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Molecular Phylogeny and Proposed Classification of the Simian Picornaviruses

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The simian picornaviruses were isolated from various primate tissues during the development of general tissue culture methods in the 1950s to 1970s or from specimens derived from primates used in biomedical research. Twenty simian picornavirus serotypes are recognized, and all are presently classified within the *Enterovirus* genus. To determine the phylogenetic relationships among all of the simian picornaviruses and to evaluate their classification, we have determined complete VP1 sequences for 19 of the 20 serotypes. Phylogenetic analysis showed that A13, SV19, SV26, SV35, SV43, and SV46 are members of human enterovirus species A, a group that contains enterovirus 71 and 11 of the coxsackie A viruses. SA5 is a member of human enterovirus species B, which contains the echoviruses, coxsackie B viruses, coxsackievirus A9, and enterovirus 69. SV6, N125, and N203 are related to one another and, more distantly, to species A human enteroviruses, but could not be definitely assigned to a species. SV4 and SV28 are closely related to one another and to A-2 plaque virus, but distinct from other enteroviruses, suggesting that these simian viruses are members of a new enterovirus species. SV2, SV16, SV18, SV42, SV44, SV45, and SV49 are related to one another but distinct from viruses in all other picornavirus genera, suggesting that they may comprise a previously unknown genus in *Picornaviridae*. Several simian virus VP1 sequences (N125 and N203; SV4 and SV28; SV19, SV26, and SV35; SV18 and SV44; SV16, SV42, and SV45) are greater than 75% identical to one another (and/or greater than 85% amino acid identity), suggesting that the true number of distinct serotypes among the viruses surveyed is less than 20.

Picornaviruses have been isolated from many vertebrate species, including humans, nonhuman primates, horses, cattle, swine, rodents, and birds (19). The family *Picornaviridae* consists of six recognized genera (*Aphthovirus*, *Cardiovirus*, *Enterovirus*, *Hepatovirus*, *Parechovirus*, and *Rhinovirus*) and three proposed genera (*Erbovirus*, *Kobuvirus*, and *Teschovirus*) (19, 31). The genera are further subdivided into species. For example, the genus *Enterovirus* is composed of eight species: *Bovine enterovirus* (BEV; 2 serotypes), *Human enterovirus A* (HEV-A; 12 serotypes), *Human enterovirus B* (HEV-B; 36 serotypes), *Human enterovirus C* (HEV-C; 11 serotypes), *Human enterovirus D* (HEV-D; 2 serotypes), *Poliovirus* (PV; 3 serotypes), *Porcine enterovirus A* (PEV-A; 1 serotype), and *Porcine enterovirus B* (2 serotypes) (19). Traditionally, picornaviruses have been classified, identified, and differentiated on the basis of physical and antigenic properties, such as acid stability and virion density, and by neutralization with specific antisera. More recently, nucleotide sequencing has been applied to picornavirus classification and its use has aided in the establishment of echoviruses 22 and 23 (renamed human parechoviruses 1 and 2, respectively) (13), Aichi virus (37), equine rhinitis B virus (36), and several porcine enteroviruses (renamed porcine teschoviruses) (4, 15, 39) as members of four new or proposed new genera (19). Our laboratory and others have shown that enterovirus VP1 sequence correlates with serotype and that VP1 sequence can be used as a molecular surrogate for antigenic typing (2, 3, 24–28). Among viruses in the *Enterovirus* genus, VP1 sequences are monophyletic with respect to serotype, species, and genus. Genetic clusters based on VP1 sequence (27) contain the same members as those based on partial 3D or VP2 sequences (14, 30), but noncapsid sequences do not always correlate with serotype. The genetic clusters correspond to the currently recognized species (19), showing that VP1 is sufficient to assign isolates to one of the recognized enterovirus species.

The nonhuman primate picornaviruses were isolated in the 1950s to 1970s from primate cell cultures during the development of general tissue culture methods, from primary cell cultures used in vaccine production, or from specimens derived from captive or wild-caught primates used in biomedical research (5, 9, 16, 18, 32). All of the 20 recognized simian picornavirus serotypes are currently classified as tentative members of the *Enterovirus* genus, but they are not yet assigned to a species (19). Eleven of the simian picornaviruses were recently characterized by partial sequencing and phylogenetic analysis of their 5′-nontranslated regions (NTRs) and polymerase (3D) genes (29). That study suggested that at least one serotype (SV18) may belong to another genus, based on the 3D sequence. However, no 5′-NTR sequence was obtained for SV18, seven additional serotypes (SA5, SV2, SV16, SV42, SV44, SV45, and SV49) were not amplified in either region, and two serotypes (N125 and N203) were not tested. Because of the high frequency of genetic recombination in noncapsid regions of the genome (21, 22, 33), it is difficult to interpret phylogenetic relationships of closely related viruses by using

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TABLE 1. Viruses analyzed

Serotype	Strain	ATCC no.	Species of origin[a]	Year	Source[b]	CPE group[c]	Reference(s)
A13	A13	VR-274	*Papio anubis*	c. 1962	Feces	NK[d]	5, 18
SA4	L79C3	VR-951	*Cercopithecus aethiops*	1954–1957	MKTC	3	23
SA5	B165	VR-952	*Cercopithecus aethiops*	1954–1957	MKTC	3	23
SV2	2383	VR-210	*Macaca mulatta*	1954	MKTC	2	12
SV4	1715 UWB	VR-287	*Macaca mulatta*	1954	MKTC	3	12
SV6	1631	VR-944	*Macaca mulatta*	1955	MKTC?	4	12
SV16	2450 SD	VR-211	*Macaca mulatta*	1955	MKTC	2	12
SV18	2481 B2	VR-212	*Macaca mulatta*	c. 1958	MKTC	2	12
SV19	M19s (P2)	VR-213	*Macaca fascicularis*	c. 1956	RS	2	9
SV26	3163	VR-290	*Macaca mulatta*	c. 1957	CNS	4	11
SV28	9128	VR-291	*Macaca mulatta*	c. 1957	CNS	3	11
SV35	A7987	VR-292	*Macaca mulatta*	c. 1957	CNS	4	11
SV42	M9 (P1)	VR-945	*Macaca fascicularis*	c. 1956	RS	2	9
SV43	OM112t (P12)	VR-946	*Macaca fascicularis*	c. 1956	RS	2	9
SV44	OM114s (P13)	VR-947	*Macaca mulatta*	c. 1956	RS	2	9
SV45	M19h (P14)	VR-948	*Macaca fascicularis*	c. 1964	RS	NK	8
SV46	OM22 (P15)	VR-949	*Macaca sp.*	c. 1964	NK	NK	8
SV49	2600 (P19)	VR-950	*Macaca mulatta*	c. 1964	RS	NK	8
N125	N125	NA[e]	*Papio cynocephalus*	c. 1972	NK	NK	32
N203	N203	NA	*Papio cynocephalus*	c. 1972	TS	NK	32

[a] *Macaca mulatta*, rhesus monkey; *Macaca fascicularis*, cynomolgus monkey; *Cercopithecus aethiops*, African green monkey (Vervet); *Papio anubis* (=*doguera*), baboon; *Papio cynocephalus*, baboon.
[b] MKTC, monkey kidney tissue culture; RS, rectal swab; TS, throat swab.
[c] According to the scheme of Hull et al. (12).
[d] NK, not known.
[e] NA, not available.

5′-NTR and 3D sequences. To better define the relationships among the simian picornaviruses and to assess their proper taxonomic classification, we have determined complete VP1 sequences for 19 of the 20 serotypes. Sequence alignments and phylogenetic analysis suggest that only 12 of the 19 are true enteroviruses, six belonging to HEV-A, one to HEV-B, and five in two proposed new species. The classification of the remaining seven viruses within the genus *Enterovirus* should be reconsidered.

MATERIALS AND METHODS

Viruses. The prototype strains of 18 of the 20 simian picornavirus serotypes were obtained from American Type Culture Collection (ATCC) (Manassas, Va.) (Table 1). Two additional strains, N125 and N203, were kindly provided by S. Kalter and R. Heberling, Esoterix, Inc., San Antonio, Tex. (Table 1). Viral RNA was extracted from cell culture supernatant using the QiaAmp Viral RNA kit (Qiagen, Santa Clarita, Calif.) either directly from the original material or following one passage in LLC-MK_2 cells (ATCC CCL 7).

Molecular characterization. Reverse transcription-PCR (RT-PCR), nucleotide sequencing, and sequence analysis were performed as described previously, using the primers listed in Table 2 (26). To determine whether the simian viruses are closely related to the human enteroviruses, all isolates were initially screened using diagnostic PCR primers that anneal at highly conserved sites in the 5′-NTR of all human enteroviruses and some rhinoviruses (38) (EV1 and EV2; Table 2). PCR amplification of a portion of VP1 was attempted for each strain by using primer pairs 012-011, 040-011, 187-222, 188-222, and 189-222 (Table 2) as described previously (25, 26). Together, primers 187, 188, 189, and 222 allow the amplification of all human enteroviruses and many nonhuman enteroviruses (25). Additional primers annealing to conserved sites in and surrounding VP1 were used for amplification and determination of complete VP1 sequences (Table 2). The sequences were compared to one another and to the VP1 sequences of other picornaviruses by using the program Gap (Wisconsin sequence analysis package, version 9.1, 1997; Genetics Computer Group, Madison, Wis.) as described previously (25, 26). The Ljungan virus VP1 sequence was generously provided prior to publication by Michael Lindberg, University of Kalmar, Kalmar, Sweden (personal communication). Other VP1 sequences were obtained from GenBank. Phylogenetic relationships were inferred using the programs DNADist and Neighbor (PHYLIP version 3.57 [PHYLIP: phylogeny inference package, version 3.5c, 1993; J. Felsenstein, University of Washington, Seattle]) and Puzzle (version 5.0 [35]). The maximum likelihood method of Kishino and Hasegawa (20), with a transition-transversion ratio of 8.0, was used to construct a distance matrix for neighbor-joining analysis. The statistical significance of phylogenies constructed using DNADist/Neighbor was estimated by bootstrap analysis with 1,000 pseudo-replicate data sets. Puzzle was executed using the distance method of Kishino and Hasegawa (20), with a transition-transversion ratio of 8.0, and the reliability of phylogenetic reconstructions was estimated by using 50,000 puzzling steps. Branch lengths of the neighbor-joining trees were calculated by the maximum likelihood method using Puzzle.

Nucleotide sequence accession numbers. The sequences reported here were deposited in the GenBank sequence database, accession no. AF326750 to AF326766 and AF414372 to AF414373.

RESULTS

A previous molecular characterization of the simian picornaviruses suggested that some of the viruses may not belong in the *Enterovirus* genus, as PCR primers expected to amplify all enteroviruses did not amplify SA5, SV2, SV16, SV18, SV42, SV44, SV45, and SV49 (29). To confirm and extend their results, we tested all 20 simian picornavirus serotype prototype strains by using our own pan-enterovirus PCR primers, EV1 and EV2 (38). Primers EV1 and EV2 amplified the same viruses as those amplified by Pöyry et al. (29), except that EV1 and EV2 successfully amplified SA5 but failed to amplify SA4 (Table 3). All primer pairs we tested (Table 2), including the 5′-NTR primers used by Pöyry et al., failed to amplify SA4 (Table 3 and data not shown). As a result, we were unable to verify the presence of amplifiable RNA, even using two independently obtained vials of SA4, using both RNA extracted from passaged virus and RNA extracted directly from the material received from ATCC.

Because enterovirus VP1 sequences have been shown to correlate with serotype and species identity (26, 27), VP1 was chosen for further genetic analyses. The amplification strategy

TABLE 2. Primers used for PCR amplification or sequencing of simian picornaviruses

Primer[a]	Sequence[b]	Gene	Position[c]
EV2	TCCGGCCCCTGAATGCGGCTAATCC	5′ NTR	446–470
EV1	ACACGGACACCCAAAGTAGTCGGTCC	5′ NTR	559–533
001	NARITAYTAYRCICAITG	VP3	2077–2094
011	GCICCIGAYTGITGICCRAA	2A	3408–3389
012	ATGTAYGTICCICCIGGIGG	VP1	2951–2970
040	ATGTAYRTICCIMCIGGIGC	VP1	2951–2970
050	GTRCTYACIAIIAGRTCYCT	2A	3483–3464
055	GGIACICAYRTIRTITGGGA	VP3	2186–2205
061	GAITGYTGICCRAAYTTTCC	2A	3372–3356
110	YTGYTCCATNGCYTCYTCRTC	2A	3802–3782
111	YTGYTCCATNGCYTCYTCYTC	2A	3802–3782
112	YTGYTCCATNGCRTCRTCYTC	2A	3802–3782
187	ACIGCIGYIGARACIGGNCA	VP1	2612–2631
188	ACIGCIGTIGARACIGGNG	VP1	2612–2630
189	CARGCIGCIGARACIGGNGC	VP1	2612–2631
222	CICCIGGIGGIAYRWACAT	VP1	2969–2951
224	GCIATGYTIGGIACICAYRT	VP3	1977–1996
238	CCIGGIWSIAAYCARTTIYTNAC	VP3	1787–1809
241	ACIGCIGCIGARACIGGNGA	VP1	2612–2631
251	GCCTAGCCTTTATCCTAG	2A	(−) SV2 group[d]
254	CTGTGCTTCAAAGTTGCTC	VP1	(−) SV2 group
281	GGCAGCGGGAGAGAACAT	VP1	2987–2970
283	TATCATCTTTCCACCACCA	2A	(−) SV2 group
302	ACIATITGGTAYCARACNGC	VP3	(+) SV2 group

[a] EV1 and EV2 are from reference 38; 011, 012, and 040 are from references 26 and 27; and 187, 188, 189, and 222 are from reference 25.
[b] Sequences are shown 5′ to 3′, using standard IUB nucleotide ambiguity codes. I, deoxyinosine.
[c] Nucleotide sequence coordinates are given relative to the sequence of the type strain of the *Enterovirus* genus, PV1-Mahoney (GenBank accession number J02281).
[d] Specific primers derived from preliminary sequence of viruses in the SV2 group. The polarity of the primer is indicated as (+) or (−).

used degenerate inosine-containing primers targeted to regions of VP3, VP1, and 2A that are highly conserved among the enteroviruses or among multiple picornavirus genera (Table 2 and Table 3). Table 3 lists the minimal primer sets needed to amplify the complete VP1 gene of each of the simian picornaviruses. For most viruses, additional primer sets were also used. Complete VP1 sequences were determined for the prototype strain of each of the recognized simian picornavirus serotypes except SA4, as noted above. The predicted VP1 proteins varied in length from 281 to 296 amino acids (843 to 888 nucleotides) based on comparisons with the VP1 cleavage sites of other picornaviruses.

The simian picornavirus VP1 nucleotide and deduced amino acid sequences were compared to one another as well as to other picornavirus VP1 sequences. Sequence comparisons used VP1 sequences of representatives of all picornavirus genera and included 68 enteroviruses, 12 aphthoviruses, 6 rhinoviruses, 4 cardioviruses, 2 parechoviruses, 2 hepatoviruses, 1

TABLE 3. Summary of PCR, sequencing, and phylogenetic analyses

Virus	PanEV PCR	VP1 primer sets[a]	VP1 length (nt)	Homologous strains	Genus-species
A13	+	224-222, 18X-11X[b]	852		EV-A
SA4	−				
SA5	+	224-222, 187-254, 012-050	843		EV-B
SV2	−	224-222, 241-11X	852		Novel genus
SV4	+	001-222, 18X-11X	879	SV28, A-2 plaque virus	EV-new species
SV6	+	224-222, 241-011	867		EV-A?
SV16	−	055-222, 241-283	879	SV42, SV45	Novel genus
SV18	−	224-222, 241-283	873	SV44	Novel genus
SV19	+	224-222, 18X-11X	864	SV26, SV35	EV-A
SV26	+	224-222, 189-061	861	SV19, SV35	EV-A
SV28	+	224-222, 18X-11X	879	SV4, A-2 plaque virus	EV-new species
SV35	+	224-222, 189-061	858	SV19, SV26	EV-A
SV42	−	224-11X	888	SV16, SV45	Novel genus
SV43	+	224-011	867		EV-A
SV44	−	055-222, 241-251	885	SV18	Novel genus
SV45	−	224-281, 241-283	879	SV16, SV42	Novel genus
SV46	+	238-240	876		EV-A
SV49	−	302-281, 241-283	876		Novel genus
N125	+	224-222, 18X-011	846		EV-A?
N203	+	224-222, 18X-011	846		EV-A?

[a] Minimum primer set needed to amplify the complete VP1 gene. For most viruses, additional primer sets were also used for sequence determination.
[b] 18X, combination of 187, 188, and 189; 11X, combination of 110, 111, and 112.

TABLE 4. VP1 sequence relationships between simian picornaviruses and other picornaviruses

Simian virus[a]	% Nucleotide identity between simian picornaviruses and other picornaviruses[e]								
	HEV-A	HEV-B	HEV-C	HEV-D	BEV[b]	PEV9	A-2[c]	HRV[d]	Other genera
A13	**59.2–52.8**	46.4–52.5	45.7–52.3	52.4–54.0	52.1–54.6	52.3	48.2	48.9–51.0	35.0–42.7
SA5	45.0–50.5	**58.4–64.6**	49.9–56.6	51.7–53.1	46.2–52.5	49.7	49.0	48.7–52.1	35.3–40.1
SV2	41.0–46.4	40.1–46.3	40.5–48.0	43.9–45.0	43.6–45.8	45.6	42.7	40.7–46.4	33.8–42.2
SV4	46.6–51.8	46.9–52.7	47.6–52.0	50.5–51.0	50.6–52.6	49.5	***84.1***	48.5–51.7	35.4–42.5
SV6	52.0–55.9	45.8–52.8	45.6–52.5	47.5–49.6	51.0–53.5	51.6	47.8	48.1–50.5	34.4–41.1
SV16	41.7–45.3	39.7–46.1	41.4–45.0	40.1–45.3	43.9–45.4	42.3	43.8	40.0–44.2	35.3–41.6
SV18	39.7–46.2	40.3–45.3	39.4–44.4	43.5–46.1	42.9–44.9	44.2	44.1	41.7–45.7	33.8–41.7
SV19	**53.5–62.1**	45.9–52.7	46.3–49.9	51.9–53.5	53.7–54.1	53.2	50.8	48.8–52.1	35.4–40.9
SV26	**57.8–62.3**	46.5–53.8	46.9–50.9	52.5–54.8	53.8–55.0	51.6	52.0	48.1–52.4	35.3–43.3
SV28	47.0–52.1	46.9–53.0	47.2–50.3	50.8–51.2	49.9–52.4	49.5	***83.8***	48.6–51.6	35.5–41.8
SV35	**55.8–62.2**	45.7–52.7	46.4–51.3	52.8–56.2	53.2–54.9	51.7	52.1	48.9–52.9	34.8–44.7
SV42	38.8–46.6	39.2–45.8	39.2–46.9	42.3–43.5	43.8–47.1	42.1	44.3	40.3–43.9	34.5–42.2
SV43	**58.9–63.1**	46.0–51.0	46.9–51.5	50.4–52.6	53.5–54.0	51.6	50.6	46.0–51.0	35.0–41.3
SV44	39.8–45.4	38.3–45.8	39.1–45.8	41.2–41.6	42.9–44.1	42.3	42.6	41.2–45.7	33.3–39.6
SV45	39.4–46.3	38.6–46.2	39.4–44.8	44.6–44.8	42.1–44.7	44.0	44.1	40.8–42.8	35.1–42.9
SV46	**59.3–62.4**	46.9–51.8	45.5–51.2	51.7–53.3	51.5–54.2	53.0	51.3	45.1–50.8	35.7–40.7
SV49	39.0–46.2	39.8–47.0	38.2–44.1	44.5–45.2	40.5–43.8	41.8	44.7	42.8–46.2	36.1–41.7
N125	50.7–56.9	44.8–49.1	45.3–51.7	49.9–51.8	48.8–53.2	48.4	48.6	47.2–50.9	33.3–40.3
N203	51.7–55.0	43.7–49.9	47.2–51.6	52.4–54.2	50.4–50.9	49.1	51.6	46.4–52.2	33.8–42.0

[a] VP1 sequence was not obtained for SA4.
[b] BEV, bovine enteroviruses. Includes BEV1, BEV2a, and BEV2b.
[c] A-2, A-2 plaque virus.
[d] VP1 sequences are available only for HRV1B, HRV2, HRV3, HRV14, HRV16, and HRV89.
[e] Comparisons of viruses of the same species are boldfaced, and comparisons of viruses of the same serotype are boldfaced and italicized.

kobuvirus, 1 teschovirus, 1 erbovirus, and 2 unassigned picornaviruses (A-2 plaque virus and Ljungan virus). Enterovirus VP1 nucleotide and amino acid pairwise identity scores may be used to determine the species or serotype identity of a virus isolate (25–28). Clinical isolates of the same serotype are usually more than 75% identical to one another in VP1 nucleotide sequence (more than 85% amino acid sequence identity). Occasionally, viruses of the same serotype may be only 70 to 75% identical to one another, but they are always more closely related to viruses of the same serotype than to viruses of another serotype (26, 28). Viruses belonging to the same species (but different serotypes) are approximately 56 to 73% identical in nucleotide sequence (55 to 85% amino acid identity), whereas those belonging to different species (but the same genus) are about 44 to 58% identical in nucleotide sequence (34 to 55% amino acid identity). Viruses of different picornavirus genera are less than 45% identical to one another (less than 34% amino acid identity) (26, 27). The simian virus nucleotide sequences are less than 65% identical to those of any other picornavirus (less than 70% amino acid identity) in all comparisons, except to A-2 plaque virus (see below), confirming that they do not represent members of recognized serotypes (Table 4). The simian viruses cluster into two distinct groups on the basis of VP1 sequence identities (Table 4): one group related to members of the genus *Enterovirus* (SA5, SV4, SV28, SV6, A13, SV19, SV26, SV35, SV43, SV46, N125, and N203) and another group distinct from all other picornavirus genera (SV2, SV16, SV18, SV42, SV44, SV45, and SV49).

For the simian viruses in the enterovirus genus, using the molecular classification scheme described above, A13, SV19, SV26, SV35, SV43, and SV46 should be assigned to HEV-A. All are 56 to 63% identical to members of species A and less than 57% identical to members of other species (Table 4). Only SV35 is not fully resolved from members of other species (55.8% identity to CA4 [HEV-A] and 56.2% identity to EV68 [HEV-D]), but its close relationship with SV19 and SV26 argues that it belongs with them in species A (Table 5). SA5 should be assigned to HEV-B (58 to 65% nucleotide [nt] identity with HEV-B viruses and less than 57% identity with members of other species) (Table 4). SV4 and SV28 are less than 53% identical to any other picornavirus other than A-2 plaque virus, indicating that SV4, SV28, and A-2 are the sole members of a new enterovirus species. SV6, N125, and N203 are 52.0 to 55.9% identical to HEV-A viruses and less than 53% identical to members of other species, suggesting that they probably represent a second new enterovirus species.

Phylogenetic relationships generally mirror those revealed by pairwise sequence comparisons, with high bootstrap support (Fig. 1). A13, SV19, SV26, SV35, SV43, and SV46 cluster together within HEV-A (Fig. 1A and B). SV6, N125, and N203 clustered together and are loosely related to HEV-A (Fig. 1A and B), but they are less than 56% identical to any HEV-A viruses (Table 4). SA5 clusters with the echoviruses and coxsackie B viruses in HEV-B (Fig. 1A and C). SV4, SV28, and A-2 plaque virus form a unique cluster in the *Enterovirus* genus (Fig. 1A and D), consistent with their pairwise identities with other enteroviruses.

The remaining simian viruses that are distinct from the enterovirus genus (SV2, SV16, SV18, SV42, SV44, SV45, and SV49) are monophyletic, forming a single cluster that is distinct from any other picornavirus genera, with 100% bootstrap support (Fig. 1A and E). Within this group, SV49 is distinct from the other viruses (100% bootstrap support), with the next-distal node branching to SV2 (100% bootstrap support). The remaining viruses are monophyletic (100% bootstrap support) and form three groups: (i) SV16 and SV45, (ii) SV42, and

TABLE 5. VP1 sequence relationships among simian picornaviruses[a]

	SV18	SV44	SV16	SV45	SV42	SV2	SV49	SV28	SV4	SV26	SV35	SV19	SV43	A13	SV46	SV6	N125	N203	SA5
SV18		91.5	74.0	75.0	74.1	68.0	58.3	33.7	33.8	35.9	36.2	36.4	35.7	37.2	36.5	35.2	45.0	45.3	37.3
SV44	95.9		72.4	74.7	74.5	66.1	58.3	33.1	33.2	36.2	36.0	36.2	35.1	36.1	36.4	34.5	44.0	43.3	36.7
SV16	84.2	82.9		77.0	72.7	68.4	57.6	34.2	34.4	36.5	37.2	37.5	37.2	38.1	37.1	35.0	45.1	42.9	36.7
SV45	84.2	84.0	92.2		76.0	69.8	58.2	33.7	33.6	36.6	36.9	36.9	38.0	35.8	38.4	35.8	46.0	45.4	35.8
SV42	86.3	85.1	85.7	87.7		66.9	55.4	36.1	36.0	36.4	36.1	36.2	37.1	38.3	36.7	36.3	44.7	46.1	35.9
SV2	71.5	70.4	73.2	73.2	71.5		56.9	36.0	36.0	37.1	37.8	37.8	38.9	37.7	37.7	35.7	45.5	44.6	35.9
SV49	49.3	50.3	50.7	51.7	51.0	53.2		32.5	32.7	35.9	36.0	36.2	35.3	35.5	33.8	34.7	47.0	47.1	34.6
SV28	27.8	27.3	27.6	27.6	28.7	27.7	27.7		98.6	46.8	46.6	46.9	47.7	47.8	48.7	48.4	50.5	49.4	47.5
SV4	27.8	27.3	27.2	27.6	28.7	27.3	26.9	98.3		46.6	46.4	46.4	47.6	47.6	48.7	48.5	50.4	49.4	47.9
SV26	30.7	30.2	30.0	30.8	29.7	29.5	29.4	43.7	43.7		93.4	87.6	72.0	68.5	64.5	56.7	58.5	55.6	47.6
SV35	30.3	29.8	30.0	30.8	30.1	29.9	29.8	43.1	43.1	96.2		90.1	70.6	67.6	63.1	54.5	58.2	57.4	47.9
SV19	29.9	29.4	30.8	31.2	30.1	29.9	30.9	44.3	43.5	95.1	95.1		72.3	66.6	63.1	54.7	57.7	56.5	47.9
SV43	30.9	30.5	31.4	32.2	31.1	30.6	30.8	44.7	44.7	84.3	82.5	83.3		65.4	63.0	54.2	55.2	55.9	46.1
A13	30.6	30.1	30.0	31.9	30.9	29.4	26.6	41.9	41.9	71.8	71.7	71.9	71.5		65.8	59.3	58.0	58.1	52.0
SV46	29.2	28.7	30.5	30.1	29.7	27.9	27.9	41.6	41.6	71.0	68.1	68.6	70.5	69.0		55.3	55.0	55.2	48.4
SV6	27.6	27.2	25.5	26.3	28.0	25.8	27.1	43.0	43.5	55.8	55.0	54.6	54.4	51.4	54.4		58.5	58.6	47.6
N125	32.6	31.4	32.5	32.8	32.5	33.5	32.2	46.5	46.9	51.8	52.0	51.2	49.1	51.3	51.6	50.2		79.7	49.5
N203	32.3	30.6	32.8	31.7	32.8	32.4	31.7	43.7	44.0	51.8	52.7	50.9	51.3	52.3	51.6	53.5	89.7		49.4
SA5	28.3	28.0	29.3	29.0	30.2	28.2	28.5	45.3	46.0	42.9	43.4	42.0	41.6	44.1	40.0	42.6	43.8	45.1	

[a] Percent identity of the nucleotide sequences is above the diagonal, and percent identity of the amino acid sequence is below the diagonal. Comparisons of viruses of the same species are boxed, and viruses of the same serotype are shaded.

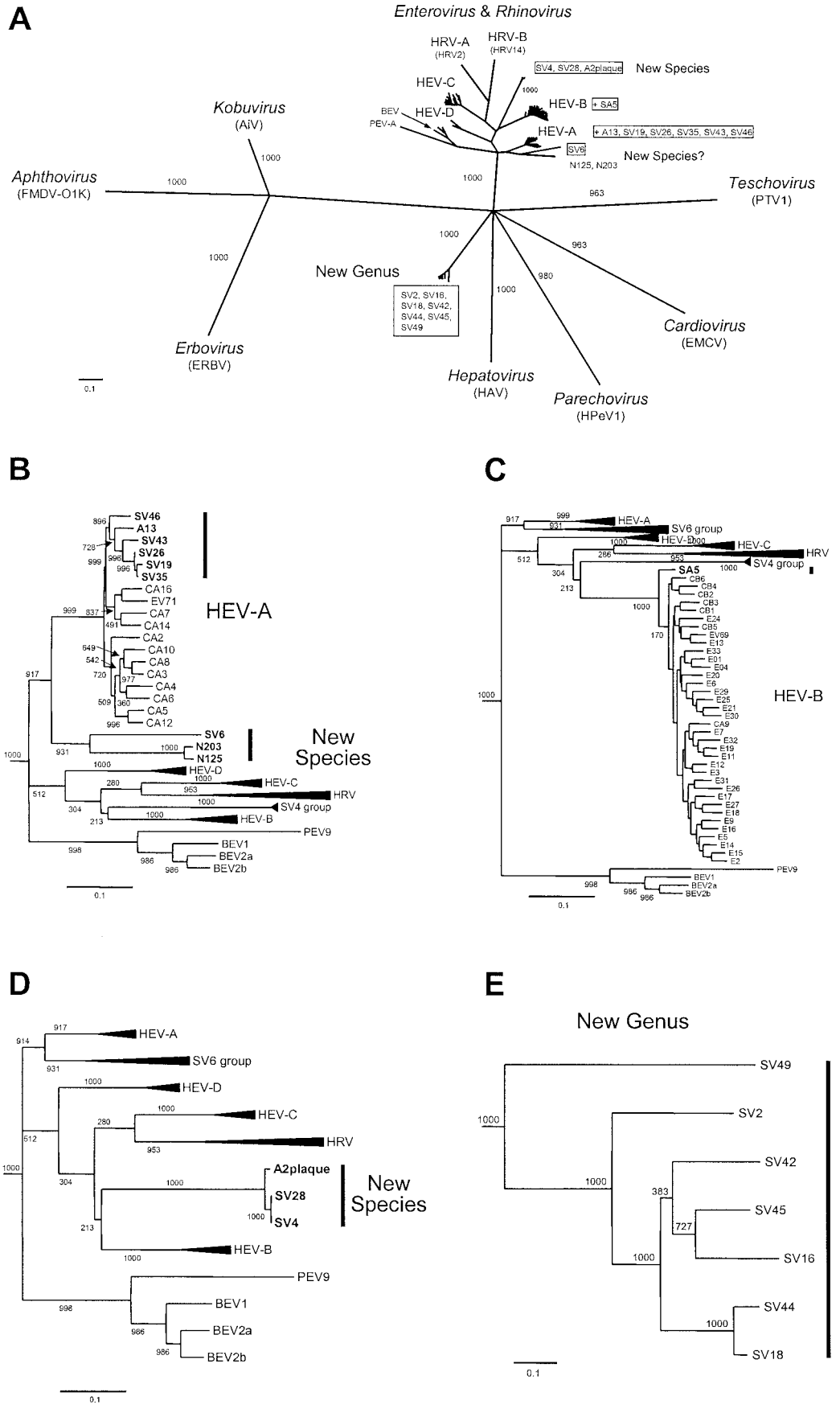
(iii) SV18 and SV44. SV18 and SV44 cluster tightly together (100% bootstrap support), consistent with their 91.5% sequence identity, but the topology of the SV16/SV45 and SV42 branches is not well supported (Fig. 1E).

Several simian virus VP1 sequences are greater than 75% identical to one another (greater than 85% amino acid identity), suggesting that the true number of distinct serotypes among the viruses surveyed is less than 20 (Tables 3, 4, and 5). The SV4 and SV28 VP1 sequences are 98.6% identical to one another (98.3% amino acid identity) (Table 5), supporting previous antigenic comparisons that identified these two viruses as variants of a single serotype (16). SV4 and SV28 are also 84.1 and 83.8% identical to A-2 plaque virus (95.9 and 95.6% amino acid identity), respectively, indicating that A-2 plaque virus is also a member of this same serotype (Table 4). N125 and N203 are 79.7% identical to one another (89.7% amino acid identity); SV19, SV26, and SV35 sequences are 87.6 to 93.4% identical to one another (95.1 to 96.2% amino acid identity); SV16, SV42, and SV45 sequences are 72.7 to 77.0% identical to one another (85.7 to 92.2% amino acid identity); and SV18 and SV44 sequences are 91.5% identical to one another (95.9% amino acid identity). The relationships among SV16, SV18, SV42, SV44, and SV45 could not be fully resolved, as they are all at least 72.4% identical to one another (84.0% amino acid identity) (Table 5).

DISCUSSION

During their initial characterization in the 1950s and 1960s, viruses of simian origin were categorized by physical properties, such as virion size, resistance to heat, plaque size, and morphology (8, 10, 12), and by the type of cytopathic effect (CPE) in tissue cultures (11). At the time, few human viruses had been well characterized, so this scheme was used to classify new agents as, for example, "adenovirus-like" or "enterovirus-like." New viruses were also characterized antigenically by neutralization, complement fixation, hemagglutination, and hemagglutination-inhibition tests. SA4, SV4, and SV28 (CPE group 3) were shown by cross-neutralization to be antigenically related to one another (8, 11, 23). Similarly, an antigenic relationship was demonstrated among SV2, SV16, SV18, SV42, SV45, and SV49, all of which belonged to CPE group 2 (8, 11). Our VP1 sequence comparisons agree with the antigenic relationships among these viruses and confirm that SV4 and SV28 are strains of a single serotype. Although we were unable to analyze the SA4 prototype strain, a previously published analysis indicated that SA4 is closely related to SV4 and SV28 (29). The partial 5′-NTR sequences of SV4 and SV28 were virtually identical to one another (29), suggesting that the two strains diverged from one another very recently. A-2 plaque virus was isolated from the icteric phase serum of a hepatitis patient

FIG. 1. Phylogram depicting the relationships of picornavirus VP1 sequences. The numbers indicate bootstrap values, out of 1,000, for the node to the right. (A) Radial neighbor-joining tree, with at least one virus from each of the six recognized and three proposed picornavirus genera (genus names in italics). For enteroviruses and rhinoviruses, the species names are also indicated. The names of the simian viruses are boxed. (B to E) Portions of the tree in panel A, redrawn as rectangular phylograms. Simian picornaviruses are indicated in bold-face type and by vertical bars to the right of the virus names. (B to D) Emphasis is on specific subbranches within the Enterovirus/Rhinovirus branch, which are drawn to the same scale. Other subbranches are collapsed for clarity (indicated by triangles at the branch tips). The length of the triangle is proportional to the genetic diversity within the collapsed subbranch.


A
Enterovirus & Rhinovirus
HRV-A
(HRV2)
HRV-B
(HRV14)
SV4, SV28, A2plaque
New Species
HEV-C
HEV-B
+ SA5
BEV
PEV-A
HEV-D
HEV-A
+ A13, SV19, SV26, SV35, SV43, SV46
SV6
N125, N203
New Species?
Kobuvirus
(AiV)
Aphthovirus
(FMDV-O1K)
Teschovirus
(PTV1)
New Genus
SV2, SV16, SV18, SV42, SV44, SV45, SV49
Erbovirus
(ERBV)
Cardiovirus
(EMCV)
Hepatovirus
(HAV)
Parechovirus
(HPeV1)
0.1
B
SV46
A13
SV43
SV26
SV19
SV35
CA16
EV71
CA7
CA14
CA2
CA10
CA8
CA3
CA4
CA6
CA5
CA12
HEV-A
SV6
N203
N125
New Species
HEV-D
HEV-C
HRV
SV4 group
HEV-B
PEV9
BEV1
BEV2a
BEV2b
0.1
C
HEV-A
SV6 group
HEV-D
HEV-C
HRV
SV4 group
SA5
HEV-B
PEV9
BEV1
BEV2a
BEV2b
0.1
D
HEV-A
SV6 group
HEV-D
HEV-C
HRV
A2plaque
SV28
SV4
New Species
HEV-B
PEV9
BEV1
BEV2a
BEV2b
0.1
E
New Genus
SV49
SV2
SV42
SV45
SV16
SV44
SV18
0.1

whose serum was also positive for hepatitis B antigen (34). The virus was originally isolated in human embryonic kidney culture and passaged in primary African green monkey kidney cells. Its close relationship to SV4 and SV28 raises the possibility that it was present in the original uninfected monkey cell cultures or that it was introduced during subsequent passages. Alternatively, it may represent the rare infection of a human by a simian enterovirus. Based on VP1 sequence analysis, SV19, SV26, and SV35 also appear to be strains of a single serotype, as do SV16, SV42, and SV45, as well as SV18 and SV44, thus reducing the number of distinct simian picornavirus serotypes from 20 to 13. SV16, SV18, SV42, SV44, and SV45 are all closely related to one another (Table 5), but additional studies are needed to determine whether they represent intratypic variants of a single serotype or a family of closely related serotypes.

Currently, picornavirus species are named according to the host with which they are most closely associated, e.g., *Human enterovirus A*, *Bovine enterovirus*, *Porcine enterovirus A*, etc. (19). The VP1 sequences of A13, SV19, SV26, SV35, SV43, and SV46 clearly places them within *Human enterovirus A* (Table 4 and Fig. 1), suggesting that the current naming convention may not be adequate to classify all enteroviruses.

The genetic relationships of the simian picornaviruses to the human enteroviruses were recently addressed by molecular methods (29). In those studies, 10 simian virus serotypes were amplified using primers specific for the 5′-NTR (A13, SA4, SV4, SV6, SV19, SV26, SV28, SV35, SV43, and SV46) and seven serotypes were amplified using 3D-specific primers (A13, SV6, SV18, SV26, SV35, SV43, and SV46). Seven serotypes (SA5, SV2, SV16, SV42, SV44, SV45, and SV49) were not amplified by either primer pair (29). With the exception of SA5, all of the serotypes that failed to amplify with both 5′-NTR and 3D primer pairs in that study are not closely related to the human enteroviruses on the basis of our VP1 sequence analysis. Our own enterovirus-specific 5′NTR primers that amplify all human enteroviruses (38) successfully amplified all of the simian picornaviruses that were enteroviruses by VP1 type (SA5, SV4, SV6, SV19, SV26, SV28, SV35, SV43, and SV46) but failed to amplify those viruses whose VP1 sequences distinguished them from the enteroviruses (Tables 3 and 4). SA4 is also likely to be a member of the *Enterovirus* genus, as described above. SV2, SV16, SV18, SV42, SV44, SV45, and SV49 appear to represent a new picornavirus genus, as phylogenetic comparisons showed that they were clearly distinct from members of the *Enterovirus* genus and from other existing picornavirus genera (Fig. 1). Although these data are consistent with previous reports, final resolution of their taxonomic status must await further detailed molecular characterization, and until then, they should be regarded as unclassified picornaviruses. These additional studies are in progress.

Comparison of our VP1 phylogeny (Fig. 1) with published simian picornavirus phylogenies based on partial 5′-NTR and partial 3D sequences (29) revealed that the three phylogenetic trees are noncongruent, suggesting that recombination has played a role in the evolution of some of the simian picornaviruses. For example, in the VP1 tree (Fig. 1), SV19, SV26, SV35, SV43, SV46, and A13 formed a single genetic cluster within HEV-A, whereas A13 was distinct from all other enteroviruses in the 3D tree (29). The A13 5′-NTR sequence was also distinct from, but related to, enterovirus 5′-NTR group I (corresponding to HEV-C and -D), while SV19, SV26, SV35, SV43, and SV46 clustered together and were related to enterovirus 5′-NTR group II (corresponding to HEV-A and B) (29). The SV18 partial 3D sequence was distinct from all enterovirus sequences, in agreement with our VP1 results (5′-NTR sequence was not obtained for SV18). Unfortunately, the simian picornavirus 5′-NTR and 3D sequences were not compared with those of genera other than *Enterovirus* (29). We were not able to make those comparisons ourselves because the sequences were not available in a public sequence database.

While it may be uncommon, picornaviruses appear to be capable of occasionally infecting a species other than the natural host(s). Antigenic and molecular comparisons have suggested that swine vesicular disease virus emerged in the past 50 years through infection of swine with the human pathogen coxsackievirus B5, followed by subsequent adaptation and evolution of the virus in the new host (1, 7, 40, 41). Serologic studies have suggested that the simian picornaviruses may infrequently infect humans, particularly those with natural or occupational exposure to wild primates (17). There is no evidence that simian picornaviruses are capable of causing disease in humans, but a number of other primate viruses, including herpesvirus B and monkeypox virus, are closely related to human pathogens and have the potential to directly cause serious disease in humans (6). The increasing encroachment of human activity on wild primate habitats may increase the risk of virus infection in species other than the natural host. Enteroviruses 70 and 71 (EV70 and EV71), agents of acute hemorrhagic conjunctivitis and hand, foot, and mouth disease, respectively, appear to have emerged as human pathogens relatively recently, but their origins remain unknown. EV70 emerged in a region of the world that is inhabited by wild primate populations, but none of the simian enteroviruses were members of HEV-D, the group that includes EV70. Likewise, none of the simian viruses were closely related to EV71, but many clearly belong to HEV-A, of which EV71 is also a member. Further studies are needed to study the natural enteroviral flora of wild primates, as very few species have been sampled to date, and to determine the potential for primate enteroviruses to infect and cause disease in humans. The availability of the relatively generic molecular reagents described here should greatly accelerate the identification and characterization of novel picornaviruses associated with human and animal disease.

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