Confirmatory Polymerase Chain Reaction Testing for *Chlamydia trachomatis* in First-Void Urine from Asymptomatic and Symptomatic Men

JAMES B. MAHONY,1,2* KATHLEEN E. LUINSTRA,1 JOHN W. SELLORS,1,3 DAN JANG,1 and MAX A. CHERNESKY1,2,4

McMaster University Regional Virology and Chlamydiology Laboratory, St. Joseph’s Hospital,1 and Departments of Pathology,2 Pediatrics,4 and Clinical Epidemiology and Biostatistics,3 McMaster University, Hamilton, Ontario L8N 4A6, Canada

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First-void urine specimens from 683 men (592 without symptoms) were tested for *Chlamydia trachomatis* by a polymerase chain reaction (PCR) with KL1 and KL2 plasmid primers and by a Chlamydiazyme enzyme immunoassay (EIA). Thirty-seven specimens were confirmed to be positive by using the EIA blocking reagent and a second set of plasmid primers (T1 and T2). By comparing unconfirmed PCR results (KL1 and KL2 primers only) with the blocked Chlamydiazyme EIA results, the sensitivity and specificity of PCR were 100% (37 of 37 specimens) and 99.5% (643 of 646 specimens), respectively. Three additional specimens were negative by EIA but positive by PCR and were confirmed to be positive with primers T1 and T2. Two of the three specimens were from men with symptoms. The confirmatory PCR assay performed equally well in detecting positive specimens from symptomatic (31 of 31) and asymptomatic (9 of 9) men. Comparison of confirmatory testing of first-void urine specimens by PCR and EIA showed that PCR was 100% sensitive (40 of 40 specimens) and that the EIA was 92.5% sensitive (37 of 40 specimens) but that the assays were equally specific (100%).

*Chlamydia trachomatis* infections in men have traditionally been diagnosed by obtaining a sample from the male urethra. Early attempts at culturing *C. trachomatis* from urine yielded poor results and suggested that there may be inhibitory substances present in urine (31). Recently, testing of first-void urine (FVU) specimens has been evaluated as a noninvasive alternative to urethral swabbing in symptomatic men. The evaluation demonstrated that testing of urine sediments by direct immunofluorescence (IF) and enzyme immunoassay (EIA) detected *C. trachomatis* with sensitivities approaching those obtained by culturing urethral specimens (3, 4, 11, 13, 26, 29, 30, 32).

Comparisons of culture, IF, and EIA have indicated that IF and EIA are generally less sensitive than culture when culture is set as the "gold standard" (4, 5, 17, 22, 28–30). Recent studies have shown that when the standard is expanded to include a combination of tests, including culture, blocked EIA, and IF, the sensitivity of culture or EIA was reduced to 80 to 90% (4, 5, 17, 22). Because of their lack of sensitivity, nucleic acid probe assays have failed to improve our ability to detect *C. trachomatis* in clinical specimens (14, 16, 21). Recently, amplification of nucleic acids by the polymerase chain reaction (PCR) has been applied to the diagnosis of *C. trachomatis* infections (1, 6–10, 20, 23, 24, 27). In PCR assays, plasmid, major outer membrane protein (MOMP), or rRNA genes have been used as targets for amplification, and PCR assays have been shown to have equal or greater sensitivities than culture and EIA (1, 19, 23, 27). We report here the use of a second PCR as a confirmatory test for the detection of *C. trachomatis* in FVU specimens from men with or without symptoms of urethritis. (This study was presented in part at the 9th International Society for Sexually Transmitted Diseases Research Meeting, Banff, Alberta, Canada, 6 to 9 October 1991, abstr. 128.)

**MATERIALS AND METHODS**

**Specimens.** Urethral swabs and FVU specimens (20 ml; the first part of the stream collected at any time of day) were collected from 91 symptomatic men presenting to a hospital-based sexually transmitted disease clinic with signs or symptoms of urethritis (discharge, dysuria, pain, or frequency). Urethral swabs and FVU specimens were collected for culture, EIA, and PCR as described previously (4, 29). Specimens for the isolation of *C. trachomatis* were collected from symptomatic men only and were transported to the laboratory at 4°C and inoculated within 24 h. Specimens for chlamydial antigen detection by EIA were collected by using the manufacturer’s collection kits. FVU specimens were also collected from 592 asymptomatic men presenting to family physicians or a university health clinic for nongenitourinary complaints as part of a prevalence study. Specimens for culture were not collected from the asymptomatic group, because urethral swabs were not indicated and we did not want to inflict any unnecessary pain. FVU specimens were collected as described previously (29) from individuals who gave informed consent under a study protocol approved by a McMaster University ethics review committee. Specimens were centrifuged and then processed by EIA or PCR as described previously (4, 29).

**Culture.** *C. trachomatis* was isolated in McCoy cell cultures by using a 96-well microculture system, iodine staining, and one blind passage as described previously (5).

**EIA.** Specimens for antigen detection were tested by the Chlamydiazyme EIA (Abbott Diagnostics, North Chicago, Ill.) according to the manufacturer’s instructions. Chlamydiazyme-positive specimens were confirmed by using the manufacturer’s blocking reagent.

* Corresponding author.
PCR. The DNA target for amplification was a 241-bp sequence of the genetically conserved cryptic plasmid extending from 727 to 967 bp downstream from the unique BamHI restriction site (19, 20). KL1 and KL2 primers amplified plasmid DNAs from all C. trachomatis serovars, which yielded HindIII digestion products of 167 and 74 bp that were resolvable on 5% polyacrylamide gels (19). FVU specimens (0.1 ml) were centrifuged for 20 min at 12,000 rpm in a microcentrifuge at room temperature, and the pellet was resuspended in 0.1 ml of PCR buffer consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, and 0.01% gelatin supplemented with 200 μg of proteinase K per ml and 1% Tween 20. Specimens were heated at 55°C for 1 h; this was followed by heating at 94°C for 10 min. A total of 20 μl of the treated urine sediment was amplified in a 100-μl reaction containing PCR buffer; 200 μM each of dATP, dCTP, dGTP, and dTTP; 2 U of Taq polymerase (AmpliTaq; Cetus); and 1 μM KL1 and KL2 primers (20). Enzymatic amplification was performed by using 35 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, as described previously (20). PCR products were analyzed by restriction endonuclease digestion and agarose gel electrophoresis or by DNA hybridization with a 32P-labeled C. trachomatis plasmid DNA probe (19). PCR-positive specimens were confirmed by a second confirmatory PCR by using primers T1 and T2; primers T1 and T2 amplify a 517-bp fragment of plasmid DNA (6). Reactions with primers T1 and T2 were prepared as described above, with the addition of 5 × 10⁻⁴ M tetraethylammonium chloride to enhance specificity (12), and the reactions were amplified for 40 cycles.

PCR control procedures. Several precautions were taken to minimize contamination of specimens by preamplified products (15, 19). These included the use of (i) separate biosafety containment hoods for preparing specimens, setting up PCRs, and analyzing products; (ii) plugged pipette tips or positive displacement pipettors; (iii) several negative controls interspersed with clinical specimens; (iv) periodic swabbing of work areas to detect amplified DNA; and (v) confirmatory PCR in resolving discordant results, but it was used judiciously.

DNA hybridization. PCR products were analyzed by Southern blotting analysis. Briefly, 10 μl of amplified DNA was electrophoresed in 2% agarose gels, transblotted onto nylon membranes (Zetabase; Bio-Rad), and reacted with a 32P-labeled chlamydia plasmid DNA probe as described previously (19).

RESULTS

Of 91 FVU specimens from symptomatic men, 29 (32%) were positive by both blocked EIA (Chlamydiazyme) and PCR, and specimens from 23 of these individuals were tested by culture and were found to be positive. Two additional FVU specimens were negative by EIA but positive by PCR, and both were from individuals with positive urethral cultures. Of the 60 specimens that were EIA and PCR negative, 54 were cultured and found to be negative. Among the asymptomatic men, 8 of 592 (1.4%) FVU specimens were positive by blocked EIA and PCR. One additional FVU specimen that was EIA negative was positive by PCR. All eight blocked EIA-positive and PCR-positive FVU specimens from the asymptomatic group plus the three discordant results (one asymptomatic, two symptomatic) were confirmed to be PCR positive by using a second plasmid PCR (T1 and T2 primers) and hybridization with a 32P-labeled chlamydial plasmid DNA probe (Fig. 1).

By combining specimens from symptomatic and asymptomatic men and using the blocked EIA as the gold standard, the sensitivity and specificity of the PCR assay described here were 100% (37 of 37 specimens) and 99.5% (643/646 specimens), respectively. By using an expanded gold standard of blocked EIA and confirmed PCR, PCR was more sensitive (100%; 40 of 40 specimens) than blocked EIA (92.5%; 37 of 40 specimens), and both assays were 100%
specific (Table 1). Inclusion of culture results (for the symptomatic group) in the expanded gold standard gave the same sensitivity and specificity. Although Chlamydiae were slightly more sensitive in the symptomatic group (93.5 versus 88.9%), PCR was 100% sensitive in both groups (Table 2). PCR performed equally well with specimens from men with or without signs or symptoms of urethritis (Table 2).

**DISCUSSION**

We tested a total of 683 FVU specimens, including 91 from symptomatic men (prevalence 32%) and 592 from asymptomatic men (prevalence 1.4%), and showed that PCR had an overall sensitivity of 100% (40 of 40 specimens) compared with an overall sensitivity of 92.5% (37 of 40 specimens) for EIA. Our PCR-positive specimens were tested in a second PCR by using a different set of primers that confirmed that these EIA-negative specimens were true positives.

Palmer et al. (24), using a MOMP gene fragment probe, reported that PCR can be used to detect C. trachomatis in urine specimens from symptomatic men. In their report, PCR was compared with Microtrak IF on 63 urethral swab and urine specimens from 37 symptomatic men attending a sexually transmitted disease clinic. Eighteen of 37 (49%) men had C. trachomatis infections diagnosed by Microtrak testing of urethral swabs. Compared with Microtrak, PCR had sensitivity and specificity of 95 and 94%, respectively, for urethral swabs and 82 and 94%, respectively, for urine specimens. In our study, we tested 91 FVU specimens from symptomatic men by PCR and EIA. By using an expanded gold standard of blocked EIA and confirmed PCR, the PCR assay had sensitivity and specificity of 100% in comparison with sensitivity and specificity of 93.5 and 100%, respectively, for EIA. For comparison, in our evaluation of 592 FVU specimens from asymptomatic men, PCR had sensitivity and specificity of 100% in comparison with sensitivity and specificity of 88.9 and 100%, respectively, for EIA. The increased sensitivity of our PCR (100%) compared with sensitivity of 82% obtained by Palmer et al. (24) could be due to several factors. First, Palmer and colleagues (24) used PCR primers to amplify a conserved region of the MOMP gene; this was followed by a second round of amplification with nested primers. On the other hand, we used a plasmid PCR to amplify DNA from the genetically conserved plasmid which is present in multiple copies (up to 10 copies per bacterium) (25). Using serial dilutions of purified DNA from serovar L2, we showed (unpublished data) that our plasmid PCR is 10 to 100 times more sensitive than a MOMP PCR similar to that used in the study of Palmer et al. (24). Second, Palmer et al. (24) used chloroform-phenol to extract DNA. In our hands, the use of DNA extraction has resulted in a decreased sensitivity of PCR, most likely because of the loss of some specimen DNA during extraction (19). Our specimens were prepared by digestion with proteinase K and Tween 20 followed by heating to 94°C. Third, we used an elevated primer annealing temperature of 55°C which not only increased the specificity of the PCR but also increased its sensitivity by eliminating primer dimer formation. We consistently obtained a sensitivity of 0.1 fg of DNA using only 35 cycles of amplification. Palmer et al. (24) reported the same sensitivity (10^-16 g) obtained after two rounds of PCR involving 70 cycles.

Investigators in several laboratories have shown that PCR can have an improved sensitivity over those of culture and EIA. Ostergaard et al. (23) compared PCR with culture in a study of 223 specimens and performed EIA on the specimens with discordant results. Their plasmid PCR had a sensitivity of 100% (26 of 26 specimens). In their study, EIA was not performed on all specimens and the sensitivity of culture was not determined. Quinn et al. (27) compared a MOMP PCR with culture in a study involving 131 endocervical specimens and reported PCR sensitivity and specificity of 100 and 98.5%, respectively. In a separate retrospective evaluation (1), those investigators reported a PCR sensitivity of 100% (46 of 46 specimens) by using culture-positive specimens. We have recently evaluated our confirmatory plasmid PCR by testing 258 endocervical and urethral specimens by culture, EIA, and PCR (19). Using an expanded gold standard of blocked EIA and confirmed PCR, the sensitivity of PCR was 100% (71 of 71 specimens), in comparison with 76% (54 of 71 specimens) for culture and 70.4% (50 of 71 specimens) for EIA. The specificity of PCR in our study was

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**TABLE 1. Comparison of the sensitivity, specificity, and positive and negative predictive values of EIA and PCR for detecting C. trachomatis in FVU specimens**

<table>
<thead>
<tr>
<th>Confirmatory test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydiazyme EIA</td>
<td>92.5 (37/40)</td>
<td>100 (643/643)</td>
<td>100 (37/37)</td>
<td>99.5 (643/646)</td>
</tr>
<tr>
<td>Plasmid PCR</td>
<td>100 (40/40)</td>
<td>100 (643/643)</td>
<td>100 (40/40)</td>
<td>100 (643/643)</td>
</tr>
</tbody>
</table>

* Performance was calculated for 683 FVU specimens from 91 symptomatic and 592 asymptomatic men by using an expanded gold standard, in which a specimen from an infected patient was positive by a confirmed Chlamydiazyme test or PCR that was confirmed by using a second set of primers. PPV, positive predictive value; NPV, negative predictive value.

**TABLE 2. Performance of EIA and PCR for detecting C. trachomatis in FVU specimens from symptomatic and asymptomatic men**

<table>
<thead>
<tr>
<th>Specimen from a patient with:</th>
<th>Test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptoms</td>
<td>EIA</td>
<td>93.5 (29/31)</td>
<td>100 (60/60)</td>
<td>100 (29/29)</td>
<td>96.8 (60/62)</td>
</tr>
<tr>
<td>PCR</td>
<td>100 (31/31)</td>
<td>100 (60/60)</td>
<td>100 (31/31)</td>
<td>100 (60/60)</td>
<td></td>
</tr>
<tr>
<td>No symptoms</td>
<td>EIA</td>
<td>88.9 (8/9)</td>
<td>100 (583/583)</td>
<td>100 (8/8)</td>
<td>99.8 (583/584)</td>
</tr>
<tr>
<td>PCR</td>
<td>100 (9/9)</td>
<td>100 (583/583)</td>
<td>100 (9/9)</td>
<td>100 (583/583)</td>
<td></td>
</tr>
</tbody>
</table>

* Performance was determined for 91 FVU specimens from symptomatic men and 592 FVU specimens from asymptomatic men by using an expanded gold standard, in which a specimen from an infected patient was positive by a confirmed Chlamydiazyme test or PCR that was confirmed by using a second set of primers. PPV, positive predictive value; NPV, negative predictive value.
100% (187 of 187 specimens). Use of an expanded gold standard provides the opportunity to calculate the sensitivity of culture, which is seldom 100% because of the liability of *C. trachomatis* transported to the laboratory under suboptimal conditions (18). Other laboratories have also adopted an expanded gold standard and have shown that culture usually has a sensitivity of 80 to 90% (4, 5, 17, 22).

PCR testing in the past has been prone to specificity problems with false-positive results resulting from carryover contamination by previously amplified DNA (15). In our laboratory, we have used separate areas for specimen processing, PCR set up, and product analysis to minimize carryover contamination and to prevent false-positive results (19). In the study described here, we improved the specificity of PCR by using a second PCR assay with a different pair of primers to confirm all positive results. Incorporation of the second PCR increased the specificity of the assay from 98.9% obtained previously to 100% obtained in this study. Bobo et al. (2) have recently used a similar approach to confirm *C. trachomatis*-positive ocular specimens in the diagnosis of eye infections in Tanzania.

In this study, we used agarose gels and ethidium bromide staining to detect PCR products. In our hands, ethidium bromide did not miss any specimens that were positive by culture or EIA. Southern blotting, although more sensitive than ethidium bromide staining, is therefore not routinely necessary for the assessment of products.

The excellent sensitivity and specificity of PCR for testing FVU specimens should establish it as the method of choice for investigating *C. trachomatis* infections in men, provided that suitable automated methods become available for detecting specific products. In this study, PCR performed equally well on urine specimens collected from men with or without symptoms. Our results for asymptomatic men should be viewed as preliminary, however, given the small number of *Chlamydia*-positive men in this study, and larger studies are therefore warranted. Since *C. trachomatis* is a reportable sexually transmitted disease and has an impact on the work loads of public health units, i.e., contact tracing and counseling, we confirm all PCR-positive specimens with a second PCR test and recommend that all laboratories confirm the results for positive specimens. PCR may prove to be useful in investigating clinical conditions thought to be associated with *C. trachomatis* in which it has been difficult to detect *C. trachomatis* in clinical specimens by culture or EIA.

REFERENCES


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