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Genetic Diversity and Recombination of Porcine Sapoviruses

Qiu-Hong Wang,[1] Myung Guk Han,[1] Julie A. Funk,[2] Gary Bowman,[2] Daniel A. Janies,[3] and Linda J. Saif[1]*

Food Animal Health Research Program, Ohio Agricultural Research and Development Center, Department of Veterinary Preventive Medicine, The Ohio State University, Wooster, Ohio 44691,[1] and Department of Veterinary Preventive Medicine, College of Veterinary Medicine,[2] and Department of Biomedical Informatics,[3] The Ohio State University, Columbus, OH 43210

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Sapoviruses (SaVs) are emerging enteric pathogens that cause diarrhea in humans and animals. Human SaVs are genetically variable and have been classified into four genogroups (GI, -II, -IV, and -V). To date, only two genetically similar porcine SaV strains have been reported that belong to GIII. To investigate the genetic diversity of porcine SaVs and their genetic relatedness to human strains, we sequenced 286 nucleotides (nt) of the RNA-dependent RNA polymerase (RdRp) region of nine porcine SaVs detected from field pig fecal samples collected in U.S. swine farms during the period from 1999 to 2003. One strain (Po/SaV/MI-QW19/2002/US) was most closely related to human GII SaVs. We also sequenced 3 kb of the viral genome, including the partial RdRp (766 to 790 nt), the complete capsid, the ORF2 and the 3′-untranslated region of four strains representative for the positive farms or for the distinct genetic clusters. From the sequence analysis of the complete capsid, we identified a potential new genogroup of porcine SaVs, with Po/SaV/OH-JJ681/00/US as the representative strain. Furthermore, two potential porcine SaV recombinants were identified. To our knowledge this is the first report of a porcine SaV strain more closely related genetically to human SaVs and the occurrence of porcine SaV recombinants. The presence of porcine SaVs more similar to human SaVs is a significant finding because of the potential for zoonotic infections or generation of porcine/human recombinants if intragenogroup human strains exist.

Sapoviruses (SaV), previously referred to as Sapporo-like viruses, are emerging enteric pathogens that cause diarrhea in humans, pigs, and mink (5, 7, 9, 10, 22). They are nonenveloped, polyadenylated single-stranded positive-sense RNA viruses and belong to the *Sapovirus* genus in the family *Caliciviridae* (7). The SaV genome is 7.3 to 7.5 kb long and contains two main open reading frames (ORFs) based on sequence analysis (8, 15, 20, 26). The ORF1 encodes a polyprotein that undergoes protease processing to produce several nonstructural proteins, including an RNA-dependent RNA polymerase (RdRp) and a capsid protein. The ORF2 encodes a small basic protein with unknown function.

Human SaVs are primarily associated with 1.8 to 9% cases of pediatric acute gastroenteritis (3, 17, 24, 28), although SaV outbreaks in adults have been described (23). Human SaVs are genetically variable. They have been classified into four genogroups (GI, -II, -IV, and -V) and at least eight genetic clusters or genotypes (GI/1 to -3, GII/1 to -3, GIV/1, and GV/1) (3, 29). Porcine SaV Cowden strain was isolated from a 27-day-old diarrheic field pig (27). It causes diarrhea and intestinal lesions in gnotobiotic pigs (10). However, only two porcine SaV strains (Po/SaV/Cowden/80/US and Po/SaV/LL14/02/US) have been reported (2, 8). They are genetically closely related sharing 96% nucleotide identity throughout the ORF1 and ORF2. The complete genome of the Cowden strain has been analyzed, and it is classified as SaV GIII (8). It is the only cultivable SaV or enteric calicivirus (2, 4, 25).

Recently, we identified porcine noroviruses, another genus of enteric caliciviruses causing diarrhea in humans and animals, that are genetically and antigenically similar to human noroviruses (33), raising questions of whether pigs may be reservoirs for emergence of human noroviruses. To investigate the genetic diversity of porcine SaVs and their relatedness to human SaVs, we chose a pair of calicivirus universal primers p290 and p110 targeting the conserved motifs "DYSKWDST" and "YGDD" of the RdRp region of caliciviruses (14, 19) to perform reverse transcription-PCR (RT-PCR) to screen for genetically variable SaVs in pigs. Nine SaVs were identified from field pig fecal samples collected from US swine farms during the period from 1999 to 2003. We further sequenced the 3′ end 3 kb, including partial RdRp, the complete capsid, and ORF2 regions of four strains representative for the positive farms or for the distinct genetic clusters. We then classified these newly identified porcine SaVs by phylogenetic analysis and recombination identification programs.

MATERIALS AND METHODS

Stool samples. A total of 377 fecal samples were collected from eight swine farms (OH farms A to E, MI farm A, and NC farms A and B) and one OH slaughterhouse from April 1999 to May 2003 and were surveyed for the presence of genetically diverse enteric caliciviruses. Nine SaV-positive pig fecal samples were identified by RT-PCR with a calicivirus universal primer pair p290/110 (14, 19), followed by sequencing of the RT-PCR products. The age and diarrhea status of the pigs from which these nine samples were obtained is summarized (Table 1). The MM280 strain (the large intestinal contents of a gnotobiotic pig) was the third passage of one field pig fecal sample performed with gnotobiotic pigs as previously reported (10). This sample was amplified in pigs because the SaV amount in the original specimen was inadequate for analysis. Fresh fecal samples or intestinal contents were placed into sterile containers and stored frozen at −20 or −70°C until tested.

* Corresponding author. Mailing address: Food Animal Health Research Program, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Ave., Wooster, OH 44691. Phone: (330) 263-3744. Fax: (330) 263-3677. E-mail: saif.2@osu.edu.

TABLE 1. Porcine sapovirus strains detected using primers p290/110 and sequenced in this study

Strain	Sample no.	Date of sampling	Swine farm	Pig age	Diarrhea	GenBank accession no.
Po/SaV/OH-II166/99/US	II166	Apr. 1999	OH D	Postweaning	No	AY974193
Po/SaV/OH-II176/99/US	II176	May 1999	OH C	Postweaning	Yes	AY974194
Po/SaV/OH-JJ259/00/US	JJ259	June 2000	OH C	Postweaning	Yes	AY826423
Po/SaV/OH-JJ681/00/US	JJ681	Dec. 2000	OH E	Postweaning	Yes	AY974192
Po/SaV/OH-LL26/02/US	LL26	Apr. 2002	OH C	Postweaning	No	AY974195
Po/SaV/MI-QW19/02/US	QW19	Dec. 2002	MI A	Finisher	No	AY826424
Po/SaV/OH-QW152/03/US	QW152	Mar. 2003	OH B	Finisher	No	AY826425
Po/SaV/OH-QW270/03/US	QW270	June 2003	NC B	Finisher	No	AY826426
Po/SaV/OH-MM280/03/US	MM280	Mar. 2003	OH B	Finisher	No	AY823308

RNA extraction. The RNA was extracted from 10% (wt/vol) fecal suspensions by using the TRIzol LS (Invitrogen, Corp., Carlsbad, CA). For amplification of the 3′-end 3-kb fragment of these samples, except for strain MM280, the extracted RNA was further concentrated and purified by using the QIAamp Viral RNA Minikit (QIAGEN, Inc., Valencia, CA). For amplification of the 3′-end 3-kb fragment of QW19, 60 ml of a 10% fecal suspension was filtered (0.45-μm pore size) and semipurified through a 40% (wt/vol) sucrose cushion by ultracentrifugation at 112,700 × *g* for 2 h. The pellets were resuspended in 1 ml of sterile Eagle minimal essential medium (Invitrogen Corp., Carlsbad, CA) for RNA extraction and purification.

RT-PCR. RT and PCR were performed separately using primer pair p290 (5′-GAT TAC TCC AAG TGG GAC TCC AC-3′) (14) and p110 (5′-ACD ATY TCA TCA TCA CCA TA-3′) (19) as previously reported but with 48°C for annealing (14). Products of primer pair p290/110 were 329 bp for SaVs. To amplify the 3′-end, 3-kb fragment of the porcine SaVs, the cDNA was synthesized by SuperScript III First-Strand cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions with primer VN_3T_{20} (5′-GAG TGA CCG CGG CCG CT_{20}-3′) (33). The first PCR was then performed by using TaKaRa Ex Taq polymerase (TaKaRa Mirus Bio, Madison, WI) with the primers p290 and VN_3T_{20}. For strains QW270 and JJ681, a second PCR was necessary to produce a visible band on agarose gel electrophoresis, using internal forward primers PSV4b (5′-GCA TGC CC(/G)T TCA CCA GTG T-3′) and PEC68 (5′-CCG CTA TAA ATT TAT TGG GTG-3′), respectively, based on the obtained 286-nucleotide (nt) RdRp region of QW270 and JJ681 strains, and the reverse primer VN_3T_{20}. After initial denaturation at 94°C for 3 min, the first 5 cycles were performed at 94°C for 30 s, 50°C for 30 s, and 72°C for 5 min, followed by another 30 cycles with a shorter elongation time of 3 min, and a final extension for 10 min at 72°C. All RT-PCR products were analyzed on agarose gel electrophoresis and stained with ethidium bromide. The RT-PCR product bands were visualized by using UV light.

Cloning and sequencing. The RT-PCR products were purified by using a QIAquick gel extraction kit (QIAGEN, Inc.). Products of primer pair p290/110 were sequenced directly using primers p290 and p110 or after cloning into pCR2.1 (T/A) vector (Invitrogen). The ~3-kb RT-PCR products were cloned by using a PCR XL cloning kit (Invitrogen). Plasmid DNA was extracted by using an alkaline lysis method. The insertion was confirmed by restriction enzyme EcoRI digestion. Three to five clones of each sample were sequenced by a primer-walking strategy. DNA sequencing was done by using BigDye Terminator Cycle chemistry and 3730 DNA Analyzer (Applied Biosystems, Foster, CA).

Sequence analysis. Sequence editing was performed by Lasergene software package (version 5) (DNASTAR, Inc., Madison, WI). Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov) with default values was used to find homologous hits (1). A hit with an expected value ≤0.001 was considered significant. Multiple sequence alignment was done by CLUSTAL W (version 1.83) at DDBJ (http://www.ddbj.nig.ac.jp). Phylogenetic analysis (neighbor-joining) with bootstrap (1,000 replicates) was conducted by using MEGA v2.1 (18). Recombinants were identified by using the recombinant identification program (RIP) (30) (http://hivweb.lanl.gov/RIP/RIPsubmit.html) and POY software

TABLE 2. Summary of sapovirus strains and representative strains for *Lagovirus*, *Vesivirus*, and *Norovirus* genera and NB-like viruses used in sequence analysis

Strain	Genus/genogroup-genotype	Abbreviation	GenBank accession no.
Hu/Sapporo/82/JP	SaV/GI-1	Sapporo	U65427
Hu/Manchester/93/UK	SaV/GI-1	Manchester	X86560
Hu/Plymouth/92/UK	SaV/GI-1	Plymouth	X86559
Hu/Lyon30388/98/FR	SaV/GI-1	Lyon30388	AJ251991
Hu/Houston/86/US	SaV/GI-1	Houston86	U95643
Hu/Parkville/94/US	SaV/GI-2	Parkville	U73124
Hu/Houston/90/US	SaV/GI-2	Houston90	U95644
Hu/Stockholm/97/SE	SaV/GI-3	Stockholm	AF194182
Hu/Mex14917/00/MX	SaV/GI-3	Mex14917	AF435813 and AF435810
Hu/London/92/UK	SaV/GII-1	London92	U95645
Hu/Lyon598/97/FR	SaV/GII-1	Lyon598	AJ271056
Hu/Bristol/98/UK	SaV/GII-1	Bristol	AJ249939
Hu/Mex340/90/MX	SaV/GII-2	Mex340	AF435812 and AF435809
Hu/Cruise ship/00/US	SaV/GII-3	Cruise ship	AY289804 and AY157863
Hu/Mc10/00/TH	SaV/GII-4	Mc10	AY237420
Hu/C12/00/JP	SaV/GII-5	C12	AY603425
Po/Cowden/80/US	SaV/GIII	Cowden	AF182760
Po/LL14/02/US	SaV/GIII	LL14	AY425671
Hu/Hou7-1181/90/US	SaV/GIV	Hou7	AF435814
Hu/Argentina39/Arg	SaV/GV	Arg39	AY289803 and AF405715
Mink/MEC/1/1999/US	SaV/G?	MEC	AF338404
Ra/RHDV/GH/1988/GE	*Lagovirus*	RHDV	M67473
Fe/FCV/F9/1958/US	*Vesivirus*	FCV	M86379
Hu/Norwalk/68/US	*Norovirus*	Norwalk	M87661
Bo/Nebraska/80/US	NB-like viruses	NB	AY082891

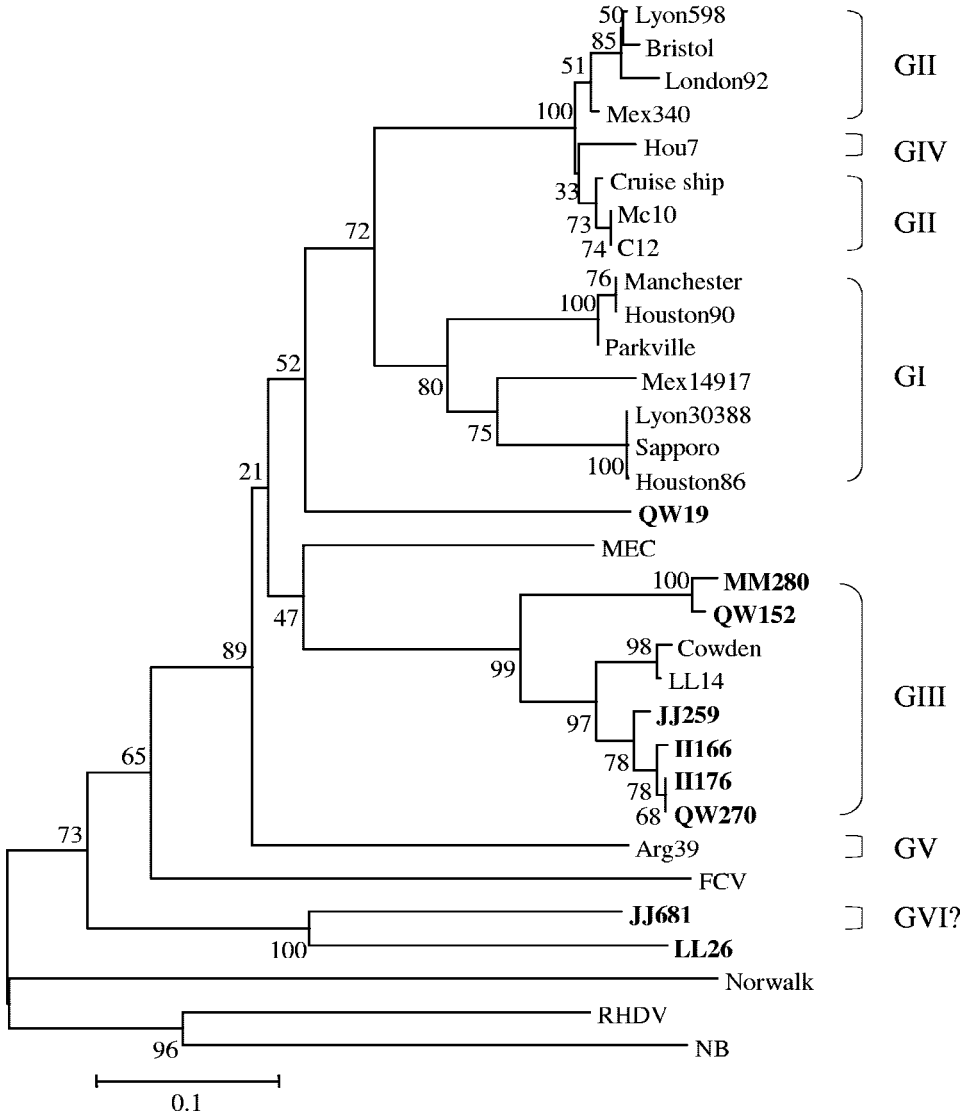


FIG. 1. Neighbor-joining phylogenetic tree based on the partial RdRp region (95 aa) of SaVs and RHDV, NB, Norwalk, and FCV strains representing *Lagovirus*, NB-like virus, *Norovirus*, and *Vesivirus* genera, respectively, in *Caliciviridae*. The nine newly identified porcine SaV strains are in boldface.

v3.0.12a (34) (http://research.amnh.org/scicomp/projects/poy.php). The classification and GenBank accession numbers of published representative strains for phylogenetic analysis are listed in Table 2.

RESULTS

Genetically diverse porcine SaVs were detected. A total of 35 from 377 samples (9%) were potentially positive by RT-PCR with the primer pair p290/110. We sequenced the RT-PCR products (317- or 329-bp products) of 23 samples, at least 1 sample from each positive farm at one sampling time. After we performed a BLAST search (blastn and blastx), we identified nine SaVs (Table 1) and seven noroviruses. Seven sequences did not have any significant hit in the nucleotide database. Because they did not contain the calicivirus RdRp conserved motif "GLPSG," they were not analyzed further. All of the SaVs and noroviruses contained the conserved amino acid motif "GLPSG" of calicivirus RdRp. The SaV-specific sequence was 286 nt encoding 95 amino acids (aa) of the RdRp region.

Neighbor-joining phylogenetic analysis was performed based on the 95-aa RdRp sequences of porcine, human, and mink SaVs. The Ra/Lagovirus/RHDV/GH/1988/GE (RHDV), Fe/Vesivirus/FCV/F9/1958/US (FCV), Hu/Norovirus/Norwalk/68/US (Norwalk), and Bo/Nebraska/80/US (NB, a potentially new genus) are representative strains of *Lagovirus*, *Vesivirus*, and *Norovirus* genera and the NB-like viruses, respectively, within the *Caliciviridae* family (7, 31) (Fig. 1). We found that JJ259, II166, II176, and QW270 grouped with the porcine SaV prototype Cowden strain and LL14 strain, sharing 91.6 to 98.9% amino acid identities. The QW152 and MM280 were similar to each other and shared 81.1% amino acid identities with the Cowden strain, whereas the QW19, JJ681, and LL26

TABLE 3. Sizes of the predicted capsid protein, the ORF1-ORF2 overlap region, the ORF2 protein, and the 3′-UTR of porcine sapoviruses

Genogroup/strain	Capsid (aa)	ORF1-ORF2 overlap (nt)	ORF2 protein (aa)	3′-UTR (nt)
GIII/Cowden	544	4	164	55
GIII/QW270	544	4	164	55
GIII/MM280	544	4	164	55
GIII/JJ259	544	4	173	55
GVI?/JJ681	554	4	168	28

strains segregated into three new branches distant from the Cowden group. Interestingly, the QW19 strain shared the highest amino acid identity (66.3%) with human GII Mc10, C12, and Cruise ship strains. In addition, the JJ681 strain shared the highest amino acid identity (63.2%) with the LL26 strain, but both showed low amino acid identities to SaVs (25.3 to 47.4%), FCV (31.6 to 33.7%), RHDV (33.0 to 34.0%), NB (32.6 to 38), and Norwalk (19.8 to 24.2%) strains.

A potential new genogroup of porcine SaVs was found based on the complete capsid sequences. To genetically classify the newly identified porcine SaVs, we chose QW270, MM280, JJ259, JJ681, QW19, and LL26 as representative strains for further analysis. Attempts to amplify the QW19 and LL26 strains, which included concentrating and purifying the viral RNA and performing second PCR with internal forward primers, were unsuccessful. We also tried but failed to detect another QW19-like strain from the 60 pig fecal samples collected at the same time from the same farm as the QW19 strain. For this RT-PCR, we used a pair of QW19-specific primers PSV6 (5′-CGG TCA TTT TGT GTG GAC TG-3′) and PSV7 (5′-ATT GCC CGT ATA AGG CAC A-3′) based on the obtained 286 nt of the RdRp region.

We successfully amplified and sequenced the 3′-end 3-kb of genome containing the partial RdRp (263 aa for the QW270, MM280, and JJ259 strains and 255 aa for the JJ681 strain), capsid protein, and ORF2 genes and the 3′-untranslated region (UTR) of the QW270, MM280, JJ259, and JJ681 strains. The sizes of the deduced capsid and the ORF2 proteins, the ORF1-ORF2 overlap region and the 3′-UTR of each newly identified strain and the porcine SaV prototype Cowden strain are summarized in Table 3. The QW270 and MM280 strains had exactly the same sizes for each region as the Cowden strain. The JJ259 strain had the same size capsid, ORF1-ORF2 overlap, and the 3′-UTR as those of the Cowden strain but not the ORF2 protein. For the JJ681 strain, only the ORF1-ORF2 overlap region was identical in size and nucleotide sequence (ATGA) to that of the other four strains; the other targeted region sizes, especially for the 3′-UTR, were highly divergent from the other porcine SaVs.

The amino acid and nucleotide percent identities of the deduced complete capsid protein sequences of the four newly identified porcine SaVs, the previously reported porcine and human SaVs, and one representative strain of each of the other four genera within the *Caliciviridae* family: RHDV, Norwalk, FCV, and NB (a potentially new genus) are summarized in Table 4. In the complete capsid region, QW270, MM280 and JJ259 strains were genetically closest to the porcine SaV prototype Cowden strain with an amino acid identity of 88 to 95%. The JJ681 strain differed genetically from these strains but showed amino acid (32 to 36%) and nucleotide (45 to 48%) identities similar to those of the different genogroups of SaVs. These percent identities were higher than those to representative strains of the other four genera: NB (23% amino acid and 40% nucleotide identities), RHDV (21% amino acid and 39% nucleotide identities), FCV (23% amino acid and 39% nucleotide identities), and Norwalk strains (17% amino acid and 34% nucleotide identities). The previously reported SaV intragenus sequence identity was somewhat higher than that noted for JJ681 strain, ranging from 37 to 49% for amino acids and 49 to 58% for nucleotides (3). The intergenus amino acid identity of caliciviruses is 15 to 27% (7, 31). A neighbor-joining phylogenetic tree based on the deduced amino acid sequences of the complete capsid (Fig. 2A) revealed that QW270, MM280, and JJ259 strains grouped with Cowden strain into GIII. The JJ681 and other SaVs segregated into different groups from a common ancestor with a high bootstrap value (99%). Based on these data, we tentatively classified the JJ681 into a new genogroup (JJ681-like, GVI?) within the *Sapovirus* genus.

Potential porcine SaV recombinants were identified. Further analysis of the deduced C-terminal ~260 aa of the RdRp region showed that all of the four newly identified porcine SaVs contained another calicivirus RdRp conserved motif "YGDD." The neighbor-joining phylogenetic tree based on the nucleotide sequence of this partial RdRp region (Fig. 2B) showed grouping results similar to those based on the capsid-tree (Fig. 2A) for JJ259 and JJ681 strains, but the RdRp and capsid assignments differed for QW270 and MM280 strains with high bootstrap values (93 to 100%). Although QW270 strain shared the highest nucleotide identity (90%) with Cowden strain in the capsid region, it shared only 83.4% nucleotide identity with Cowden strain in this partial RdRp region. However, it shared the highest nucleotide identity (96.5%) to JJ259 strain in this RdRp region. The MM280 strain shared lower nucleotide identity with Cowden strain in this RdRp region (76.2%) compared to that of the capsid protein (89.1%), which was opposite to the general phenomenon observed for caliciviruses that the RdRp region is more conserved than the capsid region.

The deduced ORF2 protein sequences of the four representative strains were also analyzed. Similar to the classification based on the capsid protein, QW270, MM280, and JJ259 were grouped into the same cluster as Cowden strain, whereas JJ681 strain again formed a new branch distant from the other SaVs. The JJ259 had a 9-aa insertion (GVTTTPKPQ) compared to the other strains, including QW270 strain in this cluster. The QW270 strain was most similar to Cowden strain sharing 95% amino acid identity, but only sharing 87% amino acid identity with JJ259 strain.

These results suggested that QW270 was a potential recombinant between JJ259-like (RdRp segment) and Cowden-like (after RdRp) SaVs and a recombination event probably occurred for MM280 and Cowden-like SaVs. To determine where the recombination occurred for QW270 and MM280/Cowden strains, we performed the RIP and POY analyses. For the RIP analysis, we placed the 3′-end 3 kb, from the C-terminal RdRp (aligned 843 nt) to just before the poly(A) tail,

of the QW270 and MM280 strains as a query sequence, respectively, and the corresponding sequences of Cowden (and JJ259) as a background sequence(s). The QW270 strain showed high homology to the JJ259 strain in the RdRp region, but it changed to shared high homology to Cowden strain in the capsid and ORF2 protein regions (Fig. 3A). The MM280 strain abruptly changed to high identity with the Cowden strain (from 68 to 90%) at the RdRp-capsid junction region (Fig. 3B). To accomplish the POY analyses, we first analyzed sequence data under a mutation only model (substitution events cost 1, and insertion/deletion events cost 2) and then demarcated regions of the sequence data considered as incongruent by RIP as potential breakpoints and analyzed the data under a mutation plus recombination model (substitution events cost 1, insertion/deletion events cost 2, and recombination events 300). Levels of tree-building replication and refinement were the same in the two analyses. We found that the combined length of the tree produced under the mutation model is 11,325 steps, whereas the combined length of the tree produced under mutation plus recombination model is 8,207 steps. The more efficient tree was achieved by reconstruction of 16 recombination events. Notable among these events were: (i) the recombination of Cowden strain (providing the RdRp) and JJ259 strain (providing the capsid) as parental strains for the progeny QW270 strain and (ii) Cowden (providing the capsid) as a parental strain for MM280 (whose RdRp is explained via mutation under these analytical conditions and taxon sampling).

We also performed nucleotide alignments around the RdRp-capsid junction region where was the potential crossover region of the potential recombination events suggested by RIP analysis (Fig. 4). This region contains the genomic-subgenomic RNA conserved 26-nt motif (7). We found that the nucleotide sequences were highly conserved among strains within each genogroup. Only one nucleotide mismatch among the Cowden, QW270, JJ259, and MM280 strains was found within the 26-nt motif.

All of these results indicated that QW270 was a potential recombinant between a JJ259-like strain (RdRp region) and a Cowden-like strain (after RdRp) and a potential recombination event occurred for MM280 and Cowden-like SaVs. We found that the RdRp of MM280 evolved via mutation.

DISCUSSION

Since the discovery of porcine SaVs in the 1980s (27), few further studies have been done to investigate their genetic diversity, the possibility of zoonotic transmission, and the disease spectrum of field strains. In the present study, we detected genetically variable porcine SaVs from swine in the United States and investigated their genetic relationship to human and mink SaVs and to the other four genera (*Lagovirus*, *Vesivirus*, *Norovirus*, and NB-like viruses) in the *Caliciviridae*.

In addition to the nine SaVs and seven noroviruses identified, seven more sequences amplified by the primer pair p290/110 at low-stringency conditions (low annealing temperature at 48°C) did not contain the calicivirus RNA polymerase conserved motif "GLPSG" and did not have any significant hit by BLAST search in the nucleotide database. These sequences probably are amplified from other existing microbes in the pig intestine and feces, which have not been fully sequenced. Elec-

TABLE 4. Summary of amino acid and nucleotide sequence identities in the complete capsid region between the newly identified porcine sapoviruses and reference calicivirus strains

Genus/genogroup genotype	Strain	% Amino acid sequence identity (% nucleotide sequence identity)												
		Sapovirus										NB-like viruses	*Lagovirus* RHDV	*Vesivirus* FCV	*Norovirus* Norwalk
		GI/1[a]	GI-2[b]	GI-3[c]	GII-1[d]	GII-2[e]	GII-3[f]	GII-4[g]	GIII[h]	GIV[i]	GV[j]				
Sapovirus/GIII	QW270	40 (50–51)	39–40 (48)	39–40 (48)	38–39 (50)	38 (49–50)	39 (50)	40 (49)	95 (90)	39 (47)	43 (51)	24 (41)	24 (42)	24 (39)	18 (38)
Sapovirus/GIII	MM280	40–41 (50)	40 (47)	39–41 (48)	39 (49)	39–40 (49–50)	40 (49)	40 (50)	91–93 (88–89)	40 (49)	43 (51)	24 (40)	24 (40)	24 (38)	19 (37)
Sapovirus/GIII	JJ259	39–40 (50–51)	38–39 (49)	39–40 (48–49)	36–37 (47–48)	38 (50)	38 (49)	38 (50)	88–90 (85–86)	39 (49)	43 (51)	23 (41)	23 (41)	24 (38)	18 (37)
Sapovirus/GVI?	JJ681	33–34 (47)	32–33 (45)	34–35 (46)	33–34 (46–47)	34–35 (46–47)	35 (47)	34 (46)	36 (47–48)	34 (46)	35 (45)	23 (40)	21 (39)	23 (39)	17 (34)

[a] GI/1 includes Sapporo, Houston86, Manchester, Plymouth, and Lyon30388.
[b] GI-2 includes Houston90 and Parkville.
[c] GI-3 includes Stockholm and Mex14917.
[d] GII-1 includes London92, Bristol, and Lyon598.
[e] GII-2 includes Mex340 and Mc10.
[f] GII-3 includes Cruise ship.
[g] GII-4 includes C12.
[h] GIII includes Cowden and LL14.
[i] GIV includes Hou7.
[j] GV includes Arg39.

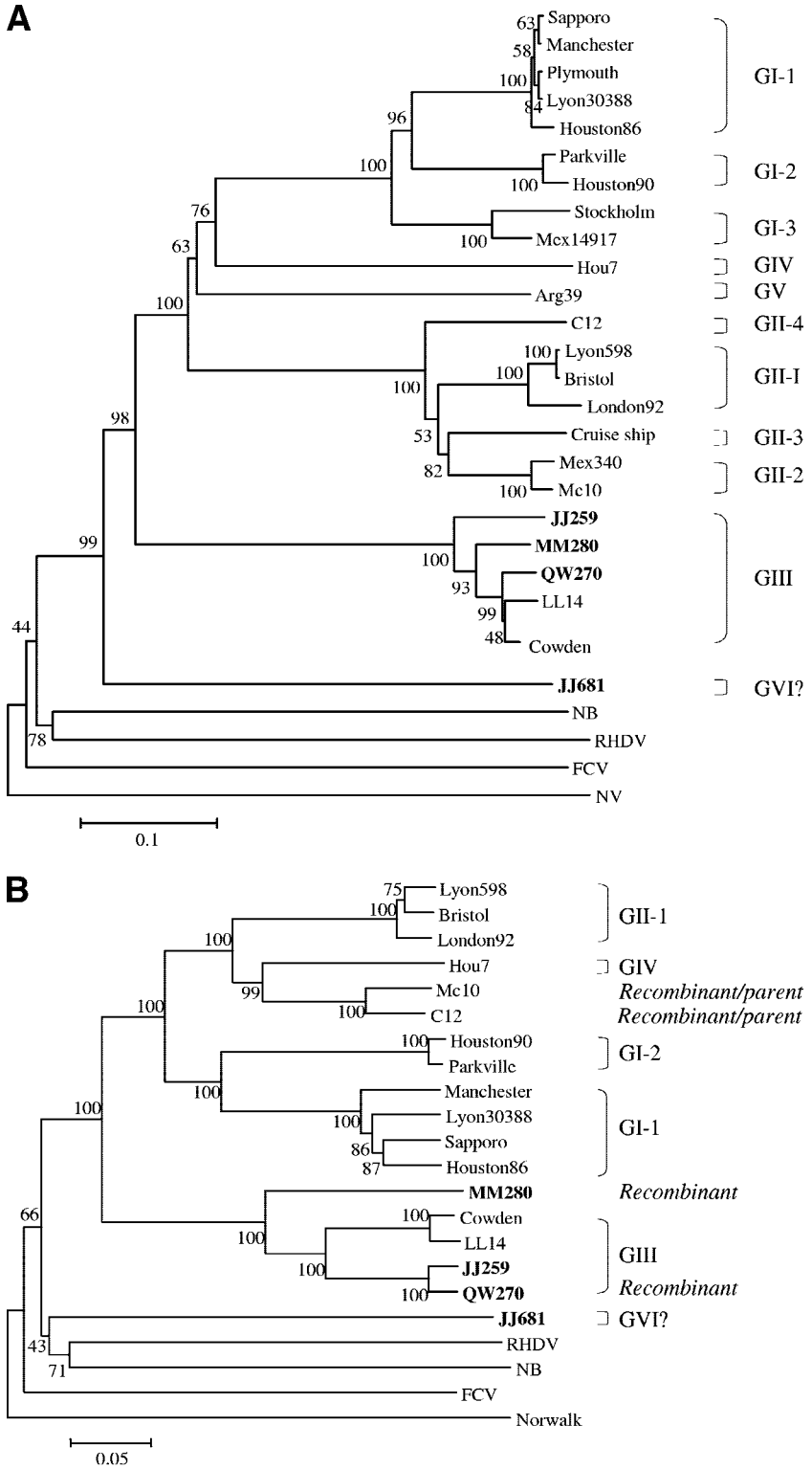


FIG. 2. Neighbor-joining phylogenetic trees of SaVs and RHDV, NB, Norwalk, and FCV strains representing *Lagovirus*, NB-like virus, *Norovirus*, and *Vesivirus* genera, respectively, in *Caliciviridae*. (A) Tree based on the deduced complete capsid amino acid sequences. (B) Tree based on the partial RdRp region (C-terminal aligned 843 nt). The SaV genogroups (G plus roman numerals) and genotypes (Arabic numbers following genogroup numbers) are indicated. The four newly sequenced porcine SaV strains are in boldface.

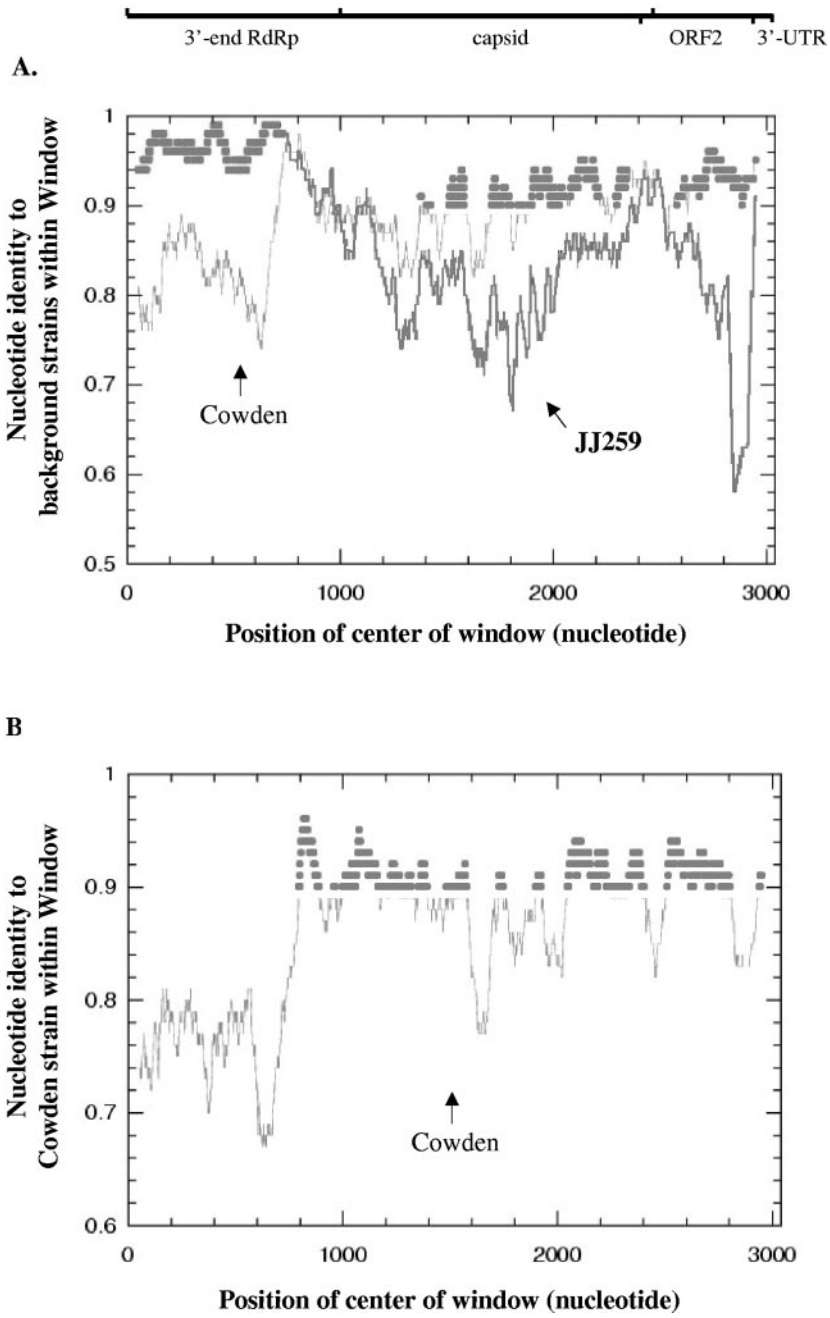
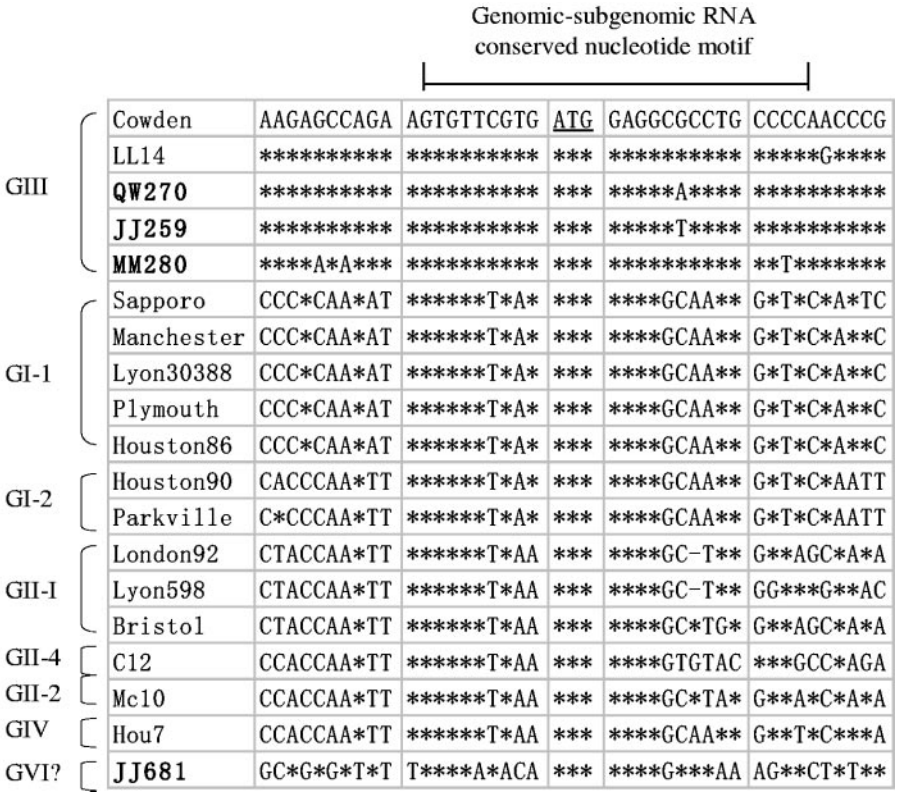


FIG. 3. Identification of QW270 and MM280 strains as potential recombinants. RIP analysis was performed based on the 3′-end 3-kb sequences, including the partial RdRp, the capsid, the ORF2, and the 3′-UTR. At each position of the window, the query sequence was compared to the background sequence(s). When the query sequence is similar to the background sequence(s), the homologous regions are indicated as thick dots on the plot. Analysis parameters were Window size of 100 and Statistical significance of 90%. The nucleotide positions of the 3′-end RdRp, the deduced capsid, ORF2, and the 3′-end UTR are indicated. (A) RIP of QW270 strain. The QW270 strain was a query sequence compared to the background Cowden and JJ259 strains. (B) RIP of MM280 strain. The MM280 strain was a query sequence and only the Cowden strain was the background.

tron microscopy examination of most of these fecal samples did not detect virus particles. These noncalicivirus sequences in pig fecal samples interfere with the amplification of norovirus or sapovirus sequences. Therefore, it is likely that the porcine noroviruses and sapoviruses may be missed if low RNA amounts are present in the samples, which is especially important in screening subclinically infected pigs. This is potentially why we only detected nine sapovirus- and seven norovirus-positive samples from 377 pig fecal samples, even under low-stringency conditions. We compared the detection limits of this broad reactive primer pair p290/110 and porcine norovirus-specific primer pair PNV7/8 (33) for the detection of porcine noroviruses and found that the sensitivity of p290/110 is 250-fold lower than PNV7/8. Because caliciviruses are genetically



GIII	Cowden	AAGAGCCAGA	AGTGTTCGTG	ATG	GAGGCGCCTG	CCCCAACCCG
	LL14	**********	**********	***	**********	*****G*****
	QW270	**********	**********	***	*****A*****	**********
	JJ259	**********	**********	***	*****T*****	**********
	MM280	****A*A***	**********	***	**********	**T*******
GI-1	Sapporo	CCC*CAA*AT	******T*A*	***	****GCAA**	G*T*C*A*TC
	Manchester	CCC*CAA*AT	******T*A*	***	****GCAA**	G*T*C*A**C
	Lyon30388	CCC*CAA*AT	******T*A*	***	****GCAA**	G*T*C*A**C
	Plymouth	CCC*CAA*AT	******T*A*	***	****GCAA**	G*T*C*A**C
	Houston86	CCC*CAA*AT	******T*A*	***	****GCAA**	G*T*C*A**C
GI-2	Houston90	CACCCAA*TT	******T*A*	***	****GCAA**	G*T*C*AATT
	Parkville	C*CCCAA*TT	******T*A*	***	****GCAA**	G*T*C*AATT
GII-I	London92	CTACCAA*TT	******T*AA	***	****GC-T**	G**AGC*A*A
	Lyon598	CTACCAA*TT	******T*AA	***	****GC-T**	GG***G**AC
	Bristol	CTACCAA*TT	******T*AA	***	****GC*TG*	G**AGC*A*A
GII-4	C12	CCACCAA*TT	******T*AA	***	****GTGTAC	***GCC*AGA
GII-2	Mc10	CCACCAA*TT	******T*AA	***	****GC*TA*	G**A*C*A*A
GIV	Hou7	CCACCAA*TT	******T*AA	***	****GCAA**	G**T*C***A
GVI?	**JJ681**	GC*G*G*T*T	T****A*ACA	***	****G***AA	AG**CT*T**

FIG. 4. Sequence alignments of the RdRp-capsid junction region of SaVs. The deduced genomic and subgenomic conserved nucleotide motif of Cowden strain is indicated by a horizontal line with two vertical bars. Asterisks indicate the identical residues to the sequence of the first line (Cowden). The start codon for the capsid protein is underlined. Six SaV genogroups (GI to VI) are indicated. The four newly sequenced porcine SaV strains are in boldface.

highly variable, this p290/110 targeting the most conserved motifs of caliciviruses remains a good way to screen for genetically new caliciviruses.

To date, the complete genomes of only five SaV strains have been sequenced (8, 15, 20, 26), including four human SaV strains (Manchester, Bristol, Mc10, and C12) and the porcine Cowden strain. The classification of SaVs based on the complete capsid gene sequences is generally accepted, although the consistency and reliability of SaV classification based on different regions, such as the complete and partial RdRp and the ORF2 protein, of the genome remains to be investigated (3, 29). In the present study, we found that the classification based on the complete capsid was consistent with that based on the larger partial RdRp (~260 aa) of most SaV strains except for the GIV/Hou7 strain and the potential recombinant strains (Mc10 or C12, QW270, and MM280) (Fig. 2). The short segment (95 aa) of the RdRp region could be used to identify genogroups but it did not reliably identify genotypes. For example, using this fragment, the GI-1/Manchester strain was distinguished from the other three GI-1 strains (Sapporo, Houston86, and Lyon30388) and clustered with GI-2/Houston90 (Fig. 1). Based on the 95-aa RdRp segment, the QW19 strain shares 66.3% amino acid identity to human GII Mc10, C12, and Cruise ship strains, and the LL26 shares 63.2% amino acid identity to the JJ681 strain. The overall intragenogroup amino acid identity of the 95-aa RdRp region of SaVs is 80 to 100%. Thus, the QW19 and LL26 strains may belong to two new genogroups. However, no further classification of the QW19 and LL26 strains was possible due to a lack of additional sequence data.

In the deduced complete capsid region, the JJ681 strain showed similar amino acid identities (32 to 36%) to each SaV genogroup (GI-GV), which were higher than those (17 to 23%) between JJ681 and representative strains (NB, RHDV, FCV, and Norwalk) of the other four genera of *Caliciviridae* or the overall intergenus amino acid identities (15 to 27%) for caliciviruses (7, 31). The JJ681 strain also had the same size and identical nucleotides in the ORF1-ORF2 overlap region as the other porcine SaVs. However, in the RdRp-capsid junction region, which is conserved within each genogroup (Table 4), the JJ681 strain differed from all of the other SaV genogroups (GI-V). Therefore, we tentatively classified the JJ681 strain as a new genogroup (JJ681-like, GVI?) within the *Sapovirus* genus. Our results indicate that porcine SaVs circulating in US swine herds are genetically diverse and comprise at least two genogroups (GIII and JJ681-like) and may comprise two other potential new genogroups (QW19-like and LL26-like).

Among porcine SaVs, the QW19 strain is genetically closest related to human GII SaVs. A similar sequence was found in GenBank (AY615810, Po/Sapo/SWECII/VA14/NET [R. van der Heide et al., unpublished]). These two strains share 100% amino acid and 84% nucleotide identities in the 286-nt (95-aa) RdRp region, the region produced as the products of primer pair p290/110. This indicates that other porcine SaVs closely

related to human SaVs exist. Whether human SaV strains similar to the QW19-like porcine SaVs circulate among swine farm workers is unknown, but such studies could provide information on the zoonotic potential of these porcine SaVs.

We tried to passage the QW19 strain in two gnotobiotic pigs orally and intranasally inoculated with QW19 fecal filtrates (Q.-H. Wang and L. S. Saif, unpublished). The purpose of the passage of the QW19 strain in gnotobiotic pigs was to examine whether this porcine SaV infects pigs efficiently and to produce enough virus for further sequence analysis. No shedding of the QW19 SaV was detected in feces by RT-PCR or by immune electron microscopy using convalescent-phase serum to this strain from one of the pigs described above. Only rotavirus particles were observed by immune electron microscopy using the convalescent antiserum. The rotaviruses were present in the original fecal sample when tested by nested RT-PCR with group C rotavirus-specific primers (6). The rotavirus infection may have interfered with replication of the SaV QW19 strain. Although 60 other fecal samples were collected and tested from the same farm (MI A) from which QW19 was identified, no other QW19-like strains were detected. Also, no calicivirus-like particles were observed in the original QW19 fecal specimen by electron microscopy (data not shown). Based on these limited results, possibly the QW19 strain was introduced only occasionally into individual pigs. Potentially, it did not replicate to high titer in the pig host (electron microscopy negative, RT-PCR weakly positive), nor did it spread efficiently in the herd, suggesting a possible alternative host origin, such as from humans, based on the phylogenetic analysis. We are now performing a prevalence study of porcine noroviruses and sapoviruses to try to detect fecal samples that contains high concentrations of QW19-like sapoviruses but not rotaviruses or other enteric viruses. Then we will try to passage the QW19-like sapoviruses again in gnotobiotic pigs through the oral/intranasal or intravenous routes.

The SaV recombinant strain (Mc10 or C12) of human origin (15) and several norovirus recombinants of human, bovine, and porcine origin have been reported (11–13, 16, 21, 32). All of the recombination events occurred in the RdRp-capsid junction region and exclusively within the genogroups of each genus, a finding consistent with the nucleotide conservation seen among strains within a genogroup in this junction region and facilitating homologous recombination. In the present study, we identified potential recombination events by two programs. The incongruence methods that are now popular to study recombination are good (and often the most effective) for identifying RNA virus recombinants (35). However, these methods are weak in that they cannot distinguish and account for whether descendant genomes arose by accumulated mutations or via recombination events. So we also used POY. POY extends the methods of phylogenetic analysis by implementing a hierarchical model that directly compares mutation versus a mixture of mutation and recombination as possible scenarios explaining the variation of genomes. We found that the QW270 was a potential recombinant strain between a JJ259-like strain (RdRp region) and a Cowden-like strain (capsid, after RdRp) of the same genotype. A recombination event probably occurred for MM280 and a Cowden-like strain, although we did not detect a strain carrying a similar RdRp but different capsid from that of the MM280 strain.

For GI, GIV, and GV human SaVs, another ORF overlapping the 5′ end of the capsid gene was proposed because there is a conserved translation initiation motif GCAAUGG at the 5′ end of this ORF (3, 29). We did not find this motif in the capsid sequences of QW270, MM280, JJ259, or JJ681 strains. It will be of interest to determine whether QW19-like porcine SaVs which are more closely related to human SaVs have this motif.

In summary, we identified genetically diverse porcine SaVs comprising at least two genogroups (GIII and JJ681-like GVI?) and possibly comprising two other potentially new genogroups (QW19-like and LL26-like). One porcine SaV strain QW19 was genetically most similar to human SaVs, suggesting the possibility of a pig reservoir for human strains or vice versa. We also identified two potential recombinant porcine SaVs (QW270 and MM280/Cowden). To our knowledge, this is the first report of porcine SaV recombinants.

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