal swab samples from two-week-old pigs with and without clinical signs were compared. The 50 samples from pigs with clinical signs had significantly lower C_t values (15.9 ± 0.59) compared to the 50 samples from non-clinical pigs (35.8 ± 0.71) ($p < .01$). All faecal samples from pigs 12 to 16 weeks old without clinical signs were negative with C_t values of equal to or higher than 40 (Figure 2).

3.5 | Determine the incidence of porcine SaV in swine herds of the United States

Totally, 234 faecal samples from pigs with diarrhoea from different states across the United States (U.S.) were examined using SaV rRT-PCR. Among them, 106 samples (45.3%) were positive with C_t values of 26.0 ± 0.5; in contrast, 128 samples were negative with C_t values equal to or higher than 40. In addition, 102 faecal samples from clinically healthy pigs from different states across the United States were also examined with SaV rRT-PCR. Among them, 44 samples (43.1%) were positive by SaV rRT-PCR with C_t values of 33.2 ± 0.9, which were significantly higher than those from pigs with diarrhoea (26.0 ± 0.5). The remaining 58 samples were negative by SaV rRT-PCR.

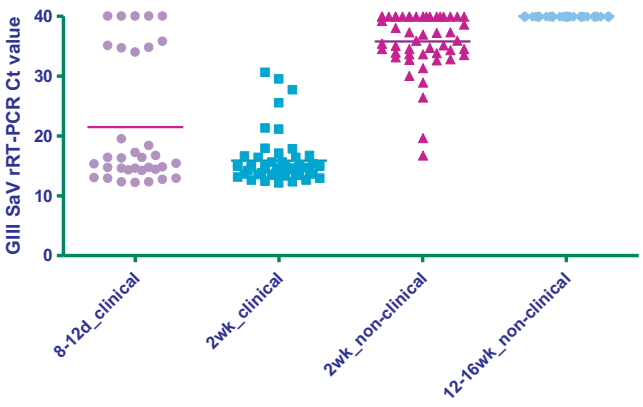

FIGURE 2 Sapovirus GIII rRT-PCR results from the outbreak farm for the 34 pigs of 8–12 days old with clinical signs, 50 pigs of 2-week-old with clinical signs, 50 pigs of 2-week-old without clinical signs, and 50 pigs between 12 and 16 weeks of age without clinical signs. The line indicates the average C_t value, and the scattered plots indicate the individual values

3.6 | Sequence comparison, phylogenetic analysis and recombination analysis

Next-generation sequencing analysis was performed with the faecal samples submitted to ISU VDL and totally 20 genome sequences from field porcine SaVs were assembled. The neighbour-joining (NJ) phylogenetic tree based on genome sequences showed that all 20 SaVs were clustered with GIII reference isolate sequences (Figure 3). The genome sequences of the six GIII SaVs that were from the outbreak farm showed 99.8% to 100% nucleotide (nt) identity, indicating that SaV was genetically relatively stable at farm level during the relatively short observation period whereas showed only 82.0%–98.0% nt identities to all other 14 GIII SaVs (Table S1).

SimPlot analysis was performed with SaV strains MW316746_IN17168-3A_2020, MW316747_IL16624_2020 and MW316757_IA4517-2_2019, in the region between ORF1 55 nt and ORF2 522 nt, to determine recombination events within the genogroup GIII (Figure 4). The strain IN17168-3A showed higher nt identity (89.8%) to the IL16624 strain in the NS-VP1 coding region (ORF1 55-5235nt), but much lower nt identity (74.8%) to IA4517-2. While in the VP2 coding region (ORF2 61-522nt), strain IN17168-3A showed higher nt identity (89.2%) to the IA4517-2 strain, but lower nt identity (82.0%) to IL16624. These results suggested that the strain IN17168-3A could be a recombinant, which may have arisen from cells/hosts co-infected with SaVs similar to the IL16624 and AI4417-2 isolates.

4 | DISCUSSION

Porcine SaV is widely detected in pig herds throughout the world (Liu et al., 2012; Reuter et al., 2010; Scheuer et al., 2013). Although experimental inoculation of isolated SaVs into gnotobiotic pigs successfully induced enteritis (Flynn et al., 1988; Guo et al., 2001), porcine SaV is usually detected in faeces from asymptomatic pigs (Collins et al., 2009) or diarrhoeic piglets with mixed infections with other viral or bacterial enteric pathogens (Dufkova et al., 2013; Mijovski et al., 2010; Scheuer et al., 2013). In this study, a metagenomics analysis detected a porcine SaV of genogroup III in faecal and intestine samples of piglets with diarrhoea. PEDV, PDCoV, TGEV or rotavirus were not detected using rRT-PCR assays and additionally, there was no significant bacterial pathogens isolated from the intestine. The newly developed rRT-PCR revealed large amounts of porcine SaV

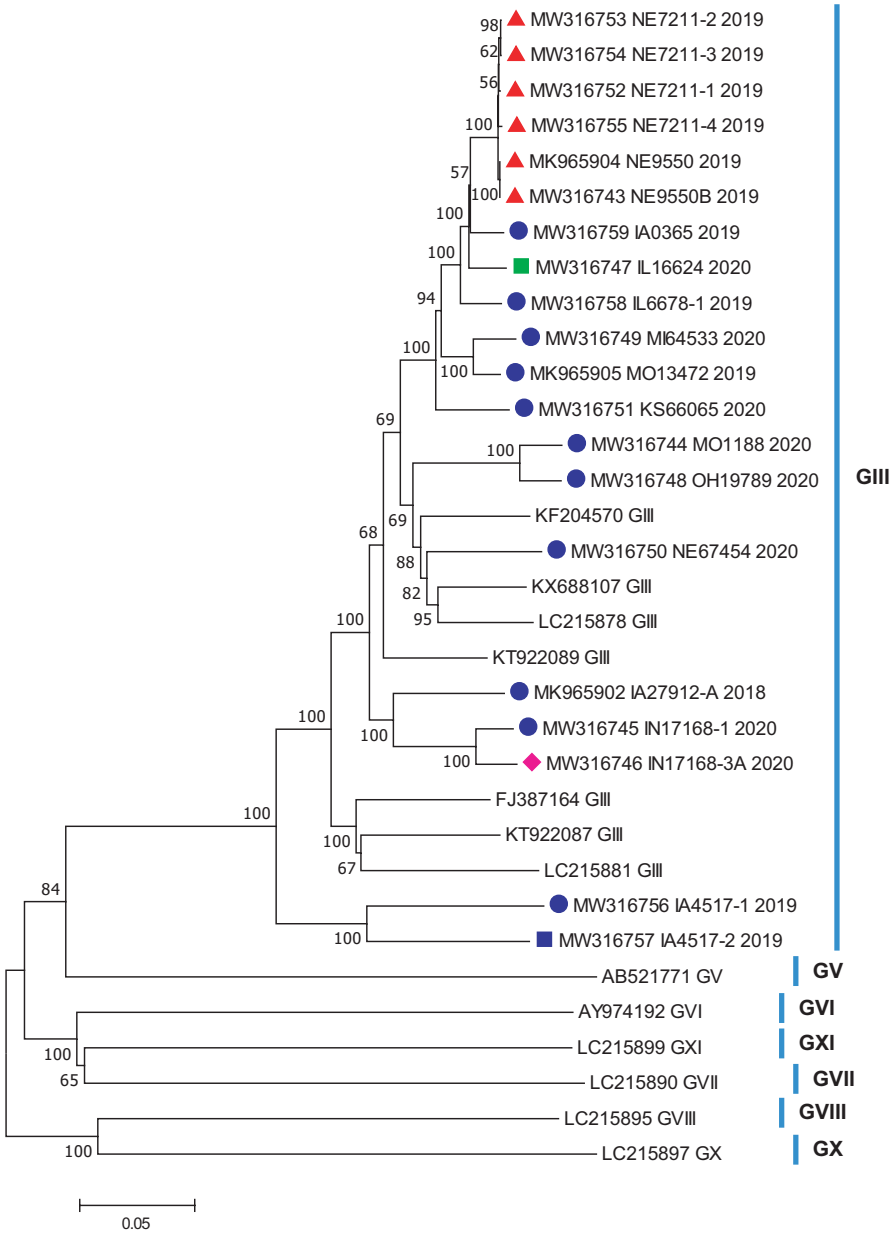

FIGURE 3 Phylogenetic analysis based on SaV genome sequences. The phylogenetic tree was constructed by the neighbour-joining (NJ) method. Significant bootstrap values are indicated as a percentage for 1000 replicates; bootstraps higher than 50 are displayed along the relative branches. ▲, the SaVs from the outbreak farm; ◆, the recombinant SaV strain; ■ and ■, potential parent strains involved in recombination; ●, other strains obtained in this study

RNA in the small intestine and the FISH assay conducted on tissue with microscopic lesions consistent with a viral enteritis further confirmed that GIII SaV was the cause of the observed clinical scenario. To the best of our knowledge, this is the first confirmation that SaV as a sole aetiological agent of enteritis and diarrhoea in naturally infected piglets in the United States has not been observed before.

In this study, a significantly sensitive (5 copy numbers per reaction) and highly specific rRT-PCR was successfully developed to detect porcine GIII SaV, the most prevalent genogroup in swine worldwide. Examination of 184 faecal samples from pigs from the outbreak farm showed that those pigs with clinical diarrhoea had significantly lower C_t values (15.9 ± 0.59) than those from clinically healthy pigs (35.8 ± 0.71) with about 2^{20} (10^6-fold) difference in quantity. We further investigated the prevalence of SaV in faecal samples of 234 pigs with diarrhoea and 102 clinically healthy pigs from different states across the United States. Our results showed that pigs with clinical diarrhoea had a comparable positive rate (45.3%) with non-clinical pigs (43.1%), which is consistent with previous reports (Collins et al., 2009; Dufkova et al., 2013). However, the SaV-positive pigs with clinical diarrhoea had significantly higher virus loads (C_t = 26.0 ± 0.5) compared to rRT-PCR positive but clinically healthy pigs (C_t =33.2 ± 0.9, with 2^7 = 128-fold difference in quantity). Most porcine SaVs have so far been reported from asymptomatic pigs and several studies reported no significant differences in the positive rates of porcine SaV between clinical and non-clinical pigs (Barry et al., 2008; Collins et al., 2009; das Merces Hernandez et al., 2014; Dufkova et al., 2013; Mijovski et al., 2010; Reuter et al., 2010). Further, it has been suggested that testing for the presence of SaV may not provide significant diagnostic value (Salamunova et al., 2018). Our results suggest that rRT-PCR is a reliable method to detect porcine SaV and that porcine GIII SaV likely plays an important role in suckling pig enteritis. This PCR provides

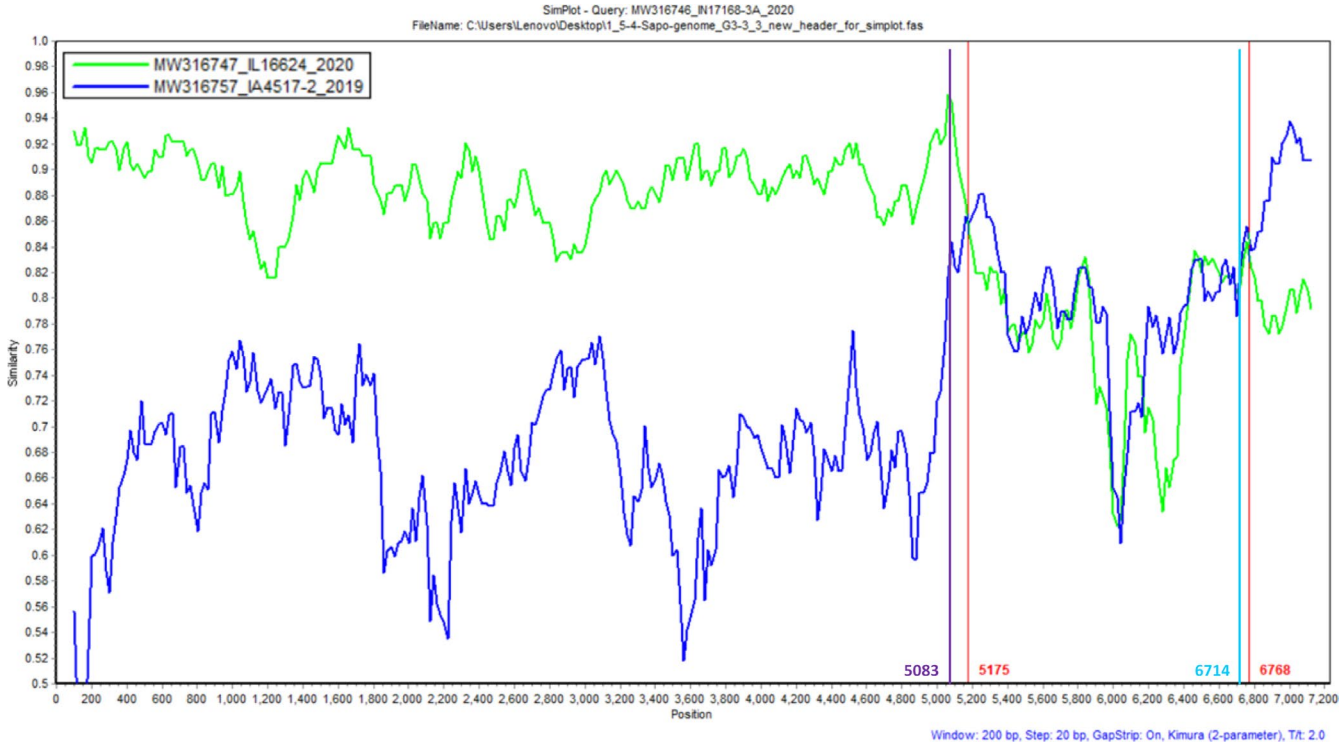

FIGURE 4 Recombination analysis. SimPlot analysis for the region from ORF1 55 nt to ORF2 522 nt of the nucleotide sequences: MW316747_IL16624_2020 (green line), MW316757_IA4517-2_2019 (blue line), and MW316746_IN17168-3A_2020 as the query sequence. The red solid lines indicate the breakpoints, 5175 nt (ORF1 5235 nt) and 6768 nt (ORF2 61 nt), in the sequence alignment; the purple dotted line indicates the border of NS7 and VP1 (5083 nt in the alignment); the turquoise dotted line indicates the border of ORF1 and ORF2 (6714 nt for the start coding position of ORF2 in the alignment)

an additional diagnostic tool that can be used in cases of suckling pig diarrhoea, particularly in those cases where other common agents are not detected. The FISH probe developed herein is another highly specific tool that can be used for direct detection of porcine SaV within intestinal lesions. It should be noted that other genogroups of SaVs could not be excluded in the samples in our study since only porcine GIII SaV was screened.

The age susceptibility of pigs to porcine SaV infection was investigated by different groups (Barry et al., 2008; Martinez et al., 2006; Valente et al., 2016). Post-weaning pigs (4–8 weeks of age) have been suggested to have a higher positive frequency of SaV (p <.05) than grower-to-finish and breeder pigs (Valente et al., 2016) and the lowest prevalence of porcine GIII SaVs was found in nursing pigs (Q. H. Wang et al., 2006). In our study, 85.5% of suckling piglets (8–12 days) were detected positive ($C_t = 21.5 \pm 3.38$) with porcine SaV infection, a decent percentage (82.0%) of pigs at 2-week-old were still positive ($C_t = 25.8 \pm 1.10$), while none of pigs at 12- to 16-week-old were rRT-PCR positive. Our results suggest that the suckling and post-weaning pigs are most susceptible to porcine SaV infection, which is consistent with the previous studies.

The genome sequences of 20 SaVs obtained in this study all belong to GIII, which is the predominant genogroup of porcine SaV worldwide (Keum et al., 2009; L'Homme et al., 2010; Nakamura et al., 2010; Wang et al., 2006; Yu et al., 2008). The genome sequences of some GIII strains shared only 85.8% nt identities. Both intra- and inter-genogroup recombinant strains have been reported for porcine SaV (Kuroda et al., 2017; Li et al., 2017; Wang et al., 2020; Wang et al., 2005) as well as human SaV (Dey et al., 2018; Kumthip et al., 2020; Lasure & Gopalkrishna, 2017; Liu et al., 2015; Tohma et al., 2020). However, most of the recombinant studies were performed with reference strains and few field strains have been investigated for recombination. In the present study, strain IN17168-3A shared a high AA identity (98.7%) with strain IL16624 in the NS-VP1 region, but a very high amino acid identity (92.8%) with strain IA4517-2 in the VP2 region. The results suggested that intragenogroup recombination may occur between field GIII strains. To the best of our knowledge, this is the first report of intragenogroup recombination within GIII SaV among field strains circulating in swine. The NS7-VP1 junction region appears to be the crossover point of the recombination event (Figure 4), which was demonstrated as well in other studies (Li et al., 2017; Oka et al., 2015; Wang et al., 2005). A conserved 26-nt motif in this region (Wang et al., 2005) is considered the transcription start signal for subgenomic RNA (Oka et al., 2015). Comparing the conserved 26-nt motif (GTGTTCGTGATGGAGGCGCCTGCCCC) in IN17168-3A with those in IL16624 and IA4517-2 strains, only one nucleotide difference was identified in IA4517-2 strain (G to A at the 18th nt). NGS analysis also identified coinfections of several (up to three) different SaV strains in the same faecal sample (data not shown) with coinfections of different strains and/or different genogroups in the same pig being a pre-perquisite for viral RNA recombination.

In summary, using metagenomics analysis, GIII porcine SaV was detected from faecal and intestine samples of piglets with enteritis that were negative for PEDV, PDCoV, TGEV, rotavirus or other bacterial enteric pathogens. Independent evidence of viral-induced microscopic lesions and in situ hybridization confirmed that porcine SaV was the cause of the observed clinical scenario, indicating it is the sole aetiological agent. A highly sensitive and specific rRT-PCR method was established for the detection of porcine GIII SaV. An epidemiological survey demonstrated that the clinical pigs had significantly higher viral loads of SaV compared with the non-clinical pigs. Further recombination analysis indicated that intragenogroup recombination occurs within the SaV GIII field isolates. Our findings provide significant insights for a better understanding of the epidemiology and pathogenicity of GIII SaV in swine.

ACKNOWLEDGEMENTS

This study was partially supported by the Swine Health Information Center (SHIC#19-220).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

Ethical statement is not applicable.

DATA AVAILABILITY STATEMENT

All data generated or analysed in this study are included in the article. Those indicated as 'not shown' are available upon request.

ORCID

Jianqiang Zhang https://orcid.org/0000-0001-7228-6202
Leyi Wang https://orcid.org/0000-0001-5813-9505
Ganwu Li https://orcid.org/0000-0002-6370-9573

REFERENCES

Alhatlani, B., Vashist, S., & Goodfellow, I. (2015). Functions of the 5' and 3' ends of calicivirus genomes. *Virus Research*, *206*, 134–143. https://doi.org/10.1016/j.virusres.2015.02.002

Barry, A. F., Alfieri, A. F., & Alfieri, A. A. (2008). High genetic diversity in RdRp gene of Brazilian porcine sapovirus strains. *Veterinary Microbiology*, *131*(1–2), 185–191. https://doi.org/10.1016/j.vetmic.2008.02.021

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics*, *30*(15), 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Burrough, E. R., Wilberts, B. L., Bower, L. P., Jergens, A. E., & Schwartz, K. J. (2013). Fluorescent in situ hybridization for detection of "Brachyspira hampsonii" in porcine colonic tissues. *Journal of Veterinary Diagnostic Investigation*, *25*(3), 407–412. https://doi.org/10.1177/1040638713485228

Chen, Q. I., Wang, L., Zheng, Y., Zhang, J., Guo, B., Yoon, K.-J., Gauger, P. C., Harmon, K. M., Main, R. G., & 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. *Virology Journal*, *15*(1), 95. https://doi.org/10.1186/s12985-018-1001-z

Collins, P. J., Martella, V., Buonavoglia, C., & O'Shea, H. (2009). Detection and characterization of porcine sapoviruses from asymptomatic animals in Irish farms. *Veterinary Microbiology*, *139*(1–2), 176–182. https://doi.org/10.1016/j.vetmic.2009.05.013

das Merces Hernandez, J., Stangarlin, D. C., Siqueira, J. A. M., de Souza Oliveira, D., Portal, T. M., Barry, A. F., Dias, F. A., de Matos, J. C. S., Mascarenhas, J. D'. A. P., & Gabbay, Y. B. (2014). Genetic diversity of porcine sapoviruses in pigs from the Amazon region of Brazil. *Archives of Virology*, *159*(5), 927–933. https://doi.org/10.1007/s00705-013-1904-3

Dey, S. K., Sumiya, M. K., Shaha, M., Haque, R., Okitsu, S., & Ushijima, H. (2018). Intragenogroup recombination in the complete genome Sequence of human sapovirus circulating in Bangladesh. *Genome Announc*, *6*(18), https://doi.org/10.1128/genomeA.00388-18

Dufkova, L., Scigalkova, I., Moutelikova, R., Malenovska, H., & Prodelalova, J. (2013). Genetic diversity of porcine sapoviruses, kobuviruses, and astroviruses in asymptomatic pigs: An emerging new sapovirus GIII genotype. *Archives of Virology*, *158*(3), 549–558. https://doi.org/10.1007/s00705-012-1528-z

Farkas, T., Zhong, W. M., Jing, Y., Huang, P. W., Espinosa, S. M., Martinez, N., Morrow, A. L., Ruiz-Palacios, G. M., Pickering, L. K., & Jiang, X. (2004). Genetic diversity among sapoviruses. *Archives of Virology*, *149*(7), 1309–1323. https://doi.org/10.1007/s00705-004-0296-9

Flynn, W. T., Saif, L. J., & Moorhead, P. D. (1988). Pathogenesis of porcine enteric calicivirus-like virus in four-day-old gnotobiotic pigs. *American Journal of Veterinary Research*, *49*(6), 819–825.

Guo, M., Hayes, J., Cho, K. O., Parwani, A. V., Lucas, L. M., & Saif, L. J. (2001). Comparative pathogenesis of tissue culture-adapted and wild-type Cowden porcine enteric calicivirus (PEC) in gnotobiotic pigs and induction of diarrhea by intravenous inoculation of wild-type PEC. *Journal of Virology*, *75*(19), 9239–9251. https://doi.org/10.1128/JVI.75.19.9239-9251.2001

Keum, H. O., Moon, H. J., Park, S. J., Kim, H. K., Rho, S. M., & Park, B. K. (2009). Porcine noroviruses and sapoviruses on Korean swine farms. *Archives of Virology*, *154*(11), 1765–1774. https://doi.org/10.1007/s00705-009-0501-y

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*(7), 1870–1874. https://doi.org/10.1093/molbev/msw054

Kumthip, K., Khamrin, P., Ushijima, H., Chen, L., Li, S., & Maneekarn, N. (2020). Genetic recombination and diversity of sapovirus in pediatric patients with acute gastroenteritis in Thailand, 2010–2018. *PeerJ*, *8*, e8520. https://doi.org/10.7717/peerj.8520

Kuroda, M., Masuda, T., Ito, M., Naoi, Y., Doan, Y. H., Haga, K., Tsuchiaka, S., Kishimoto, M., Sano, K., Omatsu, T., Katayama, Y., Oba, M., Aoki, H., Ichimaru, T., Sunaga, F., Mukono, I., Yamasato, H., Shirai, J., Katayama, K., … Nagai, M. (2017). Genetic diversity and intergenogroup recombination events of sapoviruses detected from feces of pigs in Japan. *Infection, Genetics and Evolution*, *55*, 209–217. https://doi.org/10.1016/j.meegid.2017.09.013

Lasure, N., & Gopalkrishna, V. (2017). Epidemiological profile and genetic diversity of sapoviruses (SaVs) identified in children suffering from acute gastroenteritis in Pune, Maharashtra, Western India, 2007–2011. *Epidemiology and Infection*, *145*(1), 106–114. https://doi.org/10.1017/S0950268816001953

L'Homme, Y., Brassard, J., Ouardani, M., & Gagne, M. J. (2010). Characterization of novel porcine sapoviruses. *Archives of Virology*, *155*(6), 839–846. https://doi.org/10.1007/s00705-010-0651-y

Li, H., & Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*, *25*(14), 1754–1760. https://doi.org/10.1093/bioinformatics/btp324

Li, J., Shen, Q., Zhang, W., Zhao, T., Li, Y. I., Jiang, J., Yu, X., Guo, Z., Cui, L. I., & Hua, X. (2017). Genomic organization and recombination analysis of a porcine sapovirus identified from a piglet with diarrhea in China. *Virology Journal*, *14*(1), 57. https://doi.org/10.1186/s12985-017-0729-1

Liu, G.-H., Li, R.-C., Huang, Z.-B., Yang, J., Xiao, C.-T., Li, J., Li, M.-X., Yan, Y.-Q., & Yu, X.-L. (2012). RT-PCR test for detecting porcine sapovirus

in weanling piglets in Hunan Province, China. *Tropical Animal Health and Production*, *44*(7), 1335–1339. https://doi.org/10.1007/s11250-012-0138-9

Liu, X., Yamamoto, D., Saito, M., Imagawa, T., Ablola, A., Tandoc, A. O., Segubre-Mercado, E., Lupisan, S. P., Okamoto, M., Furuse, Y., Saito, M., & Oshitani, H. (2015). Molecular detection and characterization of sapovirus in hospitalized children with acute gastroenteritis in the Philippines. *Journal of Clinical Virology*, *68*, 83–88. https://doi.org/10.1016/j.jcv.2015.05.001

Lole, K. S., Bollinger, R. C., Paranjape, R. S., Gadkari, D., Kulkarni, S. S., Novak, N. G., Ingersoll, R., Sheppard, H. W., & Ray, S. C. (1999). Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *Journal of Virology*, *73*(1), 152–160. https://doi.org/10.1128/JVI.73.1.152-160.1999

Martinez, M. A., Alcala, A. C., Carruyo, G., Botero, L., Liprandi, F., & Ludert, J. E. (2006). Molecular detection of porcine enteric caliciviruses in Venezuelan farms. *Veterinary Microbiology*, *116*(1–3), 77–84. https://doi.org/10.1016/j.vetmic.2006.03.016

Menzel, P., Ng, K. L., & Krogh, A. (2016). Fast and sensitive taxonomic classification for metagenomics with Kaiju. *Nature Communications*, *7*, 11257. https://doi.org/10.1038/ncomms11257

Mijovski, J. Z., Poljsak-Prijatelj, M., Steyer, A., Barlic-Maganja, D., & Koren, S. (2010). Detection and molecular characterisation of noroviruses and sapoviruses in asymptomatic swine and cattle in Slovenian farms. *Infection, Genetics and Evolution*, *10*(3), 413–420. https://doi.org/10.1016/j.meegid.2009.11.010

Nagai, M., Wang, Q., Oka, T., & Saif, L. J. (2020). Porcine sapoviruses: Pathogenesis, epidemiology, genetic diversity, and diagnosis. *Virus Research*, *286*, 198025. https://doi.org/10.1016/j.virusres.2020.198025

Nakamura, K., Saga, Y., Iwai, M., Obara, M., Horimoto, E., Hasegawa, S., Kurata, T., Okumura, H., Nagoshi, M., & Takizawa, T. (2010). Frequent detection of noroviruses and sapoviruses in swine and high genetic diversity of porcine sapovirus in Japan during Fiscal Year 2008. *Journal of Clinical Microbiology*, *48*(4), 1215–1222. https://doi.org/10.1128/JCM.02130-09

Oka, T., Lu, Z., Phan, T., Delwart, E. L., Saif, L. J., & Wang, Q. (2016). Genetic characterization and classification of human and animal sapoviruses. *PLoS One*, *11*(5), e0156373. https://doi.org/10.1371/journal.pone.0156373

Oka, T., Wang, Q., Katayama, K., & Saif, L. J. (2015). Comprehensive review of human sapoviruses. *Clinical Microbiology Reviews*, *28*(1), 32–53. https://doi.org/10.1128/CMR.00011-14

Ondov, B. D., Bergman, N. H., & Phillippy, A. M. (2011). Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics*, *12*, 385. https://doi.org/10.1186/1471-2105-12-385

Reuter, G., Zimsek-Mijovski, J., Poljsak-Prijatelj, M., Di Bartolo, I., Ruggeri, F. M., Kantala, T., Maunula, L., Kiss, I., Kecskemeti, S., Halaihel, N., Buesa, J., Johnsen, C., Hjulsager, C. K., Larsen, L. E., Koopmans, M., & Bottiger, B. (2010). Incidence, diversity, and molecular epidemiology of sapoviruses in swine across Europe. *Journal of Clinical Microbiology*, *48*(2), 363–368. https://doi.org/10.1128/JCM.01279-09

Saif, L. J., Bohl, E. H., Theil, K. W., Cross, R. F., & House, J. A. (1980). Rotavirus-like, calicivirus-like, and 23-nm virus-like particles associated with diarrhea in young pigs. *Journal of Clinical Microbiology*, *12*(1), 105–111. https://doi.org/10.1128/JCM.12.1.105-111.1980

Salamunova, S., Jackova, A., Mandelik, R., Novotny, J., Vlasakova, M., & Vilcek, S. (2018). Molecular detection of enteric viruses and the genetic characterization of porcine astroviruses and sapoviruses in domestic pigs from Slovakian farms. *BMC Veterinary Research*, *14*(1), 313. https://doi.org/10.1186/s12917-018-1640-8

Scheuer, K. A., Oka, T., Hoet, A. E., Gebreyes, W. A., Molla, B. Z., Saif, L. J., & Wang, Q. (2013). Prevalence of porcine noroviruses, molecular characterization of emerging porcine sapoviruses from finisher swine in the United States, and unified classification scheme for sapoviruses. *Journal of Clinical Microbiology*, *51*(7), 2344–2353. https://doi.org/10.1128/JCM.00865-13

Simpson, J. T., Wong, K., Jackman, S. D., Schein, J. E., Jones, S. J., & Birol, I. (2009). ABySS: A parallel assembler for short read sequence data. *Genome Research*, *19*(6), 1117–1123. https://doi.org/10.1101/gr.089532.108

Tohma, K., Kulka, M., Coughlan, S., Green, K. Y., & Parra, G. I. (2020). Genomic analyses of human sapoviruses detected over a 40-year period reveal disparate patterns of evolution among genotypes and genome regions. *Viruses*, *12*(5), 516. https://doi.org/10.3390/v12050516

Valente, C. S., Alfieri, A. F., Barry, A. F., Leme, R. A., Lorenzetti, E., & Alfieri, A. A. (2016). Age distribution of porcine sapovirus asymptomatic infection and molecular evidence of genogroups GIII and GIX? circulation in distinct Brazilian pig production systems. *Tropical Animal Health and Production*, *48*(1), 21–27. https://doi.org/10.1007/s11250-015-0912-6

Wang, L., Marthaler, D., Fredrickson, R., Gauger, P. C., Zhang, J., Burrough, E. R., Petznick, T., & Li, G. (2020). Genetically divergent porcine sapovirus identified in pigs, United States. *Transboundary and Emerging Diseases*, *67*(1), 18–28. https://doi.org/10.1111/tbed.13337

Wang, Q. H., Han, M. G., Funk, J. A., Bowman, G., Janies, D. A., & Saif, L. J. (2005). Genetic diversity and recombination of porcine sapoviruses. *Journal of Clinical Microbiology*, *43*(12), 5963–5972. https://doi.org/10.1128/JCM.43.12.5963-5972.2005

Wang, Q. H., Souza, M., Funk, J. A., Zhang, W., & Saif, L. J. (2006). Prevalence of noroviruses and sapoviruses in swine of various ages determined by reverse transcription-PCR and microwell hybridization assays. *Journal of Clinical Microbiology*, *44*(6), 2057–2062. https://doi.org/10.1128/JCM.02634-05

Wood, D. E., & Salzberg, S. L. (2014). Kraken: Ultrafast metagenomic sequence classification using exact alignments. *Genome Biology*, *15*(3), R46. https://doi.org/10.1186/gb-2014-15-3-r46

Yinda, C. K., Conceição-Neto, N., Zeller, M., Heylen, E., Maes, P., Ghogomu, S. M., Van Ranst, M., & Matthijnssens, J. (2017). Novel highly divergent sapoviruses detected by metagenomics analysis in straw-colored fruit bats in Cameroon. *Emerging Microbes & Infections*, *6*(5), e38. https://doi.org/10.1038/emi.2017.20

Yu, J. N., Kim, M. Y., Kim, D. G., Kim, S. E., Lee, J. B., Park, S. Y., Song, C. S., Shin, H. C., Seo, K. H., & Choi, I. S. (2008). Prevalence of hepatitis E virus and sapovirus in post-weaning pigs and identification of their genetic diversity. *Archives of Virology*, *153*(4), 739–742. https://doi.org/10.1007/s00705-008-0046-5

Zhang, J., Zheng, Y., Xia, X. Q., Chen, Q., Bade, S. A., Yoon, K. J., & Li, G. (2017). High-throughput whole genome sequencing of Porcine reproductive and respiratory syndrome virus from cell culture materials and clinical specimens using next-generation sequencing technology. *Journal of Veterinary Diagnostic Investigation*, *29*(1), 41–50. https://doi.org/10.1177/1040638716673404

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Shen H, Zhang J, Gauger PC, et al. Genetic characterization of porcine sapoviruses identified from pigs during a diarrhoea outbreak in Iowa, 2019. *Transbound Emerg Dis*. 2021;00:1–10. https://doi.org/10.1111/tbed.14087