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Discovery of a Novel Human Picornavirus in a Stool Sample from a Pediatric Patient Presenting with Fever of Unknown Origin▽

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Fever of unknown origin (FUO) is a serious problem in the United States. An unidentified agent was cultured from the stool of an infant who presented with FUO. This virus showed growth in HFDK cells and suckling mice. Using DNase sequence-independent single-primer amplification, we identified several nucleotide sequences with a high homology to Theiler's murine encephalomyelitis virus. Nearly full-length viral genome sequencing and phylogenetic analysis demonstrate that this virus is a member of the *Cardiovirus* genus of the *Picornaviridae* family.

A clinical example of fever of unknown origin (FUO) occurs when a patient presents with a temperature higher than 100.9°F that lasts more than 3 weeks as well as failure to reach a diagnosis after 1 week of patient investigation (17). Viral agents account for most of these self-limiting cases, which usually resolve within 2 weeks (5). There are four categories of potential etiology of FUO: classic, nosocomial, human immunodeficiency virus-associated, and immune deficient (5). Failure to reach a diagnosis in patients presenting with FUO is common since 20% of cases have no definitive diagnosis (5).

Two species of viruses are distinguished within the *Cardiovirus* genus in the *Picornaviridae* family of viruses. One is the Theiler viruses, which includes Theiler's murine encephalomyelitis virus (TMEV), Theiler-like virus, and Vilyuisk virus (18–20). TMEV primarily infects mice, and Theiler-like virus was isolated from a rat (18). Vilyuisk virus, believed to be responsible for a neurodegenerative disease in the Yakuts people in the Vilyuy River area in Siberia, primarily infects humans (15). Interestingly, Vilyuisk virus, the only cardiovirus suspected to cause disease in humans, is not fully sequenced. The second species is the encephalomyocarditis viruses (EMCVs), which includes mengovirus, EMCV, Maus Elberfeld virus, and Columbia SK virus (4, 16). In addition to mice, these cardioviruses also infect humans, pigs, horses, elephants, squirrels, and monkeys (1, 6, 7, 9, 12).

In this study we sought to characterize a cardiovirus that was isolated from the stool of a patient who presented with FUO. Here, we used DNase sequence-independent single-primer amplification (DNase-SISPA) to obtain genetic information about the virus. Nearly full-genome sequencing and phylogenetic analysis was also performed.

MATERIALS AND METHODS

Study subject. The patient was an 8-month-old female with FUO presenting in November 1981. A stool sample was collected at the San Diego County Public Health Department Laboratory.

Cell culture. The virus was first cultured in WI-38 cells and passed to HFDL-645 cells obtained from the State of California Viral and Rickettsial Disease Laboratory (VRDL) at the San Diego County Public Health Department Laboratory. L-645 is a line of fetal human diploid fibroblasts started and maintained by the VRDL. The sample was subsequently sent to the VRDL for further characterization. There, it was grown in in-house human fetal diploid kidney (HFDK) cells but failed to grow in primary monkey kidney, A-549, BSC, and RD cells. The virus grew in suckling mice. We did not harvest sera from any mouse for sequencing or any other analysis of Saffold virus (SAF-V).

Further characterization was carried out at the VRDL. By electron microscopy the agent had a diameter of 28 to 30 nm and appeared as a typical picornavirus. It was acid stable, was not neutralized by pooled human gamma globulin, and was temperature sensitive (no growth at 33°C).

DNase-SISPA. We screened cell-free medium that was cultured from HFDK cells. One hundred microliters of cell-free medium was filtered through a 0.22-μm-pore-size filter (Ultrafree MC; Millipore, Bedford, MA) and treated with 250 units of DNase I (Roche Diagnostics, Mannheim, Germany) to remove contaminating double-stranded DNA. DNase I-resistant nucleic acids were purified using a MagNA Pure LC DNA Isolation Kit I (Roche Diagnostics, Mannheim, Germany). Single-stranded DNA was created as follows: 50 μl of extracted nucleic acid was incubated with 240 pmol of primer A (5′-GTTTCCCAGTCACGATCNNNNNNNNN-3′) and 40 units of RNase I for 5 min at 65°C. The mixture was cooled to 25°C, and the following components were added: 7.5 μl of Stratascript buffer, 7.5 μl of 0.1 M dithiothreitol, 1 μl of 10 mM deoxynucleoside triphosphate (dNTP) mix, and 800 units of Stratascript reverse transcriptase (Stratagene, La Jolla, CA). The reaction mixture was incubated at 42°C for 30 min. Double-stranded DNA was generated by incubating 44 μl of single-stranded DNA with 5 units of Klenow fragment (exo$^-$) (New England Biolabs, Beverly, MA) and 5 μl of 10× EcoPol buffer (New England Biolabs, Beverly, MA) at 10°C for 5 min, followed by 8 min at 37°C. Twenty-five microliters of double-stranded DNA was subsequently amplified by 5 units of Takara Ex *Taq* (Takara Bio, Shiga, Japan) with 100 pmol of primer B (5′-GTTTCCCAGTCACGATC-3′) in 1× Takara buffer, 2 mM $MgCl_2$, and 0.8 mM dNTP with the following cycling conditions: 40 cycles of 94°C for 21 s, 40°C for 30 s, 50°C for 30 s, and 72°C for 1 min. Next, 44 μl of PCR product was digested with 5 units of the restriction enzyme Csp6.I (Fermentas, Hanover, MD) in 1× B$^+$ buffer (Fermentas, Hanover, MD). Twenty picomoles of adaptors composed of the hybridized oligonucleotides NBam24 5′-AGGCAACTGTGCTATCCGAGGGAG-3′ and NCsp11 5′-TACTCCCTCGG-3′ was then ligated to 15.5 μl of the restriction enzyme-digested DNA using 200 units of T4 DNA ligase (Invitrogen, Carlsbad, CA) in 1× T4 DNA ligase buffer for 10 min at room temperature. Two micro-

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TABLE 1. Growth of SAF-V in tissue culture and suckling mice

Cell type or animal	Growth[a]
HFDK	++
Suckling mice	++
HFDL	+
BSC	−
RD	−
PMK	−
A549	−

[a] ++, normal growth; +, poor growth; −, no growth.

liters of the ligation reaction mixture was used as a template in a 50-μl PCR mixture containing 25 pmol of NBam24, 1× Takara buffer, 0.8 mM dNTPs, and 2.5 units of Takara Ex *Taq*. The PCR mixture was heated to 72°C for 3 min before the addition of Takara Ex *Taq*. The mixture was then heated for an additional 5 min at 72°C to generate the primer-binding sites. Cycling conditions were as follows: 94°C for 1 min and 72°C for 3 min for 40 cycles.

Analysis of amplified DNA. Amplified PCR products were analyzed by polyacrylamide gel electrophoresis (PAGE). The distinct DNA bands seen by PAGE were excised and pooled. DNA from the pooled PAGE bands from each sample were purified and subcloned into pCR-II-TOPO (Invitrogen, Carlsbad, CA). Because excised DNA bands were pooled prior to subcloning, we were unable to relate each sequence to a particular PAGE band. Plasmids containing inserts were purified and sequenced using the T7 sequencing primer. We sequenced our cloned and uncloned PCR fragments by using a Big Dye terminator, version 3.1, sequencing kit (Amersham Pharmacia Biotechnology) and an ABI Prism 3130x genetic analyzer (Applied BioSystems) according to the manufacturers' instructions.

Amplification of the SAF-V genome. To amplify regions of SAF-V flanking the sequences that we found, we designed primers based on an alignment of TMEV (X56019), Theiler-like virus (AB090161), mengovirus (L22089), and EMCV (M81861), using Primer Premier, version 5.0, software (Palo Alto, CA). All amplicons were then sequenced using primer walking. The 5′ end of SAF-V was found using a kit for the 5′ rapid amplification of cDNA ends (Invitrogen) according to the manufacturer's instructions.

Phylogenetic analysis of the SAF-V genome. Phylogenetic analysis was performed using sequences representing full-length genomes of virus species representing all genera of the *Picornaviridae*. Sequences of all recognized individual virus species were included in the analysis except for the members of the *Enterovirus* genus, for which participation in the analysis was limited to the two most divergent members of the genus for presentation purposes. Picornavirus sequences for echovirus 5 (accession number AF083069), human rhinovirus 1B (D00239), porcine enterovirus 8 (AF406813), human hepatitis virus type A (M20273), simian hepatitis A virus (D00924), avian encephalomyelitis virus (AJ225173), Ljungan virus (AF327921), human parechovirus 2 (AJ005695), echovirus 22 (L02971), echovirus 23 (AF055846), mengovirus (L22089), EMCV (X87335), TMEV (M20562), Theiler-like virus NGS910 (AB090161), foot-and-mouth disease virus (AF308157), equine rhinovirus type 1 (X96870), Aichi virus (AB040749), porcine teschovirus (AF231769), porcine enterovirus 1 (AJ011380), equine rhinovirus 3 (AF361253), and equine rhinovirus type 2 (X96871) were retrieved from the GenBank and aligned using the CLUSTALX program (24). A neighbor-joining tree based on nucleotide distances with the Jukes-Cantor correction and pair-wise gap deletions with bootstrap resampling (100 replicates) was constructed using the MEGA3 software (13). More detailed phylogenetic analysis was performed for each genomic region (5′ and 3′ untranslated regions [UTRs], leader peptide, and the proteins VP1 to VP4, 2A to 2C, and 3A to 3D) of cardioviruses. For the Vilyuisk virus, no full-length genome sequence is available. Therefore, we used sequence M94868 in the analysis of homologous regions. Noncoding regions of cardioviruses were aligned at the nucleotide level, and coding regions were aligned at the amino acid level and then back-translated to the nucleotide sequences using the CLUSTALW program as implemented in the BioEdit program (8). For each region, phylogenetic trees were built with bootstrap resampling (100 replications) using the MEGA3 program and based on nucleotide p-distances for noncoding regions and on amino acid p-distances for coding regions. Nucleotide and amino acid similarities between the sequences of the SAF-V and other cardioviruses were calculated by the MEGA3 program. To check whether the SAF-V is a recombinant of known viruses, recombination analysis was performed using the bootscan method of the SimPlot program (http://sray.med.som.jhmi.edu/SCRoftware/).

Nucleotide sequence accession number. The sequence of the SAF-V has been deposited in the GenBank database under accession number EF165067.

RESULTS

Patient presents with FUO. In November 1981 an 8-month-old infant presented with FUO 24 days after the onset of symptoms. A stool sample was taken from the patient and cultured at the San Diego County Public Health Laboratory. Because the laboratory was unable to determine the agent, the culture was subsequently sent to the VRDL for more thorough characterization. There, a viral agent was observed that grew well in HFDK cells and in suckling mice and weakly in HFDL (human fetal diploid lung) cells and was unable to grow in PMK, A549, BSC, and RD cells (Table 1). Electron microscopy showed the virus to be 28 to 30 nm in diameter and acid stable (data not shown). However, this methodology alone was insufficient to identify the virus.

Molecular detection of new picornavirus. Different segments of the genome of this unknown virus were amplified nonspecifically using DNase-SISPA (Materials and Methods). The viral isolate that we used to acquire our sequence data was cultured in HFDK cells. DNase-SISPA is useful in that it does not require prior sequence knowledge (11). The PCR products were analyzed by PAGE (Fig. 1). This yielded several distinct bands (Fig. 1), which we subcloned and sequenced. When we analyzed these sequences with BLASTn (which searches for sequence homology on a nucleotide level), there was no significant similarity to a virus. However, using tBLASTx (which compares all possible [six-frame] translations of a nucleotide sequence against all possible [six-frame] translations of a nucleotide sequence database), all of our nucleotide sequences showed a strong similarity to Theiler-like virus, which is a member of the cardiovirus genus in the *Picornaviridae* family.

Cloning of new picornavirus. To amplify the novel picornavirus genome, which we have provisionally named SAF-V, we used several different strategies. To amplify regions of the genome surrounding the sequences that we found, we designed primers based on the DNase-SISPA acquired sequence and an

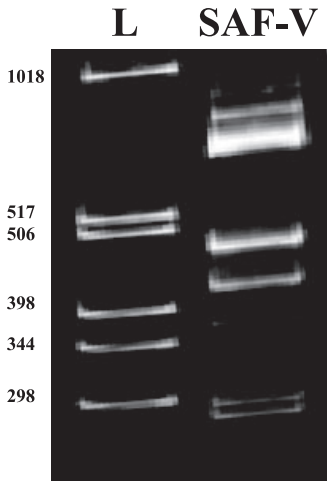

FIG. 1. DNase-SISPA amplification of tissue culture sample containing SAF-V. PCR products were analyzed on a 6.5% acrylamide gel. Lane L contains molecular size markers in base pairs.

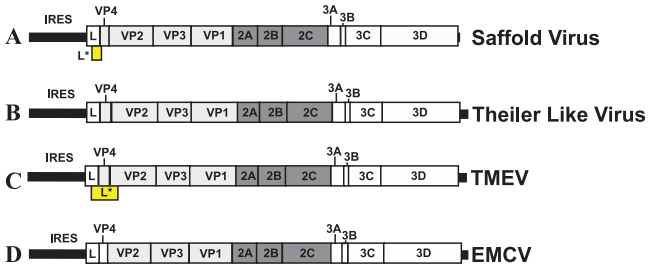

FIG. 2. Schematic representation of SAF-V (A), Theiler virus (B), TMEV (C), and EMCV (D) genomes. The boxes represent the genes that encode proteins. Solid black horizontal lines represent the UTRs. Yellow boxes represent the genes that encode the L* proteins.

alignment of well-conserved cardiovirus sequences (TMEV, Theiler-like virus, Mengovirus, and EMCV) using Primer Premier, version 5.0, software (Premier Biosoft International, Palo Alto, CA). To identify the 5′ end of the genome, we used the method for the 5′ rapid amplification of cDNA ends (Materials and Methods). The almost full-length sequence of SAF-V was 7,977 bp.

Molecular characteristics of SAF-V. The genetic organization of SAF-V is similar to that of other cardioviruses since it has one open reading frame (ORF) that has 13 cleavage sites in its genome, which should separate the viral polypeptide into the following proteins: L, VP4, VP2, VP3, VP1, 2A, 2B, 2C, 3A, 3B, 3C, and 3D (Fig. 2).

Overall, the Theiler-like virus genome had the highest nucleotide identity to SAF-V of any cardiovirus. Specifically, the 5′ UTRs of SAF-V and Theiler-like virus were most similar (85%) in nucleotide identity (Table 2). Moreover, except for VP1 of Vilyuisk virus, the proteins of Theiler-like virus had the highest amino acid identity to those of SAF-V of any of the cardiovirus proteins (Table 3).

The coding region for the L protein of SAF-V has 64% nucleotide identity to the coding regions of the L proteins of Theiler-like virus and TMEV (Table 2). The L protein of SAF-V contains the zinc finger and the acidic domains that are present in all sequenced cardioviruses (2; Fig. 3). Furthermore, the Ser/Thr domain is partially deleted and contains one threonine codon in this domain which has the potential to be phosphorylated (Fig. 3).

It has been reported that the L* protein exists in persistent strains of TMEV such as DA yet not in the neurovirulent strains GDVII and FA (17). We identified an L* protein coding region in SAF-V (Fig. 2). It is highly improbable that the C* ORF encodes a protein, because it has ACG as its start codon. In contrast to the L* protein of TMEV strain DA, which has 156 amino acids, the L* ORF of SAF-V encodes 57 amino acids and is 31% similar to the first 62 amino acids of the TMEV L* protein (data not shown). Nevertheless, we do not know if this ORF codes for a protein.

TABLE 2. Nucleotide identities between the sequences of the SAF-V and other cardioviruses

Virus	% Identity with SAF-V region or protein[a]													
	5′ UTR	L	VP4	VP2	VP3	VP1	2A	2B	2C	3A	3B	3C	3D	3′ UTR
Theiler-like virus	85	64	68	66	70	56	66	71	76	72	58	73	74	96
Vilyuisk virus	ND	64	68	66	69	60	58	ND	ND	ND	ND	ND	ND	ND
TMEV	75	63	62	65	68	58	64	64	71	65	67	69	69	89
EMCV	51	54	65	63	63	53	40	41	59	47	57	51	61	53
Mengovirus	54	49	65	64	63	55	37	44	60	50	45	51	59	60

[a] ND, no data.

TABLE 3. Amino acid identities between the sequences of SAF-V and other cardioviruses

Virus	% Identity with SAF-V protein[a]											
	L	VP4	VP2	VP3	VP1	2A	2B	2C	3A	3B	3C	3D
Theiler-like virus	69	73	74	84	58	69	74	89	80	70	81	84
Vilyuisk virus	68	73	70	81	60	25	ND	ND	ND	ND	ND	ND
TMEV	65	69	70	83	57	65	61	78	70	60	76	77
EMCV	47	64	68	68	47	23	26	58	41	35	46	62
Mengovirus	46	67	68	68	49	21	26	58	38	40	45	62

[a] ND, no data.

The EF loop structure of cardioviruses, associated with virulence, is located in the VP2 protein. Specifically, there are two loops within the larger loop structure. The first part of the SAF-V EF loop structure is unique since it has only 2 amino acid identities to other cardioviruses (Fig. 4). In contrast, the second loop of SAF-V has 7 of 18 (39%) amino acid identities to the second loops of Theiler-like virus and Vilyuisk virus (Fig. 4). Interestingly, there is an insertion in the middle of the second loop of the SAF-V EF loop, which is not found in any other cardiovirus VP2 protein (Fig. 4). The CD loop structure of cardioviruses, also associated with virulence, is located in the VP1 protein. The first of the two SAF-V CD loops has no amino acid identity to other cardioviruses, and the second half of the loop is deleted (Fig. 5). The second CD loop of Theiler-like virus and Vilyuisk virus has 47% (8/17) and 39% (7/17) amino acid identity to the second CD loop of SAF-V, respectively. Taken together this suggests that the structure of SAF-V is unlike all known cardioviruses.

Phylogenetic analysis of SAF-V. To establish the evolutionary relationship of SAF-V to other picornaviruses, phyloge-

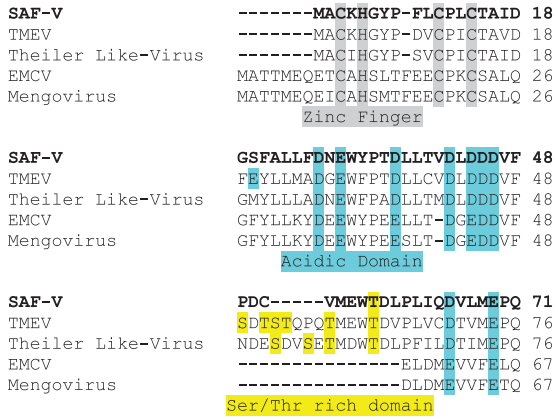

FIG. 3. Alignment of the cardiovirus leader peptides. The zinc finger domain is highlighted in gray, the acidic domain is highlighted in aqua, and the Ser/Thr domain is highlighted in yellow.

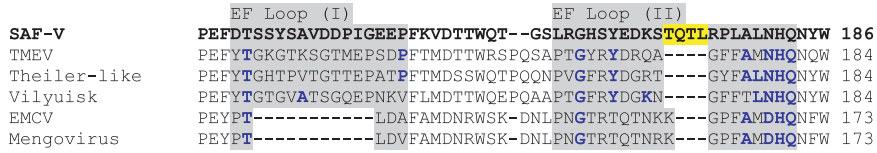

FIG. 4. Alignment of the EF loop structure amino acids in VP2 of cardioviruses SAF-V, TMEV, Theiler-like virus, Vilyuisk virus, EMCV, and mengovirus. Areas highlighted in gray are part of the EF loop structure. The area highlighted in yellow is an addition to the CD loop structure of SAF-V. Amino acids shown in blue are identical to the amino acids in SAF-V.

netic analysis of the genomes from all known members of the *Picornaviridae* family was performed (Fig. 6).

Our analysis indicated that SAF-V was closely related to the Theiler-like virus, which is a member of the genus *Cardiovirus* (Fig. 6A). The next most closely related virus was TMEV, followed by mengovirus and EMCV, all members of the *Cardiovirus* genus (Fig. 6A). Phylogenetic analyses of each genomic region confirmed these relationships (Fig. 6B). Recombination analysis using the bootscan method was also performed on SAF-V to determine if it was a recombinant of other known picornaviruses (Fig. 7). As SAF-V is a new member of the *Picornaviridae* family, there are many moderate-length genetic regions within the SAF-V genome that were similar to the Theiler-like virus and TMEV (Fig. 7). However, the short length of these regions of similarity and the low bootstrap support for these associations suggested that SAF-V was not a recombinant. Our results therefore indicated that SAF-V is a unique member of the *Picornaviridae* family that is closely related to the four members of the *Cardiovirus* genus.

DISCUSSION

Here we characterized a novel human picornavirus, which we have provisionally named SAF-V, that was cultured from the stool of an infant who presented with FUO.

Molecular evolutionary analysis demonstrated that SAF-V is a new member of the *Cardiovirus* genus in the *Picornaviridae* family (Fig. 6 and 7). In the phylogenetic analysis of the full-length genome sequences, SAF-V appeared to be most closely related to Theiler-like virus, followed by TMEV of mice and rats (Fig. 6A). The only human virus related to the Theiler viruses identified so far was the Vilyuisk virus (15). This virus, for which only short genetic regions have been sequenced, was known to be the most divergent virus within the Theiler group of viruses (Fig. 6B). Our analysis of the genetic region for which amino acid sequence of the Vilyuisk virus is available demonstrated that SAF-V is closely related to the Theiler's group (Fig. 6B). The bootstrap analysis of SAF-V demonstrated that this virus is not a recombinant of other known picornaviruses (Fig. 7).

Currently, two virus species are recognized among cardioviruses: EMCVs (mengovirus and EMCV, for which full-genome sequences are available, as well as Columbia SK and Maus Elberfeld viruses, which are not sequenced) and the Theiler virus species (TMEV, Theiler-like virus, and Vilyuisk virus) (23). The demarcation criteria in the genus are that the members of a species share greater than 70% amino acid identity in the VP1, 2C, 3C, and 3D coding regions and that they share a natural host range. While our results demonstrated that the SAF-V is related to the Theiler virus species, its amino acid similarity in the VP1 region to the recognized members of the Theiler virus species is below 70% (Table 2).

The genetic organization of SAF-V is very similar to other cardioviruses. The SAF-V structural proteins VP1, VP2, VP3, and VP4 are in the same locations as those of TMEV (Fig. 2). In addition, they are approximately the same size. Specifically, the loci of genes in SAF-V are more similar to Theiler virus species than the EMCV species (Fig. 2), which is not surprising in light of the phylogenetic data. Since SAF-V does not have a poly(C) sequence in its 5′ UTR, which is associated with virulence in EMCV (13), we would not expect SAF-V to be virulent. Conversely, the absence of a poly(C) sequence in the 5′ UTR does not preclude SAF-V virulence (14, 26).

The four loop structures that form the exterior of the cardiovirus surface are two small loops within the EF loop structure of VP2 and the two loops of the CD loop structure of VP1 (10). The EF loop structure of SAF-V is unique since the second EF loop contains a 4-amino-acid insertion, and both loops combined contain no more than 26% amino acid identity to the EF loops of other cardioviruses (Fig. 4). Similarly, the CD loop structure of SAF-V also has low homology to all other cardioviruses (0% in the first loop and 47% in the second to Theiler-like virus) as well as a 4-amino-acid deletion in the first loop. In contrast, TMEV has 63 and 44% amino acid identity to the first and second CD loops of Theiler-like virus, respectively. This suggests that the viral surface of SAF-V has a novel structure and possibly a different mechanism for infection. Furthermore, the primary sequences of the EF and CD loop structures in SAF-V do not offer any clues as to whether

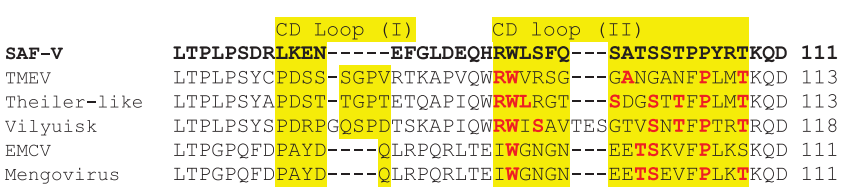

FIG. 5. Alignment of the CD loop amino acids in VP1 of cardioviruses SAF-V, TMEV, Theiler-like virus, Vilyuisk virus, EMCV, and mengovirus. Areas highlighted in yellow are part of the CD loop structure. Amino acids shown in red are identical to the amino acids in SAF-V.

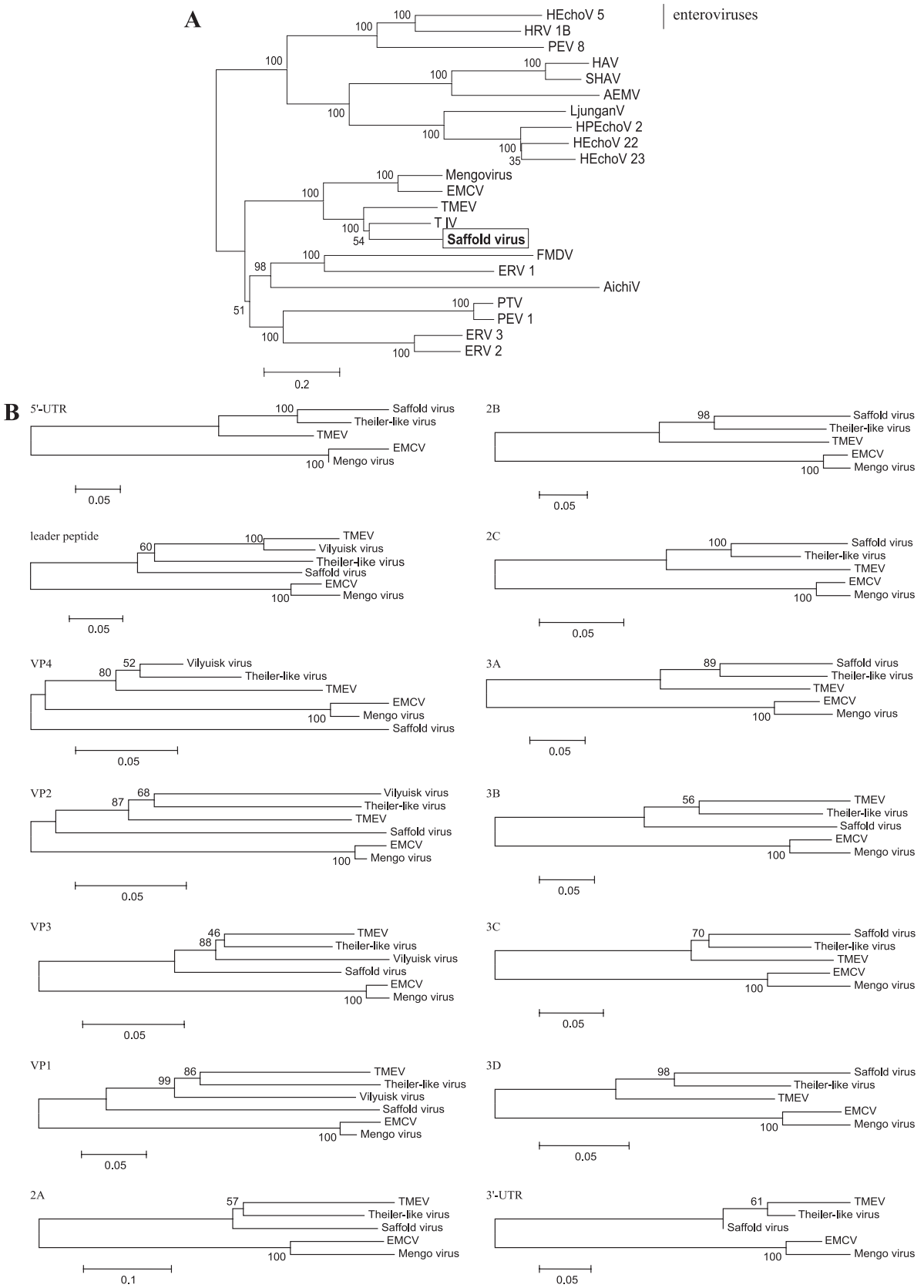

FIG. 6. Evolutionary relationships among *Picornaviridae* based on full-length genomes of picornaviruses (A) and based on sequences of each genomic region of cardioviruses (B). GenBank accession numbers are given in Materials and Methods. Abbreviations used for virus names: HEchoV 5, 22, and 23, human echoviruses 5, 22, and 23; HRV 1B, human rhinovirus 1B; PEV 1 and 8, porcine enterovirus 1 and 8; HAV, human hepatitis A virus; SHAV, simian hepatitis A virus; AEMV, avian encephalomyelitis virus; LjunganV, Ljungan virus; HPEchoV 2, human parechovirus 2; MengoV, mengovirus; T lV, Theiler-like virus; FMDV, foot-and-mouth disease virus; ERV 1, 2, and 3, equine rhinovirus 1, 2, and 3; AichiV, Aichi virus; PTV, porcine teschovirus.

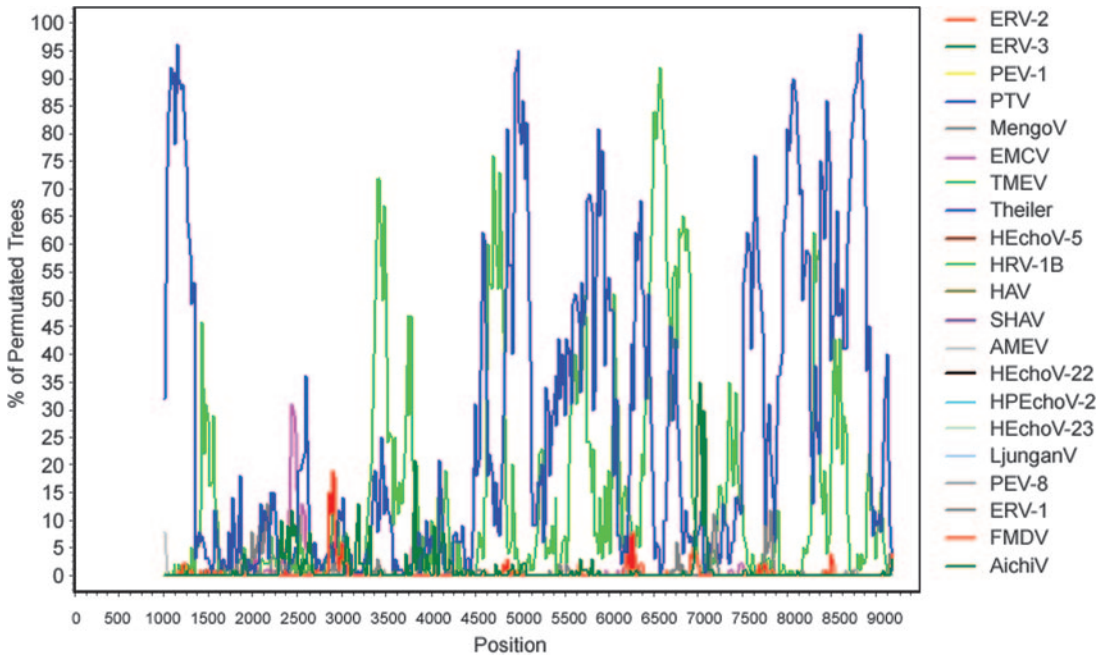

FIG. 7. Bootscan analysis of SAF-V based on full-length genomes. For virus names, see the legend of Fig. 6.

SAF-V is virulent like the GDVII strain of TMEV or persistent like TMEV strain DA.

Transmission of TMEV and EMCV occurs by the fecal-oral route (3, 22). We do not know whether SAF-V caused the patient's fever; therefore, it is also difficult to hypothesize how or when the patient was infected with SAF-V. Since TMEV is pathogenic in mice, further investigation is under way to determine the prevalence of SAF-V in humans.

Because of the length of time between the illness and our molecular characterization, we do not know much about the patient's health around the time of initial infection (e.g., characteristics of the illness and/or length of symptoms beyond the 24th day) other than the fact that the stool sample was collected 24 days after the onset of FUO, which fits the clinical definition of FUO (21). In addition, we do not know what other pathogens were tested for. However, SAF-V was the only agent isolated from the stool of the study subject. Nevertheless, we cannot claim that SAF-V is the etiological agent because we have not analyzed fecal samples from patients with FUO of known and unknown etiology.

We identified and characterized a new human virus, SAF-V, which was cultured from the stool of a pediatric patient presenting with FUO. SAF-V represents a new member of the genus *Cardiovirus* in the *Picornaviridae* family. The prevalence and pathogenicity of this virus remain to be determined.

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