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A Member of a New *Picornaviridae* Genus Is Shed in Pig Feces

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During a study of the fecal microbiomes from two healthy piglets using high-throughput sequencing (HTS), we identified a viral genome containing an open reading frame encoding a predicted polyprotein of 2,133 amino acids. This novel viral genome displayed the typical organization of picornaviruses, containing three structural proteins (VP0, VP3, and VP1), followed by seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C^{Pro}, and 3D^{Pol}). Given its particular relationship with *Parechovirus*, we propose to name it “Pasivirus” for *Parecho sister clade virus*, with “Swine pasivirus 1” (SPaV1) as the type species. Fecal samples collected at an industrial farm from healthy sows and piglets from the same herd (25 and 75, respectively) with ages ranging from 4 to 28 weeks were analyzed for the presence of SPaV1 by one-step reverse transcription (RT)-PCR targeting a 3D region of 151 bp. SPaV1 was detected in fecal samples from 51/75 healthy piglets (68% of the animals) and in none of the 25 fecal samples from healthy sows, indicating that SPaV1 circulates through enteric infection of healthy piglets. We propose that SPaV1 represents the first member of a novel *Picornaviridae* genus related to parechoviruses.

Members of the family *Picornaviridae* are small nonenveloped viruses with a genomic positive single-stranded RNA that are responsible for several human and veterinary diseases. As of 2009, the International Committee on Taxonomy of Viruses (ICTV) recognized 12 genera within the family *Picornaviridae*, namely, *Enterovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, *Parechovirus*, *Erbovirus*, *Kobuvirus*, *Teschovirus*, *Sapelovirus*, *Senecavirus*, *Tremovirus*, and *Avihepatovirus* (<http://www.picornaviridae.com>). However, recent developments in high-throughput sequencing (HTS) identified numerous *Picornaviridae* species, among which several sequences were proposed as prototypes for novel genera. At least 11 novel genera have been proposed to belong to the family *Picornaviridae* in recent literature: “Cosavirus” (7, 21) and “Salivirus” (17, 22, 37, 52) in humans, “Orthoturdivirus” and “Paraturdivirus” in wild birds (58), “Mosavirus” and “Rosavirus” in wild rodents (44), an unnamed genus in ringed seal (SePV-1) (27), an agent responsible for hepatitis in turkey poults (23), two unnamed genera harbored by bats (33), and a virus described in domestic cats (feline picornavirus [FePV]) (34). Very recently, the *Picornaviridae* study group suggested that the proposed species “seal picornavirus 1” (SePV1) be named “seal aquamavirus A1” and classified in a new proposed genus called “Aquamavirus” and that the proposed species “Turkey hepatitis virus” be classified in the proposed genus “Megrivirus.” Candidate species have also been reported within the genus *Kobuvirus* in pigs (47), dogs (28, 35), and rodents (44) and within the genus *Sapelovirus* in the California sea lion (36).

To date, viruses belonging to five genera from the family *Picornaviridae* are responsible for several diseases in domestic pigs. They are *Encephalomyocarditis virus* (genus *Cardiovirus*), *Porcine enterovirus B* (*Enterovirus*), “Porcine kobuvirus” (*Kobuvirus*), *Porcine sapelovirus* (*Sapelovirus*; formerly *Porcine enterovirus A*), and *Porcine teschovirus* (*Teschovirus*, containing only one species), which is recognized as the etiologic agent of polioencephalomyelitis, the most virulent picornaviral infection of pigs.

During a study of the fecal microbiomes from two healthy piglets using HTS, we identified a viral genome containing an

open reading frame (ORF) encoding a predicted polyprotein of 2,133 amino acids (aa) displaying the typical organization of picornaviruses. According to criteria from the ICTV (less than 40%, 40%, and 50% amino acid identities in the P1, P2, and P3 regions, respectively, for genus demarcation), this virus would represent a novel genus in the family *Picornaviridae*. Given its particular relationship with *Parechovirus*, we propose to name it “Pasivirus” for *Parecho sister clade virus*, with “Swine pasivirus 1” (SPaV1) as the type species. In the present article, we show that SPaV1 causes an acute enteric infection of young pigs and report the putative genomic organization and subsequent phylogenetic analysis of its genome.

MATERIALS AND METHODS

Fecal samples. Two fecal samples from healthy piglets (index cases) were submitted to HTS analysis. Subsequently, a prevalence survey was performed by one-step reverse transcription (RT)-PCR based on the data obtained from these index cases (for details see “Detection of SPaV1 by One-Step RT-PCR of the 3D Gene” below). This prevalence study included fecal samples from 25 healthy sows (2 years old) and from 75 healthy piglets ranging from 4 to 28 weeks old (3 or 4 piglets per age category). All the fecal samples (sows and piglets) were collected from an industrial pig farm located in the center of France in 2011.

Extraction and amplification of nucleic acids. Fecal samples were diluted (0.1 g/ml) in phosphate-buffered saline (Gibco), vigorously homogenized, and centrifuged at 12,000 × g for 25 min at 4°C. The supernatants were microfiltered (0.45 μm; Sartorius, Goettingen, Germany) to remove residual eukaryotic and bacterial cell size particles. The fecal filtrates were then treated with 0.5 U/μl of DNase I (Qiagen) for 2 h at 37°C in order to digest unprotected nucleic acids. The DNase I was inactivated

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TABLE 1 Primers designed from contigs used to acquire the genome of SPaV1

Primer name ^a	Sequence (5'–3')	Size of PCR product (bp)
SPaV1.1F	5'–GCTTTTGACCAGTGGCTCTGG–3'	531
SPaV1.1R	5'–AGCCGTAGGAGCAGCACTATG–3'	
SPaV1.2F	5'–TGATACTGCTGAATCTGGCGG–3'	566
SPaV1.2R	5'–ACCCGCAGTCAGAAAGATCAG–3'	
SPaV1.3F	5'–TCAGGTCAATGCTGCTGCAGG–3'	598
SPaV1.3R	5'–AGCTGTGAACGGTAGCAAAGG–3'	
SPaV1.4F	5'–CTAGTGTTCAGGCACGAGAG–3'	572
SPaV1.4R	5'–CTTGACAGTGTACCCGCATGG–3'	
SPaV1.5F	5'–GTTGAAACCCGATTGGCTCAC–3'	746
SPaV1.5R	5'–GGAGCCTCAGGCACTAACTTC–3'	
SPaV1.6F	5'–TATTCCTGGTCGCCATTGCGG–3'	403
SPaV1.6R	5'–CATAACATCAAGACAGGGCCAG–3'	
SPaV1.7F	5'–CCCCATTATGGGGATATTCCT–3'	807
SPaV1.7R	5'–ATTCAGGAGGGTACGATCCC–3'	
SPaV1.8F	5'–CTTCTGCTATGGAGTTGCTGG–3'	630
SPaV1.8R	5'–CCCCATACGTGGTAAAACCCT–3'	
SPaV1.9F	5'–TTGAAGGATTGTGCCACCACC–3'	900
SPaV1.9R	5'–GCTAGCGCAATAGTCGAACAC–3'	
SPaV1.10F	5'–CCTTCTTGGCCCTGCTGTTC–3'	619
SPaV1.10R	5'–GACACCATCTCCAAGGTCTCC–3'	
SPaV1.11F	5'–GGGTGCTTGACTATAATGGGTC–3'	597
SPaV1.11R	5'–TGCCAATCACAGAGTCAACCTC–3'	
SPaV1.12F	5'–TCAAGGACTAGTACCCGACAC–3'	598
SPaV1.12R	5'–CCGGAACAGCTTGCAAAGAC–3'	

^a F, forward; R, reverse.

by 10 mM EDTA at room temperature. A volume of 100 µl of each fecal filtrate was then extracted using a Nucleospin RNA virus kit (Macherey-Nagel) that allows recovery of both DNA and RNA. The nucleic acids were eluted into 50 µl of RNase-free water, and a cDNA synthesis step was performed with random hexamer primers (Superscript III RT; Invitrogen, Inc.). The two following steps, ligation of cDNA and nucleic acid amplification by bacteriophage Phi29 polymerase, were performed as previously described (5).

HTS and bioinformatics analysis. HTS and bioinformatics analysis were performed as previously described (5). Briefly, sequencing was conducted on an Illumina HiSeq-2000 sequencer (GATC Biotech AG, Konstanz, Germany) with a mean depth per sample of 29×10^6 paired-end reads 96 nucleotides (nt) in length (range, 25×10^6 to 37×10^6). The whole porcine genome (SGSC-SsCrofa9.2/susScr2) (<http://www.genome.ucsc.edu/>) was used as a reference sequence for pig sequence mapping conducted with SOAPaligner.

Viral-genome sequencing and analysis. Twelve specific primer pairs (Table 1) were designed from contigs obtained by HTS to amplify and determine the nucleotide sequence of SPaV1. All PCR amplifications were performed by using the *Taq* Core kit (MPBio, Illkirch, France) following the manufacturer's instructions. PCR products were sequenced directly using the BigDye Terminator v1.1 cycle-sequencing kit (Applied Biosystems). Sequence chromatograms from both strands were obtained on an ABI 3730 XL automated sequence analyzer (Applied Biosystems). Attempts to acquire the end of the 3D polymerase and the 3' untranslated region (UTR) were made by three methods: (i) a ligation-anchored PCR (LA-PCR) method (3), (ii) a 3' step-out rapid amplification of cDNA ends (RACE) according to the published protocol of Matz et al. (40), and (iii) a novel method using a combination of single-stranded DNA (ssDNA) circularization and rolling-circle amplification (RCA) (12). Briefly, LA-PCR involves the ligation of an oligonucleotide by T4 RNA ligase (Ambion) to the 3' end of RNA before synthesis of cDNA. This method allows reverse transcription of the nonpolyadenylated RNA virus genome. For the 3' step-out RACE, the 3' UTR of the genome was amplified with an oli-

TABLE 2 Primers used to complete the end of the SPaV1 genome

Primer name	Direction ^a	Sequence ^b (5'–3')
SPaV1–EP1	F	PHO–5'–GAGAAAGAGGGATCTCGTG CC–3'
SPaV1–P3.CircL1	R	5'–TAAGCTCATCTTTAAGGTGACAG–3'
SPaV1–P2.CircL1	F	5'–GAGAAGATTGAACAAGGCCTTAC–3'
SPaV1–P1.CircL1	F	5'–AAGAGTCTTTTGAAGCTGTTCC–3'
SPaV1–EP2	F	PHO–5'–CCACGTCAGCTCATGATAGA TG–3'
SPaV1–P3.CircL2	R	5'–GGTTACCTACCACATGCTGTG–3'
SPaV1–P2.CircL2	F	5'–TGGAATGGCTTCAGGATCACC–3'
SPaV1–P1.CircL2	F	5'–GGAGGAGGGTGTGAGTATAC–3'
SPaV1–EP3	F	PHO–5'–GTCAAGCTTGATGGTGATTAT CC–3'
SPaV1–P3.CircL3	R	5'–GCCAACTTCCATCGTCCAAC–3'
SPaV1–P2.CircL3	F	5'–GTCGGTCAGTGAGTGTTCCTCC–3'
SPaV1–P1.CircL3	F	5'–CAATTAATTCAGCAAGTATAG–3'

^a F, forward; R, reverse.

^b Primers EP (SPaV1–EP1, SPaV1–EP2, and SPaV1–EP3) are 5'–end phosphorylated (PHO).

go(dT) primer and a specific forward primer (5'–ATATGACTGTTCTTG AGGAGGAG–3'). The method based on template circularization and RCA used cDNA as the template and a specific 5'–end-phosphorylated extension primer (EP). The ssDNA was generated with Phusion High-Fidelity DNA Polymerase (New England BioLabs) and self-ligated by using the CircLigase enzyme (Epicentre Biotechnologies). This step was followed by RCA using Phi29 DNA polymerase, yielding linear concatemeric DNA, which served as the template for inverse PCR. This PCR involved two sets of primers (P2–P3 and P1–P3, listed in Table 2). The detailed protocol is available upon request.

The putative proteolytic cleavage sites were predicted by submitting the polyprotein sequence for analysis by the NetPicoRNA prediction server (<http://www.cbs.dtu.dk/services/NetPicoRNA/>). The protein sequences were aligned using Jalview 11.0 (56). The whole polyprotein of SPaV1 was used as a reference in a sliding-window analysis implemented in the RAT software (11) (see Fig. S1 in the supplemental material).

Phylogenetic analysis. All complete available amino acid sequences of the polyproteins of *Picornaviridae* were aligned in a matrix counting up to 92. Complete reference sequences were used when applicable, but the matrix was not restricted to reference sequences, and the taxon diversity was optimized by including only reference sequences for well-described genera or lower taxonomic levels. Recently described taxa were also included, as they might carry valuable information on the diversity of the corresponding groups. The sequence of a picornavirus isolated from fish (bluegill virus Montana lake) (2) was used for alignment and tree rooting (GenBank accession number JX134222). A screening of putative recombination breakpoints was performed using the RDP3 package prior to phylogenetic analysis (39). This aligned matrix was then sliced following each protein's ORF in the genome taxa, and redundant gene sequences were excluded from the analysis. Protein matrices were constituted in accordance with conserved amino acid motifs reported to be characteristic of protein starts and ends for each reference sequence. Other taxa were aligned to these reference sequences by several iterations of multialignment performed under the Muscle algorithm implemented in the Seaview software version 4.2.11 (16). Sea-AL software version 2.0a11 was also used to edit the matrices (<http://tree.bio.ed.ac.uk/software/seal/>). Reading frames were respected for subsequent analyses and phylogenetic tests. The matrices were converted back to their nucleotide sequences before computing likelihood scores and ranking the 88 model tests according to the Akaike Independent (corrected) Criterion (AICc) calculated with the jModelTest software version 0.1.1 (45). The best matrix-fitted model was then used as the tree prior in the following analyses. Other specified priors included a relaxed uncorrelated log-normal clock and the Yule speciation

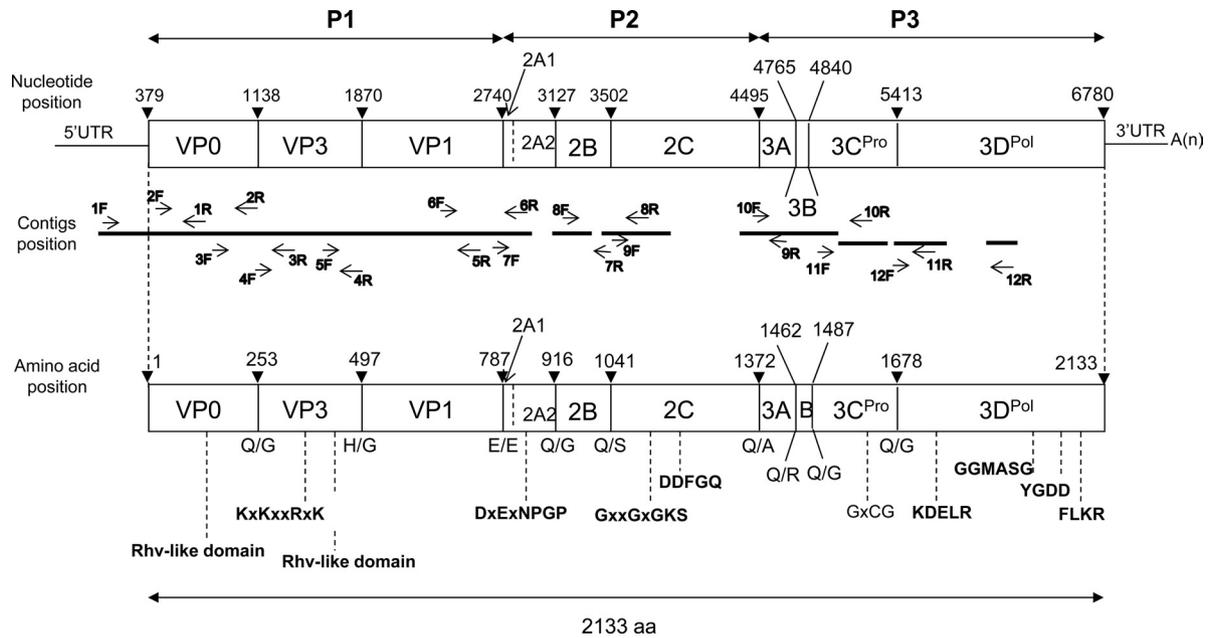


FIG 1 Schematic representation of predicted SPaV1 genome organization, including 5' UTR, 3' UTR, P1, P2, and P3 regions. Primer pairs designed from contigs and used to generate overlapping PCR products are distributed along the nucleotide sequence. Conserved motifs and predicted cleavage sites are indicated along the polyprotein.

process. Matrices were submitted to a maximum of 30,000,000 iterations in order to allow the Markov chain to converge whenever possible. These analyses were conducted using BEAST software version 1.6.1 (10). Posterior ESS values and other statistics were extracted to the output files using TreeAnnotator 1.6.1 and investigated using Tracer 1.5 from the BEAST package. The resulting trees were edited and visualized in FigTree version 1.3.1 (BEAST package).

Detection of SPaV1 by one-step RT-PCR of the 3D gene. Nucleic acids were extracted as previously described, except that no DNase treatment was applied to the fecal filtrates. Primers for the prevalence study were selected within the 3D RNA-dependent RNA polymerase gene of the SPaV1 genome by using Primer Pro 3.4 software (SPaV1.3D.151F, 5'-AA ACCATGGCCTGGTGTGCGT-3', and SPaV1.3D.151R, 5'-TGCCAATC GCAGAGTCAACCT-3'). Reverse transcription and PCR were performed using the Superscript One-step RT-PCR Platinum Taq Kit (Invitrogen) according to the manufacturer's instructions. PCR products of 151 nt were sequenced with both primers to confirm the detection and assess sequence variation.

Cell culture. The microfiltered (0.22 μ m; Sartorius, Goettingen, Germany) fecal filtrates resuspended in PBS were incubated on Vero E6 cells grown to subconfluence in minimal essential medium (MEM) supplemented with 120 μ g/ml of streptomycin, 120 units/ml of penicillin, and 10% fetal calf serum (FCS). The occurrence of any cytopathic effect (CPE) was checked on a daily basis for 12 days. The supernatants were extracted and tested by PCR following the protocol described above.

Nucleotide sequence accession number. The complete coding sequence of SPaV1 has been deposited in the GenBank database under accession number JQ316470.

RESULTS

Identification of the SPaV1 genome by HTS. Illumina sequencing generated a total of 27,146,966 reads with a mean length of 96 bp. After the host genome filtration and BLAST analysis against bacterial, viral, and generalist NCBI databases, 725 reads matching various *Picornaviridae* genomes were assembled into seven contigs (ranging from 206 bp to 3,034 bp). These contigs showed

a maximum of 42% amino acid identity with the best hits reported within the nr NCBI database: the rodent *Parechovirus* (*Ljungan virus* [LV]), the human parechoviruses type 1 (HPeV1) and type 5 (HPeV5), and *Duck hepatitis A virus* (DHV). Based on the sequences of the contigs distributed along the genome, 12 primer pairs (Table 1) were designed and used to generate overlapping PCR products validated on both WTA and cDNA products. Sequencing by the Sanger method gave a resulting sequence of 6,896 nt (after excluding the polyadenylated tract), with a 5' partial UTR of 378 nt, an open reading frame of 6,402 nt encoding a potential polyprotein precursor of 2,133 aa, and a 3' UTR of 116 nt (Fig. 1). The available SPaV1 genomic sequence showed a G+C content of 43.3%, which was similar to the values obtained for the corresponding region of the parechoviruses (HPeV1, 40%, and LV, 42%) and related clades (DHV, 43%; seal aquamavirus A1, 44%; *Porcine teschovirus 1*, 45%; and turdivirus 1, 47%). A BLASTx analysis of the complete genome of SPaV1 provided 31% amino acid identity and 50% amino acid similarity to the LV strain 145 SL. A sliding-window analysis of the polyprotein of SPaV1 showed that the identity with the members of closer genera never exceeded 50% (see Fig. S1 in the supplemental material).

Genome organization and coding region of SPaV1. The partial 5' UTR had no sequence homology to any virus recorded in GenBank. This region precedes two putative initiator methionine codons found at nucleotide positions 199 and 379. Only the initiator codon at position 379 was surrounded by an optimal Kozak context (RNNAUGG) (30) and was therefore interpreted as the start codon of the polyprotein. In picornaviruses, the polyprotein precursor is cleaved by viral protease(s) to yield the mature viral structural and nonstructural proteins. Putative cleavage sites of SPaV1 were determined by aligning the amino acid sequence with the closest known virus (LV) and submitting it to the NetPicoRNA prediction server (4). The predicted cleavage sites of the SPaV1

TABLE 3 Pairwise amino acid identities between the predicted proteins of SPaV1 and related picornaviruses

Protein ^a	SPaV1 (JQ316470)		Pairwise amino acid identity (%) between SPaV1 and the most closely related picornaviruses ^b :				
	Position	Length (aa)	LV145 SL (AF327922)	HPeV1 (FM178558)	HPeV3 (AB084913)	SPeV-1 (NC_009891)	Duck hepatitis A virus 1 (NC_008250)
VP0	1Met–Gln253	253	29.3	30.9	32.3	24.5	30.1
VP3	254Gly–497His	244	40.5	41.1	40.8	26.7	27.5
VP1	498Gly–Glu787	290	26.5	25.6	25.3	16.5	18.8
P1	1Met–Glu787	787	33.6	32.4	32.5	22.4	25.6
2A	788Glu–Gln916	119	43.6	9.9	9.6	11.9	13.0
2B	917Gly–Gln1041	125	22.7	25.0	26.4	12.5	22.1
2C	1042Ser–Gln1372	331	35.6	36.2	36.6	30.4	32.4
P2	788Glu–Gln1372	585	28.9	27.6	28.4	24.4	21.3
3A	1373Ala–Gln1462	90	18.0	24.2	22.7	1.0	18.0
3B	1463Arg–Gln1487	25	27.3	26.7	40.0	8.3	21.1
3C	1488Gly–Gln1678	191	26.1	28.6	27.7	23.3	24.4
3D	1679Gly–2133Ser	455	34.3	30.3	32.4	28.7	32.0
P3	1373Ala–2133Ser	761	29.2	29.0	29.6	24.6	27.6

^a P1, P2, and P3 (boldface), precursor protein regions 1, 2, and 3.

^b Pairwise amino acid identities were calculated with the complete 2A sequence (2A1 and 2A2).

polyprotein were consistent with that of LV's polyprotein (strain 145SL) described by Johansson et al. (25). These cleavage sites showed a molecular organization typical of picornaviruses with three structural proteins (VP0, VP3, and VP1), followed by seven nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C^{pro}, and 3D^{pol}) (Fig. 1). As observed for *Avihepatovirus*, *Enterovirus*, *Hepatovirus*, *Parechovirus*, *Tremovirus*, *Aquamavirus*, *Cosavirus*, and *Megrivirus*, SPaV1 does not contain any identifiable leader protein (L). Two predicted cleavage sites, E₇₈₇/E and Q₁₃₇₂/A, define the P1, P2, and P3 coding regions of SPaV1 (Fig. 1), which share, respectively, 17 to 34%, 17 to 29%, and 21 to 29% amino acid identities with representatives of other picornavirus genera (see Table S1 in the supplemental material). The highest identities of the polyprotein were observed with parechoviruses, and particularly with LV (33.6, 28.9, and 29.2% for P1, P2, and P3, respectively), and with seal aquamavirus A1 (22.4, 24.4, and 24.6%) and DHV (25.6, 21.3, and 27.6%) (Table 3).

The P1 coding region of SPaV1 contains the "picornavirus capsid protein domain-like" protein (pfam entry, cd00205) and is predicted to be cleaved after Q₂₅₃ (VP0/VP3), H₄₉₇ (VP3/VP1), and E₇₈₇ (VP1/2A) (Fig. 1). As observed for the related groups *Avihepatovirus*, *Parechovirus*, and *Aquamavirus* and more distant groups, such as *Porcine kobuvirus* (47, 58), "VP0" of SPaV1 is probably not cleaved into VP4 and VP2, based on sequence alignment. Similarly to parechoviruses, VP0 does not display the conserved GXXX(ST) motif for myristylation (6) (Fig. 2A), and VP1 does not contain the characteristic (PS)ALXAXETG motif. In addition, VP1 lacked the integrin binding RGD motif (involved in receptor binding), similar to HPeV3 and LV but in contrast to HPeV1 (57). Consistent with LV and in contrast with HPeVs, the VP1 protein of SPaV1 contains 2 N-terminal insertions (11 and 4 aa long) and a unique C-terminal extension of 41 aa (43 aa for LV) (Fig. 2C). Interestingly, the N-terminal extremity of the VP3 protein (40% amino acid identity to parechoviruses [Table 3]) contained the highly conserved KKKXXRXX motif at position 263 (Fig. 2B), recognized as a distinctive feature of parechoviruses (57).

The P2 polyprotein of SPaV1 was hypothesized to be cleaved after Q₉₁₆ (2A/2B), Q₁₀₄₁ (2B/2C), and Q₁₃₇₂ (2C/3A). The 2A

protein shared 43.6% amino acid identity with the LV protein, while the score falls to 13% with DHV and seal aquamavirus A1 and to 10% with human parechoviruses (Table 3). Furthermore, this 2A protein possessed the canonical cleavage site DXEXNPG₈₀₄P (48), which is present in *Avihepatovirus* and *Aquamavirus*, as well as in LV, but not in HPeV. This enzymatic cleavage releases a small 2A1 protein (17 aa) and a 2A2 protein (112 aa) with sizes similar to those of the LV proteins (20 and 135 aa, respectively). The conserved H-box and NC-box motifs, which are involved in the control of cell proliferation (24), and a putative transmembrane domain are all present in DHV and parechoviruses and absent from the 2A protein of SPaV1. The conserved GXCG motif (characteristic of a trypsin-like proteolytic activity [32]) was also absent from the 2A protein of SPaV1. As for other picornaviruses, the 2C protein displayed the NTPase motif G₁₁₈₁XXGXGKS (15) and the D₁₂₃₂DLXQ motif required for helicase activity (14). Similarly to DHV, the leucine (L) of the conserved DDLXQ motif was replaced by phenylalanine (F).

The P3 protein of SPaV1 was predicted to be cleaved after Q₁₄₆₂ (3A/3B), Q₁₄₈₇ (3B/3C), and Q₁₆₇₈ (3C/3D). Consequently, the P3 polyprotein encodes the characteristic proteins 3A, 3B (VPg, a small genome-linked protein), 3C^{pro} (protease), and 3D^{pol} (RNA-dependent RNA polymerase). A pairwise amino acid sequence analysis showed that the 3A and 3B proteins of SPaV1 share the highest identities with HPeV1 (24.2%) and HPeV3 (40%), respectively (Table 3). As observed in all picornaviruses described to date, 3B (25 aa) displayed the conserved tyrosine (Y) at position 3 from the putative N terminus. This amino acid is necessary to covalently link the 5' UTR extremity of the viral RNA to VPg, which acts as an RNA replication primer (1). Similar to those of DHV, the seal aquamavirus A1, and parechoviruses, the 3C protein of SPaV1 contained the catalytic triad formed by the amino acids H-D-C (13), found at positions 1525, 1563, and 1638, respectively. As for other picornaviruses, the GXCG (G₁₆₃₆MCG) and GXH (G₁₆₅₄LH) motifs required for proteolytic activity were identified in 3C of SPaV1 (13). In addition, 3C did not contain the RNA binding motif K[FY]RDI (18). Like all members of the family *Picornaviridae*, the 3D protein of SPaV1 displayed the four characteristic conserved motifs K₁₈₃₉DELRL, GG[LMN]PSG

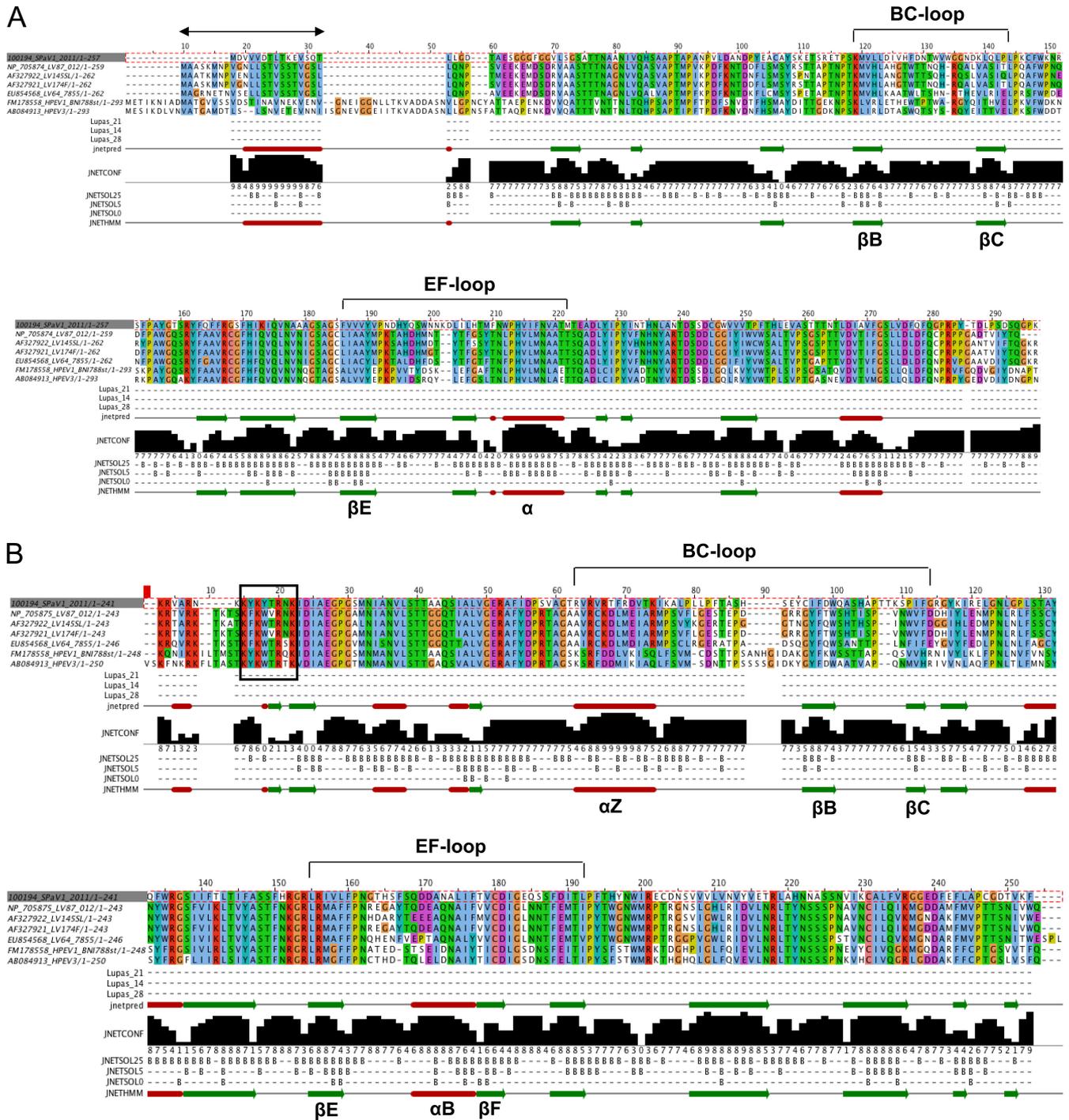


FIG 2 Two-dimensional structure predictions of the capsid proteins, VP0 (2A), VP3 (2B), and VP1 (2C), of SPaV1 aligned with representatives of parechoviruses (HPEV1 and HPEV3 and American [LV64-7855] and Swedish [LV87-012, 174F, and 145SL] strains of LV) performed with Jalview 11.0. Predicted α -helices and β -strands in SPaV1 capsid proteins are represented by red and green arrows, respectively. The positions of BC and EF loops are noted above the alignments. (A) The double-headed arrow indicates the shorter N-terminal extremity of VP0. (B) The conserved KKKXXRXX motif of VP3 is boxed. (C) VP1 showing the two N-terminal amino acid insertions (line with solid circles) and the C-terminal amino acid extension (dashed double-headed arrow).

(G₁₉₈₆GMASG, where P is replaced by A), Y₂₀₀₃GDD, and F₂₀₄₇LKR (29).

Phylogenetic analyses. No putative recombination break-point was identified in the genome of SPaV1 using RDP3 software. Based on several regions of the genome (VP0, VP3, VP1, 2C, 3C,

and 3D), members of the *Picornaviridae* cluster into three major clades: (i) the group infecting fish, used as the outgroup; (ii) the cluster composed of the genera *Parechovirus*, *Avihepatovirus*, and *Aquamavirus*, infecting birds and mammals; and (iii) the clade in which all other genera clustered (Fig. 3A to C; see Fig. S2 to S4 in

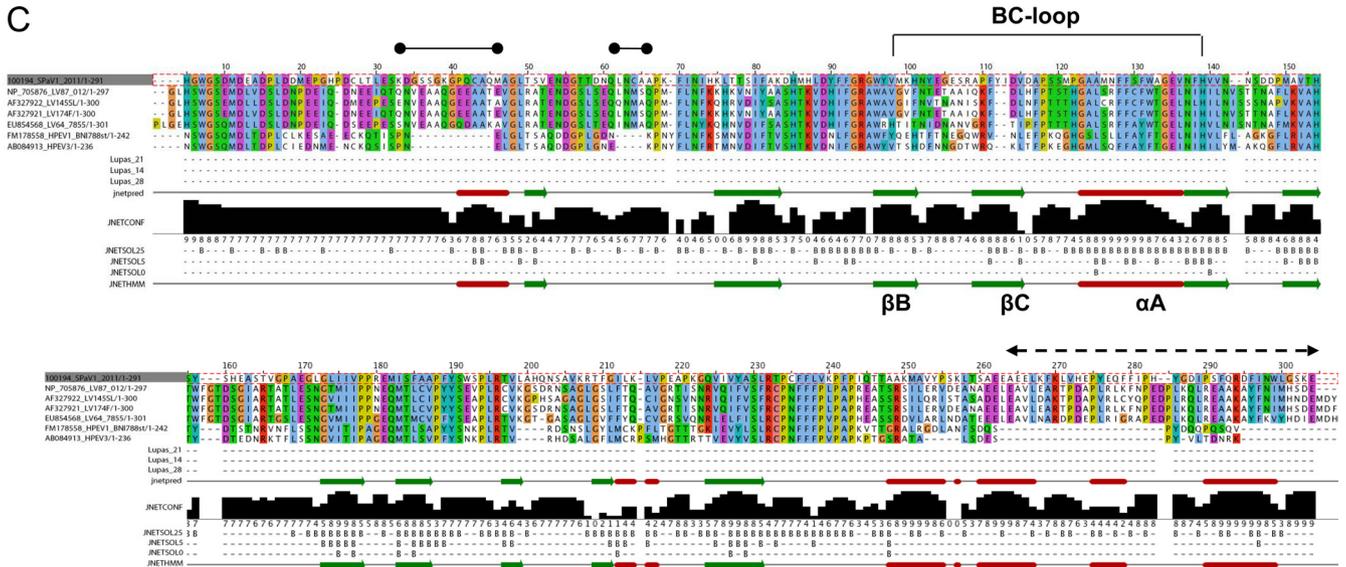


FIG 2 continued

the supplemental material). Phylogenetic analyses constantly grouped SPaV1 with *Parechovirus* and to a lesser extent with *Avihepatovirus*, *Aquamavirus*, and *Hepatovirus*, highlighting the particular relationship between these clades and the basal origin of these groups within the family *Picornaviridae*. Analysis of the non-structural proteins (2C, 3C, and 3D) identified three or four major clades in the *Picornaviridae*. Among these major clades, SPaV1 belonged to the most basal, according to the 2C protein (see Fig. S2 in the supplemental material) and the 3C protein (see Fig. S3 in the supplemental material), but diverged from the clade and rooted all other *Picornaviridae* members according to the 3D protein (Fig. 3A). Despite these differences between proteins, SPaV1 always found its origin close to the proteins of *Avihepatovirus* and *Aquamavirus* (detected in seals) and, according to the 3C protein, clustered with *Aquamavirus* in the sister clade of all parechoviruses (see Fig. S3 in the supplemental material). In contrast, analyses of the capsid proteins (VP0, VP3, and VP1) rooted parechoviruses with SPaV1 without clustering it with another taxa, making the group SPaV1-parechoviruses monophyletic (Fig. 3B and C; see Fig. S4 in the supplemental material). These capsid protein phylogenies were globally congruent, but differences noted between these fairly distinguishable topologies remained statistically well supported. Other proteins, such as 2A and 3A, did not provide significant results despite several adjustments of the priors and 30,000,000 iterations. Likewise, the 2B region remained of no interest for phylogenetic reconstruction, considering the poor significance of the alignment and low posterior probabilities obtained from analyses of these data (data not shown).

Prevalence study and genetic variation of SPaV1. Fecal samples from healthy sows and piglets from the same herd (25 and 75, respectively) with ages ranging from 4 to 28 weeks were analyzed for the presence of SPaV1 by one-step RT-PCR targeting a 3D region of 151 bp. SPaV1 was detected in fecal samples from 51/75 healthy piglets (68% of the animals) and in none of the 25 fecal samples from healthy sows. The prevalence was 45% (9/20) in piglets aged 4 to 8 weeks, 89.47% (17/19) in piglets aged 9 to 14 weeks, 88.23% (15/17) in piglets aged 15 to 20 weeks, and 52.63%

(10/19) in piglets aged 21 to 28 weeks (Fig. 4). This distribution is reminiscent of the enteric viruses transmitted after the disappearance of maternal antibodies, as observed for hepatitis E virus (8). Among the 51 fecal samples positive for SPaV1, 22 were sequenced to assess genetic diversity. Nucleotide differences between samples ranged from 0.7% to 9.3%. These results suggested the existence of a wide variety of strains at the tested industrial farm.

Cell culture. Vero E6 cells were inoculated with the fecal supernatants of the two index piglets from which the virus was identified. CPE was not observed during either the first or the second passage, and the PCRs on the supernatants were negative.

DISCUSSION

We report the nucleotide sequence and the predicted polypeptide of a novel swine picornavirus in stool samples from healthy piglets by an HTS method. A recent study has shown that RNA viruses, and more precisely *Picornaviridae*, represent the majority of the fecal virome in piglets (51). The genome of this novel virus, called SPaV1, presents the typical genome organization of a member of the family *Picornaviridae*, mixing characteristics of the two *Parechovirus* subclades: the HPEV and LV species. Interpreted in light of the phylogeny of each protein, these characteristics may reflect the common origin of parechoviruses and SPaV1. Considering the P1, P2, and the P3 proteins, SPaV1 shares less than 40% identity with LV, the closest taxon described to date. The ICTV recommends less than 40% amino acid identity in the P1 and P2 proteins and less than 50% in the P3 protein for genus demarcation in *Picornaviridae* (53). This new taxon fulfills these criteria and can therefore be considered a new genus in *Picornaviridae*.

The recent discovery of a high-ranking taxonomic level represented by SPaV1 shows that our picture of the diversity of this family is still partial. Major viral genera of the *Picornaviridae* are represented in several avian or mammalian species, and this host diversity may contribute to viral diversity, in addition to other factors, such as a typical error-prone RNA replication system (9). Overall, the diversity of both the virus family (12 genera) and hosts (fish, reptiles, mammals, and birds) depicts the dynamism of the

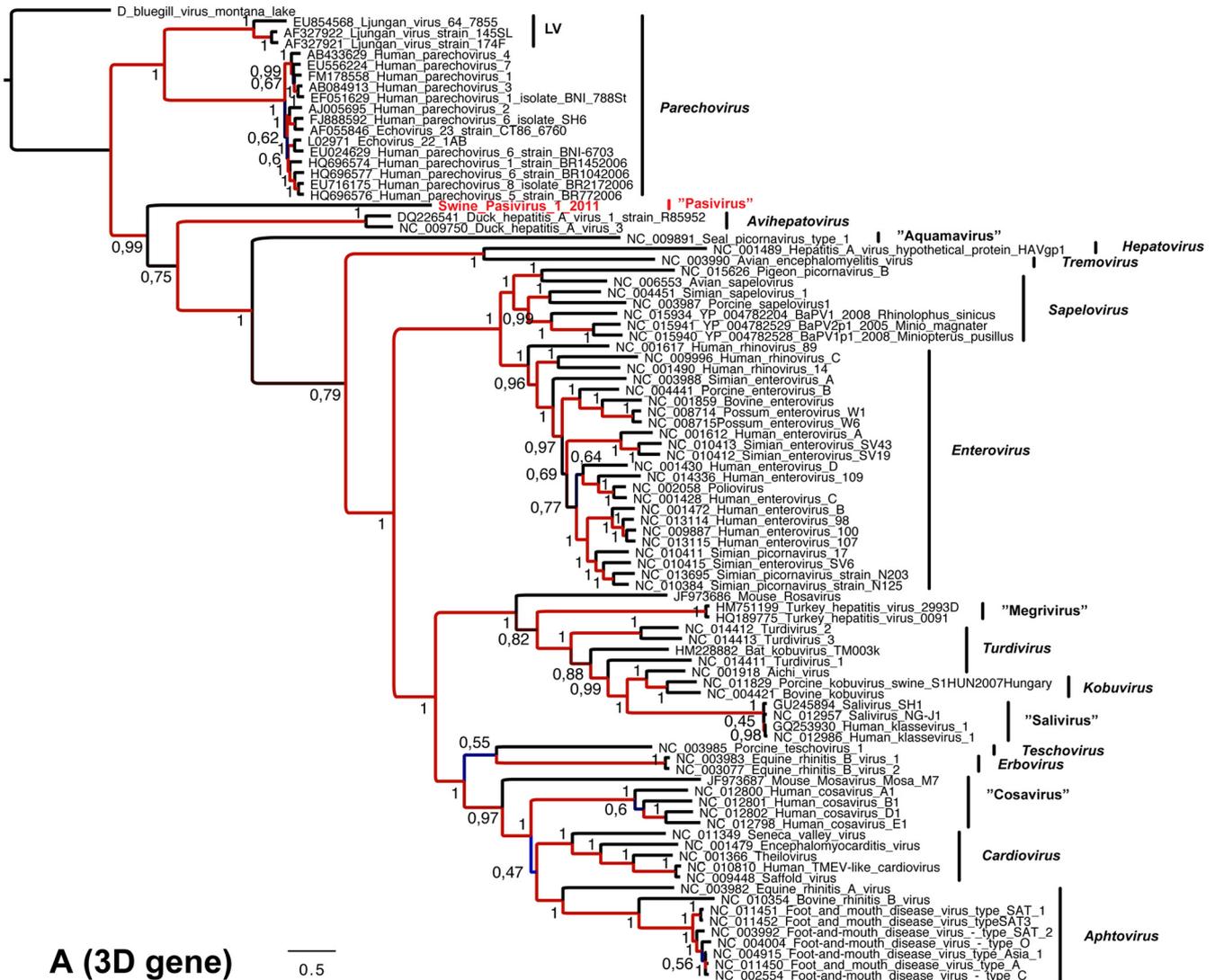


FIG 3 Phylogenetic analysis of complete VP0 (B), VP1 (C), and 3D (A) nucleotide sequences under a GTR+G model and a relaxed uncorrelated clock implemented in the BEAST package. The scale bars are expressed in numbers of substitutions per site. Posterior probabilities are reported at the nodes, and most supported nodes are highlighted in red.

evolution of the *Picornaviridae*. For the most studied groups, such as *Enterovirus*, recombinations were shown to play a master role in shaping the genome, and this was not restricted to the intraspecies level (50). Moreover, the structural and nonstructural parts of the genomes of enteroviruses were shown to evolve independently, with P1 so far being less subjected to recombination (38, 50). Although recombination between taxa and even genera might have occurred throughout the genome during the evolution of SPaV1, it seems unlikely that traces of such ancient events would remain detectable. SPaV1 and therefore the more highly ranked genus *Pasivirus* originated from one of the earliest differentiated and major clades of the *Picornaviridae* (Fig. 3A, B, and C; see Fig. S2 to S4 in the supplemental material). Given the host diversity pattern observed for several of the most studied clades, in which several viral genera sometimes clustered, it is probable that other pasiviruses may infect birds, rodents, primates, or other animals (Fig. 3A, B, and C; see Fig. S2 to S4 in the supplemental material).

The clear phylogenetic relationship between SPaV1 and the

parechoviruses is consistent with numerous similarities of these taxa. Despite representing a potential new genus of *Picornaviridae*, SPaV1 exhibits features that have been considered to be characteristic of *Parechovirus* and more specifically of LV. The low G+C percent is consistent with those of parechoviruses and related clades and contrasts with those of other *Picornaviridae*. SPaV1 contains only three capsid proteins (VP0, VP3, and VP1) exhibiting remarkable features resembling those of parechoviruses and seems to be lacking a leader protein. VP3 contains the conserved KXXXXRXX motif, considered a characteristic signature of parechoviruses (Fig. 2B). This motif belongs to a basic amino acid-rich region described as immunogenic in HPeVs (26). Moreover, VP3 of SPaV1 shares more than 40% identity with that of LV (Table 3), and the characteristics of other capsid proteins reinforce the closeness of SPaV1 and LV. Among the resemblances, the N-terminal extremity of VP0 is shorter than those of HPeVs and lacks the myristoylation site (Fig. 2A). Therefore, this site, described as mandatory for efficient viral infectivity of poliovirus

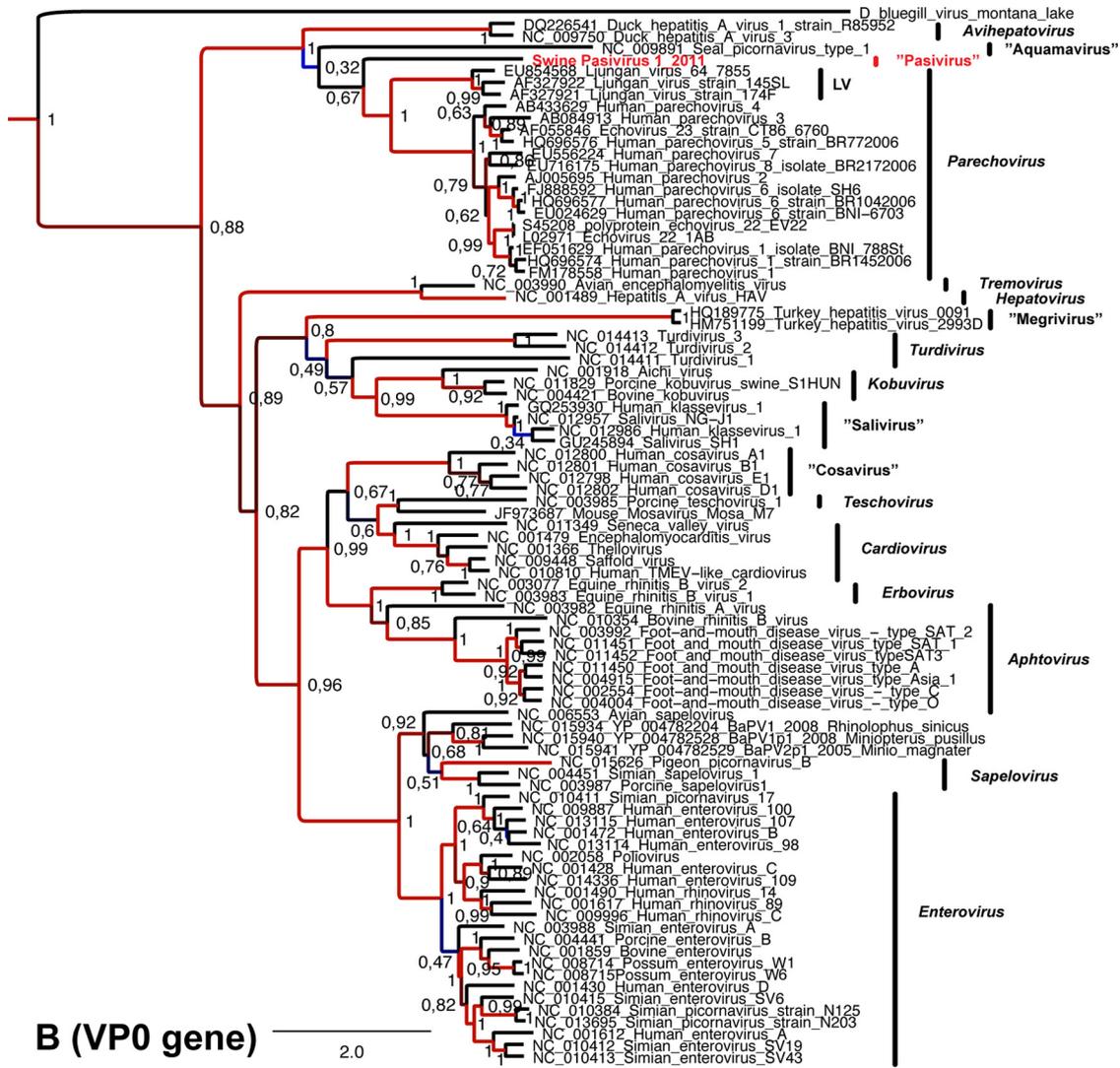


FIG 3 continued

(31), is not required for LV and SPaV1. Another capsid protein, VP1, exhibits two insertions of unknown function at the N-terminal extremity, one (11 aa) previously described in LV (25) and a second motif of 4 aa identified by multialignment of SPaV1, LVs, and HPeVs (Fig. 2C). The C-terminal extremity of VP1 contains a unique 41-aa extension (43 aa for VP1 of LV) and no RGD motif but a long C-terminal extremity (Fig. 2C). To date, RGD is the unique motif associated with viral entry mediated by integrin within parechoviruses. Among parechoviruses lacking an RGD motif, the well-studied HPeV3 has been associated with neuropathology (19). Nevertheless, no strict association between the presence/absence of RGD and the neurovirulence of parechoviruses has been demonstrated. The absence of the RGD motif implies the existence of an alternative cell receptor. In contrast with HPeVs, LV shares with SPaV1 a cleaved 2A protein, resulting in the 2A1 and the 2A2 proteins. The 2A1 protein of SPaV1 exhibits strong homology with that of LV. Due to the absence of the GXCG region, 2A lacks proteolytic activity, and SPaV1 therefore possesses a single 3C protease, as described for LV. One of the main differences between the 2A2 proteins of SPaV1 and parechoviruses is

the absence of both the H-box/NC motifs and the putative trans-membrane domain.

No pathogenicity was noted in infected piglets, which is reminiscent of the high frequency of asymptomatic infections for related parechoviruses infecting humans or animals. Nevertheless, HPeVs are pathogens frequently associated with various enteric, nervous, or respiratory syndromes in young children (49, 54). Another parechovirus, LV, was identified in bank voles (*Myodes glareolus*) in Europe and the United states (20). Interestingly, LV has been proposed as a potential environmental trigger for human type 1 diabetes on the basis of the presence of LV antibodies, while LV RNA detection remained negative, suggesting that the etiologic agent of the disease could be a cross-reactive virus (41, 42, 55). The spillover likelihood of such a virus could be greater from domestic animals than from wild animals, as seen for hepatitis E virus genotype 3, which is very prevalent but clinically silent in pigs and which frequently infects humans (43). Therefore, SPaV1 or another pasivirus could be a more relevant trigger than LV.

Major neutralizing antigenic sites have been located within exposed BC and EF loops of the capsid proteins and are therefore

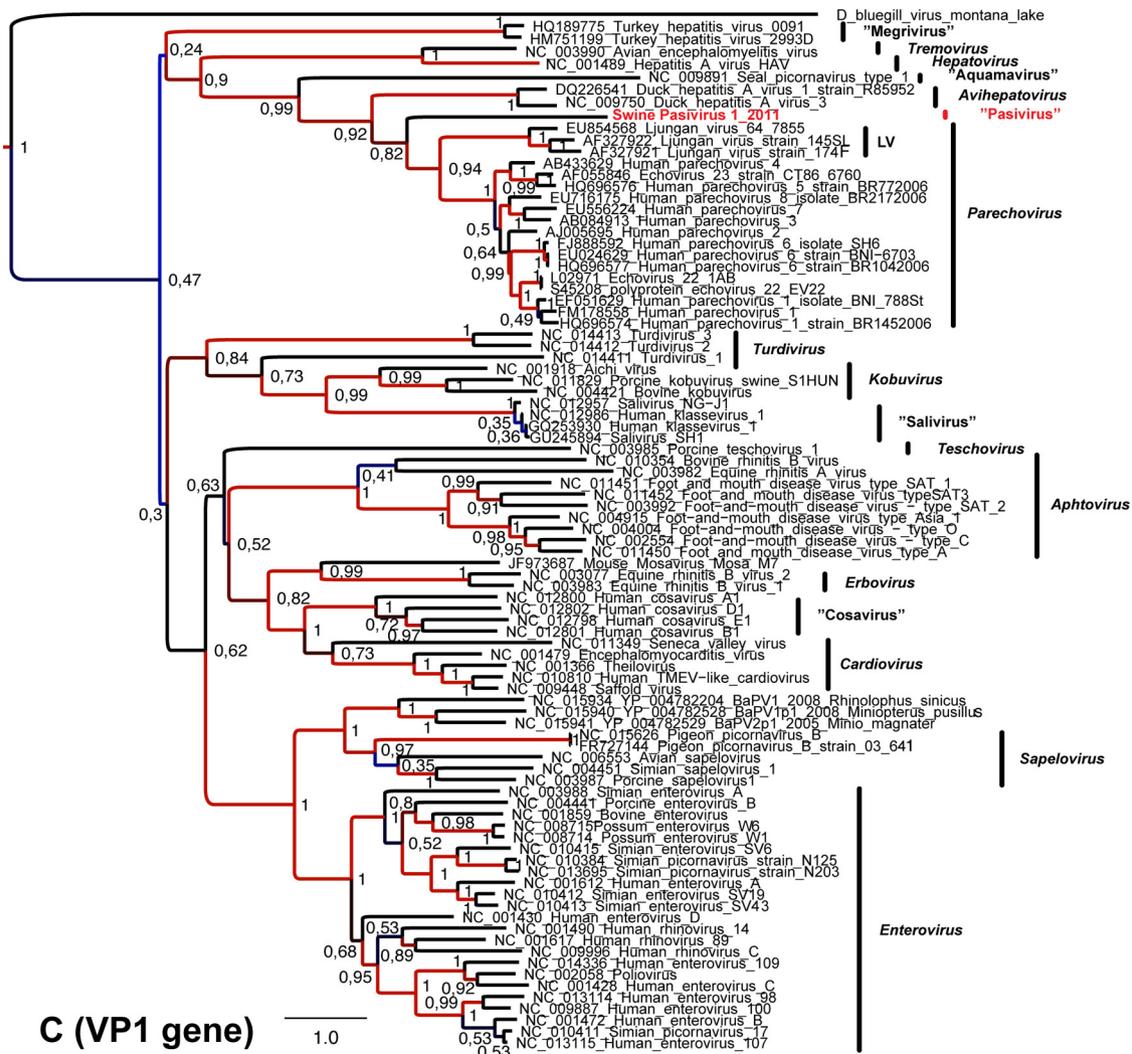


FIG 3 continued

suspected to shape the immunogenic specificity of picornaviruses (46). These BC and EF loops of SPaV1 (Fig. 2A to C) exhibit notable differences from LV and other parechoviruses, suggesting that cross-reactions are unlikely. Therefore, without experimental

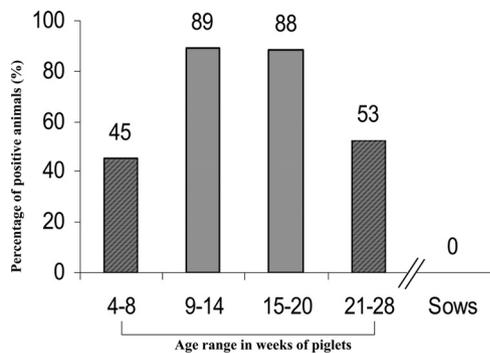


FIG 4 Prevalence of SPaV1 in fecal samples from 25 healthy sows and 75 healthy piglets ranging from 4 to 28 weeks old. The hatched bars represent weakly positive animals.

data, it is difficult to state that cross-reaction between LV and SPaV1 or another yet unknown member(s) of this new genus is impossible.

SPaV1 was identified in apparently healthy piglets, suggesting that the virus presents a silent circulation at the investigated farm. Furthermore, the detection on the same farm of several strains (0.7% to 9.3% divergent from SPaV1) suggested that swine are the natural hosts of this novel and predicted diversified genus of *Picornaviridae*. At the individual level, sequencing revealed several polymorphisms within 3D, indicating consistent variability. A better picture of SPaV1 biology would be achieved through the study of the prevalence, tropism, geographic distribution, and genetic variation of this new virus. If the zoonotic potential of SPaV1 is attested, and despite the absence of any pathogenicity in piglets, the threat to human health should be evaluated, considering its circulation in the vicinity of human populations.

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