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Characterization of novel porcine sapoviruses

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Abstract Sapoviruses are common caliciviruses known to cause enteric diseases in humans and animals. SaVs are genetically highly heterogeneous and are presently classified in five genogroups that are further subdivided in a number of genotypes. In recent years, a number of novel animal SaV strains, mostly of swine origin, have been partially characterized and proposed to represent novel genogroups or genotypes. We previously reported the detection and partial characterization of a wide range of variable and novel SaV strains of uncertain taxonomic status in Canadian swine. We now report on further genomic characterization of two novel strains to clarify their taxonomic relationship to other swine and human SaVs. Detailed analysis of different regions of their genomes, including determination of their complete capsid sequence, did not permit clear taxonomic assignment according to current criteria. This situation appears reminiscent of that of a number of SaV strains of swine origin and calls for a classification update for this calicivirus genus. We also report the detection of swine GIII SaVs for the first time in Canada.

GenBank accession numbers of all novel strains characterized in this study are indicated in the figure legends.

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Introduction

Sapoviruses (SaVs) are enteric pathogens of humans and animals and members of the family *Caliciviridae* (CV). Caliciviruses are small (27–40 nm) non-enveloped viruses containing a positive-sense single-stranded RNA of approximately 6.5–7.5 kb. The family *Caliciviridae* is presently divided into four established genera, *Norovirus*, *Sapovirus*, *Lagovirus* and *Vesivirus* [7]. Evidence has been accumulating over the past few years that more diversity exists within the family [4, 17, 23, 31]. Strains in each genus share a common genomic organization and are phylogenetically related. Sapoviruses and noroviruses cause enteric diseases in humans and animals, whereas the vesiviruses and lagoviruses cause systemic diseases in different mammals.

Sapoviruses were first discovered by electron microscopy (EM) in infant stool specimens suffering from a gastroenteritis outbreak in an orphanage in Sapporo, Japan, in 1977 [2]. SaVs are known to cause enteric diseases in humans of all ages but more so in younger children [9, 24, 28, 36]. The first complete SaV genome sequence (Manchester virus) was determined in 1995 [18]. Since then, a number of complete and partial genomic sequences have been reported from humans and animals. Like their NoV relatives, SaVs exhibit a high degree of genetic heterogeneity and have been classified based on complete capsid sequence into five genogroups, among which members of GI, GII, GIV, and GV are found to infect humans, and members of GIII are found in swine [5]. Each genogroup can be further subdivided into a number of genotypes or genetic clusters.

Porcine SaVs were first identified in faecal specimens of US piglets by EM [27]. Subsequently, pocine SaVs were reported in Europe, Asia and the Americas, suggesting

widespread distribution [14, 16, 19, 26, 35]. SaVs have been found in both diarrheic and asymptomatic pigs of different ages [3, 16, 26, 27]. The majority of porcine strains detected so far have been characterized as Cowden-like GIII SaVs [10, 14, 21, 26, 35]. However, in recent years, a number of novel and variant animal SaV strains distantly related to typical swine GIII and human strains have been detected in mink and swine [8, 16, 20, 34, 37]. Among these novel SaVs are strains that have been proposed as prototypes of novel genogroups based on pairwise comparisons of complete capsid sequences, such as strains JJ681 (GVI?), K7/Jp, K10/Jp and AB23 (GVII?), 43/06-18p3 and F19-10 (GVIII?) and F16-7 (G?) [16, 20, 34, 37]. Additional novel porcine strains have been detected in Canada and Europe [16, 26] and recently proposed by some authors to form novel genogroups GIX? and GX? based only on a short polymerase fragment [26].

We previously reported in a broad study on swine calicivirus diversity in Canada the detection and partial characterization of a number of novel SaV strains, which appeared, based on a short polymerase fragment, to be related to, but distinct from, strains JJ681 (GVI?) and K7/Jp, K10/Jp and AB23 (GVII?) [16]. Phylogenetic analysis of these atypical SaV strains using a short polymerase fragment revealed three related sub-clusters, each containing a number of strains. Further characterization of one selected strain (F16-7) representing one of the sub-clusters indicated that it might be a prototype of a novel genogroup or genotype [16]. We now report on further characterization of two additional strains representing a second sub-cluster to help clarify their taxonomical status. However, since the genogroup and genotype classification concepts are ill defined in the taxonomy of caliciviruses, and since there has been no classification update for SaVs in at least 5 years to include novel swine and mink strains, the status of these two strains, as is the case for a number of strains of swine origin characterized in recent years, remains nebulous. We also report for the first time the detection and partial characterization of GIII SaVs in Canadian swine.

Materials and methods

RNA extraction and RT-PCR amplification of SaV sequences from stool samples

Nearly 300 fecal samples were collected from farms and abattoirs in Canada between July 2005 and February 2007. Viral RNA was extracted from faecal homogenates as described previously [16]. The primer pair PEC65/PEC66 [32] was used to screen 30 samples from our collection for detection of GIII SaVs. After the initial RT at 42°C for 30 min, a denaturation step at 94°C was performed for 10 min, followed by 40 cycles of 30 s at 94°C, 60 s at 47.5°C and 60 s at 72°C. Amplification products were deposited on 1.5% agarose gels containing SYBR Safe (Invitrogen, Mississauga, ON, Canada) and visualized under UV light. Based on partial nucleotide sequences obtained previously from strains F2-4 and F8-9 [17], new specific primers were designed for 3′ RACE-PCR (F2-4 F1- 5′-CCCAAGTGGYCTYGTGTAYGAT-3′ and F2-4 F2 5′-TCAACTCAGTGTGCCACYTGAT-3′). Both primers were used in combination with primers (QT), (QO) and (QI) [30] for amplification of the 3′ end of strains F2-4 and F8-9. cDNA was synthesized using superscript III (Invitrogen, Mississauga, ON, Canada) and primer QT at 50°C for 60 min following the manufacturer's instructions. Nested PCR was performed successively using primers F2-4 F1 and F2-4 F2 in combination with primers QO and QI as described previously [16].

Cloning, sequencing and genomic analysis of SaV sequences

The RACE-PCR products were either gel-purified using a gel purification kit (Qiagen, Mississauga, ON, Canada) or purified directly on Qiagen mini spin columns (Qiagen, Mississauga, ON, canada) and cloned using a PCR TOPO 2.1 (T/A) cloning kit (Invitrogen, Mississauga, ON, Canada). Plasmid DNA from positive clones was extracted using an alkaline lysis method (midi-plasmid kit, Qiagen, Mississauga, ON, Canada). At least three clones were selected and sequenced in both directions with the Big Dye v3.1 chemistry on a 3730xl instrument from Applied Biosystems (Foster, CA, USA), using a primer-walking strategy. Sequence editing, assembly and analysis were performed using BioEdit version 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Multiple sequence alignments were performed using CLUSTAL W (version 1.6). Phylogenetic analysis was carried out using the neighbour-joining (NJ) method with the Poisson distance correction calculation for amino acids and the Kimura 2-parameter model for nucleotides using the Molecular Evolutionary Genetic Analysis (MEGA 3.1) software [15]. Confidence values at the nodes were obtained by performing 1,000 bootstrap analyses. Both within and between groups, mean pairwise amino acid identities were also calculated using MEGA 3.1.

Results

Detection of GIII SaV strains

A total of 30 samples from our collection that were previously found negative for SaVs using primers P289/P290

were screened with primer pair PEC65/PEC66, which was designed to detect GIII SaVs [32]. A total of 8 samples gave a band of the expected size, and the amplicons were sequenced, revealing fragments of 286 nucleotides and 95 amino acids in length. Since all strains were highly similar (95–100% nucleotide identity), we selected three strains originating from three different farms and from different age groups to carry out distance and phylogenetic analyses. Neighbour-joining phylogenetic analysis was performed with these three strains in addition to a selection of representative strains from the genus *Sapovirus*, including the human GI, GII, GIV and GV genogroups, mink SaV, the swine GIII genogroup and a number of unclassified swine SaV strains. Human and animal noroviruses were also included in the analysis as an outgroup (Fig. 1). Inspection of the phylogenetic tree shown in Fig. 1 revealed that strains F4-6, F10-9 and F18-5 formed a tight cluster supported by a strong bootstrap value with prototypical GIII SaV strains. Amino acid identity of the three Canadian strains was 83–95% to representatives of the GIII SaVs, which include the prototypical Cowden strain.

Novel SaV strains

To strengthen and help clarify the taxonomical status of strains F2-4 and F8-9, which formed one of the three novel SaV sub-clusters identified previously [17], additional sequence data from the 3′ end of their genomes, including the 3′ end of the polymerase gene, the complete VP1 (capsid) and VP2 genes and the 3′ UTR, were obtained. As shown in Table 1, both strains were predicted to encode a 543-amino-acid VP1 gene, which is very similar in size to those of strains K7/Jp and K10/Jp (GVII?), and a 168-amino-acid VP2 gene, which is identical in size to those of strains K7/Jp and K10/Jp (GVII?). The ORF1-ORF2 overlap for both strains was four nucleotides, which is typical of the majority of SaV strains. The 3′ UTR length of strain F2-4 was 29 nucleotides, whereas it was 33 nucleotides for strain F8-9; these are similar in length to the 3′ UTRs of strains JJ681 (GVI?), 28 nucleotides, and K7/Jp-K10/Jp (GVII?), 35 nucleotides.

Uncorrected pairwise amino acid identity and phylogenetic analysis using complete capsid gene sequences placed strains F2-4 and F8-9 closer to strains K7/Jp and K10/Jp (GVII?) (64% mean amino acid identity) than they were to strain JJ681 (GVI?) (53% mean amino acid identity) or to other prototypical SaV strains (<37% mean amino acid identity) (Table 2; Fig. 2). Strains F2-4 and F8-9 revealed 13% amino acid differences in their complete capsid genes. Pairwise amino acid identity analysis using the larger polymerase fragment (~250 amino acids) also placed strains F2-4 and F8-9 slightly closer to strains K7/Jp and K10/Jp (GVII?) (66% amino acid identity) than they were to strain JJ681 (GVI?) (64% amino acid identity). Similarly, genetic distance analysis using the complete VP2 gene sequences from F2-4 and F8-9 also placed both strains closer to strains K7/Jp and K10/Jp (GVII?) than they were to strain JJ681 (GVI?) or to any other prototypical SaV strains, consistent with the clustering performed using other genomic regions (not shown). Nucleotide alignments around the conserved polymerase/capsid junction region revealed low levels of conservation (>30% mismatches) between strains F2-4/F8-9, strains K7/Jp–K10/Jp (GVII?) and strain JJ681 (GVI?) (Fig. 3).

Despite numerous attempts and extensive efforts, we were unable to obtain additional sequence information on strains F11-7, F5-3, or F10-3, which form a third subcluster in the group of related novel porcine strains (G? Po SaV in Fig. 1).

Discussion

Like most RNA viruses, SaVs show great genetic diversity because of the poor template fidelity of their RNA polymerase [6]. The present classification scheme for SaVs, which dates back to 2004, proposes the existence of five genogroups and a number of genotypes for SaVs [5]. However, many recently characterized animal SaVs continue to have a floating status, partly because no recent update and no clear taxonomical criteria have been established with these novel strains, and also because there is limited sequence information for a number of these strains. Hence, a number of animal SaV strains have been tentatively classified into novel groups based either on partial polymerase or complete capsid gene sequences [16, 19, 20, 26, 34, 37].

The polymerase region of CVs contains conserved motifs that are ideal for primer design and molecular detection methods [1, 11, 12]. This explains why a large number of CV sequences from small polymerase regions are available in databanks. However, the reliability of the classification of CVs based solely on these short regions has been called into question by our results and those of others [13, 16, 34]. Partial polymerase sequences from a number of atypical swine SaV strains have revealed low levels of sequence conservation with prototypical SaVs, which might point towards the potential designation of novel genogroups [16, 19, 26, 34]. To strengthen their classification, further characterization of the complete capsid gene from selected strains representing novel clusters has been accomplished by some groups to help clarify their taxonomical status. This has led to the proposal of potential novel genogroups (GVI, GVII and GVIII) but has also left a number of strains in a nebulous zone [5, 16, 20, 34, 37].

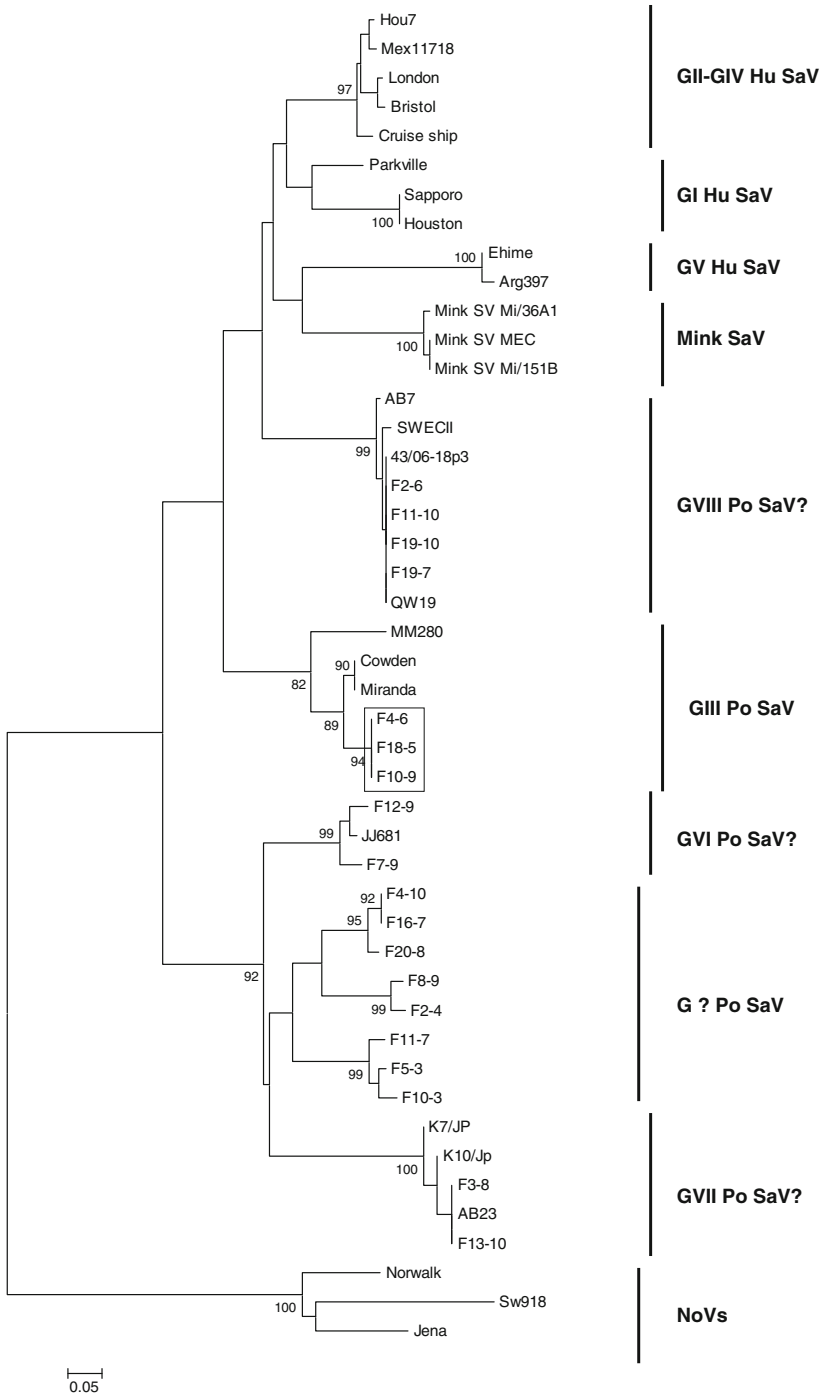

Fig. 1 Neighbor-joining phylogenetic trees of representative Canadian swine GIII sapovirus strains and other selected SaV strains based on a 95-amino-acid fragment from the RNA-dependent RNA polymerase gene region. Canadian strains sequenced in this study are *boxed*. Bootstrap values are expressed as percentages based on 1,000 replications and indicated at the branch point. Bootstrap values of ≤80% were hidden. The scale bar represents the distance expressed as aa substitutions per site. Accession numbers for the strains used in the analysis are: Norwalk (M87661), Sapporo (U65427), PEC-Cowden (AF182760), SWECII (AY615811), JJ681 (AY974192), Miranda (AY633965), K7/JP (AB221130), K10/Jp (AB221131), Jena (EU360814), Mink SV Mi/151B (AY144332), Mink SV Mi/36A1 (AY144333), Mink SV MEC (AF338404), MM280 (AY823308), Bristol (AJ249939), London (U95645), Cruise ship (AY157863), Houston/86 (U95643), Parkville (U73124), Arg39 (AF405715), Ehime (DQ058829), Mex11718 (AY157866), Hou7 (AF435814), 46/03-18p3 (EU221477), QW19 (AY826424), Sw918 (AB074893), F4-10 (EU860166), F16-7 (EU860167), F20-8 (EU860168), F8-9 (EU860169), F2-4 (EU860170), F11-7 (EU860171), F5-3 (EU860172), F10-3 (EU860173), F13-10 (EU860174), F3-8 (EU860175), F7-9 (EU860176), F12-9 (EU860177), F19-7 (EU860178), F11-10 (EU860179), F2-6 (EU860180), AB7 (FJ498795), F4-6 (GU230163), F18-5 (GU230165), F10-9 (GU230164)

Table 1 Genomic characteristics of SaVs described in this study and type strains

Genus strain genogroup	GenBank accession number	ORF1-ORF2 overlap (nt)	Capsid length (aa)	3′ ORF length (nt)	3′ UTR length (nt)
SaVs					
GI-Mex14917	AF435813	4	566	165	77
GII					
Cruise-ship	AY289804	1	559	166	98
Mex340	AF435809	4	558	166	108
GIII-PEC	AF182760	4	544	164	55
GIV-Hou7	AF435814	4	553	167	93
GV-Arg39	AY289803	4	569	166	86
GVI?-JJ681	AY974192	4	554	168	28
G?-F16-7	FJ498788	4	546	168	38
G? F2-4	**GU230161**	**4**	**543**	**168**	**29**
G? F8-9	**GU230162**	**4**	**543**	**168**	**33**
GVII?					
K7/Jp	AB221130	4	544	168	35
K10/Jp	AB221131	4	544	168	35
AB23	FJ498787	4	546	171	54
GVIII?					
46/03-18p3	EU221477	4	563	168	104
F19-10	FJ498786	4	563	168	102

Strains characterized in this study are in boldface

Table 2 Amino acid identities in the complete capsid region (VP1) between SaV strains F2-4/F8-9 and selected reference CV strains

Strain	Amino acid sequence identity range (%)								
	SaV GI	SaV GII	SaV GIII	SaV GIV	SaV GV	SaV GVI	SaV GVII	SaV GVIII	NoV
F2-4/F8-9	(33–37)	(35–37)	(33–35)	(34–37)	(34–36)	(52–53)	(63–65)	(33–34)	(16–18)

GI SaVs include Sapporo, Dresden and Houston90; GII SaVs include Chiba and Cruise ship; GIII SaVs include JJ259, PEC-Cowden and MM280; GIV SaVs include Hou7, Chiba671 and Angelholm; GV SaVs include Arg39, Ehime475 and Ishikawa; GVI SaVs include JJ681; GVII SaVs include K7/Jp and K10/Jp; GVIII SaVs include 03-18p3; NoVs include Norwalk, Southampton and Jena viruses; Genogroups GVI, GVII and GVIII are proposed genogroups

In this study, strains F2-4 and F8-9 representing a subcluster of a newly identified group of atypical swine SaV strains related to both proposed GVI and GVII genogroups were originally thought to be representatives of a novel SaV genogroup based on their partial polymerase sequences [16]. Further characterization and analysis of the complete 3′ end of their genomes clearly placed both strains closest to the proposed GVII strains. Pairwise amino acid identity analysis of the complete capsid sequence placed both strains between the genotype and genogroup cutoff values according to the current SaV taxonomic scheme [5]. Hence, the taxonomic assignment of swine SaV strains F2-4 and F8-9, like those of other swine SaV strains, remains undefined. However, as noted by Wang and Saif, this could simply be due to insufficient numbers of SaV strains that have been characterized to date compared to NoVs, which have a broader distance range of genotypes (14.3–43.8% compared to 9–30% for SaVs) [33, 38].

Furthering the conundrum about the classification of strains F2-4 and F8-9 is the analysis of the recombination hotspot situated at the polymerase-capsid junction, which has been shown previously to be highly conserved between strains belonging to the same SaV genogroup [9, 29, 34]. This region does not appear well conserved between strains F2-4, F8-9 and representatives of both proposed GVI and GVII genogroups. Since this region has been suggested to be a genogroup marker, our data suggest that strains F2-4 and F8-9 might be representatives of a novel genogroup [9, 19, 29]. Alternatively, this region might not be as conserved as previously thought among related SaV strains, and as more strains are being characterized, this might become even more evident.

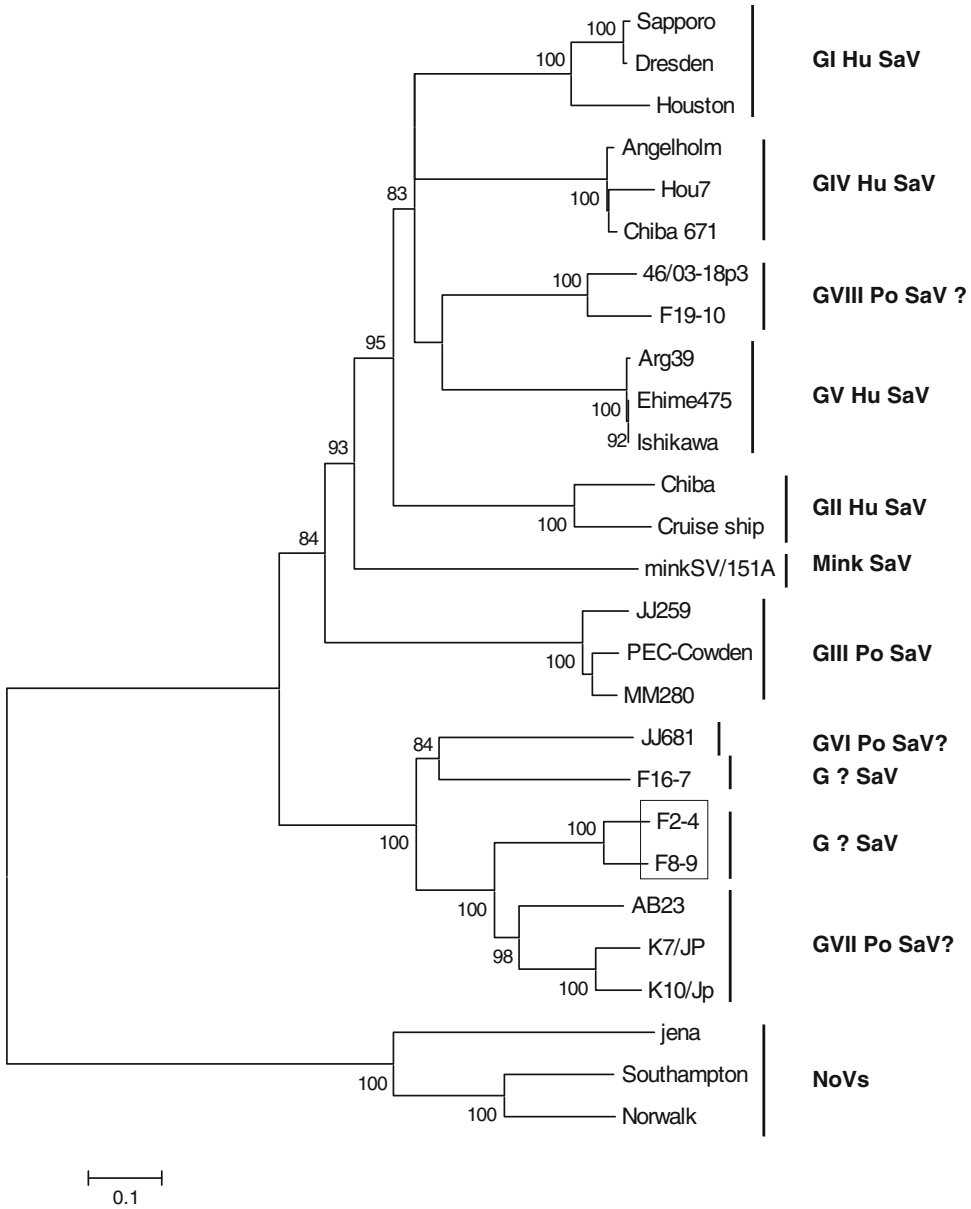

Fig. 2 Phylogenetic trees based on amino acid alignments of complete VP1 genes from SaVs. Trees were constructed by the neighbor-joining method of MEGA 3.1 using Poisson distance calculations. The confidence values at the nodes were obtained by performing 1,000 bootstrap analyses. Bootstrap values of ≤80% were hidden. The *scale* represents distance expressed as amino acid substitutions per site. Accession numbers for the strains used in the analysis are: Norwalk (M87661), PEC-Cowden (AF182760), Sapporo (U65427), Southampton (L07418), Dresden (AY694184), Cruise ship (AY157863), Houston/86 (U95643), Mink SV Mi/151B (AY144332), Chiba (AJ606690), Angelholm (DQ125334), Hou7 (AF435814), Chiba671 (AJ78649), 46/03-18p3 (EU221477), Arg39 (AF405715), Ehime 475 (DQ058829), Ishikawa (AJ786352), JJ259 (AY826423), MM280 (AY823308), JJ681 (AY974192), K7/JP (AB221130), K10/Jp (AB221131), Southampton GI-2 (L07418), Jena (EU360814), F16-7 (FJ498788), F19-10 (FJ498786), AB23 (FJ498787), F2-4 (GU230161), F8-9 (GU230162)

Therefore, the usefulness of this region as a genogroup marker remains to be confirmed.

The taxonomic situation of strains F2-4 and F8-9 is reminiscent of that of strains 43/06-18p3 and F19-10, which have been proposed to be either representatives of a novel genogroup (GVIII?) or novel GV genotypes that are distantly related to some human strains [16, 20]. Strain F16-7 is also in a similar ambiguous taxonomical situation, since it appears to be distantly related to strain JJ681 (GVI?), potentially representing a novel genotype or genogroup of SaV [16].

Recombination among CVs is known to occur predominantly between closely related strains but can also take place between more distantly related strains [22, 25].

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JJ681          GTGTACACAATGGAGGGGCC--AAAGC        GVI
F8-9           *****TGTC**********AACGGCT         G ?
F2-4           *****TGTC**********AACGGCT         G ?
F16-7          *****TGTG**********CC***AG         G ?

K7/Jp          GTGTATAAAATGGAGGGTCCTGAGGGG        GVII
K10/Jp         **************************         GVII
AB23           *******G********G*******CT         GVII
F8-9           ******GTC********G**AAC**CT        G ?
F2-4           ******GTC********G**AAC**CT        G ?
F16-7          ******GTG********G**CC*AAA*        G ?
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Fig. 3 Nucleotide alignment of the conserved 27-nucleotide motif at the polymerase-capsid junction between strains F2-4, F8-9, AB23, F16-7 and proposed GVI and GVII SaVs. *Asterisks* indicate identical residues to the top line of each alignment. The ATG initiation codon is *underlined*. The two novel Canadian strains described in this study are in *boldface*. GVI and GVII are proposed genogroups. Strains F16-7 and AB23 have an uncertain taxonomic status

Recombinant strains of caliciviruses are evidenced when two genomic regions (usually the polymerase and the capsid regions) cluster in separate groups when subjected to phylogenetic analysis. In the present study, we could find no evidence of recombination for strain F2-4 or strain F8-9, since all regions analysed clustered them closer to K7/Jp and K10/Jp strains than to any other prototypical strains.

In our previous study, although we detected a number of atypical SaV strains, we did not detect what appears to be the most common group of swine SaVs worldwide: Cowden-like GIII [14, 16, 21, 35]. One of the hypotheses put fourth to explain this observation is that the primer pair P289/P290 used in our study was not optimal for amplification of GIII strains. Consistent with this idea is the report of Wang et al., which showed that a closely related primer pair (P290/P110) was also not very efficient in amplifying GIII SaV strains [32]. In addition, analysis of the binding sites of primer pair P289/P290 to representative GIII strains revealed an important number of mismatches, particularly for reverse primer P289, which showed up to 50% mismatches compared to GIII SaV target genomes (not shown). Although definitive classification of SaV strains should be minimally based on the complete capsid sequence, phylogenetic and pairwise amino acid analysis of the short polymerase fragment can be used to tentatively classify these strains as GIII, making them the first potential Canadian SaVs to be grouped in this cluster.

In summary, we report for the first time in Canada what appears to be the most common swine SaV group: Cowden-like GIII. In addition, distance and phylogenetic analysis of a short polymerase fragment along with sequence analysis of the conserved polymerase/capsid region of strains F2-4 and F8-9 suggest that these two strains might represent a novel SaV genogroup. However, phylogenetic and distance analysis of the complete capsid gene suggest that they might instead represent a distant genotype of genogroup GVII, since the distance between representatives of F2-4/F8-9 and GVII is significantly shorter than typical SaV intergenogroup distances [5]. The data presented in this study and elsewhere [16, 20, 34, 37] underscore the ambiguity around classification of animal SaVs below the species level and also serve as a word of caution before proposing novel SaV genogroups based only on short polymerase sequences. It is hoped that as more animal SaV sequences from around the world and from informative genomic regions become available, an updated classification system will be developed for this emerging CV genus.

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