JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2007, p. 958–967 Vol. 45, No. 3
0095-1137/07/$08.00+0 doi:10.1128/JCM.01603-06

Quantitative Detection and Rapid Identification of Human Adenoviruses▽

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Received 3 August 2006/Returned for modification 24 September 2006/Accepted 23 December 2006

We have established a method of quantitative detection and rapid identification of human adenoviruses (hAdVs). Using LightCycler PCR with a primer set, we were able to amplify 554 bp of the hexon gene from each of 51 prototype strains of hAdVs. The sensitivity of LightCycler PCR was 10 copies of hAdV DNA/reaction. When LightCycler PCR was performed using a set of primers, hAdV was positive for 74.4% (99 of 133) of conjunctivitis patients and for 27.3% (81 of 297) of respiratory infection patients. We also attempted to measure hAdV in the potentially contaminated eye drops used by patients, detecting 5.4×10^2 to 1.6×10^6 copies/ml of hAdV. We determined the 350-bp nucleotide sequence of the amplified hexon gene and compared it with the sequences of the 51 prototype strains. Phylogenetic analysis based on 350 bp of the hexon gene identified 99 positive conjunctival swabs as 24 cases of AdV type 3 (AdV-3), 14 cases of AdV-4, 1 case of AdV-8, 19 cases of AdV-19a, and 41 cases of AdV-37. The 81 sequences from pharyngeal or nasal mucus swabs were identified as 29 cases of AdV-2, 18 cases of AdV-1, 18 cases of AdV-5, 12 cases of AdV-4, 2 cases of AdV-37, 1 case of AdV-3, and 1 case of AdV-6. LightCycler PCR followed by phylogenetic analysis provides an effective tool for the rapid identification of hAdVs and for studying molecular epidemiology.

Human adenoviruses (hAdVs) of the genus *Mastadenovirus* of the family *Adenoviridae* infect billions of people worldwide and cause various diseases such as conjunctivitis, respiratory infectious disease, diarrhea in infants and young children, hemorrhagic cystitis, etc. (1, 8, 33, 39, 40). Most of these diseases heal naturally, but sometimes the infection may also provoke serious illnesses such as pneumonia caused by AdV type 7 (AdV-7) or epidemic keratoconjunctivitis (EKC) due to AdV-8, -19a, or -37. These more serious outcomes occur in all age groups and can possibly trigger highly contagious nosocomial infections (5, 6, 19, 27, 42). Therefore, it is important to establish a rapid method of virological diagnosis. Furthermore, hAdV infection in immunosuppressed patients, such as graft recipients and immunodeficient patients including those with AIDS, has been a major problem in recent years and has been lethal in many cases (7, 9, 10, 17, 23, 40, 41). Nosocomial infection is also a serious problem which may require restriction of hospitalization and closing of hospital wards (15). Therefore, it is essential to monitor these viruses, and a rapid method of identifying serotypes is urgently needed.

hAdVs were initially grouped into six subgenera (A to F) on the basis of several biochemical and biophysical criteria (1, 39). In 1999, reclassification on the basis of nucleotide and deduced amino acid sequences was approved by the International Committee on Taxonomy of Viruses; under this reclassification, the 51 serotypes of hAdVs in the genus *Mastadenovirus* were grouped into six species, hAdV-A to hAdV-F (38). Virus isolation followed by a neutralization test has been the standard method of serotyping (39); however, these procedures are complicated and time-consuming, and the standardized antisera are in limited supply. Recent advances in amplifying hAdV genes and decoding nucleotide sequences have allowed us to develop a PCR-restriction fragment length polymorphism method with which we have succeeded in distinguishing 14 hAdVs including AdV-3, -4, -8, -19a, and -37, all of which cause eye infections in humans (31). Furthermore, nucleotide sequence analysis of the partial hexon genes (916 bp) of all 33 prototype strains in hAdV-D and hAdV-E allowed us to construct a database for the phylogeny-based identification of hAdVs from patients with conjunctivitis (34). However, this method requires several overlapping sequences to determine 916 bp of partial hexon genes and is limited to only hAdV-D and hAdV-E. Although several adenovirus DNA quantification methods have been developed in recent years (14, 16, 24), to the best of our knowledge, no method for the quantitative detection and identification of serotypes has yet been reported.

In the present study, we developed the LightCycler PCR for the quantitative detection and rapid phylogeny-based identification of hAdVs. We quantitatively amplified 554 bp of the hexon gene in specimens from conjunctivitis patients and from patients with acute upper respiratory tract infections and determined the 350-bp amplicon. A database comprising the 350-bp hexon genes of all 51 prototype strains of hAdV was compiled with the previously constructed 916-bp hexon gene database (30) and was used for the phylogeny-based rapid identification of hAdV serotypes.

MATERIALS AND METHODS

Virus. We obtained 51 prototype strains of hAdV from the American Type Culture Collection and the National Institute of Infectious Diseases (Tokyo,

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▽ Published ahead of print on 17 January 2007.

TABLE 1. Evaluation of the diagnostics method based on phylogenetic analysis to identify AdV serotypes

Isolate/yr	Typing by neutralization test	Typing by phylogeny			Highest-scoring heterologous prototype		GenBank accession no.
		Type	Bootstrap (%)	Identity (%)	Type(s)	Identity (%)	
TC-4822/1984	AdV-4	AdV-4	100	96.0	AdV-25, AdV-32, AdV-37, AdV-49	89.7	AB098598
TC-18040/1991	AdV-4	AdV-4	100	96.0	AdV-25, AdV-32, AdV-37, AdV-49	89.7	AB098599
4439/1998	AdV-4	AdV-4	100	96.0	AdV-25, AdV-32, AdV-37, AdV-49	89.7	AB098601
TC-7223/1986	AdV-8	AdV-8	100	99.7	AdV-29	95.1	AB099381
TC-6821/1986	AdV-8	AdV-8	100	99.4	AdV-29	94.9	AB099382
TC-26218/1996	AdV-8	AdV-8	100	99.4	AdV-29	94.9	AB099383
5363/1996	AdV-19	AdV-19a	100	100.0	AdV-25, AdV-26, AdV-38, AdV-49	97.4	AB099384
5464/1996	AdV-19	AdV-19a	100	100.0	AdV-25, AdV-26, AdV-38, AdV-49	97.4	AB099385
00402/1996	AdV-37	AdV-37	95	100.0	AdV-25	98.9	AB099386
20020/1992	AdV-37	AdV-37	95	100.0	AdV-25	98.9	AB099387
4056/1999	AdV-37	AdV-37	95	100.0	AdV-25	98.9	AB099388
MS4468/1991	AdV-1	AdV-1	100	98.6	AdV-5	90.6	AB259823[a]
MS6675/1997	AdV-1	AdV-1	100	100.0	AdV-5	90.9	AB259824[a]
MS7565/1999	AdV-1	AdV-1	100	98.6	AdV-5	90.6	AB259825[a]
MS6964/1997	AdV-2	AdV-2	85	100.0	AdV-6	98.9	AB259826[a]
MS5755/1995	AdV-3	AdV-3	91	98.6	AdV-7	97.1	AB259827[a]
MS6661/1997	AdV-3	AdV-3	91	98.6	AdV-7	97.1	AB259828[a]
MS6746/1997	AdV-3	AdV-3	91	98.6	AdV-7	97.1	AB259829[a]
MS6976/1997	AdV-3	AdV-3	91	98.6	AdV-7	97.1	AB259830[a]
MS7587/1999	AdV-3	AdV-3	91	98.6	AdV-7	97.1	AB259831[a]
KNIH Ad99/5[b]		AdV-5	100	100.0	AdV-1	90.9	AF542109
KNIH Ad99/12[b]		AdV-5	100	100.0	AdV-1	90.9	AF542116
KNIH Ad00/5[b]		AdV-5	100	100.0	AdV-1	90.9	AF542121
KNIH Ad00/12[b]		AdV-5	100	100.0	AdV-1	90.9	AF542124
KNIH Ad00/19[b]		AdV-5	100	100.0	AdV-1	90.9	AF542128
KNIH Ad01/1[b]		AdV-5	100	100.0	AdV-1	90.9	AF542130
NHRC Ad5FS 7151[b]		AdV-5	100	100.0	AdV-1	90.9	AY601635
Ton66[b]		AdV-6	96	100.0	AdV-2	98.9	Y17245

[a] Nucleotide sequence was determined in this study.
[b] The isolate year was unknown.

Japan). The AdV-19a isolate from a patient with EKC was used. These viruses were used directly for DNA extraction without further propagation, as previously described (34). Nine field isolates collected from patients with respiratory infection (three isolates of AdV-1, one isolate of AdV-2, and five isolates of AdV-3), and 11 field isolates collected from EKC patients (three isolates of AdV-4, three isolates of AdV-8, two isolates of AdV-19a, and three isolates of AdV-37) were propagated in either HEp-2 cells or HeLa cells (Table 1). These isolates were identified by a neutralization test with type-specific antisera purchased from Denka Seiken Co., Ltd. (Tokyo, Japan) and the National Institute of Infectious Diseases. These isolates have been well characterized genetically by genome typing (34). We also used seven available nucleotide sequences of AdV-5 and one of AdV-6 from GenBank.

Clinical specimens. We tested 133 swab samples collected from conjunctivitis patients, 3 samples of eye drops used by three patients with conjunctivitis, and 297 samples of pharyngeal swabs or nasal mucus collected from patients with acute upper respiratory tract infection. The conjunctivitis and pharyngeal swabs were dipped into sterilized containers containing 1 ml of physiological saline and then frozen after the cotton buds were discarded. The nasal mucus specimens were also kept frozen in sterilized containers. The eye drops were kept refrigerated.

DNA extraction. Viral DNA was extracted from 100 μl of the viral suspension and the clinical specimens using a previously described method (34). In brief, viral DNA was extracted using a Sumitest EX-R&D Kit (Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) according to the manufacturer's instructions. After a drying step, the extracted DNA was dissolved in 10 μl of 10 mM Tris (pH 8.0)–1 mM EDTA buffer.

Preparation of standard DNA for real-time PCR. The 1,004-bp fragment of the hexon gene was amplified with a pair of primers, AdTU7 (5′-GCCACCTT CTTCCCCATGGC-3′) and AdTU4′ (5′-GTAGCGTTGCCGGCCGAGAA-3′), as previously reported (34). The amplified 1,004-bp fragment of AdV-8 was inserted into pT7 Blue T-Vector (Novagen, Inc., Madison, WI), yielding the plasmid pAd8hxn, which was then amplified and purified with a QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) and dissolved in 10 mM Tris (pH 8.0)–1 mM EDTA buffer. The copy number of the pAd8hxn plasmid was calculated by measuring the optical density at 260 nm and was sorted at −80°C.

Quantitative amplification of hAdV DNA by LightCycler PCR. We compared the partial hexon sequences of 44 prototype hAdV strains and the AdV-19a strain and designed a set of PCR primers, AdnU-S′2 [nucleotides 20743 to 20762; 5′-TTCCCCATGGC(A/T/C/G)CACAA(C/T)AC-3′] and AdnU-A2 [nucleotides 21274 to 21296; 5′-TGCC(T/G)(A/G)CTCAT(A/G)GGCTG(A/G)AA GTT-3′], to amplify the short hexon region. The positions of the primers were numbered according to the complete nucleotide sequence of the AdV-2 strain (GenBank accession no. J01917). LightCycler PCR using the LightCycler-FastStart DNA Master SYBR green I (Roche Diagnostics GmbH, Mannheim, Germany) was performed according to the manufacturer's instructions. The reaction mixture contained 15 pmol of each primer, 4 mM $MgCl_2$, 2 μl of LightCycler-FastStart DNA Master SYBR green I mix, and 5 μl of extracted DNA. Each mixture was increased to a volume of 20 μl using PCR-grade water. PCR was carried out in a LightCycler Quick System 350s (Roche). The PCR protocol was as follows: 95°C for 10 min for initial activation of FastStart *Taq* DNA polymerase and denaturation of template DNA, followed by 45 cycles, each consisting of denaturation at 95°C for 10 s, annealing at 70°C for 10 s, and primer extension at 72°C for 25 s. Five microliters of PCR product was separated on 3% agarose gel, and a DNA fragment of 554 bp was identified by ethidium bromide staining. To quantify the hAdV DNA from the clinical specimens, 10-fold serial dilutions of the plasmid pAd8hxn were amplified simultaneously with LightCycler PCR. The number of copies of hAdV DNA in the clinical samples was calculated by a standard curve using pAd8hxn.

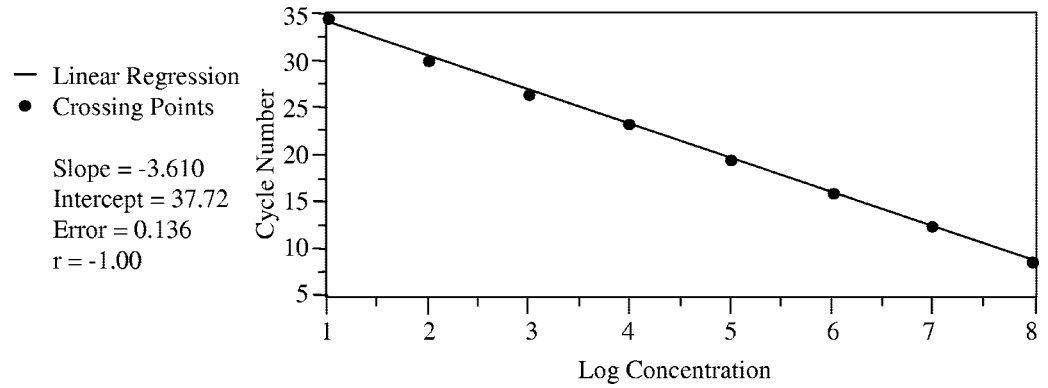

FIG. 1. A standard curve plot of the 10-fold serial dilution of pAd8hxn. Linearity is observed throughout the range from 10^1 to 10^8 copies/reaction.

Sequence analysis. The PCR products were separated on 3% agarose gel and purified using a QIAquick gel extraction kit (QIAGEN). The nucleotide sequences were determined by a 373A DNA autosequencer (PE-Applied Biosystems, Foster City, CA) with fluorescent dideoxy chain terminators (PE-Applied Biosystems), AdnU-S′2 and AdnU-A2. The sequences of clinical specimens were then analyzed and compared with the 51 prototype strains.

Phylogenetic analysis. A total of 180 nucleotide sequences from clinical specimens were compared with the 51 prototype strains using SINCA software (Fujitsu Ltd., Tokyo, Japan), and the serotypes were identified. The evolutionary distances were estimated using Kimura's two-parameter method (21), and unrooted phylogenetic trees were constructed using the neighbor-joining method (32). Bootstrap analyses were performed by 1,000 resamplings of the data sets. Bootstrap values of 70% or more were considered to be statistically significant for the grouping (13).

Nucleotide sequence accession numbers. The GenBank accession numbers of the nucleotide sequences reported in the present study are AB259820 to AB259831.

RESULTS

Amplification and quantification of hAdV DNA. The comparison of the partial hexon nucleotide sequences of the 51 prototype and AdV-19a strains demonstrated that several short stretches (19 to 23 bases) in the hexon gene are highly conserved among hAdVs. When amplification was performed by LightCycler PCR with the set of designed primers (AdnU-S′2 and AdnU-A2), the 554-bp fragment was efficiently amplified in all strains. The amplification was confirmed to be specific to hAdVs because there was no cross-amplification with a variety of heterologous pathogenic agents, such as influenza A virus (2.2×10^3 50% tissue culture infective dose [$TCID_{50}$]/reaction), influenza B virus (7.2×10^2 $TCID_{50}$/reaction), herpes simplex virus type 1 (2.2×10^3 $TCID_{50}$/reaction), herpes simplex virus type 2 (7.2×10^2 $TCID_{50}$/reaction), cytomegalovirus (1.7×10^3 PFU/reaction), *Staphylococcus aureus* (9.5×10^4 CFU/reaction), *Staphylococcus epidermidis* (1.7×10^4 CFU/reaction), *Chlamydia trachomatis* (2.0×10^4 IFU/reaction), or *Neisseria gonorrhoeae* (1.2×10^8 CFU/reaction) (data not shown).

The 10-fold serially diluted plasmid DNA, pAd8hxn, was amplified by LightCycler PCR, and a 554-bp fragment was identified by ethidium bromide staining when the sample contained more than 10 copies/reaction. When the threshold cycles were plotted against the $\log_{10}$ of the copy number of the pAd8hxn plasmid, linearity was observed over the range from 1×10^1 to 1×10^8 copies/reaction. The reliability of the standard curve was confirmed by the correlation coefficient (r) value of -1 and an error value of 0.2 or less (Fig. 1).

To determine whether the quantification was accurate, we compared primer sequences among the 51 serotypes. Forty serotypes had identical sequences, whereas 11 serotypes showed one to two differences (Fig. 2). Therefore, we selected 16 AdVs including AdV-1, -3, -4, -5, -6, -12, -18, -21, -23, -24, -29, -36, -37, -40, -41, and -19a; we then amplified 1,004 bp, cloned the fragment into a pT7 Blue T-Vector, and determined the standard curve of each strain with these 16 DNA templates. The slopes of the calibration curves of all AdVs other than AdV-18 and -41 were between -3.76 and -3.23, with an average of -3.43, and the intercepts were between 35.1 and 37.8, with an average of 36.6. The correlation coefficient r was

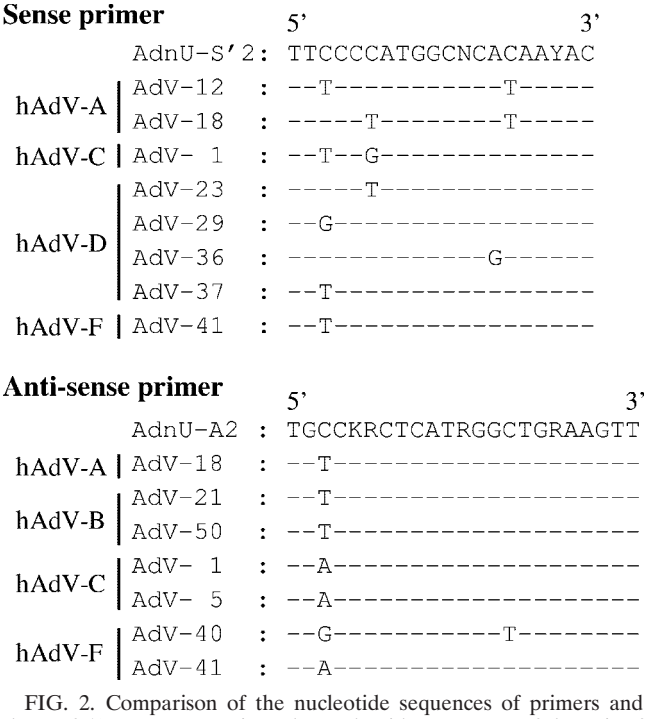

FIG. 2. Comparison of the nucleotide sequences of primers and those of 51 prototype strains. The nucleotide sequences of the pair of primers were well conserved in 40 serotypes. Only 11 serotypes showed one or two nucleotide substitutions in the primer region.

<-0.99. Likewise, the slopes determined by 14 experiments with pAd8hxn as the template were between -3.61 and -3.27, and the intercepts were between 35.7 and 37.4, with an average of 36.7, thereby demonstrating that there was no difference in the slopes determined by these different methods and that pAd8hxn is usable as the standard. The slopes of the calibration curves of AdV-18 and -41 were -3.21 and -3.43, and the correlation coefficient r was <-0.99. As the intercepts were 45.3 and 36.7, the quantifications of AdV-18 and -41 were 2×10^{-3} and 0.3 times less sensitive than those for the other serotypes. These results demonstrate that the set of primers used in this study is able to amplify accurately all hAdV except AdV-18 and -41.

Inhibition control. To determine whether an inhibitor included in the sample caused a false negative for amplification, we added 2.5×10^3 copies of pT7 Blue T-Vector as an inhibition control (IC) into residual adenovirus-negative samples and reamplified the samples using two set of primers, the pair M13RP1 (5′-CAGGAAACAGCTATGACC-3′) and −20M13 (5′-GTAAAACGACGGCCAG-3′) and the pair AdnU-S′2 and AdnU-A2. All adenovirus-negative samples were PCR positive. The melting temperature of the PCR products derived from the IC was 94°C, whereas the melting temperatures from the samples containing a viral load of 10^2 to 10^4 were 83.5 to 90.5°C. There was no significant difference in viral loads determined with and without the IC (data not shown).

Phylogenetic analysis. We previously determined the nucleotide sequences of the partial hexon genes (916 bp) of all 33 prototype strains in hAdV-D and hAdV-E (34). In the present study, we used the residual hAdV prototype strains—AdV-6 in hAdV-C, AdV-31 in hAdV-A, and AdV-50 in hAdV-B—to complete the database based on 350 bp of the hexon gene of hAdV; these strains were not available from the GenBank database. Alignment of the partial hexon nucleotide sequences was performed as described previously (34). The hAdVs were segregated into six major clusters, A to F (data not shown). Each cluster corresponded well to the six newly designated human hAdV species, A to F (38).

The 51 prototype strains and the AdV-19a strain showed 73.4 to 99.7% (average, 87.3%) identity with the exception of AdV-11, AdV-35, AdV-21, and AdV-50. Cluster A consisted of three serotypes, AdV-12, -18, and -31, whose nucleotide identity ranged from 81.1% (between AdV-18 and AdV-31) to 88.3% (between AdV-12 and AdV-31), with an average identity of 84.5%. In cluster B, which included AdV-3, -7, -11, -14, -16, -21, -34, -35, and -50, nucleotide identity ranged from 83.4% (between AdV-7 and AdV-16) to 99.7% (between AdV-11 and AdV-34, between AdV-11 and AdV-35, and between AdV-34 and AdV-35), with the exception of four prototype strains. The nucleotide sequences between AdV-11 and AdV-35 and between AdV-21 and AdV-50 were identical. The average nucleotide homology in cluster B was 90.3%. Cluster C included four serotypes, AdV-1, -2, -5, and -6, and the nucleotide identity ranged from 87.7% (between AdV-1 and AdV-6 and between AdV-5 and AdV-6) to 98.9% (between AdV-2 and AdV-6), with an average of 90.5%. Cluster D consisted of 32 serotypes, AdV-8 to -10, -13, -15, -17, -19, -20, -22 to -30, -32, -33, -36 to -39, -42 to -49, and -51, whose nucleotide identity ranged from 91.7% (between AdV-8 and AdV-19a) to 99.7% (between AdV-24 and AdV-38, between AdV-25 and AdV-38, and between AdV-32 and AdV-38), with an average of 97.3%. AdV-19a showed only 95.7% nucleotide identity with the AdV-19 prototype AV-587, which was first reported in 1955 in Saudi Arabia (8), and did not form a monophyletic cluster with AdV-19. Cluster F consisted of two serotypes, AdV-40 and -41, and the nucleotide identity between them was 90.0%. These results indicate that phylogenetic analysis based on partial nucleotide sequences is capable of distinguishing the prototype strains of hAdVs other than AdV-11 and -35 and AdV-21 and -50.

Evaluation of phylogeny-based serotyping. It has been reported that EKC is caused by four serotypes, AdV-4, -8, -19a, and -37, and that acute upper respiratory tract infection is caused primarily by nine serotypes, AdV-1, -2, -3, -4, -5, -6, -7, -14, and -21. These viruses present indistinguishable clinical manifestations.

To evaluate the reliability of the phylogeny-based identification of hAdVs using 350 bp of the hexon gene, we used three isolates each of AdV-4, -8, and -37 and two isolates of AdV-19a from hAdV-D and hAdV-E (Table 1). All isolates had been previously identified by a neutralization test with type-specific antisera and well characterized genetically by both genome typing and phylogenetic analysis based on 916 bp of the hexon gene (34). When the 350 bp of the hexon gene from the three AdV-8 isolates were compared with those of the 51 prototype strains and the AdV-19a strain, all isolates showed 99.4 to 99.7% nucleotide identity with the prototype strain of AdV-8 and a difference of at least >4.0% was seen between AdV-8 (Trim) and the heterologous prototype strain (AdV-29) (Table 1). The two isolates of AdV-19 showed only 95.7% nucleotide identity with the prototype strain of AdV-19 (AV-587); however, these isolates showed nucleotide sequences identical to those of AdV-19a. The second-highest nucleotide identity was seen between the isolates and AdV-25, -26, -38, and -49 (Table 1). The three isolates of AdV-37 showed the highest identity with the homologous prototype strain of AdV-37 (GW), and at least a 1.0% difference was seen between AdV-37 and the heterologous prototype strain (AdV-25) (Table 1). Therefore, the present phylogenetic analyses based on 350 bp of the hexon gene successfully segregated these isolates into cluster D and separated them into distinct monophyletic clusters with their respective prototype strains, AdV-8, -19a, and -37 (Fig. 3). When the three AdV-4 isolates were analyzed, they showed the highest identity with the prototype strain AdV-4 (RI-67) and were segregated into cluster E with 100% bootstrap support (Fig. 3). These results indicate that phylogeny-based clustering with the partial hexon nucleotide sequences is capable of identifying clinical isolates from conjunctivitis patients.

We used three isolates of AdV-1 and one isolate of AdV-2 from hAdV-C and three isolates of AdV-3 from hAdV-B. These were isolated from pharyngeal swabs and were identified by the neutralization test with type-specific antisera. At least an 8.0% difference was seen between the AdV-1 prototype strain (Adenoid 71) and the heterologous prototype strains (Table 1). The AdV-2 isolate showed 100% nucleotide identity with the prototype strain of AdV-2 (Adenoid 6), with a 2.1% difference from the heterologous prototype strains (Table 1). The AdV-3 isolates showed at least a 1.5% difference between the prototype strain of AdV-3 (GB) and the heterologous prototype strains (Table 1). We also used nucleotide sequences of

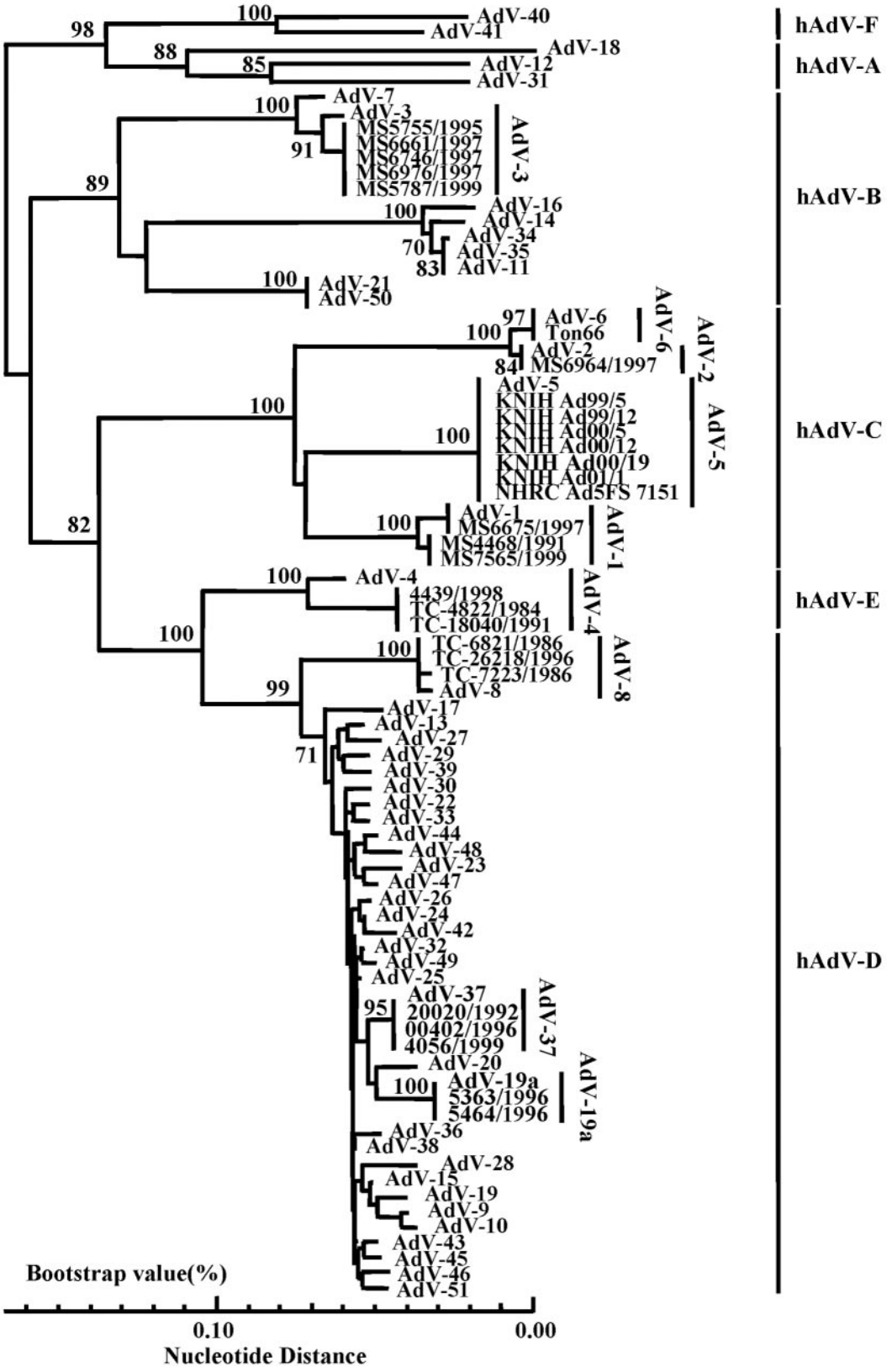

FIG. 3. Evaluation of phylogeny-based identification. The 350-bp sequence of a partial hexon gene of the isolates was analyzed along with the sequences of prototype strains and the AdV-19a strain by the neighbor-joining method. The numbers at the nodes are percentages of 1,000 bootstrap pseudoreplicates containing the cluster distal to the node.

TABLE 2. Type identification of hAdV DNA detected in conjunctival swabs and eye drops used by patients with conjunctivitis by phylogenetic analysis

Typing by phylogeny			No. of identical nucleotide sequences	No. of copies/ml[a]			Highest-scoring heterologous prototype		Representative sample (isolate/yr)
Source and virus type	Bootstrap (%)	Identity (%)		Minimum	Maximum	Mean	Type(s)	Identity (%)	
Conjunctival swab									
AdV-3	88	99.1	18	2.0×10^5	2.2×10^{10}	9.9×10^6	AdV-7	97.7	C058/2003
AdV-3	88	98.9	4	1.4×10^6	7.6×10^7	2.1×10^4	AdV-7	97.1	C064/2003
AdV-3	88	98.6	1			5.6×10^7	AdV-7	97.7	C082/2003
AdV-3	88	98.9	1			9.6×10^7	AdV-7	97.4	H1009-6/2003
AdV-4	100	96.0	14	7.0×10^4	5.6×10^8	2.7×10^7	AdV-25, AdV-32, AdV-37, AdV-49	89.7	C003/2003
AdV-8	87	99.7	1			4.2×10^8	AdV-29	94.6	H1009-16/2003
AdV-19a	100	100	19	2.0×10^6	2.4×10^9	4.2×10^7	AdV-25, AdV-26, AdV-38, AdV-49	97.4	C001/2003
AdV-37	70	100	35	4.8×10^2	2.0×10^8	1.3×10^6	AdV-25	98.9	C004/2003
AdV-37	70	99.7	2	1.3×10^4	1.6×10^6	8.1×10^5	AdV-25	98.3	C023/2003
AdV-37	70	99.7	1			3.3×10^6	AdV-24, AdV-25	98.6	C075/2003
AdV-37	70	99.4	3	2.4×10^5	2.7×10^6	5.2×10^5	AdV-25	98.3	C095/2003
Eye drops									
AdV-4	100	96.0	1			1.8×10^4	AdV-25, AdV-32, AdV-37, AdV-49	89.7	
AdV-8	100	98.9	1			4.2×10^8	AdV-37	94.6	
AdV-19a	100	100	1			5.4×10^2	AdV-25, AdV-26, AdV-38, AdV-49	97.4	

[a] For AdV-3, the range was 2.0×10^5 to 2.2×10^{10}, with a mean of 1.3×10^7. For AdV-37, the range was 4.8×10^2 to 2.0×10^8, with a mean of 1.3×10^6.

AdV-5 and -6, which are available from GenBank, finding 100% nucleotide identity with their respective prototype strains, AdV-5 (Adenoid 75) and AdV-6 (Tonsil 99) (Table 1). All isolates of AdV-3 were segregated into cluster B along with the prototype, and all isolates of AdV-1, -2, -5, and -6 were segregated into cluster C along with their prototypes, with >84% bootstrap support (Fig. 3). These results indicate that phylogeny-based clustering with 350 bp of partial hexon nucleotide sequences can successfully identify clinical isolates from patients with respiratory infection.

Identification of hAdV DNA from clinical specimens. (i) Conjunctival swabs. We detected hAdV DNA in 99 (74.4%) of 133 conjunctivitis patients. When the 350-bp sequences from patients were compared with those of the 51 prototype strains and the AdV-19a strain, 24 sequences showed the highest nucleotide identity with AdV-3, having at least 0.9 to 1.5% higher nucleotide identity than with other prototype strains (Table 2). Thus, they formed a distinct cluster with the prototype strain of AdV-3 in cluster B (Fig. 4A). Sixty-one sequences showed the highest identities with prototype strains in cluster D, 1 sequence showed the highest identity (99.7%) with AdV-8, 19 sequences showed the highest identity with the AdV-19a strain (100%), and 41 sequences showed the highest identity with the AdV-37 prototype strain (99.4 to 100%) (Table 2). Each sequence showed at least 0.9 to 5.1% higher nucleotide identity with the prototype strains in its cluster than with other prototype strains. These sequences were segregated into cluster D and formed a distinct cluster with the prototype strains with which they showed the highest identity (Fig. 4A). Fourteen sequences showed the highest identity (96.0%) with the AdV-4 prototype strain, showing at least 6.3% higher nucleotide identity than with other prototype strains (Table 2); they formed a distinct cluster with AdV-4 in cluster E (Fig. 4A).

(ii) Pharyngeal swabs and nasal mucus specimens. We detected hAdV DNA in 81 (27.3%) of 297 pharyngeal swabs and nasal mucus samples. One sample showed the highest nucleotide identity (98.6%) with the AdV-3 prototype strain, showing at least 1.5% higher nucleotide identity than with other prototype strains (Table 3). This sample formed a distinct cluster with the prototype strain of AdV-3 in hAdV-B (Fig. 4B). Sixty-six samples showed the highest nucleotide identity with the prototype strains in cluster C: 18 sequences showed the highest nucleotide identity with the AdV-1 prototype strain (98.6 to 99.7%), 29 with AdV-2 (100%), 18 with AdV-5 (99.7 to 100%), and 1 with AdV-6 (100%). Each of these sequences showed at least 1.1 to 11.7% higher nucleotide identity with the prototype strains in its cluster than with other prototype strains (Table 3).

These results prove that phylogeny-based clustering with 350-bp nucleotide sequences is a powerful tool for the rapid identification of hAdV.

Quantification of hAdV DNA. (i) Conjunctival swabs. The viral load in 99 conjunctival swabs was determined by LightCycler PCR. The virus load of AdV-3 from 24 swabs ranged from 2.0×10^5 to 2.2×10^{10} copies/ml (mean, 1.3×10^7 copies/ml) and that of AdV-4 from 14 swabs ranged from 7.0×10^4 to 5.6×10^8 copies/ml (mean, 2.7×10^7 copies/ml). The virus load of AdV-8 from 1 swab was 4.2×10^8 copies/ml, the virus load of AdV-19a from 19 swabs ranged from 2.0×10^6 to 2.4×10^9 copies/ml (mean, 4.2×10^7 copies/ml), and the virus load of AdV-37 from 41 swabs ranged from 4.8×10^2 to 2.0×10^8 copies/ml (mean, 1.3×10^6 copies/ml) (Table 2). The median virus load of 99 samples was 1.3×10^7 copies/ml (range, 4.8×10^2 to 2.2×10^{10} copies/ml). These data show that conjunctival swabs from patients with adenoviral conjunctivitis contain a high viral load and may cause mass infection such as nosocomial or family infection.

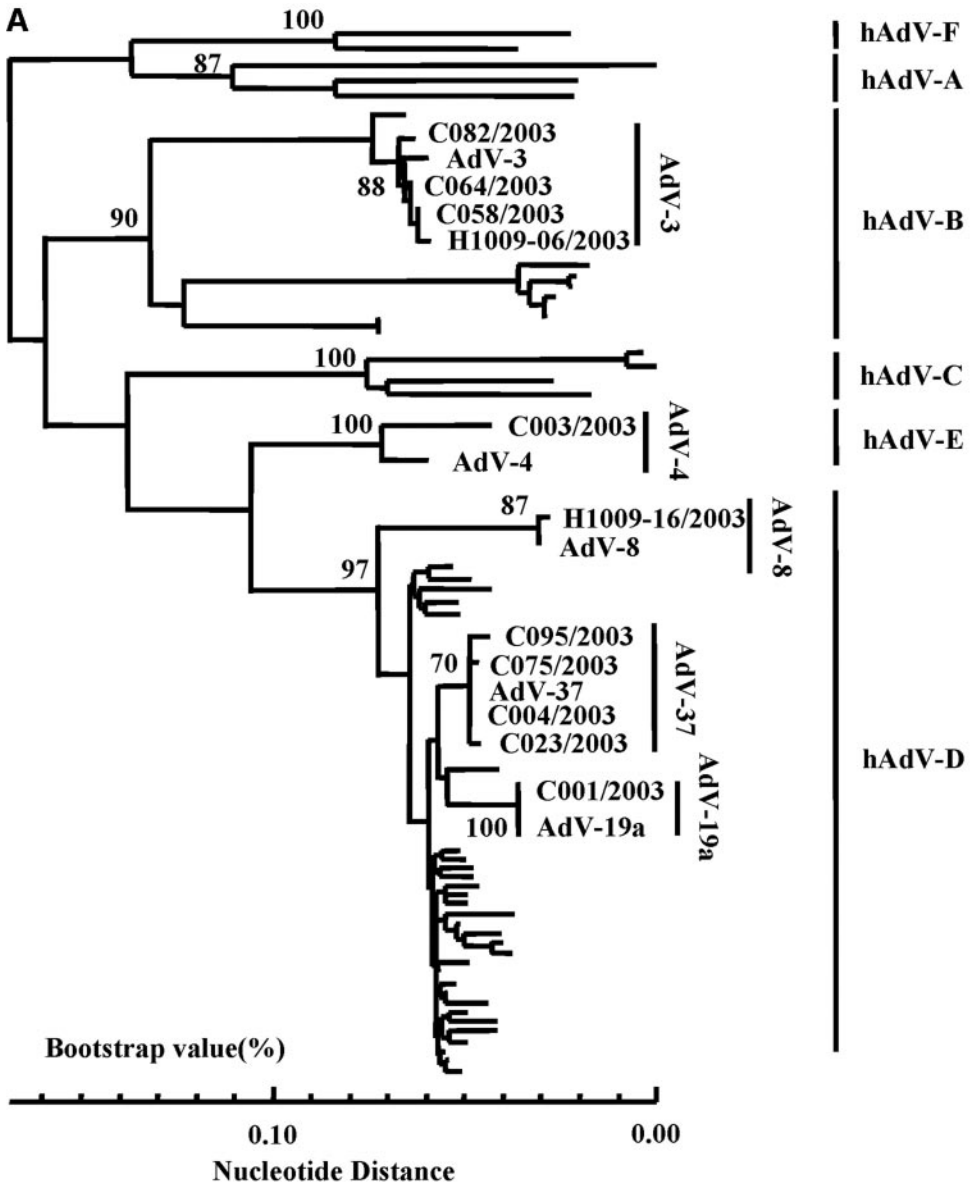

FIG. 4. Phylogenetic analyses of AdVs from patients with conjunctivitis (A) and acute upper respiratory tract infection (B). The 350-bp sequence of a partial hexon gene of representative samples was analyzed by the neighbor-joining method together with the 51 prototype strains of hAdV and the AdV-19a strain. The numbers at the nodes are percentages of 1,000 bootstrap pseudoreplicates containing the cluster distal to the node.

(ii) Pharyngeal swabs and nasal mucus specimens. The viral load in 297 swabs or nasal mucus was determined. The virus load of AdV-1 from 18 samples ranged from 3.3×10^2 to 4.0×10^7 copies/ml (mean, 6.0×10^4 copies/ml) and that of AdV-2 from 29 samples ranged from 4.4×10^1 to 3.1×10^8 copies/ml (mean, 4.7×10^4 copies/ml). The virus load of AdV-5 from 18 samples was from 3.2×10^2 copies/ml to 1.2×10^8 copies/ml (mean, 9.1×10^5 copies/ml) (Table 3). The median virus load of 81 samples was 7.1×10^5 copies/ml (range, 4.4×10^1 to 3.1×10^8 copies/ml).

(iii) Eye drops. Uchio et al. showed that contaminated eye drop solution may be a causative vehicle of adenoviral conjunctivitis (37). In the present study, we quantified hAdV DNA in eye drop bottles used by patients with conjunctivitis. LightCycler PCR showed that the viral load in eye drops ranged from 5.4×10^2 to 1.6×10^6 copies/ml (AdV-19a, 5.4×10^2 copies/ml; AdV-4, 1.8×10^4 copies/ml; and AdV-8, 1.6×10^6 copies/ml) (Table 2). The nucleotide homologies of isolates with their prototype strains were 96.0% for AdV-4, 98.9% for AdV-8, and 100% for AdV-19a. The present phylogenetic analysis demonstrated that two hAdVs, which formed distinct clusters with AdV-8 and AdV-19a, were segregated into cluster D. Additionally, one hAdV was segregated into cluster E with AdV-4 prototype strains (data not shown). These results show that eye drops used by patients with adenoviral conjunctivitis contain a high viral load and may cause nosocomial or family infection.

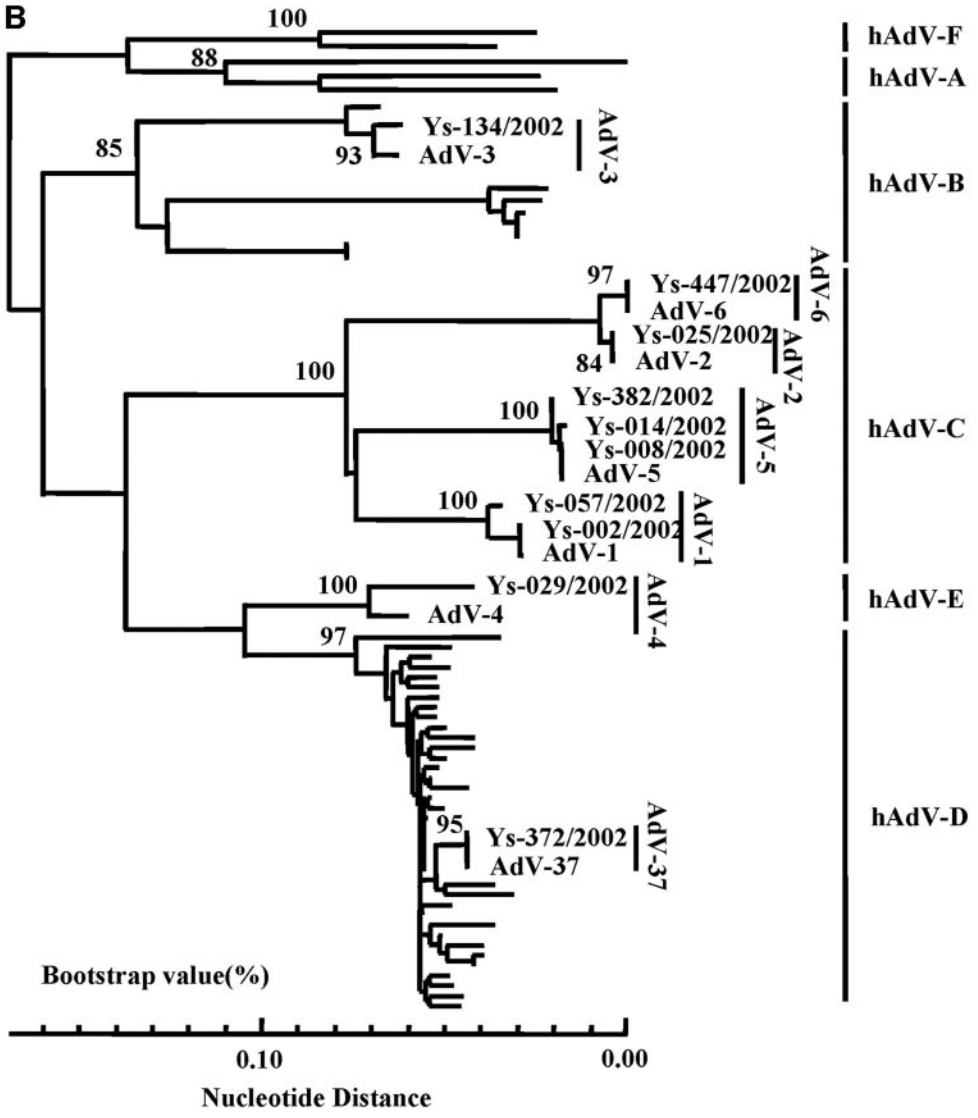

FIG. 4—*Continued*.

We also performed experiments using diluted conjunctival swabs, pharyngeal swabs, nasal mucus, and eye drops that contained 10^1 to 10^5 copies/reaction of the genome from AdV-1, -2, -3, -4, -5, -6, -8, -37, and -19a, and the same results were obtained (data not shown).

DISCUSSION

In conventional virus isolation with cell culture, the serotyping of hAdVs by neutralization is time-consuming, requiring 2 to 4 weeks. Various rapid identification techniques replacing this virus isolation have been developed: for example, detection of hAdV DNA using hybridization (9, 18, 22), serotype differentiation using type- or group-specific primers (2, 4, 20, 30, 43), PCR-restriction fragment length polymorphism (3, 11, 20, 25, 31), and gene sequencing (25, 34, 35). An adenovirus quantification method using real-time PCR has also been proposed (14, 16, 24) and has also been tested for other viruses such as hepatitis C virus, hepatitis B virus, herpes simplex virus, Epstein-Barr virus, etc. (12, 26, 28, 29). However, to the best of our knowledge, no genetic testing method allowing the quantification and identification of hAdV serotypes has been developed to date. In the present study, we developed such a method. Our proposed method makes use of LightCycler PCR and phylogenetic analysis to perform quantification of DNA of all but 2 of 51 serotype strains of hAdV using a single set of designed primers and to identify hAdV DNA of each serotype within 24 h. LightCycler PCR using a set of primers in the hexon region allows the amplification of a partial hexon gene from all 51 prototype strains and the AdV-19a strain as well as from clinical specimens. The sensitivity of this real-time PCR is as high as 10 copies/reaction, which is similar to that of the other adenovirus quantification methods that have previously been reported (14, 16). Moreover, phylogenetic analysis based on 350-bp sequences in the hexon region grouped the 51 prototype strains and the AdV-19a strain into six species, hAdV-A

TABLE 3. Type identification of hAdV DNA detected by phylogenetic analysis of pharyngeal swabs and nasal mucus samples from patients with acute upper respiratory tract infection

Typing by phylogeny			No. of identical nucleotide sequences	No. of copies/ml[a]			Highest-scoring heterologous prototype		Representative sample (isolate/yr)
Type	Bootstrap (%)	Identity (%)		Minimum	Maximum	Mean	Type(s)	Identity (%)	
AdV-1	100	99.7	17	3.3×10^2	4.0×10^7	2.5×10^4	AdV-5	91.1	Ys-002/2002
AdV-1	100	98.6	1			7.9×10^6	AdV-5	90.6	Ys-057/2002
AdV-2	84	100	29	4.4×10^1	3.1×10^8	4.7×10^4	AdV-6	98.9	Ys-025/2002
AdV-3	93	98.6	1			2.2×10^8	AdV-7	97.1	Ys-134/2002
AdV-4	100	96.0	12	1.1×10^6	1.5×10^8	1.7×10^7	AdV-25, AdV-32, AdV-37, AdV-49	89.7	Ys-029/2002
AdV-5	100	100	12	3.2×10^2	1.2×10^8	9.1×10^5	AdV-1	90.9	Ys-008/2002
AdV-5	100	99.7	4	1.1×10^3	6.5×10^7	4.7×10^6	AdV-1	90.6	Ys-014/2002
AdV-5	100	99.7	2	1.4×10^3	1.0×10^7	5.0×10^6	AdV-1	90.6	Ys-382/2002
AdV-6	97	100	1			1.6×10^7	AdV-1	88.3	Ys-447/2002
AdV-37	95	100	2	2.1×10^2	5.9×10^6	3.0×10^6	AdV-25	98.9	Ys-372/2002

[a] For AdV-1, the range is 3.3×10^2 to 4.0×10^7, with a mean of 6.0×10^4. For AdV-5, the range is 3.2×10^2 to 1.2×10^8, with a mean of 9.1×10^5.

to hAdV-F. These results are identical to those obtained previously in a study using 916 bp in the hexon region, with the exception of AdV-50 (34). Recently, de Jong et al. (8) discovered two new serotypes of adenovirus, AdV-50 and AdV-51. Unfortunately, due to a laboratory mistake, the labeling of these two strains appears to have been reversed. Therefore, we have corrected AdV-50 to -51 in hAdV-D and AdV-51 to AdV-50 in hAdV-B (de Jong, personal communication).

The advantage of using the 350-bp nucleotide sequence for phylogenetic analysis is that only one sequencing reaction with a primer (AdV-S2′ or AdV-A2) is required, while three or four primers were needed to determine the 916-bp nucleotide sequence for phylogenetic analysis.

We detected hAdV DNA from 27.3% of the specimens of pharyngeal swabs and nasal mucus taken from patients with upper respiratory tract infection. According to data compiled in 2002 by the Tokyo Metropolitan Research Laboratory of Public Health (available at http://idsc.tokyo-eiken.go.jp/epid/2002/tbkj2304.html), hAdV hexon genes were detected in 25.9% of samples from patients with upper respiratory tract infection. This is virtually the same detection rate as that obtained in the present study with real-time PCR. We also detected hAdV DNA in 74.4% of conjunctival swabs taken from patients with conjunctivitis. Thus, the hAdV infection rate for conjunctivitis seems to be high. A point-of-care test based on an immunochromatographic test to detect hAdV is available from several commercial diagnostic kit companies and is currently used in many hospitals and clinics in Japan. This method is capable of detecting adenovirus in a few minutes; however, it has been shown to be unable to detect hAdV from one-third of conjunctive swabs from conjunctivitis patients (36). These swabs contained 10^1 to 10^6 copies/ml of hAdV. In addition, the present data also showed that the eye drops used by conjunctivitis patients were contaminated with hAdV DNA. These false-negative swabs from the immunochromatographic test and eye drops used by patients nevertheless contain active virus and may cause nosocomial or family infection.

The technique described in the present study makes it possible to quantitatively detect hAdV DNA of all serotypes with high sensitivity and to identify hAdV serotypes within 24 h. Thus, it is an extremely useful and accurate method for the rapid and specific diagnosis of hAdV-infected patients as well as for molecular epidemiological studies of hAdV infection.

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