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ORIGINAL ARTICLE

A novel human adenovirus hexon protein of species D found in an AIDS patient

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Abstract To date, human adenoviruses are classified into 53 types (types 1–51 and types 53 and 54), which have been grouped into six species named A through F, and the recently identified type 52 has been proposed as member of a new species, G. Type classification is based on type-specific epitopes within loop 1 (L1) and loop 2 (L2) of the hexon protein, which contain seven hypervariable regions that are responsible for type specificity. In this paper, we present the characterization of an adenovirus strain isolated from a male AIDS patient in Cordoba, Argentina. This strain was found to be a member of species D by genomic *Sma* I restriction analysis. Sequencing of the L1 and L2 regions of the hexon gene and immunological characterization by virus neutralization revealed this hexon to be unique and distinct from the previously identified hexons of types within species D. A seroepidemiologic study in the human population of Cordoba showed that this strain was not endemic in the local human population.

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Introduction

Adenoviruses have nonenveloped virions with icosahedral symmetry and a double-stranded DNA genome of approximately 36 kbp. The capsid is composed of three proteins and contains 252 capsomers: 12 vertex capsomers composed of a penton base, from which projects a fiber attachment protein, and 240 hexon capsomers. Each hexon capsomer is a homotrimer of the hexon protein, the most abundant constituent of the virion [27, 32].

There are 53 types of human adenovirus (types 1–51 and types 53 and 54), which have been grouped into six species named A through F; and a recently identified novel type, type 52, has been proposed to comprise a new species, G [6, 19–21, 29, 34, 35]. Type classification is based on type-specific epitopes within loop 1 (L1) and loop 2 (L2) of the hexon protein, which contain seven hypervariable regions that are responsible for type specificity. Adenovirus type identification can be carried out by viral neutralization (VN), genomic DNA restriction enzyme analysis (DREA), and PCR amplification and sequencing of the L1 and L2 coding regions in the hexon gene. In addition to the major type-specific determinants within the L1 and L2 regions, adenoviruses contain a minor type-specific antigenic determinant on the fiber knob [9, 23].

Adenoviruses are recognized as the etiologic agents of a wide spectrum of infectious syndromes in humans. Adenovirus infections in the immunocompetent host are usually limited by the immune system. In contrast, in the immunocompromised host, adenoviruses take advantage of the immune deficiency to establish persistent infections, which are often disseminated, and potentially life-threatening. Types involved in these patients are generally characteristic of the nature of the immunosuppression, e.g. transplant recipients are affected principally by serotypes

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of species B and C. In contrast, the species and types involved in infections in AIDS patients are quite diverse and characteristic of the site of isolation. In stools, species D serotypes are predominant and may be excreted for weeks or months after initial infection [3, 8, 16]. Strikingly, the species D isolates from stools of AIDS patients represent new types and atypical or intermediate strains [6, 17, 19, 29]. Indeed, nine out of the last ten recognized types (types 43–50 in species D and type 51 in species B) were first isolated from stool samples from AIDS patients since the mid-1980s, indicating an association of the emergence of adenovirus serotypes with concurrent HIV infection [6, 19, 29]. Regarding that notion, it has been suggested that the long-term adenovirus infection seen in AIDS patients and the potential for coinfection by more than one type could provide the opportunity for mutations within a type or for recombination between coinfecting types. However, other biological factors, such as disease manifestation, susceptibility and transmission have also been suggested as the cause of this emergence [4]. Despite these hypotheses, there is not a clear explanation for the emergence of novel adenovirus types in AIDS patients.

With the continuing and increasing HIV pandemic, it has been speculated that more new adenovirus types will be detected among immunocompromised AIDS patients [6]. In this paper, we describe an adenovirus strain of species D named in our laboratory as Ad-Cor-96-487, which was isolated from a stool sample of a male AIDS patient. From results obtained from the genomic and immunologic characterization (L1 and L2 sequencing, DREA and VN), the hexon of Ad-Cor-96-487 strain was found to be unique and distinct from the previously identified hexons of types within species D. A seroepidemiologic study was carried out in order to estimate the circulation of this strain in the local human population.

Materials and methods

Patient and specimen

In February 1996, one stool sample was collected from a 31-year-old male AIDS patient who was hospitalized for treatment of opportunistic infections in a public hospital in Cordoba, Argentina. This sample was sent to our laboratory to be included in a study of enteric viral prevalence in people infected with HIV [14].

Adenovirus detection in stool sample and isolation

Adenovirus was detected in the stool sample by Adenolex (Orion diagnostica, Finland), a latex agglutination test designed to detect human adenoviruses [15]. An adenovirus was isolated following inoculation of the stool sample into Hep-2 and Graham 293 cells. Both cell lines were subcultured and maintained in Earle's MEM supplemented with 10% of fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (200 U/ml), streptomycin (200 µg/ml), and Fungizone (1 µg/ml). For inoculation, the same medium without FBS was used. The adenovirus strain grew well in both cell lines, yielding crude cell lysates with infectious virus titers up to 10^3 50% tissue culture infective dose per ml ($TCID_{50}$/ml) in Hep-2 cells and up to 10^5 $TCID_{50}$/ml in Graham 293 cells. The isolated strain was named Ad-Cor-96-487.

Genome purification and DNA restriction enzyme analysis (DREA)

Confluent monolayers of Graham 293 cells inoculated with the adenovirus-positive stool sample were observed for cytopathic changes, and when extensive cytopathic effect was evident, viral DNA was extracted and purified by the procedure of Shinagawa et al. [30]. Briefly, after removal of the medium, cells were lysed by the addition of 2 ml of a solution containing 1% SDS, 10 mM Tris–HCl, 10 mM EDTA, pH 7.5. High-molecular-weight (cellular) DNA was then precipitated by the addition of 0.5 ml of 5 M NaCl. Viral DNA in the supernatant was extracted with buffer–saturated phenol and precipitated with 2 volumes of cold ethanol. The viral DNA was resuspended in deionized water, treated with proteinase K (250 µg/ml), extracted with buffer–saturated phenol and precipitated with 2 volumes of cold ethanol and 0.1 volume of 3 M sodium acetate. Cellular RNA was removed by RNase treatment (RNase One, Promega) and the viral DNA was precipitated and stored at −20°C until use. For adenovirus identification by DREA (DNA restriction enzyme analysis), *Sma*I restriction enzyme (Promega) was utilized according to the manufacturer's protocol. Digestion fragments were resolved using a 5% polyacrylamide gel and detected using silver stain. The whole-genome restriction pattern obtained for the Ad-Cor-96-487 strain was compared with published *Sma* I restriction patterns for Ad1 to Ad51 prototype strains.

PCR amplification of hexon loops 1 and 2

The region of the hexon gene encoding loops 1 and 2 was amplified by a nested PCR described by Takeuchi et al. [31]. The first set of primers included HX5-1 (forward primer), 5′-AAGATGGCCACCCCCTCGATGATGCCG CAGT-3′, and HX3-1 (reverse primer), 5′-CACTTATGT GGTGGCGTTGCCGGCCGAGAACGG-3′. The reaction was done in a total volume of 20 µl containing 10× PCR buffer, 400 mM of each deoxynucleoside triphosphate

(dATP, dGTP, dCTP, and dTTP), 0.2 uM of each primer, 0.6 μl of 50 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase (Invitrogen). The cycling parameters consisted of a total of 40 cycles of denaturing at 98°C for 10 s followed by annealing and extension at 65°C for 6 min. The second PCR primer set included HX5-3 (forward primer), 5′-CACATCGCCGGACAGGATGCTTCGGAGTA-3′, and HX3-4 (reverse primer), 5′-GTGTTGTGAGCCATGGG GAAGAAGGTGGC-3′. The reaction was done in a total volume of 20 μl containing 10× PCR Buffer, 200 mM of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), 0.2 μM of each primer, 0.6 μl of 50 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase (Invitrogen). The cycling parameters consisted of a total of 40 cycles of denaturing at 94°C for 1 min, annealing at 40°C for 1 min, and extension at 72°C for 2 min. After each PCR reaction, a 10-μl aliquot was subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide, and the bands (2.8 kpb for the first step and 1.8 kpb for the second step) were visualized using a UV transilluminator.

PCR amplification of the fiber knob region

The region of the fiber gene encoding the fiber knob was amplified in a one-step PCR described by Madisch et al. [23]. The primers used were as follows: FiDL (forward primer), 5′-ATGTCTCACTCAAGGTGGGA-3′, and ADD2 (reverse primer), 5′-GCTGGTGTAAAAATCAATAAA GA-3′. The reaction was done in a total volume of 100 μl containing 10× PCR buffer, 10 μM of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), 0.1 μM of each primer, 3 μl of 50 mM $MgCl_2$ and 5 U of *Taq* DNA polymerase (Invitrogen). The cycling parameters consisted of a total of 40 cycles of denaturing at 94°C for 2 min, annealing at 56°C for 20 s, and extension at 72°C for 1 min. After PCR, 10 μl of each reaction mixture was subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide, and the band (1.0 kpb) was visualized using a UV transilluminator.

DNA sequencing

PCR fragments were generated three times independently and sequenced in order to eliminate sequencing errors. The PCR products were purified and sequenced by Macrogen Inc (Seoul, Korea) using an ABI3730 XL DNA Analyser.

Sequence and phylogenetics analysis

Sequences from the loop 1, loop 2 and fiber knob regions were compared by sequential pairwase alignment using the Clustal algorithm in the BioEdit software package. Phylogenetic relationships were inferred from the aligned nucleic acid sequences by the neighbor-joining method in the Clustal X program. Support for specific tree topologies was estimated by bootstrap analysis with 1,000 pseudo-replicate data sets. For making multiple alignments of L1, L2 and fiber knob regions, the following GenBank sequences were used: for the hexon gene: HAdV-C1 (X67709), -C2 (AJ293903), -B3 (X76549), -E4 (X84646), -C5 (AF542130), -C6 (X67710), -B7 (Z48571), -D9 (AF161562), -D10 (AB023548), -B11 (AY163756), -A12 (X73487), -B14 (AB018425), -B16 (X74662), -D17 (AF108105), -D19 (AF161565), -B21 (AJ012091), -B34 (AB052911), -D23 (AB023552), -D26 (AB023554), -A31 (X74661), -B35 (AB052912), -D37 (AB023555), -F40 (X51782), -F41 (X51783), -D45 (AB023556), -D46 (AB023557), -D47 (AB023558), and -D48 (U20821), -D53 (FJ169625), -D54 (AB333801) and -G52 (DQ923122); for the L1 region:-B14 (AB018425), -B50 (AJ864518), -D13 (AJ749847), -D15 (AJ821891), -D20 (AJ749848), -D25 (AJ749849), -D27 (AJ749850), -D28 (AJ749851), -D29 (AJ749852), -D30 (AJ749853), -D32 (AJ749854), -D33 (AJ749855), -D36 (AJ749856), -D38 (AJ749857), -D39 (AJ749858), -D42 (AJ821893), -D43 (AJ821894), -D44 (AJ749859), -D49 (AJ821895), -D51 (AJ821896) and Ad-Cor-96-487 strain (GU012021). For the L2 region: HAdV-A18 (AJ821897), -B14 (AJ749860), -B50 (AJ749861), -D13 (AJ745880), -D15 (AJ745881), -D20 (AJ745882), -D22 (AJ745883), -D25 (AJ745884), -D27 (AJ745885), -D28 (AJ745886), -D29 (AJ745887), -D30 (AJ745888), -D32 (AJ745889), -D33 (AJ745890), -D36 (AJ745891), -D37 (AJ745892), -D38 (AJ745893), -D39 (AJ745894), -D42 (AJ745895), -D43 (AJ745896), -D44 (AJ745897), -D49 (AJ745898), -D51 (AJ745899) and Ad-Cor-96-487 strain (GU012021). For the fiber-knob-encoding region, the following sequences were employed: HAdV-C1 (AB108423), -C2 (J01917), -B3 (X01998), -C5 (M18369), -C6 (AB108424), -B7 (AF104384), -D8 (AB162768), -D9 (X74659), -B11 (AY163756), -A12 (X73487), -B14 (AB065116), -D15 (X72934), -B16 (U06106), -D17 (Y14241), -D19 (U69131), -D28 (Y14242), -B21 (U06107), -A31 (X76548), -B34 (AB073168), -B35 (U32664), D37 (U69132), -E4 (X76547), -F40 (M28822), -F41 (M60327) and fiber knob sequences of HAdV-A18 (AJ841699), -B50 (AJ811465), -D10 (AJ811442), -D13 (AJ811443), -D20 (AJ811444), -D22 (AJ811445), -D23 (AJ811446), -D24 (AJ811447), -D25 (AJ811448), -D26 (AJ811449), -D27 (AJ811450), -D29 (AJ811451), -D30 (AJ831473), -D32 (AJ811452), -D33 (AJ811453), -D36 (AJ811454), -D38 (AJ811455), -D39 (AJ811456), -D42 (AJ811457), -D43 (AJ811458), -D44 (AJ811459), -D45 (AJ811460), -D46 (AJ811461), -D47 (AJ811462), -D48 (AJ811463), -D49 (AJ811464), -D51 (AJ811460) and Ad-Cor-96-487 strain (GU012020).

Neutralization assays for antigenic analysis

The Ad-Cor-96-487 strain was investigated serologically by viral neutralization assay (VN) using polyclonal antisera directed against prototype strains of adenoviruses 8, 9, 10, 13, 15, 17, 29, 33, 43, 44, 45, 46 and 47. VN tests were conducted on Hep-2 cells grown in 96-well microplates. Type-specific antisera were inactivated at 56°C for 30 min and serially diluted twofold, 50 µl per well with four replicate wells per dilution. A working dilution of virus (Ad-Cor-96-487 strain) containing 100 $TCID_{50}$ in 50 µl was added to each well, and the plates were incubated at 37°C in 5% CO_2 for 1 h. During the incubation period, Hep-2 cells were trypsinized and resuspended at 5×10^4 cells per ml. After the incubation, 100 µl of cell suspension was added to each well. After mixing the contents of each well, the solutions in wells were mixed, and the plates were incubated at 37°C in 5% CO_2 for 6 days. After 6 days, the medium was removed and cells were stained with crystal violet solution (1.46 g crystal violet, 50 ml ethanol, 300 ml formaldehyde, 650 ml distilled water). The neutralization titer was calculated as the maximum dilution of antiserum that completely inhibited viral replication.

Serologic population study

A seroprevalence study of the Ad-Cor-96-487 strain was carried out by VN as described above using sera collected from the Cordoba population. Serum samples were obtained from 244 individuals: 80 children (5–11 years old; median age, 7.3 years), 44 adults from the general population (19–46 years old; median age, 27.5 years) and 120 HIV seropositive adults (25–47 years old; median age, 32.7 years).

Results

Genomic restriction enzyme analysis

DREA of the Ad-Cor-96-487 strain using *Sma* I revealed a pattern of 15 bands that are characteristic of species D adenoviruses, confirming its classification into this species (Fig. 1). The restriction pattern obtained was distinct from those of prototype strains of all human adenoviruses of species D (data not shown).

Nucleotide sequence analysis of the hexon and fiber knob

The L1, L2 and fiber knob sequences of the Ad-Cor-96-487 strain were compared with the corresponding sequences of

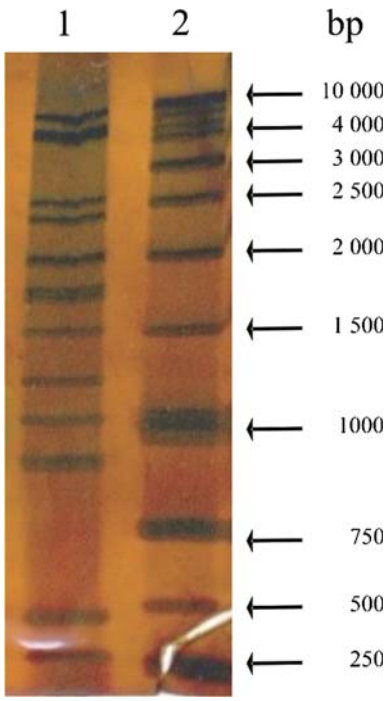

Fig. 1 *Sma*I restriction pattern of adenovirus full−length genome. *Lane 1*, Ad-Cor-96-487 strain. *Lane 2*, molecular size marker, 1-kb DNA ladder

prototype strains of types of species D adenoviruses (Table 1). In the hexon sequences, the highest homology was with serotype 33: 87.3 and 78.7% for loop 1 and 2, respectively. The nucleotide divergence between the Ad-Cor-96-487 strain and the type 33 prototype strain was also mirrored in the amino acid sequence, with homologies of 82.6 and 60% for L1 and L2 (data not shown). It must be pointed out that the amino acid divergence was displayed along the full length of the coding regions analyzed. The second-highest homology in L1 was with type 38 (79.7%), which was significantly lower than the homology between Ad-Cor-96-487 and the type 33 prototype strain. In L2 the second-highest homology was with types 15, 30 and 49 in loop 2 (71.7%), which is also lower than the homology with the type 33 prototype strain in this region (Table 1). On the other hand, the Ad-Cor-96-487 fiber knob sequence had the highest homology with the type 29 prototype strain (98.2%) (Table 2).

Phylogenetics analysis

Phylogenetic analysis based on the hexon loops showed segregation of the Ad-Cor-96-487 strain into species D. The Ad-Cor-96-487 strain is most closely related to type 33 (Figs. 2, 3). However, the distance between Ad-Cor-96-487 and the type 33 prototype is characteristic of distances between other species D types, and the phylogenetic

Table 1 Percent nucleic acid identity of loops 1 and 2 of the Ad-Cor-96-487 strain compared to all known types of species D adenoviruses

Type	8	9	10	13	15	17	19	20	22	23	24	25	26	27	28	29	30
Loop 1	74.2	73.6	71.5	72.8	76.3	67.1	74.6	75.6	75.7	72.4	77.8	73.3	73.8	73.7	76.7	77.0	76.6
Loop 2	66.5	69.7	71.5	66.2	**71.7**	59.1	70.4	68.0	69.4	69.7	69.9	69.9	68.8	68.7	66.8	**72.0**	**71.7**
Type	32	33	36	37	38	39	42	43	44	45	46	47	48	49	50	53	54
Loop 1	75.4	**87.3**	77.8	73.5	**79.7**	76.4	78.0	78.1	75.0	73.3	79.0	75.0	76.7	74.9	77.7	72.7	72.0
Loop 2	70.9	**78.7**	68.4	67.1	69.6	67.2	71.2	65.9	71.0	68.5	66.8	67.5	68.5	**71.7**	69.8	68.6	67.7

Highest identities are marked in bold

Table 2 Percent nucleic acid identity of the fiber knob of the Ad-Cor-96-487 strain compared to all known types of species D adenoviruses

Type	8	9	10	13	15	17	19	20	22	23	24	25	26	27	28	29	30
Fiber knob	66.7	67.9	68.7	58.9	61.2	69.6	68.2	66.7	62.0	67.5	59.4	86.7	67.5	66.0	65.2	**98.2**	57.6
Type	32	33	36	37	38	39	42	43	44	45	46	47	48	49	50	53	54
Fiber knob	66.3	66.3	64.2	68.2	64.9	65.9	61.7	66.2	63.2	65.6	66.3	66.6	63.3	64.9	63.7	67.1	67.4

Highest identities are marked in bold

distance between type 33 and Ad-Cor-96-487 in the L2 region is greater than that between any other established adenovirus prototype strains.

Viral neutralization

Since the Ad-Cor-96-487 strain shared the highest homology with the type 33 prototype virus, viral neutralization assays were conducted to examine the antigenic relationship between these strains. Antiserum specific for type 33 that neutralized the homologous type 33 strain with a reciprocal titer of 512 did not react with the Ad-Cor-96-487 (titer <8), demonstrating that these strains were antigenically distinct. Antisera against other species D types (types 8, 9, 10, 13, 15, 17, 29, 43, 44, 45, 46 and 47) available in our laboratory were also tested against the Ad-Cor-96-487 strain. Antisera specific for types 8, 9, 10, 13, 15, 17, 43, 45 and 47 did not neutralize the Ad-Cor-96-487 strain (neutralizing titers <8). On the other hand, antisera specific for types 44 and 46 neutralized the Ad-Cor-96-487 strain with a low titer (titer of 8), while antiserum specific for type 29 neutralized the Ad-Cor-96-487 strain with a titer of 32. However, the homologous titer of prototype adenovirus type 29 was 512.

Seroprevalence study

Sera obtained from children and seronegative and seropositive HIV adults were assayed for specific antibody to Ad-Cor-96-487. As a control, the sera were assayed in parallel for antibody specific for adenovirus type 5, which is endemic worldwide. All sera were negative for antibody specific for Ad-Cor-96-487; however, antibody specific for type 5 was prevalent in all three populations (Table 3).

Discussion

Adenovirus type classification is based on neutralization epitopes on the surface of virion, and it has been shown that hypervariable regions encoded by L1 and L2 of the hexon gene are important contributors to these epitopes [5, 12]. Thus, the diversity of L1 and L2 nucleotide sequences determines the diversity of adenovirus types. Historically, adenovirus types were classified by viral neutralization assay using reference antisera, but with the development of molecular biology techniques, the use of comparisons with prototype sequences has been adopted for adenovirus typing [24, 26, 28, 31, 33, 37], and recently, phylogenetic analysis was incorporated by the International Committee on Taxonomy of Viruses as a criterion for type definition [2]. Therefore, the use of phylogenetic distances of the L1 and L2 regions for classification and identification of adenovirus types is the method of choice [7, 23]. A criterion for typing using L1 and L2 sequencing was recently proposed by Madisch et al. [23] in which a nucleotide divergence of <2.5 and <2.4% from L1 and L2 prototype sequences, respectively, is needed for precise typing. Moreover, these authors demonstrated that a wild strain with a divergence in the L2 amino acid sequence ≥1.2% to the nearest heterologous type was a candidate for classification as a new type. In this paper, we described molecular characteristics of the Ad-Cor-96-487 strain, which shows a high nucleotide divergence in the L1 and

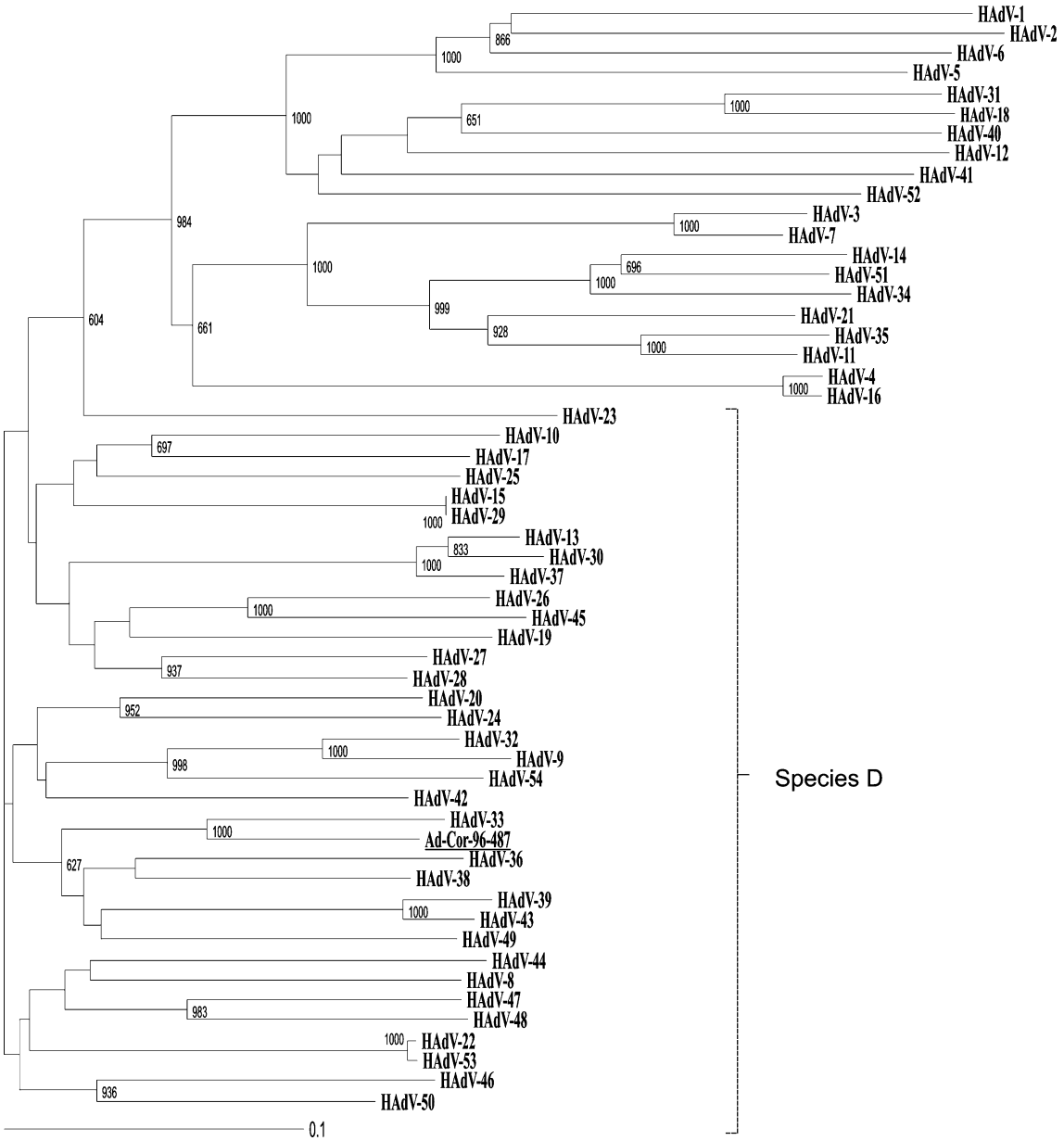

Fig. 2 Phylogenetic analysis of the nucleic acid sequences of Ad-Cor-96-487 strain L1 hexon together with those the 54 prototype strains of human adenoviruses. The tree was generated by the neighbor–joining method. Prototype strains representative of each serotype are identified at the ends of the branches. The Ad-Cor-96-487 strain is underlined to show its position in the species D cluster. Bootstrap confidence levels (1,000 replicates) are shown at the *branch nodes*. The *scale bar* indicates the branch length, expressed as the expected number of substitutions per site

L2 regions from all currently classified human adenovirus types. This strain belongs to species D, as determined by *Sma*I DREA, but its pattern was distinct from those of prototype species D types. Moreover, phylogenetic trees of both L1 and L2 confirmed that the Ad-Cor-96-487 clustered with adenovirus species D prototype strains. Nucleotide sequence homology with the nearest species D type (HAdV-33) was 87.3 and 78.7% for loops 1 and 2, respectively. In amino acid sequence, the divergence with the HAdV-33 was also high; 17.4 and 40% for loops 1 and 2, respectively. Therefore, using molecular criteria, the Ad-Cor-96-487 strain could not be typed and moreover showed a divergence from the species D prototype sequences sufficient to suggest that strain Ad-Cor-96-487 could be a new type. In comparison, two recently described types, HAdV-50 and 51, have 16 and 20% nucleic acid sequence divergence in the L2 region from the nearest heterologous type (HAdV-34 for HAdV-50 and HAdV-15 and 19 for

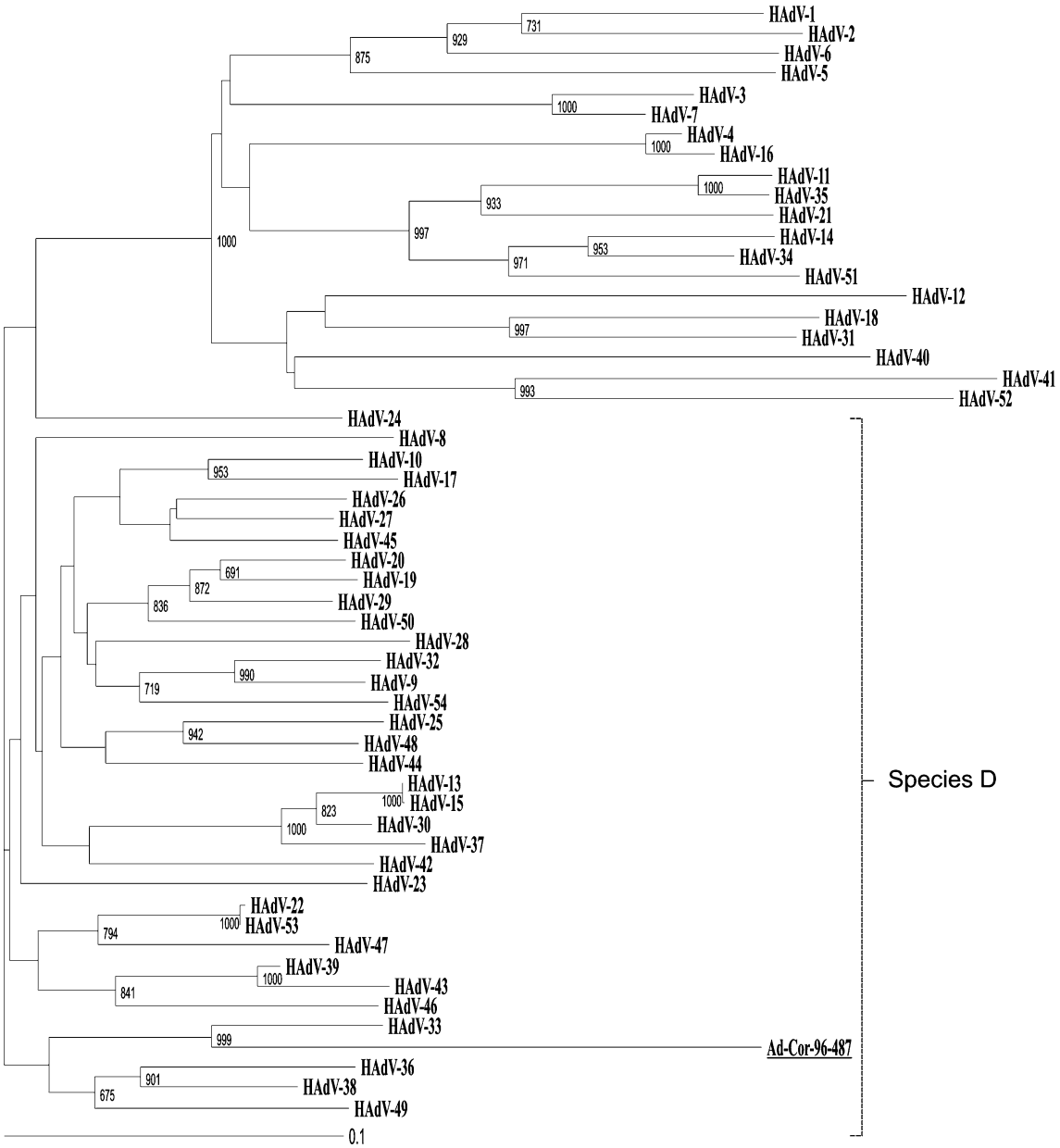

Fig. 3 Phylogenetic analysis of the nucleic acid sequence of the Ad-Cor-96-487 strain L2 hexon together with those the 54 prototype strains of human adenoviruses. The tree was generated by the neighbor−joining method. Prototype strains representative of each serotype are identified at the ends of the branches. The Ad-Cor-96-487 strain is underlined to show its position in the species D cluster. Bootstrap confidence levels (1,000 replicates) are shown at the *branch nodes*. The *scale bar* indicates the branch length, expressed as the expected number of substitutions per site

Table 3 Seroprevalence of the Ad-Cor-96-487 strain in groups of seronegative and seropositive individuals

Group	*n*	Median age (range)	Type 5 prototype strain	Ad-Cor-96-487 strain
Children HIV−	80	7.3 (5–11)	58.7% (47/80)	0% (0/80)
Adults HIV−	44	27.5 (19–46)	54.5% (24/44)	0% (0/44)
Adults HIV+	120	32.7 (25–47)	32.5% (39/120)	0% (0/120)

HIV + HIV seropositive, *HIV*− HIV seronegative, *n* number of individuals in the group

HAdV-51) [23]. We also compared the L1 and L2 hexon sequences of Ad-Cor-96-487 strain with non-human adenovirus sequences available in GenBank (data not shown). In this match, we found a lack of significant nucleotide sequence identity with any previously published non-human adenoviruses, so the Ad-Cor-96-487 strain is not an animal adenovirus.

In agreement with direct nucleotide sequence comparisons, phylogenetic distance analysis supported the conclusion that the hexon protein of the Ad-Cor-96-487 strain

is distinct from all human hexon proteins previously reported, revealing that the L2 sequence of the Ad-Cor-96-487 strain is the most divergent among the types within species D. The phylogeny of the Ad-Cor-487-96 strain sequences indicated a monophyletic origin with type 33. In regard to this monophyletic origin, it could be suspected that the Ad-Cor-96-487 strain is a variant of type 33. In order to determine if this was the case, we performed virus neutralization assays with specific antisera against type 33. The result obtained (no neutralization) showed that strain Ad-Cor-96-487 is not a variant of type 33. Therefore, based on the hexon sequence data, DREA and neutralization test results, we conclude that the Ad-Cor-96-487 strain corresponds to a novel human adenovirus hexon protein of species D that might represent a new type of species D human adenovirus.

Besides L1 and L2 of the hexon, the fiber knob possesses type-specific antigenic determinants [9]. From a type-identification perspective, fiber knob sequencing is clearly secondary to L1 and L2 sequencing, but it is important for predicting the immunogenic properties of an isolated wild strain. As determined by sequencing, the Ad-Cor-96-487 strain possesses a knob fiber region with closest identity (98.2%) to the corresponding region of HAdV-29. This result suggests that a homologous recombination event involving a HAdV-29 and a previously unknown adenovirus type, occurred in the history of the Ad-Cor-96-487 strain. In this regard, it is important to point out that identical or closely related sequences of the fiber knob to known types are not contradictory to the identification of a new type, since other adenovirus types have almost identical fiber knob regions, for example, HAdV-44 and HAdV-48 or HAdV-19 and 37.

In an attempt to characterize the immunogenic properties of the Ad-Cor-96-487 strain more extensively, we performed VN assays on this strain using polyclonal reference antisera specific for representative types of species D adenoviruses. In these assays, most of the reference antisera failed to neutralize 100 $TCID_{50}$ of the Ad-Cor-96-487 strain (antisera specific for types 8, 9, 10, 13, 15, 17, 43, 45 and 47), demonstrating no antigenic relationship between the Ad-Cor-96-487 strain and these types. On the other hand, very weak reactivities were observed with reference antisera to types 44, 48 and 29. In this regard, weak cross-reactivities have been observed between types that are members of the same species [18, 36]. Ad-Cor-96-487 strain neutralization by type 29 antiserum could also be explained by the fact that the knob fiber is closely related to that of the type 29 fiber in sequence. It has been shown that antifiber antibodies can neutralize viruses with a homologous fiber sequence in vitro [13]. It should be pointed out that our study had the limitation that the Ad-Cor-96-487 strain was not tested against a full panel of hyperimmune sera to all known types. Despite this limitation, the low nucleotide sequence identity between Ad-Cor-96-487 and all of the serotypes in species D, it seems unlikely that this strain belongs to a previously recognized adenovirus type. This notion is supported by recent reports in which adenovirus typing is based primarily on DNA sequencing and phylogenetic analysis. [22, 23, 35].

Out of 54 human adenovirus types recognized to date, 34 of them belong to species D. Some members of this species are common worldwide and have been implicated as causative agents of respiratory, conjunctival and keratoconjunctivital illness, e.g., HAdV-8, HAdV-9, HAdV-19, HAdV-37 [1, 10, 25]. In contrast, most other human adenovirus types, especially the AIDS-related types (types 43-51), are completely uncharacterized in terms of pathogenicity and epidemiology. Consequently, we considered it important to study the possible circulation of the Ad-Cor-96-487 strain, which bears a novel antigenic hexon protein, in the local human population. For this purpose we conducted a seroprevalence survey for Ad-Cor-96-487 strain-specific antibodies in sera obtained from healthy children and HIV-seropositive and seronegative adults. Of a total of 244 individuals investigated, none of them showed the presence of Ad-Cor-96-487 strain-specific antibodies as assayed by virus neutralization. This result suggests that Ad-Cor-96-487 circulates only in a limited population or that specific immunity against Ad-Cor-96-487 generated during infection is too low to be detected by a neutralization assay. It is known that virus neutralization assays are limited by sensitivity; therefore, a more sensitive method for detection of antibodies to the Ad-Cor-96-487 strain is likely needed. However, it can be inferred that the Ad-Cor-96-487 strain is not endemic in the local population, since it is estimated that seroprevalence to the endemic types ranges from 25 to 82%, while, on the other hand, seroprevalence to types involved in sporadic outbreaks ranged from 0 to 6% [11].

In conclusion, we present in this paper an adenovirus strain that possesses a hexon protein that is divergent from the hexon proteins of the rest of the known human adenovirus types. This could be consistent with the isolation of a new type of human adenovirus. The recent identification of this novel adenovirus hexon protein and several other types, particularly from AIDS patients, underscores the importance of understanding adenovirus diversity and shows that adenoviruses are in constant evolution.

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