Rapid Detection and Typing of Oculopathogenic Strain of Subgenus D Adenoviruses by Fiber-Based PCR and Restriction Enzyme Analysis

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Purpose. To develop a new detection and typing method of oculopathogenic strains of subgenus D adenoviruses directly from conjunctival scrapings by a combination of polymerase chain reaction (PCR) and restriction enzyme analysis (REA).

Methods. A new PCR method using primer pairs of AF2/AR2, which are specific for the fiber genes, were developed to amplify 1150-bp products from nine oculopathogenic prototypes of subgenus D adenoviruses. Amplicons were cleaved with three restriction enzymes: *Dde*I, *Hin*fI, and *Rsa*I. Clinical specimens of 102 conjunctival scrapings were also evaluated by this PCR method. Restriction patterns of prototypes were used for the typing of clinical samples. Detection limit was determined by the PCR amplification of a known amount of purified adenovirus serotype 8 DNA.

Results. A novel PCR method based on the fiber genes allowed the amplification of nine oculopathogenic serotypes of subgenus D (Ad8, Ad9, Ad15, Ad17, Ad19, Ad22, Ad28, Ad37, and Ad39). As little as 38.4 fg of adenovirus type 8 could be detected by this method. Positive results were obtained from 48 of 102 samples (47%) by both hexon- and fiber-based PCR, whereas only 29 of 102 (28.4%) yielded positive results by culture isolation/neutralization test (NT). All positive specimens (29 samples) of culture isolation and PCR-RFLP methods showed positive results by our new fiber-based PCR method, and no positive products were detected from other subgenus of adenovirus or nonadenoviral DNA.

Conclusions. A newly developed fiber-based PCR-REA method for the detection and typing of adenoviruses is faster than any former PCR methods. This all-in-1-day detection and typing method will be quite useful to the rapid diagnosis of subgenus D adenovirus infection. (*Invest Ophthalmol Vis Sci.* 2001;42: 2010–2015)

Adenoviruses (Ads) cause a wide range of clinical diseases that affect respiratory, ocular, and gastrointestinal systems. Ocular adenoviral infections exhibit varieties of clinical presentations, such as epidemic keratoconjunctivitis (EKC), pharyngoconjunctival fever (PCF), nonspecific follicular conjunctivitis, and chronic papillary conjunctivitis.[1] EKC is a clinical disease entity characterized by severe bilateral conjunctivitis with substantial corneal and extraocular involvement. The incubation period is 8 to 10 days. Other clinical symptoms may include edema of the eyelids, photophobia, and lacrimation. Superficial erosion of the cornea occasionally develops that can ultimately cause corneal infiltration and opacity. Thus, blurring of vision may persist for months and occasionally for years.[2] Adenoviral keratoconjunctivitis is transmitted through direct contact with the infected area and frequently causes community epidemics. Although not blinding, the ocular infections with the above-mentioned character create discomfort that leads to the decreased quality of life and even possibly economic damages.[3]

Human adenovirus consists of a large family of 51 described serotypes, classified into 6 subgenera (A–F) on the basis of biochemical, immunologic, and morphologic criteria.[4,5] Thirty-two serotypes constitute subgenus D, and several of these have been significantly associated with ocular infections.[6] For example, serotypes 8, 19, and 37 are the most common causative agents of EKC. Occasionally, Ad9, Ad15, and Ad22 have also been reported as a cause.[7] On the other hand, Ad9, Ad15, Ad17, and Ad28 are known to induce relatively mild follicular conjunctivitis.[4] Ad3, Ad4, and Ad7, which belong to other subgenus, also cause conjunctivitis and pharyngoconjunctival fever.[4] Even though an antivirals medicine, Cidofovir (Baush and Lomb Pharmaceuticals, Inc., Tampa, FL), is in a preclinical trial and has been found to be a promising medicine against subgenus C adenoviruses (Ad1, Ad2, and Ad5), it is not clinically available at this moment.[8,9] Clinical diagnosis are usually made based on the patient's history, clinical examination, and if needed, a laboratory test detecting the viral antigen in an ocular swab sample or by culture and neutralization test (NT).[10]

For detection of adenovirus directly from conjunctival swab, a new test kit (immunochromatography; SAS Adenotest; SA Scientific Inc., San Antonio, TX) is currently available in developed countries. Yet, the sensitivity of this test is approximately 54.7%, and a negative test result does not always rule out the possibility of adenoviral infection.[11] As long as 2 to 4 weeks are needed for the identification by culture isolation NT. Recent development of PCR-based rapid serotype identification methods such as hexon-based PCR restriction fragment length polymorphism (RFLP), type-specific PCR, and PCR with direct sequencing of products offer a sensitive and specific laboratory test similar to culture isolation and NT. They are only good for the detection of common EKC strains (Ad8, Ad19, and Ad37) from subgenus D, and 2 to 3 days are required for the whole procedure. Therefore, a more simple, more rapid, and more sensitive method was in demand to identify these oculopathogenic serotypes of subgenus D.

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TABLE 1. Primers for PCR

Primer*	Polarity	Position†	Sequence (5′-3′)
AF2	+	66-83	5′ CGG GTG GAA GAT GAC TTC 3′
AR2	−	1188-1205	5′ CGT GCT GGT GTA AAA ATC 3′

* The nucleotide position refers to the fiber sequences of Ad 8. GenBank accession number for fiber gene is X74660.

† Forward primer is located in 5′ coding region and reverse primer is located in the 3′ noncoding region of the fiber gene.

In this study, we developed a new PCR method based on the fiber gene for rapid, accurate detection and typing of the subgenus D adenoviruses directly from conjunctival scrapings.

MATERIALS AND METHODS

Prototypes and Clinical Specimens

Adenovirus prototypes Ad8, Ad19, Ad37, Ad22, Ad28, and Ad39 were kindly provided from National Institute of Infectious Disease (NIID, Tokyo) and Ad9, Ad15, and Ad17 were provided from Umea University (Umea, Sweden).

For the clinical study, ocular samples were collected in an eye clinic in South Kyushu, Japan, between March 1998 and February 2000 from patients diagnosed with EKC or acute conjunctivitis. Specimens were scraped from the lower palpebral conjunctiva and collected, respectively, in 1 ml of phosphate-buffered saline for PCR and in 1 ml of viral transport medium (1% fetal calf serum in Eagle's MEM, 60 mg/l penicillin G, and 40 mg/l gentamicin) for culture isolation. These samples were preserved at −30°C until the analyses. This study was approved by the Ethical Committee of the University of Tokyo, School of Medicine. The research plan followed the tenets of the Declaration of Helsinki.

Preparation of Viral DNA

Prototypes. Viral DNA was prepared by phenol/chloroform extraction and ethanol precipitation. Briefly, 200 μl of culture fluid and the same volume of lysis buffer [10 mM Tris-HCl (pH 7.6) 5 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 200 μg/ml Proteinase K] were mixed in a microcentrifugation tube and incubated for 1 hour at 37°C. Then 20 μg of RNase A (Boehringer-Mannheim, Mannheim, Germany) was added to the tube and incubated at 37°C for another hour. Then, the same volume of phenol-chloroform (phenol, 100 μl; chloroform, 100 μl) was added to the mixture for 10 minutes and centrifuged at 8000*g* for 10 minutes. This extraction process was repeated again. Finally, the supernatant containing genomic DNA was precipitated in 500 μl of 100% ethanol. After drying, the pellet was dissolved in 30 μl of TE buffer.

Clinical Samples. DNA from clinical samples was prepared for PCR by two protocols.

In the first protocol, DNA was prepared by guanidine thiocyanate glass powder methods as described previously.[12] Briefly, 200 μl of clinical sample and the same volume of 1,2,2-trichloro-1,2,2-trifluoroethane were mixed in a microcentrifugation tube. After vortexing for 20 minutes, the mixture was centrifuged at 5500*g* for 10 minutes. Two hundred microliters of 6 M guanidine thiocyanate was added to 200 μl of the supernatant and vortexed for 5 minutes. Glass powder (4.8 μl; Asahi Glass Co Ltd., Tokyo, Japan) was added to the tube and mixed well for 20 minutes After centrifugation at 220*g* for 2 minutes, the supernatant was removed, and the pellet was washed three times with 800 μl washing buffer (Asahi Glass Co., Ltd.). After the last wash, 400 μl of 100% ethanol was added, and the pellet was dissolved and then further centrifuged at 8000*g* for 5 minutes. The supernatant was removed, and the pellet was dried in a vacuum centrifuge at 55°C to remove all the residual 100% ethanol. Then 24 μl distilled water was added to dissolve the pellet. The sample was heated in a heating block at 65°C for 10 minutes and then centrifuged at 8000*g* for 10 minutes. Supernatant that contained genomic DNA was stored at −30°C until use.

In the second protocol, 10 μl of clinical sample collected in phosphate-buffered saline was heated directly at 97°C for 15 minutes in a thermal cycler before adding PCR reagent.

Detection of Adenovirus

Primers. Primers were selected for PCR on the basis of the alignment of the fiber gene sequences (GenBank accession number for fiber gene X74660 [Ad8], U69130 [Ad19], U69132 [Ad37], X74659 [Ad9], and X72934 [Ad15]) from human adenovirus serotypes Ad8, Ad19, Ad37 Ad9, and Ad15, respectively. The pair of primers AF2/AR2 was used to amplify approximately 1150 bp in the general PCR. The primer positions corresponded to 66 to 83 bp (AF2) and 1188 to 1205 bp (AR2) of Ad8 fiber gene (Table 1).

Polymerase Chain Reaction. One-microliter aliquots of DNA prepared from prototype and 10 μl from clinical samples prepared by heat method were used as the DNA template. The negative control tube contained 10 μl of double-distilled water. Amplification reactions were conducted in 50 μl reaction mixtures containing 0.5 μM each of the primer pair, 200 μM of each deoxynucleoside triphosphate (dNTP), 5 μl of 10-fold concentrated buffer, and 1.25 U of *Taq* polymerase (Boehringer-Mannheim). Thermal cycling was conducted for 35 cycles (94°C, 1-minute denaturation, 50°C, 1-minute annealing, and 72°C, 2-minute extension; 72°C, 7-minute final extension) in a thermal cycler. Five microliters of reaction products was analyzed in a 1.5% agarose gel and stained with ethidium bromide (1 μg/ml).

Specificity of Detection. Specificity of the test was determined using other subgenus of adenoviruses and nonadenoviral DNA from other agents of conjunctivitis. These agents included herpes simplex type I and type II, enterovirus, and *Chlamydia trachomatis*.

Limits of Detection. A known amount of Ad8 purified DNA was amplified after serial dilution to obtain a theoretical range of virus particle 10^{10} to 10^{1} per reaction mixture; 0.384 fg of Ad DNA corresponds to a single copy of linear double-stranded DNA that is approximately 35,000 bp. After PCR amplification, 5 μl of product was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide.

Culture Isolation. All clinical samples were seeded to a confluent monolayer of Hep2 or CaCo2 cells that had grown in a 24-well plate and examined for 10 days before the next passage. Samples were passaged four times in a 24-well plate. If there was no cytopathic effect (CPE) after four passages, samples were considered to be cell culture negative.

Typing of Adenovirus

Restriction Enzyme Analysis (REA). REAs were performed using positive PCR products with three restriction enzymes: *Dde*I, *Hin*fI, and *Rsa*I (Boehringer-Mannheim). Briefly, 5 μl of PCR-amplified DNA product was incubated with 10 units of restriction endonucleases in 15 μl of reaction mixture and at a designated temperature (recommended by manufacturer for each restriction endonuclease) for 3 hours. After digestion, 10 μl of the reaction mixture was mixed with 3 μl of loading buffer (60% glycerol, 0.25% bromphenol blue, and 0.25%

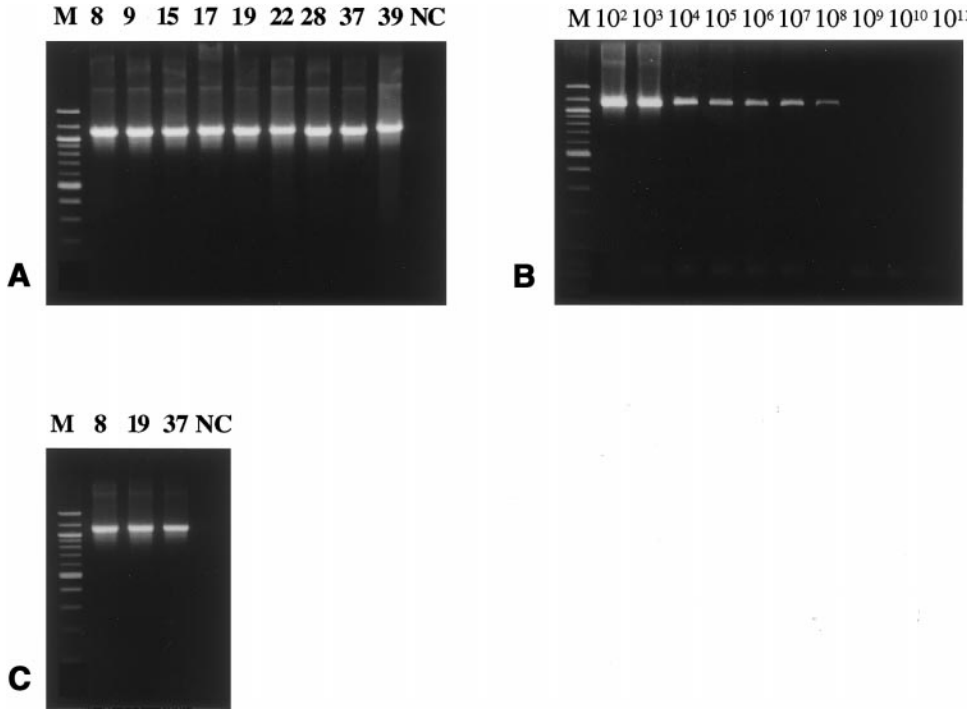

FIGURE 1. (**A**) PCR amplification of fiber gene from adenovirus prototypes (Ad8, Ad9, Ad15, Ad17, Ad19, Ad22, Ad28, Ad37, and Ad39). All prototypes are approximately 1150 bp except Ad39, which is approximately 1200 bp. Lane designation refers to oculopathogenic strain of subgenus D. *Lane M,* molecular size markers (New England Biolabs 100-bp DNA ladder); *lane NC,* negative control. (**B**) PCR-amplified products obtained from serially diluted adenovirus type 8 purified DNA. (**C**) PCR amplification of fiber gene from clinical specimens (Ad8, Ad19, and Ad37). Lane designation refers to serotypes Ad8, Ad19, and Ad37, respectively. *Lane M,* molecular size markers (New England Biolabs 100-bp DNA ladder); *lane NC,* negative control.

xylene cyanol) and then run in 1.5% horizontal agarose gel at 100 V for 50 minutes in a 50 mM Tris-borate-EDTA buffer (pH 8.0). After electrophoresis, the gel was stained with ethidium bromide. The bands were visualized under UV light.

Hexon-based PCR-RFLP. For hexon-based, PCR-RFLP after nested PCR, 956-bp positive PCR products were digested with three restriction enzymes: *Hae*III, *Hin*fI, and *Eco*T14I, and restriction patterns were compared with a prototype pattern for typing.

Neutralization Test. Cell culture–positive samples were typed by using antiadenoviral serum.

RESULTS

DNA Preparation

Both guanidine thiocyanate glass powder method and direct heating of clinical samples yielded enough DNA for the following PCR test. The glass powder method, though, was more expensive and time consuming than direct heating of the clinical samples (data not shown).

Specificity and Limits of Detection

The primer pair AF2/AR2 amplified approximately 1150-bp products from nine prototypes of subgenus D (Ad8, Ad9, Ad15, Ad17, Ad19, Ad22, Ad28, Ad37, and Ad39 in fiber gene-based PCR; Fig. 1A). The specificity of the PCR was tested against DNA from other subgenus of adenovirus and nonadenoviral DNA (see Materials and Methods). No amplified products were identified, indicating a high specificity of the test.

After serial dilution and amplification of purified Ad8, DNA could be detected at a dilution of $1:10^8$. This represent 38.4 fg of adenovirus DNA, which correspond to 100 genome copies (Fig. 1B).

Ad Detection

Our PCR gave a positive result in 48 of 102 clinical samples (47%; Fig. 1C), whereas only 29 of 102 (28.4%) were identified positive by culture isolation and NT. Hexon-based PCR produced the same results as fiber gene-based PCR.

Ad Typing by REA

Nine prototypes were digested with three restriction enzymes (*Dde*I, *Hin*fI, and *Rsa*I), and all prototypes except Ad22 for *Rsa*I were completely digested (Fig. 2A). Polymorphic restriction patterns of three enzymes could be clearly differentiated the nine prototypes. *Dde*I discriminated well all the serotypes except Ad8 and Ad9. In these two serotypes, the upper and lower restriction fragments are very close to each other, making it difficult to tell the position. In case of Ad8, the upper and lower restriction fragments are 584 and 551 bp, respectively. In case of Ad9, the fragments are 584 and 557 bp, respectively. *Hin*fI discriminate all the serotypes except Ad19 and Ad37. Both have the same restriction pattern for *Hin*fI. Ad19 and Ad37 have same restriction sites for *Rsa*I as well. The upper two fragments are 492 and 477 bp, respectively, and they appear to be a single fragment. Ad22 do not have any restriction site for *Rsa*I.

After REA, 48 PCR positive samples were identified as Ad8 (45/48), Ad19 (2/48), and Ad37 (1/48; Fig. 2B). Hexon-based PCR-RFLP produced the identical results to fiber gene-based PCR-REA. So far all the positive results of culture-NT and PCR-RFLP were met with our new PCR-REA method (100% sensitive). Comparative results of the three methods are shown in Table 2.

DISCUSSION

Adenovirus keratoconjunctivitis is a common viral ocular infection in Japan, with its peak incidence in the summer. Saitoh-Inagawa et al.[13] and Ishii et al.[14] conducted a comparative study to investigate viral conjunctivitis in three cities of East Asia: Sapporo, Japan; Kaoshiung, Taiwan; and Busan, South Korea. The proportion of adenoviral conjunctivitis in Sapporo, Kaoshiung, and Busan were 52%, 46%, and 55%, respectively. Common serotypes of subgenus D Ad8, Ad19, and Ad37 were the predominant agents of keratoconjunctivitis. According to the National Surveillance System of Infectious Diseases in Japan, the main etiologic agent of viral conjunctivitis was ade-

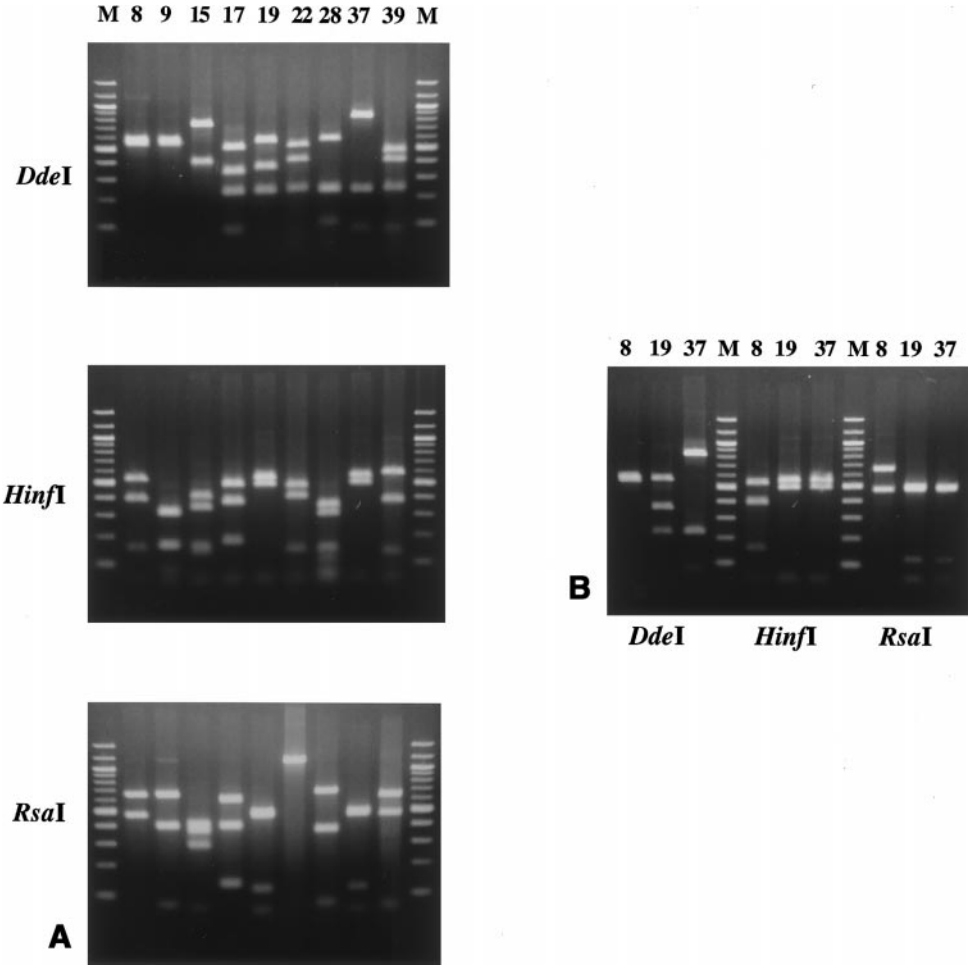

FIGURE 2. (**A**) Nine prototypes of subgenus D adenovirus were digested with *Dde*I, *Hin*fI, and *Rsa*I. Lane designation refers to serotype of adenovirus. *Lane M,* molecular size markers (New England Biolabs 100-bp DNA ladder). (**B**) Clinical samples of subgenus D adenovirus were digested with *Dde*I, *Hin*fI, and *Rsa*I. DNA was prepared by heat method. Lane designation refers to serotype of adenovirus. *Lane M,* molecular size markers (New England Biolabs 100-bp DNA ladder).

novirus. Furthermore, predominant agent of EKC belongs to subgenus D group.[15] A recent study showed that Ad37 is the major cause of EKC in the northern part of Japan, whereas Ad8 in the southern part.[16] Our study in South Kyushu of Japan revealed that Ad8 was detected as a major agent of keratoconjunctivitis, with almost negligible numbers of Ad19 and Ad37. For the last few years, Ad9 and Ad17 were recovered from the patients with keratoconjunctivitis. This warrants that these less common serotypes may lead to an outbreak in the future.

Culture-NT is considered to be the "gold standard" for the identification of adenovirus. However, 2 to 4 weeks are required for the whole procedure. Furthermore, a successful cell culture heavily depends on a careful transport and storage procedure. Occasional cross-reaction among the serotypes often leads to a wrong identification of isolates. Therefore, PCR-based identifications of common serotypes, such as type-specific PCR, PCR-RFLF, and PCR with direct DNA sequencing, were in demand as a rapid, alternative methods to culture-NT.[16–18] Type-specific PCR targets type-specific sites in the hypervariable region of the hexon gene. Because of the genetic variation of the target region among the different strains of the same serotype, this approach is prone to false-negative failure. For hexon-based PCR-RFLP, a nested PCR procedure is required. Identification of serotype is done by PCR and DNA sequencing that targets the hypervariable region of the hexon, where sequence of the prototypes and clinical samples are aligned to type the clinical samples. Because there are genetic variation in the hypervariable region, this method tends to be unrefined and requires expensive instrumentation and technical skill. Furthermore, these methods above only identify common EKC strains (Ad8, Ad19, and Ad37). Three days are required to yield a result. If less common serotypes (Ad9, Ad15, Ad22, and Ad39) are causing an outbreak of EKC, rapid agent identification and epidemic control may be difficult through these procedures. Our new method offers some advantages over previously described rapid identification methods, regarding its simplicity, rapidity, and cost-effectiveness. It could identify a wide range of nine serotypes that includes not only common and less-common agents of EKC, but also other serotypes associated with nonspecific follicular conjunctivitis.

Heat-mediated rupture of viral capsids to expose viral DNA for enzymatic action has been described.[20] Direct heat treatment of clinical samples at 97°C for 15 minutes was found to be equally effective as the guanidine thiocyanate glass powder method, which is rather expensive and time consuming. The higher success rate of the heat method may be due to the less inhibitory substances found in conjunctival swabs.[10] Therefore, our study used the direct heat method for all clinical samples. Very sensitive results of our fiber gene-based PCR using DNA extracted by the direct heat method indicate that this simple extraction method is quite useful.

Fiber genes of subgenus D are targeted for designation of the primers that are not significantly homologous among other subgenus.[21] The nucleotide sequence of the fiber gene contains 5′ noncoding region, fiber-coding region, and a 3′ noncoding region. Regarding designation of the primers, the fiber gene sequences of Ad8, Ad9, Ad15, Ad19, and Ad37 derived from GenBank were compared. The forward and reverse primers, AF2/AR2 are located in the 5′ coding and the 3′ noncoding regions and are found to be conserved among the serotypes

Table 2. Comparison of Subgenus D Adenovirus Detection

Sample	Year of Collection	PCR-REA	PCR-RFLP	Culture
1	1998	Ad37	Ad37	Ad37
2	1998	19	19	19
3	1998	19	19	19
4	1998	8	8	(−)
5	1998	8	8	8
6	1998	8	8	(−)
7	1998	8	8	8
8	1998	8	8	(−)
9	1998	8	8	(−)
10	1998	8	8	8
11	1998	8	8	(−)
12	1998	8	8	8
13	1998	8	8	(−)
14	1998	8	8	(−)
15	1998	8	8	8
16	1998	8	8	8
17	1998	8	8	(−)
18	1998	8	8	8
19	1998	8	8	(−)
20	1998	8	8	(−)
21	1998	8	8	8
22	1998	8	8	8
23	1998	8	8	8
24	1998	8	8	8
25	1999	8	8	(−)
26	1999	8	8	8
27	1999	8	8	(−)
28	1999	8	8	(−)
29	1999	8	8	(−)
30	1999	8	8	(−)
31	1999	8	8	(−)
32	1999	8	8	8
33	1999	8	8	8
34	1999	8	8	(−)
35	1999	8	8	8
36	1999	8	8	8
37	1999	8	8	8
38	1999	8	8	8
39	1999	8	8	8
40	1999	8	8	(−)
41	1999	8	8	8
42	1999	8	8	8
43	1999	8	8	8
44	1999	8	8	8
45	1999	8	8	8
46	1999	8	8	(−)
47	1999	8	8	8
48	1999	8	8	8

(−), Culture isolation negative.

described here. Lengths of the fiber gene between the primers vary among the serotypes. The amplified product is approximately 1150 bp long and yields very little variation in lengths among the serotypes, although these differences do not help visual recognition of serotypes by PCR.

This long amplicon has multiple restriction sites for different restriction enzymes. We chose the most appropriate three restriction enzymes. When the amplified product is digested with three restriction enzymes (*Dde*I, *Hin*fI, and *Rsa*I), different serotypes could be discriminated by their polymorphic patterns. All serotypes were digested well except Ad22, which has no restriction site for *Rsa*I.

The reliability of this new method was evaluated using clinical specimens. In comparison with culture isolation and hexon-based PCR method, our PCR method has a sensitivity of almost 100%. REA was performed on PCR positive samples, and the results were completely identical with those obtained by NT after cell culture isolation and hexon-based RFLP method.

The specificity of the PCR method was determined against other subgenus of adenovirus and nonadenoviral DNA. The lack of amplified products indicated a high level of specificity of the test.

The minimum limit of the detection level of our PCR was 10^2 copies of viral DNA. It is not known how many copies of viral DNA are needed to induce conjunctivitis. However, the minimum limit of 10^2 copy level is more sensitive than culture isolation.[17]

DNA preparation by heating and a single-round PCR enabled us to prepare a result in 1 day. Our new fiber-based PCR-REA method could detect and type nine serotypes of subgenus D adenoviruses within 1 day compared with the need of 2 to 4 weeks for conventional cell culture isolation-NT and 3 days with the hexon-based PCR-RFLP method. Our method, thus, can provide a faster, more sensitive, cost-effective tool for detecting several types of subgenus D adenoviruses.

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