A Novel Adenovirus Species Associated with an Acute Respiratory Outbreak in a Baboon Colony and Evidence of Coincident Human Infection

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ABSTRACT **Adenoviruses (AdVs) are DNA viruses that infect many vertebrate hosts, including humans and nonhuman primates. Here we identify a novel AdV species, provisionally named "simian adenovirus C (SAdV-C)," associated with a 1997 outbreak of acute respiratory illness in captive baboons (4 of 9) at a primate research facility in Texas. None of the six AdVs recovered from baboons (BaAdVs) during the outbreak, including the two baboons who died from pneumonia, were typeable. Since clinical samples from the two fatal cases were not available, whole-genome sequencing of nasal isolates from one sick baboon and three asymptomatic baboons during the outbreak was performed. Three AdVs were members of species SAdV-C (BaAdV-2 and BaAdV-4 were genetically identical, and BaAdV-3), while one (BaAdV-1) was a member of the recently described SAdV-B species. BaAdV-3 was the only AdV among the 4 isolated from a sick baboon, and thus was deemed to be the cause of the outbreak. Significant divergence (<58% amino acid identity) was found in one of the fiber proteins of BaAdV-3 relative to BaAdV-2 and -4, suggesting that BaAdV-3 may be a rare SAdV-C recombinant. Neutralizing antibodies to the other 3 AdVs, but not BaAdV-3, were detected in healthy baboons from 1996 to 2003 and staff personnel from 1997. These results implicate a novel adenovirus species (SAdV-C) in an acute respiratory outbreak in a baboon colony and underscore the potential for cross-species transmission of AdVs between humans and nonhuman primates.**

IMPORTANCE **Adenoviruses (AdVs) are DNA viruses that infect many animals, including humans and monkeys. In 1997, an outbreak of acute respiratory illness from AdVs occurred in a baboon colony in Texas. Here we use whole-genome sequencing and antibody testing to investigate new AdVs in baboons (BaAdVs) during the outbreak, one of which, BaAdV-3, came from a sick animal. By sequence analysis, BaAdV-3 may be a recombinant strain that arose from a related BaAdV found in baboons nearby in the colony (who were not sick) and yet another unknown AdV. We also found antibodies to these new BaAdVs in baboons and staff personnel at the facility. Taken together, our findings of a new AdV species as the cause of an acute respiratory outbreak in a baboon colony underscore the ongoing threat from emerging viruses that may carry the potential for cross-species transmission between monkeys and humans.**

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Many emerging infectious diseases in humans, including those caused by Ebola virus and H5N1 avian influenza, are zoonotic (1). Given the close phylogenetic relationship between humans and nonhuman primates (NHPs), humans are especially vulnerable to cross-species infections from pathogens harbored in apes and monkeys (2). The risk of disease transfer between NHPs and humans may be greatest in hot spots such as the forests of central and west Africa and the Amazon basin, where humans come into frequent contact with a diverse range of closely related species of NHPs (2). Zoos and research facilities housing captive NHPs also represent settings in which cross-species transmission of emerging pathogens can occur (3–5).

Adenoviruses (AdVs), first isolated from human adenoidal tissue (6), are double-stranded DNA viruses that naturally infect a broad range of vertebrate hosts, including humans and NHPs (7–9). In humans, infections caused by AdVs include conjunctivitis, gastroenteritis, hepatitis, myocarditis, and pneumonia (7, 10–16). Members of the genus *Mastadenovirus*, which encompass the AdVs infecting primates, have been classified by the International Committee on Taxonomy of Viruses (ICTV) to include the 7 human adenovirus (HAdV) species (HAdV-A to HAdV-G) and 1 simian AdV species (simian adenovirus A [SAdV-A]) (17). Recently, members of a phylogenetically distinct AdV species, SAdV-B, were also discovered in fecal samples from asymptom-

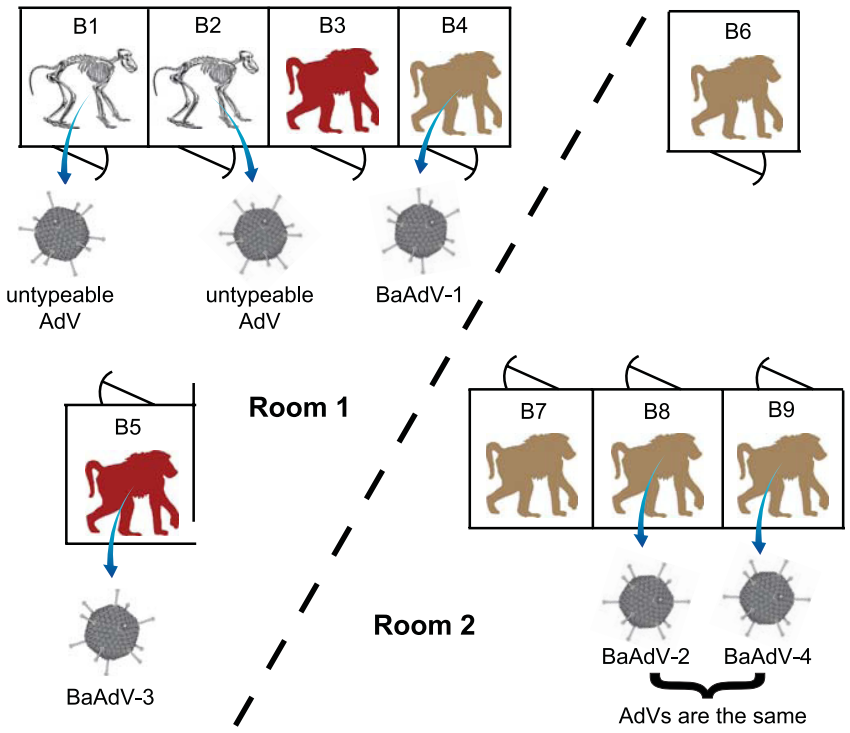


FIG 1 Epidemiological features of the 1997 baboon adenovirus outbreak. Map of the baboon nursery during the 1997 outbreak with cages situated in two separate rooms, showing the locations of baboons who died from pneumonia (skeleton), baboons who became clinically ill with respiratory symptoms but survived (red), and asymptomatic baboons (brown). The novel AdVs genetically characterized in this study (BaAdV-1 to BaADV-4) were isolated from nasal swabs from both sick (B5) and asymptomatic (B4, B8, and B9) baboons.

atic captive rhesus monkeys (18). Although AdVs are conventionally thought to exhibit a very narrow host range due to coevolution with their respective hosts (7, 17), there is mounting evidence supporting the potential for cross-species transmission of AdVs between monkeys and humans. AdVs identified in fecal samples from NHPs were found to share a remarkable similarity to human strains and could be classified phylogenetically into the conventional "human" species HAdV-A to HAdV-E (19, 20). Large-scale serological surveys have detected antibodies to simian AdVs in humans living in monkey-endemic regions (21, 22). Furthermore, we previously described a novel AdV, titi monkey adenovirus (TMAdV), as the cause of a fatal outbreak of pneumonia and hepatitis in a colony of New World titi monkeys, which was also associated with a cross-species respiratory infection in a scientist investigating the outbreak and a household family member (3).

In 1997, an outbreak of fatal pneumonia occurred in infant olive baboons (*Papio hamadryas anubis*) housed at the Texas Biomedical Research Institute (TBRI). Here we investigate the cause of the outbreak and identify by sequencing and *de novo* genome assembly the presence of a novel AdV species, provisionally named "simian adenovirus C (SAdV-C)," in both sick and asymptomatic baboons. In addition, we present clinical, epidemiological, and serological evidence that members of species SAdV-C and SAdV-B have the capacity to cause cross-species infections in humans and monkeys.

RESULTS

A 1997 outbreak of fatal pneumonia in a baboon colony. In February of 1997, 4 of 9 infant baboons at the TBRI developed an acute respiratory infection of unknown etiology shortly after being isolated from birth in preparation for a research study on respiratory syncytial virus (Fig. 1). The first two cases (Fig. 1, baboon 1 [B1] and B2) died 13 and 5 days after the onset of symptoms despite treatment with empiric broad-spectrum antibiotics and supportive care. Symptoms began with sneezing and rapidly worsened with development of lethargy, >20% weight loss, abnormally low body temperature, and dyspnea. White blood cell (WBC) counts were normal, but a few atypical lymphocytes were present. Chest radiographs revealed a bilateral interstitial pneumonia. Initial bacterial cultures of bronchoalveolar lavage fluid and blood samples were positive for methicillin-sensitive *Staphylococcus aureus* (MSSA) and rare *Kluyvera ascorbata* (23) in only one of the two cases.

On necropsy immediately after death, the lung tissue from both cases was noted to be hemorrhagic with patchy regions of consolidation and significant neutrophilic infiltration. Notably, intranuclear inclusions (presumably from AdV infection) were evident throughout the respiratory epithelium and were most evident in the major airways. The final pathological diagnosis was bronchointerstitial pneumonia, probably viral in etiology, with accompanying tonsillitis, lymphadenitis, and mild liver necrosis (in one baboon). Tests from lung tissue were negative for *Bordetella pertussis*, *Chlamydophila* spp., *Mycoplasma* spp., *Ureaplasma* spp. *Legionella* spp., and hantavirus. Adenovirus was isolated in cell culture from bronchoalveolar lavage fluid and lung tissue samples from both fatal cases. The isolates were untypeable by virus neutralization testing at an off-site laboratory using sera reactive to members of species HAdV-A to HAdV-F and SAdV-A.

Although clinical samples from the two fatal cases were unavailable for further analysis, two other animals in the room were noted to be sneezing and coughing around the same time cases B1 and B2 presented with fatal pneumonia (Fig. 1, B3 and B5). Adenovirus was isolated from a nasal swab collected from one of these two symptomatic individuals during the 1997 outbreak (Fig. 1, room 1, BaAdV-3). Nasal swabs were also collected from the sole remaining asymptomatic baboon in the same room as the symptomatic cases (Fig. 1, room 1, B4) and from asymptomatic baboons in a nearby but separate room (Fig. 1, room 2, B8 and B9). From these nasal swabs, 3 additional AdV isolates were obtained (Fig. 1, BaAdV-1, BaAdV-2, and BaAdV-4). The two symptomatic baboons in room 1, cases B3 and B5, were quarantined and recovered completely within 1 week with supportive care, including

TABLE 1 Tropism of baboon adenovirus (BaAdV) isolates in human and monkey cells

Cell or cell line	CPE[a] of virus in cells:		
	BaAdV-1	BaAdV-2/-4	BaAdV-3
Human			
A549 (human epithelial lung adenocarcinoma)	+++	+++	+++
HFDL (human fetal diploid lung)	−	−	++
HFDK (human fetal diploid kidney)	−	−	++
Old World monkey			
PMK (primary rhesus monkey kidney)	+++	+++	+++
Vero (African green monkey kidney)	+++	+++	+++
CyMK (cynomolgus monkey kidney)	+++	+++	+++
New World monkey			
B95a (marmoset monkey lymphoblastoid)	−	−	−

[a] +++, strong cytopathic effect (CPE); ++, moderate CPE; −, no CPE.

intravenous fluids, oxygen administration, and empirical antibiotics. None of the 4 baboon AdV isolates was typeable as HAdV species A to F or SAdV-A by neutralization testing done in 1997.

Cell culture tropism of novel baboon adenoviruses. We attempted to culture the 4 BAdVs isolated from sick and asymptomatic baboons during the 1997 outbreak in a variety of human and monkey cell lines. The majority of cells and cell lines tested resulted in productive infection as determined by the extent of cytopathic effect (CPE) (Table 1). All 4 BAdVs grew efficiently in cells from other Old World monkey species (rhesus, cynomolgus, and African green monkeys). In 2012, we tested the BaAdVs for growth in human cell lines; all 4 grew efficiently in the human lung adenocarcinoma A549 cell line, which is commonly employed in isolation of human AdVs (24). Notably, unlike the other 3 AdV strains, BaAdV-3 was also successfully cultured on two additional human cell lines. No growth was observed in lymphoblastoid B95a cells from marmosets, which are New World monkeys.

***De novo* assembly and whole-genome phylogenetic analysis of novel baboon adenoviruses (BaAdV-1 to BaAdV-4).** In 2012, we sought to further characterize the 4 AdVs isolated from baboons by whole-genome sequencing and phylogenetic analysis. The sequences of the AdV hexon, DNA polymerase, and fiber were initially recovered by Sanger sequencing. To sequence the entire AdV genome, early passaged cultures corresponding to the 4 BaAdV isolates were subjected to next-generation "deep" sequencing on an Illumina HiSeq 2000 (Fig. 2). Out of 32.9 to 45.2 million raw reads, from 61.3% to 93.1% of the genome was assembled *de novo* for each of the 4 AdV strains. Specific PCR and Sanger sequencing were then used to confirm the *de novo* assembly and to fill in the gaps. The genomic assemblies revealed that all 4 baboon AdV strains retained the major core adenoviral proteins, and similar to AdVs in species SAdV-B, -F, and -G, all 4 strains contained two fiber proteins (16, 25–28).

By pairwise analysis of nucleotide identity (Fig. 3A), BaAdV-1, the strain isolated from an asymptomatic baboon in the outbreak room (Fig. 1, room 1, B4), was found to be closely related to a previously described SAdV-B strain isolated from a stool sample from a captive rhesus monkey (SAdV-A1335, GenBank accession number JN880456, 97.8% identity) (18). Strains BaAdV-2 and BaAdV-4, both isolated from asymptomatic baboons housed away from the outbreak location in a separate room (Fig. 1, room 2, B8 and B9), were 100% identical to each other, while strain BaAdV-3, isolated from a symptomatic baboon positioned near the two baboons who died from adenoviral pneumonia (Fig. 1, room 1, B5), was 91.2% identical to strains BaAdV-2/-4 (Fig. 3B). The closest relatives to BaAdV-2/-4 and BaAdV-3 were members of AdV species F and G (Fig. 3B and 4). Phylogenetic analysis of the individual hexon, penton base, DNA polymerase, and fiber proteins and amino acid pairwise identity comparisons revealed that BaAdV-1 is a member of the SAdV-B species (Fig. 4 and 5A), while BaAdV-2/-4 and BaAdV-3 appear to be members of a new species intermediate between SAdV-F and SAdV-G, provisionally named "simian adenovirus C (SAdV-C)" (Fig. 4 and 5B). Both BaAdV-2/-4 and BaAdV-3, like members of the SAdV-A and SAdV-B species, contained the RGD motif in the penton base protein which mediates binding to α_v integrins during virus internalization (29).

Pools of hyperimmune rabbit antisera containing neutralizing antibodies to the 41 HAdV prototype strains (representing species A to F) were tested against the BaAdVs. No neutralization was observed at a starting serum dilution of 1:8 with the exception of the species F (HAdV-40 and -41) antisera pool that showed low-level neutralization (1:16) with BaAdV-1 (SAdV-B) and BaAdV-2/-4 (SAdV-C). Sera from control baboon B107 that was positive for neutralizing antibody to BaAdV-1 and BaAdV-2/-4 did not neutralize species G HAdV-52.

Notably, despite sharing 91.2% overall nucleotide identity across the genome (Fig. 3B), the sequence of BaAdV-3, the only AdV among the 4 that had been isolated from a symptomatic baboon, diverged significantly from that of BaAdV-2/-4 in the short fiber 1 gene. While the other major adenoviral proteins were >90% identical, the short fiber 1 of BaAdV-3 shared only 58% amino acid identity relative to that of BaAdV-2/-4 (Fig. 5B). Similarity and bootscanning plots of BaAdV-3 relative to BaAdV-2/-4 (species SAdV-C) and related AdVs in species SAdV-A, -F, and -G suggested high likelihood of a recombination event involving the short fiber 1 gene (Fig. 6). However, if the short fiber 1 gene of BaAdV-3 is the result of a recombination event, the presumptive AdV donor strain has yet to be identified, as the BaAdV-3 short fiber 1 lacked any closely related phylogenetic neighbors (~58% to BaAdV-2/-4 and ≤50% amino acid identity to other sequenced fiber proteins in GenBank).

Seroprevalence of BaAdV-1, BaAdV-3, and BaAdV-2/4 in baboons and humans. Of note, many staff members at the TBRI had anecdotally reported experiencing "flu-like" symptoms around the time of onset of the 1997 baboon outbreak. To investigate the possibility that a cross-species transmission event, either zoonotic (from baboon to human) or anthroponotic (from human to baboon), may have occurred, preoutbreak and postoutbreak sera from potentially exposed human staff personnel at the TBRI (Ta-

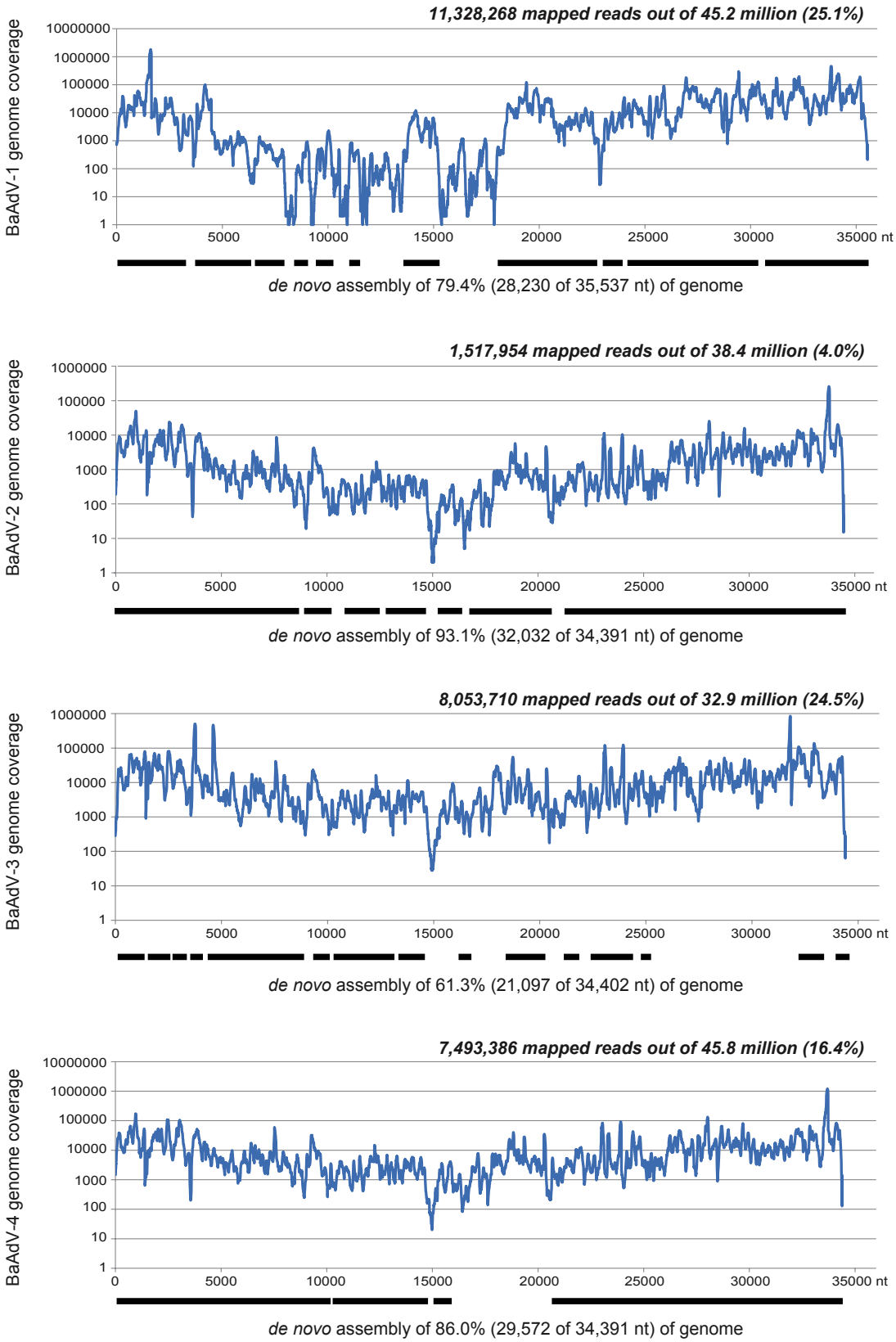


FIG 2 Genomic coverage of 4 novel baboon adenoviruses (BaAdVs) by deep sequencing. Part of the viral genome was recovered directly from deep sequencing reads using a *de novo* assembly approach (black bars). After completion of the genome and confirmation by Sanger sequencing, deep sequencing reads are mapped to the corresponding AdV genome. The coverage (*y* axis) achieved at each position along the genome (*x* axis) is plotted on a logarithmic scale. Abbreviation: nt, nucleotide.

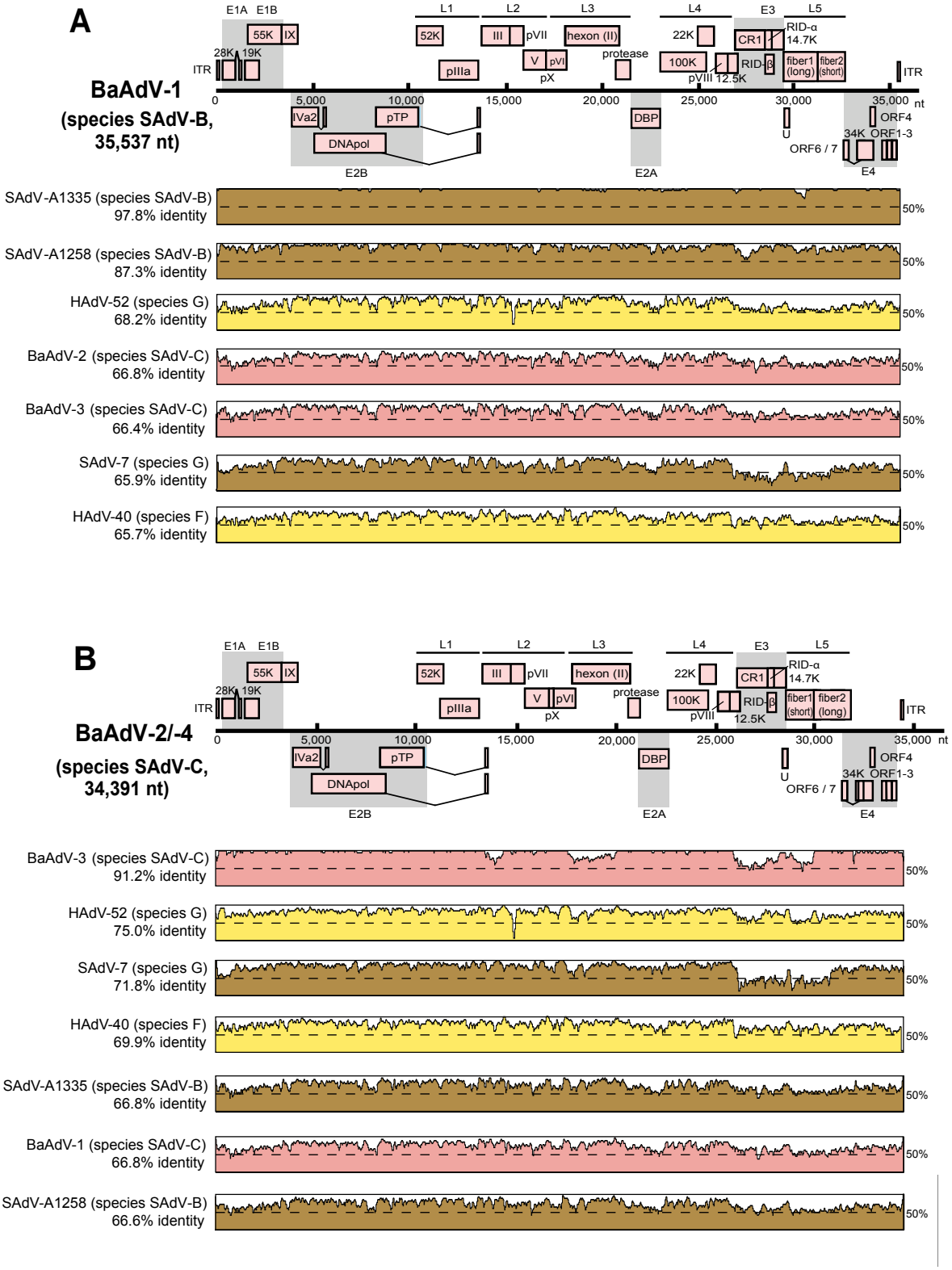


FIG 3 Genome organization of BaAdV-1 and BaAdV-2/-4 and pairwise alignment with related adenoviruses. (A and B) Maps of the genome organization corresponding to two baboon AdVs, BaAdV-1 (A), a species SAdV-B AdV, and BaAdV-2/-4 (B), a novel species SAdV-C AdV, are shown. Boxes above the central black line represent genes on the forward strand, while boxes below the black line represent genes on the reverse strand. Early region genes are shaded in gray. The scanning nucleotide pairwise identities of BaAdV-1 (A) and BaAdV-2/-4 (B) relative to selected related human (yellow), simian (brown), or novel baboon (pink) AdVs are shown ranked in order of decreasing overall percent identity. The *x* axis refers to the nucleotide position along the genome. Abbreviations: ITR, inverted terminal repeat; pTP, precursor terminal protein; ORF, open reading frame; DNApol, DNA polymerase; DBP, DNA binding protein; CR1, complement receptor 1; RID-α, receptor internalization and degradation.

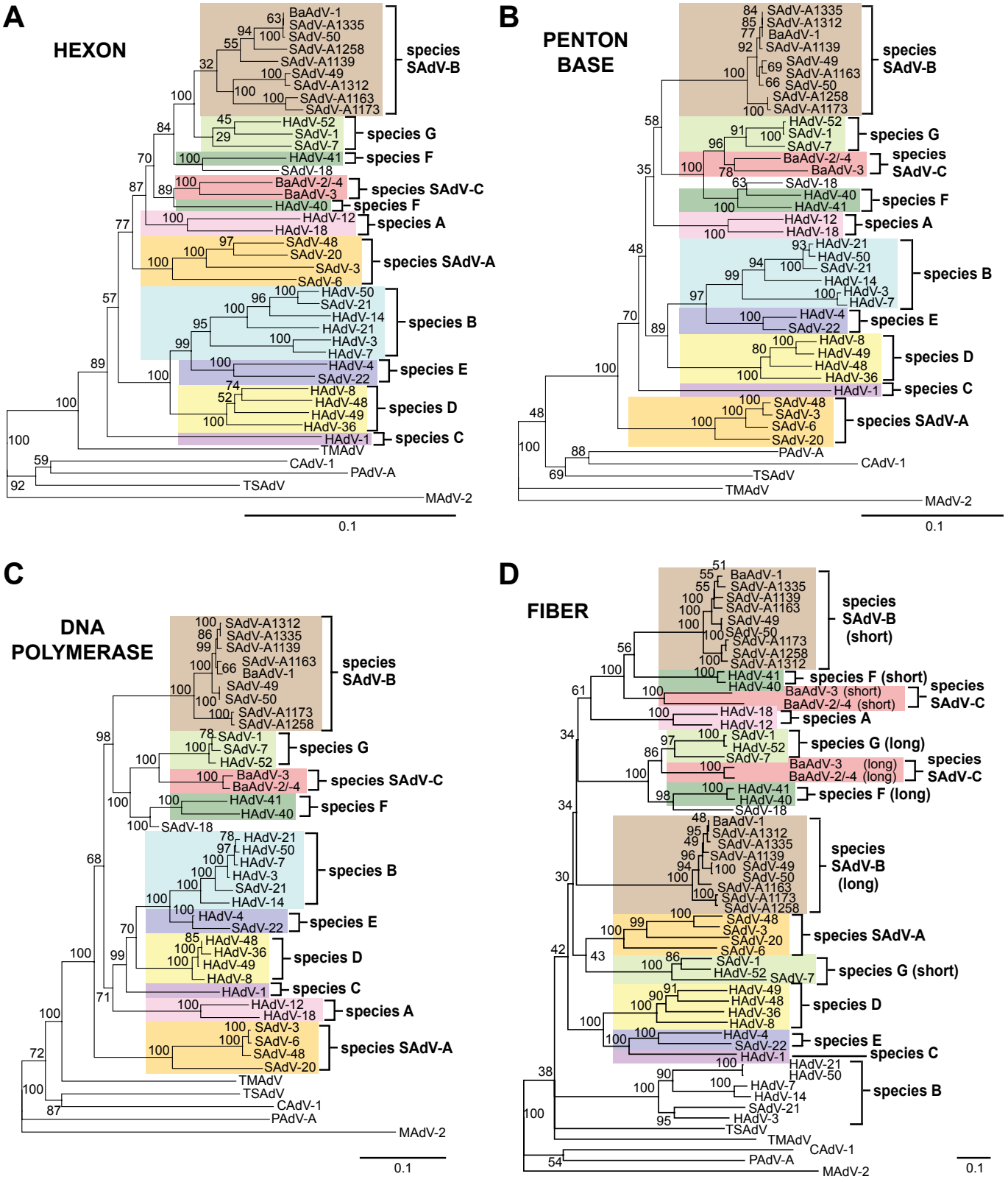
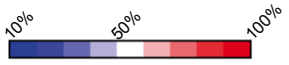


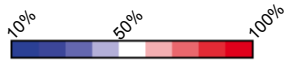
FIG 4 Amino acid phylogenetic analysis of BaAdV-1, BaAdV-2/-4, and BaAdV-3 relative to other adenoviruses. (A) Hexon; (B) penton base; (C) DNA polymerase; (D) fiber. Representative primate AdVs in species A to G, SAdV-A, and SAdV-B, and nonprimate AdVs, were included in the phylogenetic analysis. Bayesian support levels are displayed at each branching point. The 4 novel BaAdVs identified in this study and the proposed "species SAdV-C" designation are highlighted in red. Scale bars indicate the number of amino acid substitutions per site. Abbreviations and GenBank accession numbers are given in the text.

A **Amino Acid Pairwise Identity against BaAdV-1 (species SAdV-B)**



	E1B-55K	DNApol	pTP	penton base (III)	hexon (II)	DBP	fiber1 (long)	fiber2 (short)
BaAdV-2/-4 (baboon, species SAdV-C)	56%	74%	80%	83%	86%	53%	37% (fiber1) 34% (fiber2)	32% (fiber1) 35% (fiber2)
BaAdV-3 (baboon, species SAdV-C)	57%	74%	80%	82%	85%	53%	40% (fiber1) 36% (fiber2)	32% (fiber1) 34% (fiber2)
SAdV-A1335 (rhesus, species SAdV-B)	100%	99%	100%	100%	100%	99%	95%	99%
SAdV-3 (rhesus, species SAdV-A)	43%	69%	78%	79%	80%	59%	33%	
SAdV-18 (vervet)	58%	76%	79%	85%	89%	56%	36%	
TMAdV (titi)	36%	63%	68%	68%	78%	36%	27%	
HAdV-12 (human, species A)	53%	71%	77%	83%	84%	53%	41%	
HAdV-14 (human, species B)	41%	73%	76%	74%	79%	47%	28%	
HAdV-1 (human, species C)	45%	75%	78%	70%	78%	49%	37%	
HAdV-8 (human, species D)	43%	75%	75%	77%	80%	48%	28%	
HAdV-4 (human, species E)	49%	77%	73%	75%	80%	43%	27%	
HAdV-40 (human, species F)	56%	74%	76%	83%	84%	55%	40% (fiber1) 36% (fiber2)	34% (fiber1) 35% (fiber2)
HAdV-52 (human, species G)	59%	76%	80%	83%	93%	55%	33% (fiber1) 35% (fiber2)	35% (fiber1) 37% (fiber2)
MAdV-2 (mouse)	25%	47%	37%	56%	67%	31%	24%	

B **Amino Acid Pairwise Identity against BaAdV-2/-4 (species SAdV-C)**



	E1B-55K	DNApol	pTP	penton base (III)	hexon (II)	DBP	fiber1 (short)	fiber2 (long)
BaAdV-1 (baboon, species SAdV-B)	56%	74%	80%	83%	86%	53%	37% (fiber1) 32% (fiber2)	34% (fiber1) 35% (fiber2)
BaAdV-3 (baboon, species SAdV-C)	98%	98%	99%	91%	92%	99%	58%*	93%
SAdV-A1335 (rhesus, species SAdV-B)	56%	75%	80%	83%	86%	53%	37% (fiber1) 32% (fiber2)	34% (fiber1) 35% (fiber2)
SAdV-3 (rhesus, species SAdV-A)	43%	69%	77%	79%	81%	50%	32%	
SAdV-18 (vervet)	67%	79%	89%	88%	86%	62%	57%	
TMAdV (titi)	36%	63%	69%	67%	77%	37%	28%	
HAdV-12 (human, species A)	56%	72%	80%	79%	84%	62%	38%	
HAdV-14 (human, species B)	47%	74%	77%	74%	79%	52%	22%	
HAdV-1 (human, species C)	49%	73%	79%	69%	76%	52%	34%	
HAdV-8 (human, species D)	47%	76%	77%	75%	86%	52%	27%	
HAdV-4 (human, species E)	54%	77%	74%	76%	80%	48%	31%	
HAdV-40 (human, species F)	67%	77%	83%	86%	89%	62%	30% (fiber1) 54% (fiber2)	45% (fiber1) 29% (fiber2)
HAdV-52 (human, species G)	72%	85%	93%	91%	85%	72%	33% (fiber1) 29% (fiber2)	28% (fiber1) 61% (fiber2)
MAdV-2 (mouse)	25%	46%	38%	58%	65%	31%	21%	

*highest identity between the short fiber1 protein of BaAdV-3 and that of any other AdV

FIG 5 Amino acid pairwise identities of BaAdV-1 and BaAdV-2/-4 relative to other adenoviruses. Comparisons are made against representative human, simian, and murine AdVs. The amino acid pairwise identity table is displayed as a heat map; colors ranging from blue to white to red correspond to pairwise identities of 10 to 100% (color bar). The black cells denote AdVs that lack short fibers.

ble 2, human 1 [H1] to H6) were tested for antibodies to BaAdV-1, BaAdV-3, and BaAdV-2/-4 by virus neutralization in a blinded fashion. As additional controls for baseline seroprevalence, we also tested sera from a random selection of 5 young children and available sera from 10 baboons born approximately the same time as affected baboons with pneumonia, but not part of the 1997 outbreak. Significantly, 5 of 6 (83%) and 6 of 6 (100%) personnel, while seronegative prior to the outbreak, had evidence of neutralizing antibody titers to BaAdV-1 and BaAdV-2/-4, respectively, after the outbreak. The highest neutralizing antibody response, 1:160, corresponded to the researcher in closest contact with sick baboons during the 1997 outbreak (Table 2, H1). Interestingly, no neutralization to BaAdV-3 was observed in any of the staff personnel. The specificity of the neutralization assays was further

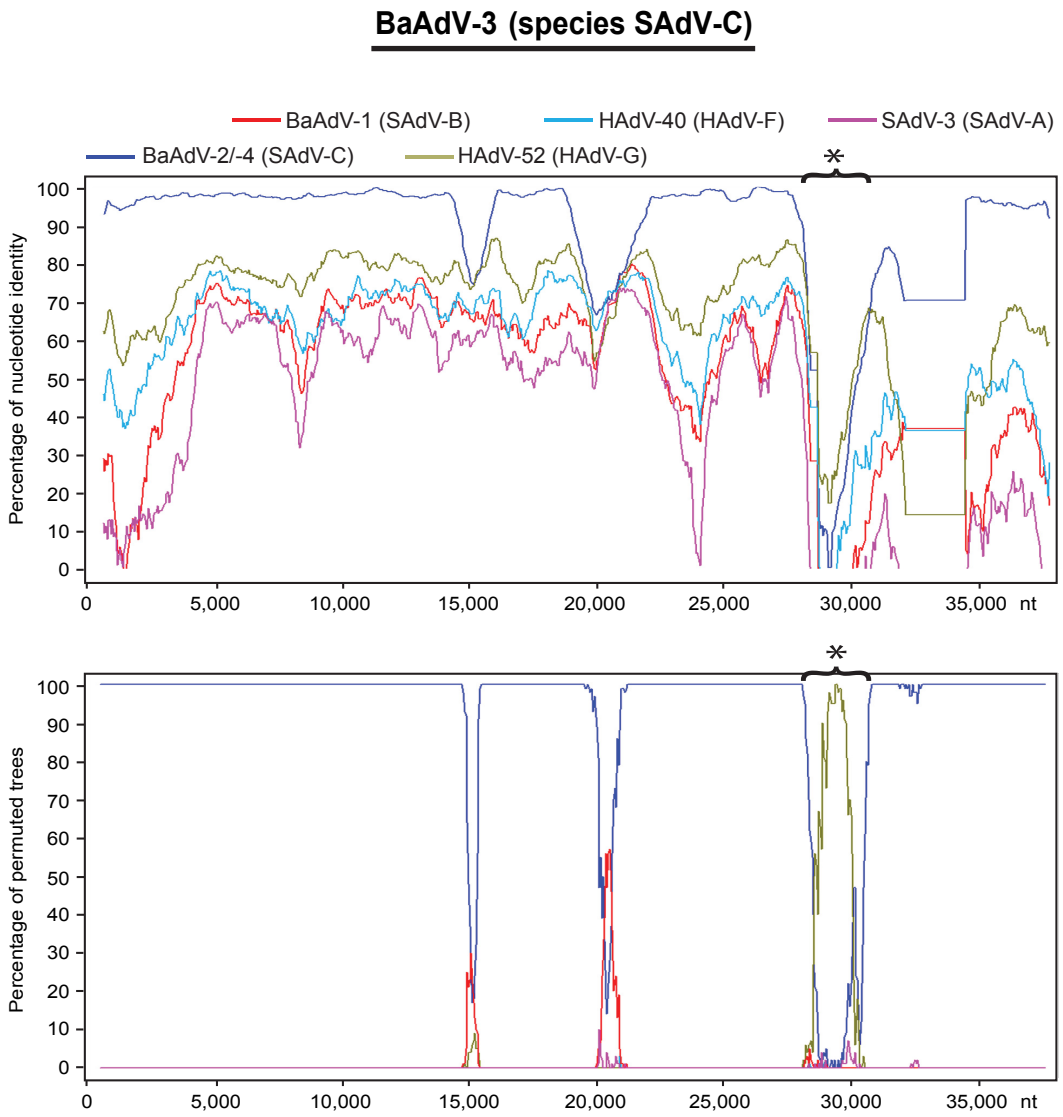


FIG 6 Evidence for recombination in species SAdV-C adenovirus BaAdV-3. Similarity (top) and bootscanning (bottom) plots of AdVs in species SAdV-A, SAdV-B, SAdV-C, -F, and -G relative to BaAdV-3 are shown. Bootscanning analysis reveals likely recombination breakpoints in the region corresponding to the divergent short fiber 1 gene (asterisk). The *x* axis refers to the nucleotide position. The genome organization map is annotated in the same fashion as in Fig. 2. Abbreviation: nt, nucleotide.

confirmed by the screening of 5 epidemiologically unassociated human children under 5 years of age, all of whom were negative for neutralizing antibodies to BaAdV-1, BaAdV-2/-4, and BaAdV-3. Among 11 healthy baboons in the colony who were not part of the 1997 outbreak, 4 of 11 (36%) (from sera collected in 2002–2003) and 3 of 11 (27%) (from sera collected in 1996 and 2003) harbored antibodies to BaAdV-1 and BaAdV-2/-4, respectively (Table 2, B101 to B111). Very little to no neutralization was observed to BaAdV-3; neutralizing antibody at 1:8 titer was detected in only one baboon, B108, who also had a titer of 1:64 to closely related strain BaAdV-2/-4.

DISCUSSION

An outbreak of rapidly fatal adenoviral pneumonia in infant baboons occurred in 1997 at the Primate Research Center of the TBRI. The diagnosis of primary AdV infection was supported by the presence of atypical lymphocytes in the peripheral circulation, hemorrhagic and necrotic lesions in the lung and liver, and intranuclear inclusions in bronchial epithelium, with subsequent confirmation by direct isolation of AdV from lung tissue. Two of four baboons presenting with acute respiratory infection (50%) died in the 1997 outbreak. Although the number of deaths is small, the case fatality rate of 50% is high for AdV infections, which typically cause much lower mortality rates in susceptible human children of <15% (30–32). One explanation for the high death rate may be the concurrent presence of bacteria such as MSSA in at least one of the 2 baboons, which may have predisposed AdV-infected baboons to severe and potentially fatal bacterial superinfections of the lung (33). It is also possible that newborn baboons are highly susceptible in general to severe infection from AdVs, which are known to be associated with more severe disease in humans who are immunocompromised, elderly, or very young (7, 34).

TABLE 2 Human and baboon serum antibody neutralization titers with baboon adenovirus (BaAdV) isolates

Serum specimen[a]	Date (mo/day/yr) collected[c]		Serum antibody neutralization titer[c] with:					
			BaAdV-1		BaAdV-2/-4		BaAdV-3	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Laboratory staff[b]								
H1	1/20/1993	5/6/1997	−	1:128	−	1:128	−	−
H2	8/30/1988	5/6/1997	−	1:64	−	1:64	−	−
H3	1/20/1993	5/6/1997	−	1:64	−	1:64	−	−
H4	3/16/1993	5/6/1997	−	1:32	−	1:32	−	−
H5	1/20/1993	5/6/1997	−	−	−	1:64	−	−
H6	8/30/1988	5/6/1997	−	1:64	−	1:64	−	−
Baboon controls								
B101	NA	6/9/2003	NA	−	NA	−	NA	−
B102	6/1/1996	9/4/2002	−	1:8	−	−	−	−
B103	7/1/1996	NA	−	NA	1:8	NA	−	NA
B104	8/10/1996	NA	−	NA	−	NA	−	NA
B105	NA	12/31/2001	NA	−	NA	−	NA	−
B106	NA	7/15/2002	NA	−	NA	−	NA	−
B107	NA	6/24/2003	NA	−	NA	−	NA	−
B108	NA	2/21/2003	NA	1:8	NA	1:64	NA	1:8
B109	NA	5/22/2003	NA	1:8	NA	1:8	NA	−
B110	NA	5/20/2004	NA	−	NA	−	NA	−
B111	NA	12/04/2003	NA	1:8	NA	−	NA	−

[a] Sera from 5 epidemiologically unrelated young children were also tested to serve as negative human controls. All lacked neutralizing antibodies to BaAdV-1, BaAdV-2/-4, and BaAdV-3 at a 1:8 screening dilution.
[b] H1 to H6, laboratory staff at the Texas Biomedical Research Institute (TBRI) during the outbreak; B101 to B111, asymptomatic baboons housed at the TBRI from 1996 to 2004.
[c] Sera were collected before (Pre) and after (Post) the 1997 outbreak. −, negative antibody titer at 1:8 screening dilution; NA, not available.

The AdV strains isolated from lung tissue from the two baboons who died from pneumonia were untypeable by virus neutralization testing for AdVs in HAdV species A to F and SAdV-A, including HAdVs in species B, C, and E that are typically associated with human respiratory disease and pneumonia (35). This finding raised the possibility that the causative agent of the outbreak may be a novel AdV strain of unknown pathogenicity. Since tissue and primary cultures from the two fatal cases had been sent out to off-site laboratories in 1997 and were unavailable for further analysis, we sought to characterize AdVs isolated from other symptomatic and/or asymptomatic baboons (BaAdVs) involved in the 1997 outbreak. Four AdV isolates (BaAdV-1 to BaAdV-4) were successfully cultured from nasal swabs in 1997, of which only one, BaAdV-3, was derived from a baboon with acute respiratory symptoms. Similar to the two AdV strains from dead baboons, the 4 isolates were untypeable at the time of the outbreak for HAdV species A to F and SAdV-A by virus neutralization testing (although subsequent repeat neutralization testing against HAdV species A to G in 2012 found a low level of serological cross-reactivity between BaAdV-1 or BaAdV-2/4 and members of species HAdV-F).

To further characterize these untypeable AdVs, the genomes corresponding to all 4 isolates were recovered by a combined deep sequencing, traditional Sanger sequencing, and *de novo* assembly approach (Fig. 2). BaAdV-1 was found to be a member of the recently described SAdV-B species (18), but BaAdV-2, BaAdV-3, and BaAdV-4 (identical to BaAdV-2) were found to represent members of a potentially novel species (Fig. 3B and 4). Both BaAdV-2/-4 and BaAdV-3 meet one of the main ICTV criteria for a new AdV species by exhibiting >10% phylogenetic distance from their nearest AdV neighbors, members of human and adenoviral species F and G (Fig. 3B) (17). The absence of cross-neutralization with BaAdV-3 and very weak cross-neutralization with BaAdV-2/-4 and the HAdV-F AdVs also support the contention that these BaAdVs are novel serotypes. Thus, we propose the designation "simian adenovirus C (SAdV-C)" for this new species, in accordance with previously established taxonomic criteria for adenoviruses and following the identification of the SAdV-A and SAdV-B species (17, 18, 36–38).

Interestingly, BaAdV-3, despite sharing 91.2% overall nucleotide identity to BaAdV-2/-4 (Fig. 3B), exhibits little to no cross-reactivity with BaAdV-2/-4 in testing of both human and baboon sera (Table 2). The basis for this serological specificity may be the sequence of the short fiber 1, which diverges significantly in BaAdV-3 relative to both BaAdV-2/-4 and all other sequenced AdVs (≤58% identity at the amino acid level) (Fig. 5B). Bootscan analysis suggests that BaAdV-3 may have arisen from a recombination event involving a species SAdV-C AdV such as BaAdV-2/-4 and an as-yet unidentified donor strain harboring the divergent BaAdV-3 short fiber 1 (Fig. 6). Since productive infection by BaAdV-3, but not BaAdV-2/-4, was observed in two human fetal cell lines (Table 1), we speculate that this donor strain may potentially be a human adenovirus, or at least human-tropic. A search for this presumptive AdV donor strain is now under way.

Anecdotal reports of "flu-like" symptoms in staff members around the time of the 1997 baboon outbreak precipitated an investigation of serological responses to BaAdV-1, BaAdV-2/-4, and BaAdV-3 in present-day baboons in the colony and potentially exposed staff personnel at the TBRI. Neutralizing antibody titers to BaAdV-1 (species SAdV-B) and BaAdV-2/-4 (species SAdV-C) were detected in both baboons and humans (Table 2). Although it is possible that these titers may reflect borderline cross-reactivity with human species F AdVs, we deem it more likely that BaAdV-1 (species SAdV-B) and BaAdV-2/-4 (species SAdV-C) are serologically distinct from HAdV-F, given the low hexon and fiber homology (Fig. 5), and the high rates of serocon-

version and magnitude of antibody titer rise in exposed staff personnel over a relatively brief 4-year period (Table 2). Thus, our serological results may provide the first glimpse of zoonotic transmission of presumptive simian AdVs in species SAdV-B and the novel species SAdV-C to exposed humans. However, as we cannot rule out the possibility of cross-reactivity to known human AdVs at this time, broader prevalence studies of seroreactivity to BaAdVs in the human populations will be needed to confirm these findings.

Several lines of evidence support the conclusion that BaAdV-3, a novel species SAdV-C AdV, is the most likely cause of the pneumonia outbreak in 1997. First, BaAdV-3, a member of SAdV-C, was the only sequenced AdV that was isolated from a sick baboon with acute respiratory symptoms. Second, this SAdV-C-infected baboon was in a cage situated nearby and in the same room as the two baboons that died from pneumonia and untypeable AdVs could be isolated from lung tissue. Third, aside from untypeable AdVs isolated directly from lung tissue from both fatal cases, the only other infectious agents isolated were MSSA and *Kluyvera ascorbata* in one of the 2 dead baboons. Collectively with the serological testing showing little to no seroreactivity to BaAdV-3 in asymptomatic control baboons and human staff researchers, these data suggest that BaAdV-3 may be a rare pathogenic SAdV-C recombinant whose emergence precipitated the outbreak. Further studies, including experimental infections with SAdV-C viruses in baboons and more extensive assessment of seroprevalence in human and monkey populations are needed to investigate this possibility.

MATERIALS AND METHODS

Ethics statement. This study was performed in strict accordance with the *Guide for Care and Use of Laboratory Animals* (39). The use and care of all animals followed policies and guidelines established by the Institutional Animal Care and Use Committee (IACUC) and the Institutional Biohazards Committee of the TBRI. The protocol for the maintenance and breeding of the baboon study was approved by the TBRI IACUC. No specific animal research protocol was drafted for this study, as only excess clinical samples were analyzed for diagnostic purposes. Extensive veterinary care was provided to all animals affected by the outbreak to minimize pain and distress. Written informed consent was obtained from the staff of the TBRI for analysis of their samples and analyzed under protocols approved by the University of California, San Francisco (UCSF) institutional review board (IRB). Any potentially identifying information has been provided with the explicit permission of the individual involved.

Biosafety. Analysis of necropsy tissues and cultivation of the novel baboon AdVs described in this study were performed under biosafety level 2 (BSL-2) conditions as approved by the Institutional Biohazards Committees of the TBRI and Centers for Disease Control and Prevention (CDC). Nucleic acid extractions of AdV cultures were performed at the University of California, San Francisco in BSL-2 facilities specifically certified by the UCSF Biosafety Committee for handling novel simian AdVs. Serological analysis of human and baboon sera was performed in a BSL-2 laboratory at the California Department of Public Health (DPH).

Outbreak management and investigation. The baboon outbreak lasted approximately 3 weeks from 21 February to mid-March of 1997. Affected baboons were quarantined immediately after development of respiratory symptoms. The two baboons with fatal cases died or were humanely euthanized 5 and 13 days after the onset of clinical signs. Daily reports on clinical and epidemiological parameters were tracked and recorded by veterinary and management staff. In response to the outbreak, all incubator rooms were decontaminated with paraformaldehyde gas. Cages, walls, floors, and all exposed work area surfaces were cleaned with 2.6% buffered glutaraldehyde (Metricide) or bleach. Disposable protective suits and gloves were worn at all times when feeding or otherwise in contact with infant baboons for a period of at least 2 weeks. Hematological testing and cultures for bacteria, mycoplasma, and fungi were performed at the University of Texas Health Science Center at San Antonio (UTHSCSA). Samples were also tested for respiratory syncytial virus (RSV), influenza virus, parainfluenza, human adenovirus, and herpesviruses, including cytomegalovirus (CMV), at the UTHSCSA. In cultures manifesting cytopathic effects, the presence of adenovirus was confirmed by immunofluorescence microscopy with an anti-hexon antibody conjugate. Respiratory samples were sent out to an off-site laboratory and tested for *Bordetella pertussis*, *Chlamydophila* spp., *Mycoplasma* spp., *Ureaplasma* spp., *Legionella* spp., and hantavirus. AdV typing by virus neutralization testing using sera reactive against HAdV species A to F and SAdV-A viruses was performed at Viral Reference Laboratories, Inc., in San Antonio, TX, in 1997. Culturing of baboon AdVs was performed at the UTHSCSA, CDC, and California DPH.

Pathology. Gross and histopathological analyses of necropsy tissues were performed by a board-certified veterinary pathologist at the Primate Research Center of the TBRI. Necropsy tissues were fixed in 10% formalin and embedded in paraffin. Five-micron-thick sections were then cut using a microtome, stained with hematoxylin and eosin (H&E), and examined by light microscopy.

Nucleic acid extraction. Two hundred microliters of sample was first passed through a 0.4-μm-pore-size filter to remove bacteria and cellular debris and then treated with RNase (Invitrogen, Carlsbad, CA). Total nucleic acid was then extracted from cultured AdV supernatant using commercially available kits (Qiagen, Valencia, CA).

Virus cultivation. All inoculations of monkey cells (PMK, or primary monkey kidney; CyMK, or cynomolgus monkey kidney; and Vero [African green monkey kidney]) were made using primary nasal swab specimens. Inoculations of human cells and cell lines were made with P1 virus after a single passage in monkey cells. The cells or cell lines were grown in media consisting of Hank's medium (for A549 cells) or Dulbecco's modified Eagle's medium (DMEM) (for other cells) supplemented with 1× nonessential amino acids (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (FBS), 100 U of penicillin/ml, and 100 μg of streptomycin/ml. After 80 to 90% confluence was achieved, cell culture media were changed to maintenance media with 2% FBS and inoculated with 200 μl of clinical sample or 100 μl of passaged viral supernatant. Prior to inoculation, nasal samples were clarified by centrifugation at 4,000 × *g* for 10 min; lung tissue was homogenized using a tissue homogenizer in 5 volumes of buffer. Prior to passaging, cell culture supernatant was subjected to 3 freeze-thaw cycles and clarified as described above. Viral replication was monitored over 2 weeks by visual inspection under light microscopy for CPE. Supernatants of viral cell lysates were quantified using an endpoint dilution assay.

Deep sequencing library preparation. Deep sequencing libraries were prepared for whole-genome AdV sequencing using a variation of the TruSeq protocol (Illumina, San Diego, CA) (3). Briefly, nucleic acid extracts were randomly amplified to cDNA using a round A/B procedure as previously described (40, 41), and then digested using the restriction enzyme BpmI (New England Biolabs, Ipswich, MA) for 2 h at 37°C, followed by end repair and A-tailing with Klenow and *Taq* polymerase, respectively (Invitrogen, Carlsbad, CA). Size selection targeting 200- to 300-bp fragments was then performed using AMPure beads, followed by attachment of adapters containing 6-nucleotide barcode tags using DNA ligase. Final libraries were quantified using the Bioanalyzer DNA 12000 chip (Agilent) and SYBR Fast quantitative PCR (qPCR) system (KAPA Biosystems), pooled into a single lane, and sequenced on an Illumina HiSeq 2000 instrument (100-bp paired-end sequencing).

***De novo* viral genome assembly.** Raw deep sequencing reads were initially trimmed by removal of adapters, primers, and low-complexity/low-quality sequences. *De novo* assembly of partial AdV genomes was performed using the PRICE assembler (42). Despite high overall coverage (Fig. 2), *de novo* assembly of the entire AdV genome was not achieved

because of (i) the significant degree of variation in coverage (especially for BaAdV-1) and (ii) the fact that PRICE is a "seed-based" assembler that assembles into contiguous regions and not a true *de novo* assembler. All gaps were subsequently filled by PCR using specific primers designed from assembled regions followed by Sanger sequencing. PCR and Sanger sequencing were also used to confirm the correctness of the assembly. After assembly of the full viral genomes corresponding to the 4 BAdV isolates, trimmed deep sequencing reads were then mapped to the AdV genome using Geneious software (43) with the following parameters (no gaps allowed, maximum mismatches allowed per read of 5%, and maximum ambiguity of 1).

Structural features and phylogenetic analysis. The predicted coding regions in BaAdV genomes were identified using the fully annotated genome sequences of species F and G AdVs in GenBank as a reference. First, each BaAdV genome was aligned to the most similar reference genome in GenBank, followed by identification of open reading frames (ORFs) using Geneious. The selection of ORFs required the presence of an ATG start codon, a 100-amino-acid (aa) minimum size, and a match to a corresponding ORF in an annotated AdV reference genome. The GT-AG intron start-stop signal was used to predict the splice donor and acceptor sites for spliced genes. To confirm the accuracy of the predicted coding regions, each identified ORF was then aligned using BLASTx to a reference database consisting of all adenoviral proteins in GenBank. Whole-genome nucleotide pairwise identity plots (window size of 100) and amino acid pairwise identity calculations were performed in Geneious. Similarity and bootscanning plots were generated using Simplot (44), with a window size of 1,000 bp and a step size of 50 bp.

To construct the amino acid phylogeny trees corresponding to the hexon, penton base, DNA polymerase, and short/long fiber proteins, the translated protein sequences corresponding to representative human and simian AdVs in species A to G, SAdV-A, and SAdV-B, as well as nonprimate AdVs, were first downloaded from GenBank. Multiple sequence alignments were then performed using the FFT-NS-i×1000 algorithm of MAFFT at default parameters (45). A phylogenetic tree was constructed in Geneious using the Jukes-Cantor neighbor-joining method and 5,000 bootstrap replicates and a support threshold percentage of 25, using murine adenovirus 2 (MAdV-A) as an outgroup.

Baboon adenovirus neutralization assay (human and baboon sera). Viral stocks of BaAdV-1, BaAdV-2, and BaAdV-3 were generated by passaging in Vero E6 cells, aliquoted, and quantitated by endpoint dilution. To perform the virus neutralization assay, 100 μl of viral supernatant mixed with serum or control serum was incubated for 1 h at 37°C. After incubation, the mixture was inoculated into wells containing 4,000 Vero E6 cells per well and incubated at 37°C and 5% CO_2. The cells in the wells on the plate were observed every other day for evidence of CPE. For cells in wells that showed inhibition of viral CPE at the screening dilution of 1:10, the corresponding serum samples were diluted 2-fold from 1:8 to 1:128 and then retested. The reciprocal of the highest dilution where replicate well monolayers showed no CPE was taken as the neutralizing antibody titer.

Human adenovirus A to F cross-neutralization assay (rabbit typing sera). Five pools of rabbit hyperimmune reference sera at the California DPH, collectively containing antibodies to human AdV species A to E, were available for testing. An individual rabbit serum sample reactive to HAdV-40 and HAdV-41 was also available for cross-neutralization testing of HAdV-F. For each pool, rabbit sera at a screening dilution of 1:8 and 100 μl of viral supernatant at a 50% tissue culture infective dose ($TCID_{50}$) of 10^3/ml were mixed and inoculated into Vero E6 cells. Cells in wells on the plate were observed every other day for 2 weeks for evidence of CPE. For cells in wells that showed inhibition of viral CPE at the screening dilution of 1:8, the corresponding serum samples were diluted 2-fold from 1:8 to 1:128 and then retested.

Human adenovirus G indirect cross-neutralization assay (baboon sera). Since neutralizing reference sera to human HAdV-52 (species G) was not available, the serum sample from baboon B108, shown previously to be positive for neutralizing antibody to species SAdV-B and SAdV-C AdVs (Table 2), was tested for cross-neutralization of HAdV-52 in an indirect neutralization assay. To perform the assay, 100 μl HAdV-52 supernatant at a $TCID_{50}$ of 10^3/ml was mixed with serum from baboon B108 at the screening dilution of 1:8 and used to inoculate Vero E6 cells. The cells in wells were observed every other day for 2 weeks for evidence of CPE.

Nucleotide sequence accession numbers. GenBank accession numbers for the adenoviral sequences used in Fig. 3 to 5 are as follows: CAdV-1 (canine adenovirus 1), AC_000003; HAdV-1, AC_000017; HAdV-3, DQ086466; HAdV-4, AY458656; HAdV-7, AC_000018; HAdV-8, AF532578; HAdV-12, NC_001460; HAdV-14, FJ822614; HAdV-18, GU191019; HAdV-21, AY601633; HADV-36, GQ384080; HAdV-40, NC_001454; HAdV-41, DQ315364; HAdV-48, EF153473; HAdV-49, DQ393829; HAdV-50, AY737798; HAdV-52, DQ923122; MAdV-2 (murine adenovirus 2), NC_014899; PAdV-3 (porcine adenovirus A), AC_000009; SAdV-1, AY771780; SAdV-3, NC_006144; SAdV-6, JQ776547; SAdV-7, DQ792570; SAdV-18, FJ025931; SAdV-20, HQ605912; SAdV-21, AC_000010; SAdV-22, AY530876; SAdV-48, HQ241818; SAdV-49, NC_015225; SAdV-50, HQ241820; SAdV-A1139, JN880448; SAdV-A1163, JN880449; SAdV-A1173, JN880450; SAdV-A1258, JN880451; SAdV-A1312, JN880454; SAdV-A1335, JN880456; TMAdV (titi monkey adenovirus), HQ913600; TSAdV (tree shrew adenovirus), NC_004453. The complete genome sequences of BaAdV-1, BaAdV-2, BaAdV-3, and BaAdV-4 (genetically identical to BaAdV-2) have been submitted to GenBank (accession numbers KC693021 to KC693024). Deep sequencing reads have been submitted to the NCBI Sequence Read Archive (accession number SRA067619).

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