Adenovirus Polymerase Chain Reaction Assay for Rapid Diagnosis of Conjunctivitis

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PURPOSE. To evaluate newly designed primers in a polymerase chain reaction (PCR) for the detection of adenovirus DNA in conjunctival swabs.

METHODS. Oligonucleotides were derived from the adenovirus hexon gene and modified such that a maximum of only two mismatches occurred with adenovirus types 2 through 5, 7, and 16. Specificity was determined against adenovirus types 2 through 4, 7, 8 through 11, 14, 19, 37, 40, and 41 and from non-adenoviral DNA and the sensitivity by PCR amplification of purified adenovirus type 2 DNA. The assay was compared retrospectively with cell culture and a PCR with different primers on 59 stored conjunctival swab samples. The new PCR also was used prospectively in comparison with cell culture on 2743 conjunctival swabs.

RESULTS. The 140-bp product was amplified from all the adenovirus serotypes tested except types 40 and 41, which have not been isolated from the eye. There were no amplified products from the non-adenoviral DNA tested. With adenovirus type 2 DNA, despite two deliberate mismatches, 40 copies of the target were detectable after PCR and ethidium bromide-staining. In the retrospective study, 51 of 55 (92.7%) were positive by this new PCR compared with 42 of 55 (76.4%) by the older PCR and 40 of 55 (72.7%) by cell culture. In the prospective study, the new PCR detected 386 of 415 (95%) adenovirus-positive specimens compared with 248 of 415 (59.8%) by cell culture. Of 167 specimens positive for herpes simplex virus by cell culture, none were positive by the adenovirus PCR.

CONCLUSIONS. PCR with the newly designed primers shows a much increased sensitivity over cell culture and previous PCRs for the detection of adenoviruses in conjunctival swabs. (Invest Ophthalmol Vis Sci. 1999;40:90–95)
swabs were from patients presenting with conjunctivitis and showing symptoms and signs consistent with a HSV or adenovirus etiology.

Sample Preparation
DNA was extracted from the viral transport medium with lysis buffer. Briefly, equal volumes (75 μl) of sample and lysis buffer (20 mM Tris-hydrochloric acid [pH 8.3], 2 mM EDTA, 1% Triton X-100, 0.002% sodium dodecyl sulfate, and 500 μg/ml proteinase K) were incubated at 56°C for 2 hours and then boiled for 10 minutes. For a small number of specimens, an alternative procedure was used in which 100 μl of the viral transport medium was mixed with 50 μl 30% polyethylene glycol (PEG-9000; Sigma Chemical, St. Louis, MO) in 3 M sodium chloride and kept on ice for 30 minutes. After centrifugation (10,000g, 15 minutes), the supernatant was removed, and the pellets were resuspended in 20 μl of 10 mM Tris-hydrochloric acid (pH 7.6), 0.5% Nonidet P-40 and incubated at room temperature for 10 minutes before adding 5 μl directly to the PCR mixture.

PCR Conditions
The reaction mixture for the PCR was composed of 10 mM Tris-hydrochloric acid (pH 8.3), 50 mM potassium chloride, 200 μM each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 0.2 μM primers, 1.25 U Taq DNA polymerase (Advanced Biotechnologies Ltd, Epsom, UK), and 5 μl appropriate DNA sample or sterile distilled water, as contamination control, to form a final volume of 50 μl. One drop of mineral oil (Sigma) overlay was added to each reaction mixture to prevent evaporation. The assay was performed on a Programmable Dri-Block Gene Ataq thermal cycler (Pharmacia LKB, Uppsala, Sweden) using one cycle each of 94°C for 7 minutes, 55°C for 1 minute, and 72°C for 1.5 minutes followed by 40 cycles each of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minute. The amplification products were analyzed by electrophoresis in 6% polyacrylamide gels, and the anticontamination measures in all the PCRs were as previously described. They included separate rooms for preparation of the reaction mixtures, for preparation and addition of DNA extracts, and for product analysis. Plugged pipette tips were used throughout.

Control DNA Samples
The control DNA samples were composed of adenovirus type 2 genomic DNA (GIBCO-BRL, Paisley, Scotland, UK) and DNA extracted from adenovirus strains derived from clinical isolates. DNA extracted from HSV type 1 strain Syn17 infected Vero cells; purified plasmid designated pC1.2 from Chlamydia trachomatis strain L2/434; and human fibroblast DNA.

Statistical Analysis
The results of the various assays of clinical specimens were compared using McNemar’s test. P < 0.05 was considered significant.

RESULTS

Primer Design
The two adenovirus primer pairs (H1 and H2, JCH1 and JCH2) evaluated on eye swabs in this laboratory had nucleotide sequences identical to those of adenovirus types 2 and 5 but showed, respectively, 4, 5, 3, and 2 mismatches with adenovirus type 3 (Fig. 1). This included a mismatch at the 3′ terminus of primer H2, a site crucial in the binding of Taq polymerase. Thus, the apparently lower sensitivity of PCRs using primers H1 and H2 rather than JCH1 and JCH2 for detection of subgenus B adenoviruses, and the suboptimal performance of the former primers with the same strains even after lowering of the annealing temperature to 40°C suggested that further improvement in the diagnostic performance of adenovirus DNA amplification required the design of a novel oligonucleotide pair showing minimal nonterminal sequence mismatching with the genomes of as many adenovirus types as possible. The recent release of partial sequences of hexon DNA for the subgenus B and E adenoviruses types 3, 4, 7, and 16 (PC/Gen CD-ROM, Release 14.0; Intelli Genetics, Oxford, UK) offered the possibility of deriving primers showing minimal mismatches with these sequences and those of the subgenus C adenoviruses types 2 and 5. Sequences of the new adenovirus primer set evaluated in this study (ADRJC1 and ADRJC2; Fig. 2) were derived from the highly conserved DNA region coding for the carboxyl end of the monomeric protein II that forms the trimeric pseudohexagonal base of the adenovirus hexon. This is the same region from which JCH1 and JCH2 primers were derived, and, indeed, ADRJC2 has considerable overlap with JCH1. The new primers yielded a product size of 140 bp. Mismatches were deliberately introduced into the primer sequence such that each primer had a maximum of two nonterminal mismatches when compared with known DNA sequences of the hexon proteins of adenovirus types 2 through 5, 7, and 16 (Fig. 2).

Sensitivity and Specificity of the PCR
The detection limit with primer set ADRJC1 and ADRJC2 was 40 molecules adenovirus type 2 genomic DNA per reaction mixture. The adenovirus PCR amplified control subgenus B (types 3, 7, 11, and 14), C (type 2), D (types 8 through 10, 19, and 37), and E (type 4) DNA but not subgenus F (types 40 and 41), control HSV, C. trachomatis plasmid or human DNA.

Retrospective Detection of Adenovirus
Of the 59 lysis buffer-extracted conjunctival swabs tested retrospectively using ADRJC1 and ADRJC2, 54 were from swabs positive for adenovirus by culture, or PCR, or both with the H1 and H2 primer pair at the time of their collection (Table 1). The remaining 5 extracts were negative by both of these tests. The new PCR detected 15 positive samples that were culture-negative and a total of 9 positive samples that were negative with the H1 and H2 primer pair. There were also 4 culture-positive specimens that were negative by both PCR primer sets. If all adenovirus culture-positive and all PCR-positive, culture-negative eye swabs were considered to indicate ocular adenovirus infection, then the new primer pair had the highest sensitivity. Thus, for ADRJC1 and ADRJC2, the sensitivity was 51 of 55 (92.7%), higher than H1 and H2 at 42 of 55 (76.4%, P = 0.004), and at culture at 40 of 55 (72.7%, P = 0.019). The sensitivities of culture and the PCR of primer pair H1 and H2 were not significantly different (P = 0.845).

Prospective Analysis of Clinical Samples
All eye swabs sent to the diagnostic virology laboratory for virus isolation over a 2-year period were subjected also to the
adeno
virus PCR. A total of 2743 specimens were tested over this period. These data are shown in Table 2, and an example of the PCR results is shown in Figure 3. The adeno
virus PCR detected 167 positive samples that were negative by culture compared with 29 culture-positive samples that were missed by the PCR. These 29 specimens were examined further. Four of the isolates were chosen at random (adenovirus type 3 = 3) and adenovirus type 4 (n = 11) and subjected to PCR. All four isolates were positive, indicating that failure to detect them in the original virus transport medium was not due to a sequence heterogeneity with the new primer pair. Of the 29 original specimens, 23 were available for retesting. After repeat extraction with lysis buffer, these specimens remained negative by PCR. However, when 15 of them were treated using the PEG precipitation method, 14 became positive by PCR, suggesting the presence of PCR inhibitors that were not being removed by the lysis buffer extraction, or both. The specimen that remained negative by PCR after both extraction procedures was an adenovirus type 1. If all the specimens that were adenovirus culture-positive or lysis buffer-extracted PCR-positive were taken to indicate ocular infection, then PCR was much more sensitive, detecting 386 of 2743 (14.1%) specimens. HSV was isolated from 167 of 2743 (6.1%) of the specimens.

**DISCUSSION**

The design of primers ADRJC1 and ADRJC2 achieved only two nonterminal mismatches when compared with the hexon DNA sequences of six adenovirus types (2 through 5, 7, and 16) representative of three of the four subgenera infecting the eye (B, C, and E). Despite these deliberate mismatches, the PCR was still capable of detecting 40 copies of adenovirus type 2 DNA. In the retrospective studies, the ADRJC1 and ADRJC2 primer pair identified 9 positive samples (8 of which were also culture-positive) that were not detected by the H1 and H2 primer pair, presumably reflecting the difference in primer-target mismatching of the two pairs of primers. An alternative approach would be the use of degenerate primers. However, these tend to be more useful in circumstances in which target copy numbers are relatively high because they can lack sensitivity. In addition, they require a more complex thermal cycling program and, because of the multiplicity of primers, can result in nonspecific product bands on the gel. Overall, adenovirus was isolated from 248 of 2743 (9.0%) of conjunctival swabs compared with the PCR, which detected adenovirus DNA in 386 of 2743 (14.1%) specimens. HSV was isolated from 167 of 2743 (6.1%) of the specimens.
for a busy routine diagnostic laboratory when tests are simple and straightforward.

The new PCR is significantly more sensitive than culture for the detection of adenovirus in conjunctival swabs. This may reflect a loss of viable virus during transport to the laboratory, a very slow growth rate of some strains of adenovirus, toxicity of the samples, and, occasionally, bacterial contamination of the cell cultures. The possibility of contamination of the PCR in culture-negative, PCR-positive specimens is unlikely because of the negative results of the contamination controls included in each PCR run, the technical precautions taken to minimize DNA carryover, the random distribution of these specimens, and the fact that of 167 swabs culture-positive for MSV, none were positive by the adenovirus PCR. Also, the culture-positive, PCR-negative specimens remained PCR-negative when reextracted with lysis buffer.

**TABLE 1. Retrospective Comparison of Virus Culture and Polymerase Chain Reaction with Either H1/H2 or ADRJC1/ADRJC2 Primer Pairs for Detection of Adenoviruses in Conjunctival Swabs**

<table>
<thead>
<tr>
<th>Virus Culture</th>
<th>H1/H2</th>
<th>ADRJC1/ADRJC2</th>
<th>No. of Specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>28</td>
</tr>
<tr>
<td>+</td>
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<td>+</td>
<td>8</td>
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<td>−</td>
<td>−</td>
<td>4</td>
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<td>+</td>
<td>+</td>
<td>14</td>
</tr>
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<td>−</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>−</td>
<td>4</td>
</tr>
</tbody>
</table>

+ = positive; − = negative (for adenoviruses).
and only became PCR-positive when concentrated by PEG precipitation.

In view of the latter observation we considered replacing the lysis buffer extraction method by PEG precipitation. In a small pilot study (results not shown), fewer positive specimens were detected with PEG than with lysis buffer. There is no perfect extraction system for PCR, and different methods remove a different range of nonspecific inhibitors. The pilot study suggests that those found in eye swabs are more likely to be removed by lysis buffer than PEG, but neither method is 100% successful. In addition, the PEG procedure requires more manipulations than the lysis buffer method, which is simpler and the one we continue to use.

The single-target PCR using primers ADRJC1 and ADRJC2 could replace H1/H2 PCR and cell culture for detection of adenoviruses in conjunctival swabs. No false-negative PCR results were obtained, confirming the success of the simple anticontamination measures used\(^{11,14}\) and the applicability of DNA amplification as a technique for the routine diagnosis of ocular infections. In addition, the PCR result can be available for the physician within 8 hours of the laboratory receiving the specimen compared with 1 to 4 weeks for conventional cell culture. However, we do continue to use cell culture for conjunctival swabs to detect HSV. After 1 week, by which time HSV will have become apparent, cultures are discarded except for those inoculated from swabs positive by the adenovirus PCR, where incubation is continued for a further 3 weeks. This enables us to isolate many of the adenoviruses detected by PCR that can then be typed for epidemiologic studies.

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**References**