Multiplex Polymerase Chain Reaction for Diagnosis of Viral and Chlamydial Keratoconjunctivitis

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PURPOSE. To develop a multiplex polymerase chain reaction (PCR) for the detection of adenovirus, herpes simplex virus, and Chlamydia trachomatis in conjunctival swabs.

METHODS. Oligonucleotide primers for detection of the 5 agents were combined in one reaction and evaluated for optimal performance using control DNAs of adenovirus type 2, herpes simplex virus, and C. trachomatis plasmid. The multiplex PCR was evaluated prospectively against its corresponding uniplex PCRs, virus isolation, Chlamydia Amplicor PCR, and an immunodot assay technique (immune dot blot test) in a total of 805 conjunctival swabs from patients with suspected viral and chlamydial keratoconjunctivitis.

RESULTS. The multiplex PCR was as sensitive as uniplex PCRs for the detection of the agents in clinical specimens. In the prospective study, 48 of 49 (98%) clinical specimens were positive for adenovirus by the multiplex PCR compared with 26 of 49 (53%) by adenovirus isolation. For herpes simplex virus detection, the multiplex PCR had a sensitivity of 92% (34/37) compared with 94.5% (35/37) by cell culture. The multiplex PCR produced identical results to the Amplicor PCR (21/21; 100%) compared with 71% (15/21) by the immune dot blot test.

CONCLUSIONS. With clinical specimens the multiplex PCR was as sensitive as its respective uniplex PCRs but more sensitive than adenovirus isolation and as sensitive as herpes simplex virus isolation or C. trachomatis Amplicor PCR. It has the potential to replace several diagnostic tests with consequent savings in cost. The test also reduces the risk of misdiagnosis by the clinicians. (Invest Ophthalmol Vis Sci. 2000;41:1818–1822)

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denovirus, herpes simplex virus (HSV), and Chlamydia trachomatis are common causes of keratoconjunctivitis (KC).1–3 Outbreaks of adenoviral KC occur throughout the world.3 HSV KC occurs in all countries and is the most common infectious cause of unilateral corneal blindness in the developed world.5 In the West, chlamydial KC is caused by C. trachomatis serovars D to K, and up to 90% of patients with chlamydial conjunctivitis experience concurrent genital infection.6–9

Several reports have demonstrated the diagnostic advantages of various laboratory techniques for optimal management of viral and chlamydial KC,10–20 but conventional laboratory techniques such as cell culture and antigen detection methods can be inefficient due to lack of sensitivity, specificity, and/or speed.21 During the last decade, however, several studies have concluded that polymerase chain reaction (PCR)--based laboratory investigation is a valuable approach for achieving reliable diagnosis of viral and chlamydial KC.21 In this article, we describe a simple and sensitive multiplex PCR, which detects adenovirus, HSV, and C. trachomatis in eye swabs from cases of KC.

METHODS

Clinical Specimens

A total of 805 eye swabs from 541 patients with suspected viral or chlamydial KC was tested prospectively for the presence of adenovirus, HSV, and C. trachomatis within 1 week of receipt. Of the 805 specimens, 456 in virus transport medium (Hanks’ balanced salt solution, pH 7.4, 10% [vol/vol] fetal calf serum, 2.5% [wt/vol] sodium bicarbonate, 200 IU/ml penicillin G, 200 mg/ml streptomycin, and 5 mg/ml amphotericin B) had investigation requests for viral etiology, whereas 349 specimens in sucrose phosphate (2SP) transport medium (8 mM KH2PO4, 12 mM K2HPO4, 0.2 M sucrose, 2.5 µg/ml amphotericin B, 50 µg/ml streptomycin, and 100 µg/ml vancomycin) had chlamydial investigation requests. All the specimens were tested for the three agents by the multiplex PCR and its corresponding uniplex PCRs. The 456 viral specimens were also tested for the presence of adenovirus and HSV by virus isolation, and the remaining 349 chlamydial specimens were tested for C. trachomatis by the Amplicor PCR and Immune Dot Blot (IDB) test.

Virus Isolation

Cell lines (Vero, HEL 229, and Hep-2) were immediately inoculated with the untreated eye swabs and maintained for up to 4 weeks to permit the detection of adenovirus and HSV.22–24
Multiplex PCR and IDB Test

Chlamydial swabs were tested using the Amplicor PCR assay (COBAS AMPLICOR; Roche Diagnostic Systems, Indianapolis, IN). This test targets the C. trachomatis plasmid but uses different primers to those incorporated in our multiplex PCR.

The IDB test was performed as described previously. Briefly, 0.4 ml of the specimen was digested with 250 μg/ml proteinase K for 60 minutes at 56°C and then heated to 95°C for 15 minutes. The treated specimen was added to a nitrocellulose membrane in a dot blot manifold, and the bound chlamydial lipopolysaccharide was then detected with an 125I-labeled genus-specific mouse monoclonal antibody.

Sample Preparation

DNA was extracted from all specimens, including negative material as extraction negative controls, using lysis buffer. Briefly, equal volumes (75 μl) of sample and lysis buffer (20 mM Tris–HCl [pH 8.3], 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. In addition, a few specimens were also extracted by guanidinium thiocyanate (GuSCN) and 30% polyethylene glycol (PEG).

Uniplex PCRs

All PCRs were carried out adhering to stringent precautions to avoid contamination. In a 50-μl PCR mixture, all uniplex PCRs contained 1× PCR buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl, 0.01% [wt/vol] gelatin), 1.5 mM MgCl₂, 2.2 μM of each primer of the primer pairs ADRJC1/ADRJC2 (adenovirus PCR), YS1/YS2 (HSV PCR), and KL1/KL2 (C. trachomatis PCR; Table 1) and 5 μl of appropriate DNA sample or sterile distilled water as a contamination control. The reaction was overlaid with 2 drops of mineral oil to prevent evaporation. The assays were performed on a Programmable Dri-Block PCH-1 (Techne, Cambridge, UK) using one cycle each of 94°C for 7 minutes, 55°C for 1 minute, and 72°C for 1.5 minute 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 8% polyacrylamide gels.

Multiplex PCR

The reaction conditions of the multiplex PCR were identical to those described for the uniplex PCRs except for the use of 2.5 U amplitaq DNA polymerase, 2.2 mM MgCl₂, the presence of equimolar concentrations (0.2 μM) of each primer of the primer pairs (ADRJC1/ADRJC2, YS1/YS2, and KL1/KL2), and the use of 50 cycles.

### TABLE 1. Properties of Oligonucleotide Primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Designation (mer)</th>
<th>Sequence (5′→3′)</th>
<th>Amplicon (base pair)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus hexon</td>
<td>ADRJC1 (29)</td>
<td>GACATGACTTTCGAGGTCGATCCCATGGA</td>
<td>140²⁸</td>
</tr>
<tr>
<td></td>
<td>ADRJC2 (26)</td>
<td>CCGGCTGAGAAGGGTGTGGCGAGGTA</td>
<td>211²⁹</td>
</tr>
<tr>
<td>HSV DNA polymerase</td>
<td>YS1 (18)</td>
<td>GACATGACTTTCGAGGTCGATCCCATGGA</td>
<td>140²⁸</td>
</tr>
<tr>
<td></td>
<td>YS2 (18)</td>
<td>CCGGCTGAGAAGGGTGTGGCGAGGTA</td>
<td>211²⁹</td>
</tr>
<tr>
<td>C. trachomatis plasmid</td>
<td>KL1 (20)</td>
<td>TCCGGACGAGT TAGAAGA</td>
<td>241²⁵¹</td>
</tr>
<tr>
<td></td>
<td>KL2 (20)</td>
<td>AATCAATGCGGGGATTGGT</td>
<td>241²⁵¹</td>
</tr>
</tbody>
</table>

### Control DNA Samples

Control samples were composed of adenovirus type 2 DNA, HSV type 1 DNA (Life Technologies, Paisley, UK), and the plasmid DNA (pCtL2) of the *C. trachomatis* strain L2/434.

### Statistical Analysis

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

### RESULTS

#### Sensitivity and Specificity

The development and optimization of both uniplex and multiplex PCRs required screening for optimal primers and adjustment of PCR conditions such as enzyme concentration and cycling profile. The primers ADRJC1/ADRJC2, YS1/YS2, and KL1/KL2 were found satisfactory and generated PCR products that can be differentiated on gel electrophoresis (Table 1). In addition, the primer pair ADRJC1/ADRJC2 demonstrated great inclusivity for detection of adenovirus serotypes representing all subgroups found in eyes.

In the presence of a single target, the multiplex PCR produced detection limits of 4 × 10³, 3 × 10², or 10² copies of adenovirus, HSV, or *C. trachomatis* plasmid control DNAs, respectively, whereas detection limits of 10³ copies of adenovirus or HSV DNA and 10⁴ copies of *C. trachomatis* plasmid DNA were achieved when all targets were present in the same reaction tube. The latter detection limits were unchanged when only two targets (adenovirus–HSV, adenovirus–*C. trachomatis*, or HSV–*C. trachomatis*) were added in the mixture. The detection limits of the uniplex PCRs were 40 copies of adenovirus type 2 DNA, 3 copies of HSV DNA, and 10 copies of *C. trachomatis* plasmid DNA.

#### Prospective Analysis of Clinical Samples

A total of 805 eye swabs sent to the diagnostic virology laboratory for virus isolation (456 viral specimens) or *C. trachomatis* detection (349 chlamydia specimens) was tested by the multiplex PCR and its corresponding uniplex PCRs. The data obtained are shown in Table 2, and an example of the multiplex PCR results is shown in Figure 1. The multiplex PCR was compared with virus isolation in the 456 viral specimens and with Amplicor PCR and IDB test in the 349 chlamydial specimens.

In all the specimens (n = 805), both the multiplex and uniplex PCRs produced identical results. Of the viral specimens (n = 456), 49 specimens were positive for adenovirus (25 by cell culture and multiplex PCR, 23 by multiplex PCR
only, and in 1 sample by cell culture only), and 37 specimens were positive for HSV (32 were positive by both cell culture and multiplex PCR, 2 specimens by the multiplex PCR, and 3 were positive by cell culture). In addition, the multiplex PCR detected *C. trachomatis* in 10 specimens from patients who had equivalent chlamydia swabs.

In the chlamydial specimens (*n* = 349), *C. trachomatis* was detected in 21 specimens by the multiplex PCR, the uniplex PCR, and the Amplicor PCR. Of the positive specimens, 15 were also positive by the IDB test, which was reported negative or equivocal in the remaining 6 specimens. The multiplex PCR and uniplex PCR detected viral DNA in 47 chlamydial specimens (45 as adenovirus and 2 as HSV).

If all the positive samples by any of the techniques were considered true positives, the performance of the uniplex and multiplex PCRs is identical. For adenovirus detection, the sensitivity of the multiplex PCR is 98% (48/49), significantly more sensitive than cell culture (53%; 26/49; *P* < 0.0001). For HSV detection, the multiplex PCR has a sensitivity of 92% (34/37), and the corresponding figure for cell culture is 94.5% (35/37; *P* = 1.00). With respect to *C. trachomatis* detection, the multiplex PCR and the Amplicor PCR have 100% (21/21) sensitivity, whereas that of the IDB test is only 71% (15/21; *P* > 0.05).

All the specimens analyzed (*n* = 805) were received from a total of 541 patients with clinical suspicion of viral and/or chlamydial KC. Of these patients, viral etiology was suspected in 227 patients, chlamydial etiology in 128 patients, and viral or chlamydial etiology in the remaining 186 patients (Table 3). In the viral etiology group (*n* = 227), adenovirus was detected in 11 patients and HSV in 33 patients. In the group with clinical suspicion of chlamydial etiology (*n* = 128), *C. trachomatis* was detected in 8 patients. Interestingly, adenovirus DNA was detected in 10 patients of this group by the multiplex and the uniplex PCRs. In the patients with viral or chlamydial etiology (*n* = 186), adenovirus was detected in 32 patients, HSV in 2 patients, and *C. trachomatis* in 10 patients.

## DISCUSSION

Adenovirus, HSV, and *C. trachomatis* multiplex PCR fulfills a rational approach in that all the agents are system (ocular tissue)- and symptom (KC)-specific and could be of use in all cases of KC. A reduction in the sensitivity of the multiplex PCR to detect control DNAs was observed when compared with its corresponding uniplex PCR. However, in clinical specimens,
the multiplex and the uniplex PCRs produced identical results for the detection of all agents but were significantly more sensitive than adenovirus isolation, or as sensitive as HSV isolation or Amplicor PCR for the detection of C. trachomatis.

The superiority of PCR to adenovirus isolation using the primer pair ADRJC1/ADRJC2 has been reported by us previously28 and is probably due to the loss of viable virus during transport, a very slow growth rate of some strains of adenoviruses, and technical pitfalls in virus isolation because of toxicity or bacterial contamination.54 Nevertheless, both the multiplex and uniplex PCRs failed to detect either viruses in a few specimens in which virus isolation was reported positive. This could be due to the occurrence of false-positive virus isolation results due to cross contamination, although PCR inhibition55–57 cannot be ruled out. For prevention of the latter, the simple lysis buffer used as an extraction method, as described previously,24,29,36 proved satisfactory because none of the samples that were culture positive but PCR negative, became positive when re-extracted by alternative extraction methods (GuSCN and PEG).

A few reports have claimed the possibility of coinfection by adenovirus and HSV.59–60 adenovirus and C. trachomatis,41 or HSV and C. trachomatis42 in cases of KC. None of the samples tested in this study showed any sign of coinfection by any of the techniques. However, the possibility of coinfection would favor the use of the PCR over virus isolation because PCR would detect, for example, adenovirus DNA in the presence of HSV DNA, whereas cell culture would lead to production of an early HSV cytopathic effect destroying the cell cultures and thereby preventing the growth of adenovirus.58

A total of 641 (80%) eye swabs remained negative for adenovirus, HSV, and C. trachomatis. This apparently high proportion is partly due to the policy of the local eye hospital. Patients who present with what, on clinical grounds, is regarded as typical adenovirus KC are not swabbed. Also, other forms of conjunctivitis such as those of allergic origin may have contributed to the high percentage of the negatives in this study. Other possible explanations include the presence of, for example, adenoviruses that are not amplifiable by the primer pair ADRJC1/ADRJC2 or the occurrence of C. trachomatis strains or other chlamydia that are plasmid-free.52,45,44

### Table 3. Detection of Adenovirus, HSV, and C. trachomatis in 541 Patients with Clinical Suspicion of Viral and/or Chlamydial KC

<table>
<thead>
<tr>
<th>Investigation Requested</th>
<th>Agent Detected</th>
<th>No. of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral only (227 patients)</td>
<td>Adenovirus</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>HSV</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>C. trachomatis</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>183</td>
</tr>
<tr>
<td>Chlamydial only (128 patients)</td>
<td>Adenovirus</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>HSV</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C. trachomatis</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>110</td>
</tr>
<tr>
<td>Viral and chlamydial (186 patients)</td>
<td>Adenovirus</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>HSV</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>C. trachomatis</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>142</td>
</tr>
<tr>
<td>Total (541 patients)</td>
<td>Adenovirus</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>HSV</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>C. trachomatis</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>435</td>
</tr>
</tbody>
</table>

The ability of even experienced clinicians to diagnose acute conjunctivitis is limited. The multiplex PCR detected adenovirus DNA in 10 patients who had clinical suspicion of chlamydial KC. The diagnostic advantages of multiplex PCRs, which include cost-effectiveness and detection of pathogens that are not suspected clinically, are highlighted in this study and suggest its usefulness in large and small centers and in the community to non-specialist staff. Laboratory charges of the different laboratory techniques used in this study were approximately $66 for virus isolation, $28 for the Amplicor PCR, $25 for the IDB test, $36 for the multiplex PCR, and $35 for each uniplex PCR. Thus, for complete viral and chlamydial laboratory investigation of each specimen, the cost by using standard techniques would be $118 using a combination of virus isolation, Amplicor PCR, and IDB test; $106 using the uniplex PCRs; and only $36 using the multiplex PCR.

In conclusion, the multiplex PCR described here is sensitive and cost-effective. The test allows simultaneous screening for the three pathogens in a single eye swab within a maximum of 7 hours, and the methodology has the potential to replace routine time-consuming and costly diagnostic techniques for viral and chlamydial KC.

### References

15. Kowalski RP, Gordon YJ, Romanowski EG, Araullo–Cruz T, Kinchington PR. A comparison of enzyme immunoassay and polymerase