Rapid Diagnosis of Adenoviral Conjunctivitis by PCR and Restriction Fragment Length Polymorphism Analysis

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To detect and identify adenovirus (Ad), we used a combination of PCR and restriction fragment length polymorphism (RFLP) analysis. Nested PCR with two primer sets that hybridize to the conserved region for hexon proteins of 14 prototypes of Ad, Ad serotype 1 (Ad1) to Ad8, -11, -14, -19, -37, -40, and -41, amplified a 956-bp DNA fragment. The amplified fragments from the 14 prototypes were completely differentiated with a combination of three restriction endonucleases, EcoT141, HaeIII, and HindIII. We applied this new method for 127 samples of conjunctival scrapings from patients with conjunctivitis and compared the results with those obtained with the combination of culture isolation and a neutralization test (NT). PCR gave a positive result in 69 of 127 cases (54.3%), while only 61 of the 127 samples (48.0%) tested positive by culture isolation. Compared with isolation, the PCR method had a sensitivity of 100% (61 of 61). Positive PCR samples were further classified as Ad37 (59.5%), -3 (31.9%), -11 (4.3%), -8 (2.9%), and -4 (1.4%) by PCR-RFLP analysis. Of eight samples that were PCR positive and culture isolation negative, six were Ad37 and two were Ad8 by PCR-RFLP analysis. These differentiations of isolation-positive samples were identical to the results obtained by the NT. It took only 3 days to detect and identify Ad by PCR-RFLP analysis, whereas it took at least 3 weeks by culture isolation and NT. Our newly developed method of detecting and typing human Ad by PCR-RFLP analysis is more sensitive, accurate, and rapid than the conventional method of culture isolation and an NT.

Materials and Methods

Study group and specimens. The conjunctival scrapings used in this study were collected from June to October 1994 in hospitals in five cities of Japan (Sapporo, Morioka, Ashigara, Matsuyama, and Kumamoto) from 127 patients whose diagnoses were acute conjunctivitis, epidemic keratoconjunctivitis, pharyngconjunctival fever, or acute follicular conjunctivitis. The specimens were scraped from the lower palpebral conjunctiva with two cotton swabs and collected in two kinds of transport medium (4.5 ml), one for PCR (10 mM Tris [pH 8.0], 1 mM EDTA) and another for cell culture isolation (0.5% bovine serum albumin, 2.5% boulion, 0.01% gentamycin).

Ad1 to -8, -11, and -19 were obtained from the National Institute of Health (Tokyo, Japan). Ad14, -37, -40, and -41 were obtained from the American Type Culture Collection (Rockville, Md.). Ad1 to -8, -11, -14, and -19 were grown on a monolayer culture of HeP-2 cells in Eagle's minimum essential medium (MEM) (Nissui, Tokyo, Japan) supplemented with 2% fetal calf serum (CSL Limited, Parkville, Australia) and antibiotics. Ad37 was inoculated onto a monolayer culture of HEL cells, and Ad40 and -41 were inoculated onto a monolayer culture of 293 cells. Both of these cell types were in MEM supplemented with 2% fetal calf serum and antibiotics.

DNA preparation. (i) Clinical specimens. For DNA lysis, a 200-μl volume from a 4.5 ml PCR sample was centrifuged for 30 min at 12,000 × g. DNA was extracted from the pellet in 95 μl of lysis buffer (10 mM Tris-Cl [pH 8.3], 1 mM EDTA, 0.5% Tween 20) and 2 μl of proteinase K (15.5 mg/ml) for 1 h at 55°C, and then the solution was heated for 10 min at 95°C.

(ii) Ad prototypes. For DNA extraction, 200 μl of the supernatant of Ad prototypes (serotypes 1 to 8, 11, 19, 37, 40, and 41) was incubated in 174 μl of extraction buffer (0.1 M Tris-Cl [pH 8.0]), 0.01 M EDTA, 1% sodium dodecyl...
sulfate)-25.8 µl of proteinase K (15.5 mg/ml) for 4 h at 42°C. After phenol-
chloroform extraction and ethanol precipitation, the pellet was suspended in 50 µl of Tris-EDTA buffer (10 mM Tris [pH 8.0], 1 mM EDTA). The amount of suspension diluted 20-fold was determined spectrophotometrically, on the basis of a ratio of 50 µg/ml at an optical density at 260 nm of 1.0, and the DNA suspension was diluted to 10 pmol/µl.

**Ad detection. (i) Primers.** The choice of primers for nested PCR was based on comparisons of the hexon gene sequences derived from Ad2, -5 (-5), -40 (13), and -41 (12). The pair of primers for the first PCR (AdTU7 and AdTU4') was used to amplify a 1,004-bp product corresponding to bp 20,734 to 21,737, and the pair of primers for the second PCR (AdnU-S' and AdnU-A) was used to amplify a 956-bp product corresponding to bp 20,743 to 21,698 of the sequenced Ad2 genome (5) (Table 1).

(ii) PCR. The primers used for one-step amplification were AdTU7 and AdTU4', and those used for two-step amplification were AdnU-S' and AdnU-A. The first PCR amplification was performed with 10 µl volumes of prepared conjunctival-scrapping suspensions, undiluted or diluted 100-fold in sterilized water. PCR amplifications were conducted in 50-µl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 µM each deoxynucleoside triphosphate (i.e., dATP, dGTP, dCTP, and dTTP), 0.5 µM each primer needed for the specific reaction, and 1 U of thermostable Taq DNA polymerase (Roche Diagnostic Systems, Branchburg, N.J.). PCR was carried out in a Cetus 9600 thermal cycler (Perkin-Elmer Cetus, Emeryville, Calif.) by using a cycle of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and primer extension at 72°C for 2 min, for 36 cycles. After the last cycle, samples were extended at 72°C for 7 min.

As a positive control in all experiments, 1 pg of Ad5 DNA was used per reaction mixture. PCR mixtures with DNA of scrapings from unaffected eyes were used as negative controls.

The nested-PCR amplification was performed with 10 µl of one-step-PCR-amplified suspensions, undiluted or diluted 100-fold in sterilized water. This PCR amplification was conducted by using the same protocol as used for the first reaction. After the nested-PCR amplification, 5 µl of the reaction mixture was subjected to electrophoresis on a 3% agarose gel containing 0.5 µl of ethidium bromide per ml. Specificity of the nested PCR was evaluated by using nonadenoviral DNA samples from several causative agents of conjunctivitis. Viruses used were herpes simplex virus type 1 and 2 and cytomegalovirus, and bacteria used were *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *Corynebacterium diphtheriae*, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Chlamydia trachomatis*, and *Candida albicans*.

To establish the sensitivity of the nested PCR, further amplifications of known amounts of Ad3, -4, -8, -11, -19, and -37 DNAs were performed. After phenol-
chloroform extraction and ethanol precipitation, the DNA suspensions were diluted in 11 steps to obtain a theoretical range of virus particles from 10⁹ to 1 per reaction mixture. 0.384 fg of Ad DNA corresponds to a single copy of linear double-stranded DNA of about 35,000 bp. After the PCR amplification, 5 µl of the reaction mixture was subjected to electrophoresis on a 3% agarose gel containing 0.5 µl of ethidium bromide per ml.

(ii) Culture isolation. Ad cultures were performed at the Mitsubishi Kagaku Bio-Clinical Laboratories, Inc. (Tokyo, Japan), by using HEp-2 cells. HEL cells, and HeLa cells in tissue culture or microtiter plates. Of the 127 specimens, samples 1 through 90 were inoculated onto HEp-2 cells and HEL cells and samples 91 through 127 were inoculated onto HEp-2 cells, HEL cells, and HeLa cells. Cell cultures that did not show CPE but were PCR positive were passed 2 times, and those that were both CPE and PCR negative were passed 2 times. The CPE-negative samples were classified as cell culture negative. Infected cells were identified by the immunofluorescent-antibody technique with Ad monoclonal antibody (Chemicon International Inc., Temecula, Calif.).

**Ad typing. (i) PCR-RFLP analysis for Ad prototypes.** When 956-bp amplified products of the 14 prototypes were tested by our PCR-RFLP method, there were 7, 12 and 6 digestion patterns for EcoT14I, *Hae*III, and *Hin*II, respectively, and the 14 prototypes were completely differentiated by the combination of digestion patterns of these three endonucleases (Fig. 2).

(ii) PCR-RFLP analysis for clinical specimens. PCR-RFLP analysis was performed for 69 samples (54.3%) that were PCR positive, and they were identified as Ad37 (59.5% [41 of 69]), Ad3 (31.9% [22 of 69]), Ad11 (4.3% [3 of 69]), Ad8 (2.9% [2 of 69]), and Ad4 (1.4% [1 of 69]). Of eight samples that were...
PCR positive and cell culture negative, six were Ad37 and two were Ad8.

(iii) NT for cell culture-positive specimens. The 61 specimens that were culture isolation positive were identified as Ad37 (57.4% [35 of 61]), Ad3 (36.1% [22 of 61]), Ad11 (4.9% [3 of 61]), and Ad4 (1.6% [1 of 61]). The results of PCR-RFLP analysis of the 61 cell culture-positive samples were identical to the results of the NT.

**DISCUSSION**

We have established a new method to sensitively, accurately, and rapidly detect and identify human Ad from conjunctival scrapings by PCR-RFLP analysis. This method could completely differentiate 14 prototypes of Ad. The reliability of the new method was evaluated by using clinical specimens. In comparison with culture isolation, the PCR method had a sensitivity of 100% (61 of 61), showing that the method is highly accurate and sensitive. We also performed RFLP with PCR-positive clinical specimens, and the results were completely identical to those obtained by the NT following cell culture isolation.

To check the reaction of our PCR method with other microorganisms, we tested the nonadenoviral DNA samples listed in Materials and Methods and normal conjunctival tissue. The lack of amplified products demonstrated the high level of specificity of our primer sets.

The minimum limit of detection of our nested-PCR assay was $10^3$ copies of viral DNA (data not shown). Since it is unknown how many copies of adenoviral DNA are necessary to cause conjunctivitis, further research is desirable. However, the minimum limit of detection of $10^3$ copies was sufficient because the PCR assay was more sensitive than culture isolation.

The designation of primers for PCR in this study was based on comparison of the hexon gene sequences derived from Ad2, -5 (5), -40 (13), and -41 (12). Allard et al. (1) and Kinchington et al. (7) amplified 308- and 306-bp sequences of Ad DNA, respectively, which are partially located in the $\beta$-barrel-forming P1 domain within the 5' region of the hexon (11). On the other hand, Hierholzer et al. (6) amplified a 161-bp sequence of Ad DNA that is partially located in the P2 domain within the 3' region of the hexon (11). Our amplified fragment includes sequences of both domains (11) and is longer than those obtained by the other investigators. The aims of this research were to identify conjunctivitis-causing serotypes among six kinds (Ad3, -4, -8, -11, -19, and -37) and to differentiate Ads by digestion patterns of the long amplified fragment in PCR-RFLP analysis. In addition, Allard et al. (1) and Pring-Åkerblom et al. (10) developed type-specific PCR amplification assays. It is useful to differentiate Ad40 from Ad41, or to differentiate these two enteric Ads from other types of Ad. Since we know six serotypes that cause conjunctivitis, it is difficult to choose specific primers for each serotype. The PCR-RFLP assay that amplifies a long fragment consisting of many restriction endonuclease sites is probably a more useful method for identifying adenoviral conjunctivitis.

It has been reported that the fiber region contains one type-specific determinant, $\gamma$, which reacts with hemagglutination inhibition antibody (9). The results of PCR analysis with the fiber sequence might be different from ours with the hexon, which is responsible for Ad neutralization (9), and so further research is needed.

Ad is the most common agent of infectious conjunctivitis in Japan. The standard method for diagnosis of adenoviral conjunctivitis has been generally considered to be a combination of cell culture isolation and an NT. In contrast to the situation with stool, sputum, or urine samples, little proteinaceous materials inhibiting PCR exist in conjunctival-swab samples. Thus, direct PCR-RFLP analysis is probably the best way to diagnose infectious conjunctivitis. The PCR method not only is stable with respect to the biological properties, such as adaptability of cell culture isolation, but also is highly reproducible. Our PCR-RFLP method can accurately detect Ad that was not detected by the conventional method, for example, Ad37 and -8 in this study. Our method also has the advantage of detecting and typing Ad from clinical specimens in a shorter period than that required for cell culture. We intend to apply our PCR-RFLP analysis for Ad surveillance to study the worldwide epidemiology of adenoviral conjunctivitis.

Yamadera et al. (15) reported that 91% of viruses isolated from patients with conjunctivitis in the Ad surveillance study were Ads, whereas the PCR-positive rate for Ads was 54% in this study. Although our rate was lower, the purpose of this study was to evaluate the sensitivity of the PCR-RFLP method for clinical specimens collected from patients with acute follicular conjunctivitis and was not to survey adenoviral epidemiology. The difference was based on the kinds of pathogens and diseases; for example, allergic conjunctivitis was included in our study.

In our report, Ad37 was the predominant type and no Ad19 was found. We used the Ad19 prototype in this PCR-RFLP assay, whereas the results might be more confirmatory with Ad19a, which is a more significant cause of conjunctivitis than the prototype, which is no longer isolated. We will proceed with further research.

In conclusion, our new method of typing human Ad directly from conjunctival scrapings by PCR-RFLP analysis is more
sensitive, accurate, and rapid than the conventional cell culture isolation and NT.

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