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# Multiplex PCR Assay for Rapid Identification of Oculopathogenic Adenoviruses by Amplification of the Fiber and Hexon Genes

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**Eye infections caused by adenovirus (Ad) often result in nosocomial infections and community epidemics with significant rates of morbidity. No antiviral agent effective against Ad is yet available for clinical use. Therefore, early diagnosis is still the mainstay for patient management and the prevention of epidemics. A multiplex PCR assay based on amplification of a combination of the fiber and hexon genes which can identify the six important oculopathogenic serotypes of Ads (Ad serotype 3[Ad3], Ad4, Ad7, Ad8, Ad19, and Ad37) in a single-tube amplification reaction was developed. Ad serotypes could be distinguished by the different amplicon sizes. The assay correctly identified prototype strains as well as isolates in clinical specimens. In comparison with a previously described PCR-restriction fragment polymorphism method, our assay gave unequivocal results for clinical specimens. Our multiplex PCR has the potential to serve as a rapid and cost-effective tool for the typing of important ocular Ads.**

Human adenovirus (Ad) comprises 51 serotypes that are divided into six species (formerly subgenera A to F) (3) and is associated with a variety of diseases that mainly affect the respiratory, ocular (eye), and gastrointestinal systems (6, 7, 23). The eye infections caused by Ads include epidemic keratoconjunctivitis (EKC), pharyngoconjunctival fever, and nonspecific follicular conjunctivitis. Among these ocular disease entities, EKC is a highly contagious disease that is frequently spread nosocomially and that frequently causes community epidemics (9, 16). Merely six serotypes, Ad serotype 3 (Ad3), Ad4, Ad7, Ad8, Ad19, and Ad37, account for as many as 89% of cases of adenoviral conjunctivitis (22, 29). In patients with EKC, severe bilateral conjunctivitis with substantial corneal and extraocular manifestations results in absence from work and school, with the associated negative impacts on the commercial sector and the educational system (5, 16, 24). No antiviral agent effective against strains responsible for EKC is available. Thus, rapid and accurate methods for confirmation of the clinical diagnosis and identification of the strains that cause EKC are needed so that measures against the spread of the disease can be taken early in the course of infection.

Culture of Ads in various cell lines and observation of a cytopathic effect are routinely used for virus detection, and the neutralization test (NT) is routinely practiced for identification of the serotype (11, 27). In some cases the hemagglutination inhibition assay is performed, in addition to NT (10, 20). Although the methods mentioned above are sensitive, a lack of rapidity makes them impractical in clinical situations. Nowadays, with the advent of PCR, a variety of Ad serotype identification methods have been developed, such as type-specific PCR, a combination of PCR and restriction enzyme analysis, and typing by sequencing of the PCR product (17, 19, 21, 25). None of these methods are simple and rapid. In all cases nested PCR is necessary to increase the sensitivity of the type-specific PCR and to obtain a sufficient amount of DNA, required both for the cycle sequencing reaction in DNA sequencing analysis and for restriction endonuclease analysis by PCR-restriction fragment length polymorphism (RFLP) analysis. Identification of serotypes by direct sequencing of the PCR product is not only expensive but also requires technical skill and instrumentation. Despite the proven value of the PCR-RFLP method in molecular epidemiological studies, interpretation of the restriction patterns of nested PCR products digested with multiple enzymes could occasionally be cumbersome.

The hexon and fiber proteins are the major capsid proteins of Ad. Hypervariable regions of the hexon protein carry neutralization epitopes that react with neutralizing antibody in NT, and the fiber protein is responsible for hemagglutination. Together, they determine the serotype specificity of Ad (4, 15). Therefore, the genes encoding the hexon and fiber proteins are genomic sites suitable for analysis for the genetic discrimination of Ad serotypes. In our previous study (1), the fiber gene was successfully used as a site for the type-specific identification of species C Ads. To enhance our previous effort and address the problems associated with the existing methods for the identification of ocular Ads, a combination of primers that specifically amplify the fiber and hexon genes was used to discriminate common EKC-causing strains of Ads by multiplex PCR. The accuracy and reproducibility of the test were confirmed with field isolates and directly with clinical samples that were previously tested by culture-NT or PCR-RFLP analysis.

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## MATERIALS AND METHODS

**Prototypes, field isolates, and clinical specimens.** Prototype Ad strains were obtained from the American Type Culture Collection (ATCC; Rockville, Md.). Twenty-seven field isolates of the following serotypes from the National Institute

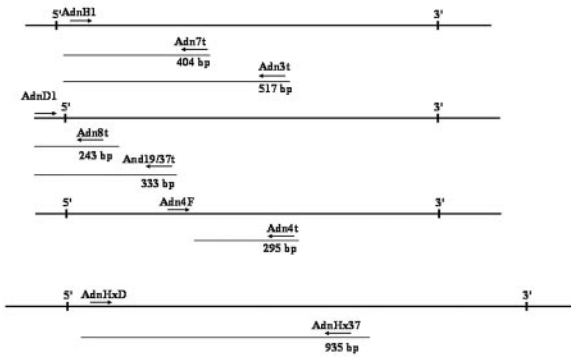


FIG. 1. Fiber gene sequence-based forward primers AdnB1, AdnD1, and Adn4F are specific and common for species B (Ad3, Ad7), D (Ad8, Ad19, and Ad37), and E (Ad4), respectively. The reverse primers Adn3t, Adn4t, Adn7t, and Adn8t are specific for Ad3, Ad4, Ad7, and Ad8, while Adn19/37t is common for both Ad19 and Ad37. The hexon gene-based primer set AdHxD-AdHx37t is specific for Ad37.

of Infectious Diseases (Tokyo, Japan) were used in this study: Ad3, nine samples; Ad4, one isolate; Ad7, two isolates; Ad8, nine isolates; Ad19, two isolates; and Ad37, four isolates. In addition, 35 Ad-positive conjunctival scrapings from patients with EKC caused by the following Ads, as identified by the PCR-RFLP method, were used: Ad3, 2 samples; Ad4, 1 sample; Ad8, 22 samples; Ad19, 4 samples; and Ad37, 6 samples.

**Preparation of viral DNA. (i) Prototypes and field isolates.** Viral DNA was prepared by phenol-chloroform extraction and ethanol precipitation, as described previously (1). In a microcentrifugation tube, 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, 200 μg of proteinase K per ml) were mixed and incubated for 1 h at 37°C. Then, 20 μg of RNase A (Roche, Mannheim, Germany) was added to the tube and the mixture was incubated at 37°C for another hour. After incubation, the contents were extracted with same volume of a phenol-chloroform mixture (phenol, 100 μl; chloroform, 100 μl) for 10 min and, centrifuged at 8,000 × *g* for 10 min. This extraction process was repeated once. Finally, the genomic DNA in the supernatant was precipitated with 500 μl of absolute ethanol. After the pellet was dried, it was dissolved in 30 μl of distilled water.

**(ii) Clinical specimens.** DNA extraction was carried out with a magnetic particle separator (Toyobo, Osaka, Japan). Briefly, 150 μl of a clinical sample, 750 μl of lysis buffer, and 40 μl of magnetic beads were placed in a microcentrifuge tube and the contents were mixed with a micromixer for 10 min to attach the genomic DNA to the magnetic beads. Subsequently, the beads with DNA were separated from the rest of the fluid with a magnet stand. Then, the beads were washed once with 900 μl of washing buffer. After the beads were washing twice with 70% alcohol, they were dried. Thirty microliters of double-distilled water was added, and the mixture was heated at 56°C for 10 min. The DNA was separated and collected in a microtube.

**Detection of adenovirus. (i) Primers.** Primer selection was based on the alignments of the human Ad fiber gene and the human Ad hexon gene. The GenBank accession numbers of the fiber gene sequences of the different human Ad serotypes were as follows: Ad3, m12411 and x01998; Ad7, z48954 and m23696; Ad11, l08232; Ad14, ab065116; Ad16, u06106; Ad21, u06107; Ad34, u10271; Ad35, u32664 and u10272; Ad4, x76547; Ad8, x74660; Ad9, x74659; Ad15, x72934; Ad17, af108105; Ad19, u69130, u69131, and x94485; Ad28, y14242; and Ad37, u69132 and x94484. The GenBank accession numbers of the hexon gene sequences of the different human Ad serotypes were as follows: Ad8, x74663; A9, x74664; Ad10, ab023548; Ad15, x74666; Ad19, x98539; Ad22, ab023551; and Ad37, x98360. Three sets of primers specific for the fiber gene and one set of the primers specific for the hexon gene were designed to detect the serotypes belonging to species B (Ad3 and Ad7), D (Ad8, Ad19, and Ad37), and E (Ad4). The forward primers were common within a species, and the reverse primers were type specific (Fig. 1; Table 1).

**(ii) PCR.** PCR amplification was carried out with 50-μl reaction mixtures containing viral DNA (2-μl aliquots of DNA from prototype and field isolates and 10-μl aliquots of DNA from clinical samples), 5 μl of 10-fold-concentrated buffer, 0.2 μM each primer, 200 μM each deoxynucleoside triphosphate, and 1.25 U of *Taq* polymerase (Roche). DNA extracted from the conjunctival scraping of a healthy eye was used as a negative control. The assays were performed in a programmable heat block (model 9600-R; Perkin-Elmer, Norwalk, Conn.). Thermal cycling consisted of preliminary denaturation for 3 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, with a final product extension at 72°C for 7 min. Five microliters of the reaction product was mixed with 1 μl of loading buffer (60% glycerol, 0.25% bromphenol blue, 0.25% xylene cyanol), and then the mixture was run on a 1.5% horizontal agarose gel (containing ethidium bromide at 1 μg/ml) at 100 V for 50 min in 50 mM Tris-borate-EDTA buffer (pH 8.0). The bands were visualized with a UV transilluminator and photographed with a charge-coupled-device camera.

**(iii) Specificity of detection.** The specificity of the test was determined by performing the test with other rare oculopathogenic Ads and non-Ad DNA from other agents of conjunctivitis, such as herpes simplex virus types 1 and 2, enterovirus, and *Chlamydia trachomatis*.

**(iv) Limits of detection.** The limit of detection of our multiplex PCR assay for each serotype was determined by amplification of a known amount of a serial 10-fold dilution of purified DNA from Ad3, Ad4, Ad7, Ad8, Ad19, and Ad37. Then, the formula of Uchio et al. (26) was used to calculate the minimum number of Ad genome copies amplified. After PCR amplification, 5 μl of product was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

TABLE 1. Oligonucleotide primers for multiplex PCR

| Serotype | Primer | Polarity | Position[a] | Sequence | Amplicon size (bp) |
|---|---|---|---|---|---|
| Ad3 | AdnB1 | + | 292–312 | 5′-TAC CCY TAT GAA GAT GAA AGC-3′ | 517 |
| | Adn3t | − | 808–786 | 5′-TGT TTT TAA ATA AGG TGT TAA CG-3′ | |
| Ad7 | Adn7t | − | 2623–2601 | 5′-GCC ATT ATT TGA CAG TTG GCT GT-3′ | 404 |
| Ad4 | Adn4F | + | 695–716 | 5′-ACC AGT AGT ACA GAA ACA GGA G-3′ | 295 |
| | Adn4t | − | 989–968 | 5′-CTT GAG CAC TGC TTA CTG TGC C-3′ | |
| Ad8 | AdnD1 | + | 1–22 | 5′-AAG GGA TGT CAA ATT CCT GGT C-3′ | 243 |
| | Adn8t | − | 243–225 | 5′-CCT TGA GTG ATA CAT TTT G-3′ | |
| Ad19 | Adn19/37t | − | 333–309 | 5′-GCA AGC TCA AGT TTT TTA TCA GTA T-3′ | 333 |
| | AdHxD | + | 34–51 | 5′-AGC TTC AAA CCC TAC TCG-3′ | |
| Ad37 | AdHx37t | − | 968–946 | 5′-ACT CCG CCT CCT GTT TGT ACA GG-3′ | 935 |

[a] The nucleotide positions of AdnB1 and AdnD1 refer to those in the fiber gene sequences of Ad3 (GenBank accession number x01998) and Ad8 (GenBank accession number x74660), respectively. The nucleotide positions of the other primers are located in the fiber gene sequences of the respective prototypes. The nucleotide positions of AdHxD and AdHx37t refer to those in the hexon gene sequence of Ad37 (GenBank accession number x98360).

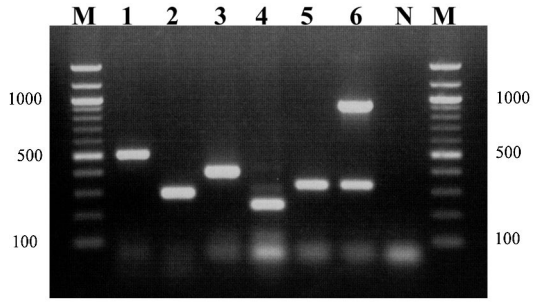


FIG. 2. PCR amplification of fiber gene from prototype serotypes (Ad3, Ad7, Ad4, Ad8, Ad19, and Ad37). Lanes 1 to 6, prototype strains of Ad3, Ad4, Ad7, Ad8, Ad19, and Ad37, respectively; lanes M, molecular size markers (100-bp DNA ladder; New England Biolabs); lane N, negative control. Values to the left and right of the gel are in base pairs.

**Comparison of PCR assays.** The hexon gene-based PCR-RFLP method described previously (21) was selected to validate our PCR method. By the PCR-RFLP method, a nested PCR is followed by digestion of the 956-bp positive PCR product with three restriction enzymes, HaeIII, HinfI, and EcoT14I. For typing, the restriction patterns of the different strains are compared with the pattern of the prototype.

**Culture and typing of virus.** All clinical samples were seeded onto a confluent monolayer of A549 or Caco2 cells that had been 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 after four passages, the samples were considered cell culture negative. For typing by NT, viral titers were determined in a 96-well microtiter plate containing a confluent monolayer of A549 cells. Twenty-five microliters of 100 tissue culture infective doses of virus was incubated at 37°C for 1 h with 25-μl volumes of serially twofold-diluted rabbit antiserum obtained from ATCC and inoculated in A549 cells. The Ad type was determined with antiserum (ATCC), which completely inhibited viral growth.

## RESULTS

**Multiplex PCR assay. (i) Prototype viruses.** The fiber gene sequences are 100% homologous between Ad19a and Ad37. Therefore, another set of primers specific for the hexon gene of Ad37 was designed for discrimination of these two serotypes. The combination of four primer sets (three primer sets specific for the fiber gene and one primer set specific for the hexon gene) allowed amplification of products of the appropriate length from six prototype viruses (Ad3, Ad4, Ad7, Ad8, Ad19, and Ad37) (Fig. 2). Ad3, Ad4, Ad7, Ad8, and Ad19 (or Ad37) gave products of 517, 295, 404, 243, and 333 bp, respectively. The hexon gene sequence-based primers yielded an additional 935-bp amplicon for Ad37. The presence of double amplicons in Ad37, one of 333 bp from the fiber gene and one of 935 bp from the hexon gene, clearly distinguished Ad37 from Ad19a.

**(ii) Field isolates and viruses in clinical samples.** Similar to the prototype isolates, 27 field isolates (Table 2) yielded clearly visible PCR products of the expected lengths (Fig. 3). All 35 clinical specimens, including the 9 culture-negative ones (Table 3), also had positive results. Thus, our fiber gene-based PCR successfully identified all the field isolates as well as the viruses in the clinical specimens.

**Specificity and limits of detection.** No amplified products were identified when the specificity of the PCR was tested by performing the PCR with other Ad and non-Ad DNA samples.

TABLE 2. Comparison of types determined by fiber gene-based multiplex PCR assay with hexon gene-based PCR-RFLP method performed with Ad isolates

| Isolate | Original serotype identification | Fiber gene-based multiplex PCR result | Hexon gene-based PCR-RFLP result |
|---|---|---|---|
| YC 64-222 | Ad3 | Ad3 | Ad3 |
| YC 64-224 | Ad3 | Ad3 | Ad3 |
| YC 64-225 | Ad3 | Ad3 | Ad3 |
| YC 64-227 | Ad3 | Ad3 | Ad3 |
| YC 64-232 | Ad3 | Ad3 | Ad3 |
| YC 64-235 | Ad3 | Ad3 | NI[a] |
| YC 64-238 | Ad3 | Ad3 | Ad3 |
| YC 88-87 | Ad3 | Ad3 | Ad3 |
| YC 86-88 | Ad3 | Ad3 | Ad3 |
| YC 86-163 | Ad4 | Ad4 | Ad4 |
| YC 92A-383 | Ad7 | Ad7 | NI |
| YC 95C-Bal | Ad7 | Ad7 | Ad7 |
| Miya-7 | Ad8 | Ad8 | Ad8 |
| Miya-10 | Ad8 | Ad8 | Ad8 |
| Miya-12 | Ad8 | Ad8 | Ad8 |
| Miya-15 | Ad8 | Ad8 | Ad8 |
| Miya-22 | Ad8 | Ad8 | Ad8 |
| Miya-32 | Ad8 | Ad8 | Ad8 |
| Miya-35 | Ad8 | Ad8 | Ad8 |
| Miya-44 | Ad8 | Ad8 | Ad8 |
| Miya-48 | Ad8 | Ad8 | Ad8 |
| YC 85-80 | Ad19 | Ad19 | Ad19 |
| YC 85-81 | Ad19 | Ad19 | Ad19 |
| YC 81-116 | Ad37 | Ad37 | Ad37 |
| YC 81-117 | Ad37 | Ad37 | Ad37 |
| YC 82-132 | Ad37 | Ad37 | Ad37 |
| YC 85-204 | Ad37 | Ad37 | Ad37 |

[a] NI, not identified.

After serial 10-fold dilution of purified Ad3, Ad4, Ad7, Ad8, Ad19, and Ad37 DNA, amplified products could be detected at a dilution of $1:10^8$, which represents 38.4 fg of Ad DNA. This quantity of DNA corresponds to 100 genome copies (data not shown).

**Comparison of multiplex PCR assay with PCR-RFLP method.** Our multiplex PCR method yielded the same results (100%) as the PCR-RFLP method for the identification of the viruses in 35 clinical specimens. It also correctly identified all

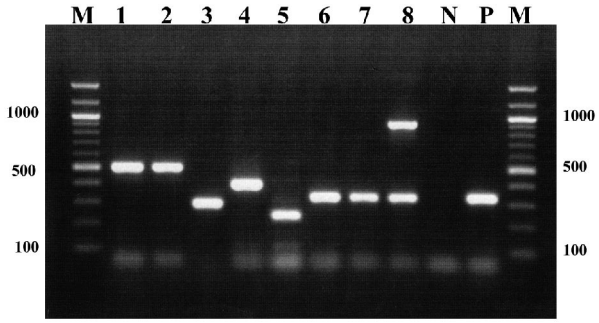


FIG. 3. PCR amplification of fiber gene from field isolates. Lanes 1 to 8, Ad3 (strain YC 64-222), Ad3 (strain YC 64-227), Ad4 (strain YC 86-163), Ad7 (strain YC 95c-Bal), Ad8 (strain Miya-32), Ad19 (strain YC 85-80), Ad19 (strain YC 85-81), and Ad37 (strain YC 81-117), respectively; lane N, negative control; lane P, positive control (Ad19); lanes M, molecular size markers (100-bp DNA ladder; New England Biolabs). Values to the left and right of the gel are in base pairs.

TABLE 3. Comparison of fiber gene-based multiplex PCR assay with hexon gene-based PCR-RFLP method performed with clinical specimens

| Strain | Hexon gene-based PCR-RFLP result | Fiber gene-based multiplex PCR result | Culture-NT result |
|---|---|---|---|
| M 98-5 | Ad3 | Ad3 | Ad3 |
| M 98-6 | Ad4 | Ad4 | Ad4 |
| M 98-8 | Ad8 | Ad8 | –[a] |
| M 98-9 | Ad8 | Ad8 | –[a] |
| M 98-11 | Ad19 | Ad19 | Ad19 |
| M 98-13 | Ad3 | Ad3 | Ad3 |
| M 98-17 | Ad8 | Ad8 | – |
| M 99-25 | Ad8 | Ad8 | – |
| M 99-27 | Ad8 | Ad8 | – |
| M 99-34 | Ad19 | Ad19 | Ad19 |
| M 99-40 | Ad8 | Ad8 | Ad8 |
| M 99-45 | Ad8 | Ad8 | Ad8 |
| M 99-51 | Ad8 | Ad8 | Ad8 |
| M 99-57 | Ad8 | Ad8 | Ad8 |
| M 99-61 | Ad8 | Ad8 | Ad8 |
| M 99-62 | Ad8 | Ad8 | – |
| M 99-63 | Ad8 | Ad8 | – |
| M 99-64 | Ad8 | Ad8 | Ad8 |
| M 99-65 | Ad8 | Ad8 | Ad8 |
| M 99-71 | Ad8 | Ad8 | – |
| M 99-72 | Ad8 | Ad8 | Ad8 |
| M 99-73 | Ad8 | Ad8 | Ad8 |
| M 99-74 | Ad8 | Ad8 | Ad8 |
| M 99-75 | Ad8 | Ad8 | Ad8 |
| M 99-77 | Ad8 | Ad8 | – |
| M 99-79 | Ad8 | Ad8 | Ad8 |
| M 99-87 | Ad8 | Ad8 | Ad8 |
| Tok-3 | Ad37 | Ad37 | Ad37 |
| Tok-8 | Ad37 | Ad37 | Ad37 |
| Tok-9 | Ad37 | Ad37 | Ad37 |
| Tok-12 | Ad37 | Ad37 | Ad37 |
| Tok-13 | Ad37 | Ad37 | Ad37 |
| Tok-23 | Ad19 | Ad19 | Ad19 |
| Tok-55 | Ad19 | Ad19 | Ad19 |
| Tok-202 | Ad37 | Ad37 | Ad37 |

[a] –, culture isolation negative.

27 field isolates. However, the PCR-RFLP method was unable to identify the restriction patterns of two isolates (isolates YC 64-235 and YC 92A-383).

## DISCUSSION

A highly sensitive and specific multiplex PCR assay based on the combination of the fiber and hexon genes has been developed for the rapid identification of important strains of Ads that cause EKC, namely, Ad3, Ad4, Ad7, Ad8, Ad19, and Ad37. A simple glance at the amplicon size and number is enough to determine the serotype without the need to rely on further restriction enzyme analysis or sequencing of the PCR product. This one-step, single-tube amplification reaction not only minimizes the chance of contamination but also saves time and is cost-effective.

The nucleotide sequences of the fiber genes of the different Ad serotypes share low degrees of homology (18). This unique characteristic was used to design primers specific for the six serotypes (Ad3, Ad4, Ad7, Ad8, Ad19, and Ad37) belonging to three different species, species B, D, and E. Each forward primer is specific for a particular species, and the sequence of that primer is shared among the serotypes within the species. The mismatch of the nucleotide sequences at the 3′ end of the reverse primer allowed the discrimination between homologous DNA sequences. Among the different genome types of Ad19, only Ad19a is associated with EKC.

Our method correctly identified all the field isolates by detection of amplicons of the expected lengths. The viruses in all clinical samples, including both isolation-positive and isolation-negative samples, were also clearly identified. All the isolation-negative samples contained Ad8, which could not be isolated possibly due to the fastidious nature of this serotype (13). Other reasons why the culture-negative samples were PCR positive may have been the loss of viable virus during any of the processes involved with sample collection, storage, and transport. For the clinical samples, the results of our assay were identical to those of the PCR-RFLP method. However, two isolates could not be typed by the PCR-RFLP method due to noninterpretable restriction patterns. Both patterns were different from the previously described prototype patterns. This result indicates that our method is more sensitive than culture isolation and more specific than the PCR-RFLP method.

The specificity of the assay was determined in tests with the DNA of other rare ocular Ads and non-Ads. The lack of amplified products indicated that the assay has a high level of specificity.

In comparison to other type-specific multiplex PCR methods (8, 14, 28), our assay offers some distinctive characteristics, such as the fact that (i) our assay is primarily a fiber gene-based method, whereas others are based on the hexon gene; (ii) the new method targets ocular Ads, while other assays have mainly targeted the respiratory Ads; and (iii) the minimum limit of detection of our PCR was $10^2$ copies of viral DNA, suggesting that it has a higher sensitivity than other methods. With the use of quantitative PCR, it may be possible to detect even smaller numbers of DNA copies; but the sophisticated PCR machine required for quantitative PCR is expensive and is still generally beyond the reach of many laboratories. Again, although primers specific for rare strains of ocular Ads are not included in this assay, this fact still does not diminish the potential utility of the assay, as only six epidemic strains are responsible for about 90% of ocular infections.

In EKC, Ads are usually spread from contaminated ophthalmic instruments, such as tonometers, used to measure ocular pressure, and eye drops shared by patients. Nosocomial spread among inpatients may even necessitate temporary closure of an ophthalmic unit, and outpatient clinics may be the cause of community epidemics. The clinical presentations of patients with eye diseases caused by herpes simplex virus, enteroviruses such as enterovirus 70 and the coxsackie A virus serotype 24 variant, and chlamydia occasionally mimic those of patients with keratoconjunctivitis caused by Ad (2, 9, 12). Therefore, in ophthalmic practice, rapid identification of Ads directly from ocular specimens is also important for proper therapy. By our method, 1 day is enough to obtain a result for clinical specimens, and our method will provide a convenient tool to prevent the spread of the disease as well as epidemiological study of important ocular Ad serotypes.

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