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# Multiplex PCR for Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in Genitourinary Specimens

JAMES B. MAHONY,[1,2]* KATHY E. LUINSTRA,[1] MARK TYNDALL,[1] JOHN W. SELLORS,[3] JACK KREPEL,[4] AND MAX CHERNESKY[1,2,5]

*Regional Virology and Chlamydiology Laboratory[1] and Departments of Pathology,[2] Pediatrics,[5] and Clinical Epidemiology and Biostatistics,[3] McMaster University, Hamilton, and Gamma North Peel Laboratory, Toronto,[4] Ontario, Canada*

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**We developed a multiplex PCR (M-PCR) assay for the simultaneous detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. M-PCR employed *C. trachomatis*-specific primers KL1-KL2 and *N. gonorrhoeae*-specific primers HO1-HO3 and produced products of 241 and 390 bp, respectively. PCR products were easily detected by agarose gel electrophoresis and confirmed by Southern hybridization using labelled oligonucleotide probes. M-PCR had a sensitivity of 10 fg of *C. trachomatis* and *N. gonorrhoeae* DNA (equivalent to 1 to 2 genome copies). M-PCR detected the presence of *C. trachomatis* and *N. gonorrhoeae* DNA in 15 male urethral and 12 female endocervical specimens, 3 of which were positive for *C. trachomatis*, 18 of which were positive for *N. gonorrhoeae*, and 6 of which were positive for both organisms. M-PCR was evaluated further by testing 200 male first void urine (FVU) specimens, of which 18 were positive by *C. trachomatis* PCR and Chlamydiazyme and 4 were positive by *C. trachomatis* PCR but negative by Chlamydiazyme. All 22 FVU specimens were positive by a confirmatory PCR using a second plasmid target and were positive by M-PCR. Ten of 11 men with cultures that were positive for *N. gonorrhoeae* had FVU specimens that were positive by both *N. gonorrhoeae* PCR and M-PCR. Two other men with negative *N. gonorrhoeae* urethral cultures had FVU specimens that were positive by *N. gonorrhoeae* PCR, by two confirmatory *N. gonorrhoeae* PCR assays using 16S rRNA and cytosine methyltransferase primers, and by M-PCR. The sensitivity of M-PCR for detecting *C. trachomatis* was 100% (22 of 22 specimens), compared with 81.8% (18 of 22 specimens) for enzyme immunoassay. Sensitivity of M-PCR for *N. gonorrhoeae* was 92.3% (12 of 13 specimens), compared with 84.6% (11 of 13 specimens) for urethral culture. The specificity of M-PCR was 100% for both *C. trachomatis* (178 of 178 specimens) and *N. gonorrhoeae* (187 of 187 specimens). M-PCR testing of FVU specimens provided a sensitive and noninvasive method for detecting *C. trachomatis* and *N. gonorrhoeae* infection in men.**

Genitourinary tract infections due to *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are a major cause of morbidity in sexually active individuals (8). In North America *N. gonorrhoeae* rates are on the increase and *C. trachomatis* infection is now the most common bacterial sexually transmitted disease (STD), with asymptomatic carriage rates in the general population of 2 to 5% (8, 12).

Over the past 15 years the diagnosis of *C. trachomatis* and *N. gonorrhoeae* infections has been largely dependent on traditional methods such as culture, enzyme immunoassay (EIA), and direct fluorescent antibody staining for *C. trachomatis* and culture, Gram smear, and biochemical tests for *N. gonorrhoeae*. The last 5 years have seen major improvements in our ability to detect these STDs, first with the advent of newer technologies such as DNA hybridization and nucleic acid amplification and second with the testing of noninvasive urine specimens (2, 4–7, 9–11, 14, 16–20, 22–25). PCR and, more recently, ligase chain reaction (3, 9) have been developed for the detection of *C. trachomatis* and *N. gonorrhoeae*, and evaluations of the first commercial tests are now appearing (2, 7, 14, 16, 20). Nucleic acid amplification tests have generally been more sensitive than traditional tests for the detection of *C. trachomatis* (7, 10, 14, 16, 19, 20, 22, 23), but they have not been extensively evaluated for *N. gonorrhoeae*. Although multiplex PCR (M-PCR) has been applied to the detection of multiple viruses in clinical specimens (1, 21, 27, 29), this approach to diagnosis has only recently been applied for the detection of bacterial STDs (13, 24). We report here the development of an M-PCR for the simultaneous detection of *C. trachomatis* and *N. gonorrhoeae* in genitourinary specimens and show that M-PCR is more sensitive than traditional tests for these STDs.

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Twenty-seven endocervical and urethral specimens submitted to a private laboratory in Toronto for chlamydial testing were selected for the development of the M-PCR assay. Two hundred additional urethral swabs and first void urine (FVU) specimens (20 ml) were collected from men presenting with nongonococcal urethritis to the Nairobi City Commission Special Treatment Clinic. These specimens were collected during June 1992 by two clinic physicians who were asked to refer only those men with clinical findings consistent with nongonococcal urethritis as determined by using an algorithm which selected men with nonpurulent urethral discharge or symptoms of dysuria with no evidence of urethral discharge (28). All men were between the ages of 17 and 40 years and were not under antibiotic treatment. Two urethral swabs and an FVU specimen were collected for culture, EIA, and PCR as described previously (19, 20, 25). The FVU specimen was collected first. The first swab was used for isolation of *N. gonorrhoeae*, and the second was used for the detection of *C. trachomatis* antigens by EIA. FVU specimens were centrifuged and processed for

* Corresponding author. Mailing address: Regional Virology Laboratory, St. Joseph's Hospital, 50 Charlton Ave. East, Hamilton, Ontario L8N 4A6, Canada.

*C. trachomatis* antigen detection by EIA and PCR as described below. The study was approved by the University of Nairobi's Scientific and Ethical Review Committee.

Culture for *N. gonorrhoeae* was performed by inoculation of urethral swabs directly onto modified Thayer Martin medium incubated in a 5% $CO_2$ atmosphere. Characteristic *N. gonorrhoeae* colonies were confirmed by oxidase and carbohydrate utilization tests.

FVU specimens were tested for chlamydial antigen by the Chlamydiazyme EIA (Abbott Diagnostics, North Chicago, Ill.) according to the manufacturer's instructions. Chlamydiazyme-positive specimens were confirmed with the manufacturer's blocking reagent. *C. trachomatis* PCR-positive, EIA-negative discordant specimens were tested by direct fluorescent antibody staining with the Syva Microtrak reagent after concentration of urine by centrifugation at 16,000 × *g* for 10 min. Detection of two or more elementary bodies was considered a positive result.

DNA was extracted from laboratory strains of *N. gonorrhoeae* as described by Ho et al. (11). DNA was extracted from Renografin-purified *C. trachomatis* elementary bodies as described previously (19). Briefly, 0.1-ml aliquots of elementary bodies were pelleted in a microcentrifuge for 20 min at 12,000 rpm, and the pellets were resuspended in 0.1 ml of PCR buffer and treated with proteinase K and Tween 20 as previously described for FVU specimen sediments (17). Chlamydial DNA was then purified by phenol-chloroform extraction followed by ethanol precipitation and was dissolved in Tris-EDTA buffer.

PCR was performed with either extracted DNA or processed Chlamydiazyme specimens as described previously (18). FVU specimens were processed for Chlamydiazyme testing according to the manufacturer's instructions, and 10 μl in specimen diluent buffer was tested by PCR in a 100-μl final reaction volume. Some Chlamydiazyme specimens were stored at −20°C for up to 1 month. For the detection of *C. trachomatis*, KL1-KL2 primers, which amplify a 241-bp fragment of the genetically conserved plasmid, were used (19). All positive specimens were tested by a confirmatory PCR using a second set of plasmid primers, T1-T2 (17). For the detection of *N. gonorrhoeae*, HO1-HO3 primers, which amplify a 390-bp fragment of the *cppB* gene on the 4.2-kb cryptic plasmid, were used (11). The conditions for *N. gonorrhoeae* PCR were modified as follows: 2.5 mM $MgCl_2$, 0.01% gelatin, 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C for 40 cycles on a Perkin-Elmer model 480 so that they were the same as those used for *C. trachomatis* PCR with the KL1-KL2 primers (19). The specificity of the HO1-HO3 primers has been demonstrated by *Msp*I cleavage of the 390-bp amplicon into 250- and 140-bp fragments (11). Two *N. gonorrhoeae* PCR-positive FVU specimens from urethral culture-negative men were confirmed by two other PCRs using 16S rRNA primers and an *N. gonorrhoeae*-specific oligonucleotide probe (performed by Linda Chui, University of Alberta Hospitals, Edmonton, Alberta, Canada) or SS01-SS02 primers for the cytosine methyltransferase gene and an immobilized internal oligonucleotide capture probe (SS06) in a 96-well microtiter plate format (performed by Maurice Rosenstraus, Roche Molecular Systems, Branchburg, N.J.) (data not shown). PCR products were analyzed by agarose gel electrophoresis and Southern blot hybridization using fluorescein-11-dUTP-labelled oligonucleotide probes prepared with terminal deoxynucleotidyl transferase by using an ECL 3′-oligolabelling and detection kit (Amersham Life Sciences, Arlington Heights, Ill.). The KL3 probe, specific for the *C. trachomatis* 241-bp PCR product (19), and JMGC3 probe (5′-ATA TAC CTA GCA AGC TCC AC-3′), derived from the published plasmid sequence (15) and specific for the 390-bp *N. gonorrhoeae* PCR product, were detected by enhanced chemiluminescence (ECL; Amersham). Extensive precautions were employed for all PCR assays to prevent carryover contamination (17, 19). These included the use of (i) designated biosafety containment hoods for preparation of specimens and setting up of PCRs and a separate area for analysis of 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) a confirmatory PCR used judiciously for resolving discordant results.

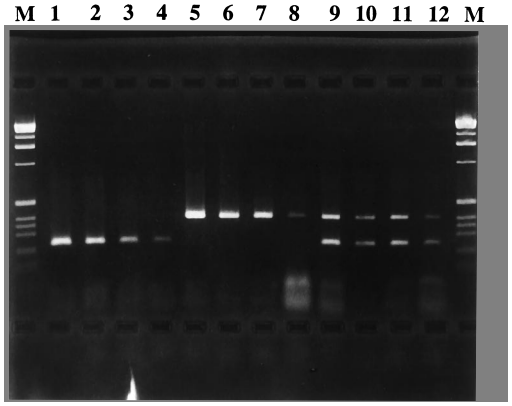


FIG. 1. Detection of *C. trachomatis* and *N. gonorrhoeae* DNA by M-PCR. A 50-μg solution containing 1 μg each of *C. trachomatis* and *N. gonorrhoeae* DNA was serially diluted to achieve dilutions containing 10 pg, 1 pg, 100 fg, and 10 fg of DNA. An aliquot of each dilution was tested by *C. trachomatis* PCR using KL1-KL2 primers (lanes 1 to 4), *N. gonorrhoeae* PCR using HO1-HO3 primers (lanes 5 to 8), and M-PCR using both primer sets (lanes 9 to 12). Outside lanes contain DNA markers ranging from 134 bp to 12.2 kb (Gibco BRL, Gaithersburg, Md.).

The optimal conditions for M-PCR were established by varying the concentrations of $MgCl_2$, deoxynucleoside triphosphates (dNTPs), and primers at different annealing temperatures. The optimal conditions determined were an annealing temperature of 55°C, primer concentrations of 0.5 μM, an $MgCl_2$ concentration of 2.5 mM, and dNTP concentrations of 200 μM. Under these optimal conditions the total analytical sensitivity of M-PCR was 10 fg of total DNA, equivalent to 1 to 2 genome copies for both *C. trachomatis* and *N. gonorrhoeae* and equal to the sensitivities of the individual *C. trachomatis* PCR and *N. gonorrhoeae* PCR assays (Fig. 1, lane 12). Figure 1 demonstrates the specificity of the primers used in M-PCR; KL1-KL2 primers (lanes 1 to 4) amplified only *C. trachomatis* DNA and HO1-HO3 primers (lanes 5 to 8) amplified only *N. gonorrhoeae* DNA in the presence of DNA from both organisms.

Figures 2 and 3 demonstrate the ability of M-PCR to correctly detect *C. trachomatis* and *N. gonorrhoeae* when present in the clinical specimens. Three endocervical specimens were tested by M-PCR and single PCR, and products were analyzed by Southern hybridization using oligonucleotide probes for *C. trachomatis* and *N. gonorrhoeae* (Fig. 2). The first endocervical specimen was positive for *C. trachomatis* by both *C. trachomatis* PCR (lane 2) and M-PCR (lane 3); the second endocervical specimen was positive for *N. gonorrhoeae* by both *N. gonorrhoeae* PCR (lane 4) and M-PCR (lane 6); and the third endocervical specimen was positive for *N. gonorrhoeae* by *N. gonorrhoeae* PCR (lane 7), *C. trachomatis* by *C. trachomatis* PCR (lane 8), and both *C. trachomatis* and *N. gonorrhoeae* (lane 9) by M-PCR. The M-PCR results for an additional 16

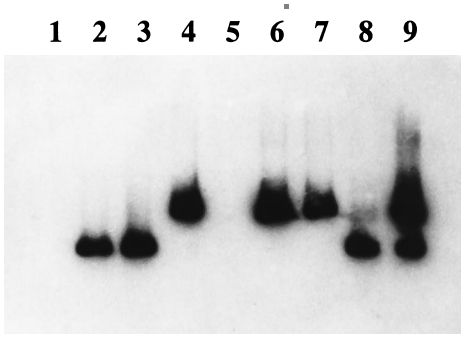


FIG. 2. Southern hybridization of three M-PCR-positive endocervical specimens. One *C. trachomatis*-positive specimen (lanes 1 to 3), one *N. gonorrhoeae*-positive specimen (lanes 4 to 6), and one specimen positive for both *C. trachomatis* and *N. gonorrhoeae* (lanes 7 to 9) were tested by *N. gonorrhoeae* PCR (lanes 1, 4, and 7), *C. trachomatis* PCR (lanes 2, 5, and 8), and M-PCR (lanes 3, 6, and 9). PCR products were probed with fluorescein-11-dUTP-labelled oligonucleotide probes for both *C. trachomatis* (KL3) and *N. gonorrhoeae* (JMGC2) as described in the text.

specimens (3 endocervical specimens and 13 male FVU specimens) are shown in Fig. 3, lanes 9 to 24. Six specimens were positive for *C. trachomatis* (specimens in lanes 10, 12, 14, and 16 to 18), nine specimens were positive for *N. gonorrhoeae* (lanes 9 to 13, 15, 16, 18, and 23), and four specimens were positive for both *C. trachomatis* and *N. gonorrhoeae* (lanes 10, 12, 16 and 18). The results of M-PCR testing obtained for 27 specimens including 15 male urethral swabs and 12 endocervical specimens are summarized in Table 1. M-PCR correctly identified all 15 male urethral swabs, 13 of which were positive for *N. gonorrhoeae* alone and 2 of which were positive for both *C. trachomatis* and *N. gonorrhoeae*, and all 12 endocervical specimens, 5 of which were positive for *N. gonorrhoeae*, 3 of which were positive for *C. trachomatis*, and 4 of which were positive for both *C. trachomatis* and *N. gonorrhoeae.*

M-PCR was further evaluated by testing 200 FVU specimens from men attending an STD clinic. Eighteen men had paired urethral swabs and urine specimens that were positive for *C. trachomatis* by Chlamydiazyme assay. An additional four specimens were positive for *C. trachomatis* by *C. trachomatis* PCR (Table 2). All 22 urine specimens were confirmed positive by direct fluorescent antibody staining and by a confirmatory PCR using a second plasmid target. M-PCR detected *C. trachomatis* in all 22 positive urine specimens, and compared with *C. trachomatis* PCR, M-PCR had a sensitivity of 100% (22 of 22 specimens) and a specificity of 100% (178 of 178 specimens) (Table 2). By comparison EIA testing of urethral swabs and FVU specimens was 81.8% (18 of 22 specimens) sensitive. There were 11 men with urethral swabs that were culture positive for *N. gonorrhoeae*, and 10 of these had matched FVU specimens that were positive by *N. gonorrhoeae* PCR. Two additional men with negative urethral cultures had FVU specimens that were positive by *N. gonorrhoeae* PCR and were confirmed positive using two sets of primers, one targeting the *N. gonorrhoeae* cytosine methyltransferase gene and the other targeting the 16S rRNA gene. M-PCR detected *N. gonorrhoeae* in all 12 of the 12 FVU specimens determined to be positive by *N. gonorrhoeae* PCR. With an expanded reference standard of an infected patient positive by culture or confirmed PCR, urethral culture had a sensitivity of 84.6% (11 of 13 specimens) and urine *N. gonorrhoeae* PCR and M-PCR had sensitivities of 92.3% (12 of 13 specimens). The specificities of culture and M-PCR were 100% (187 of 187 specimens). None of the 200

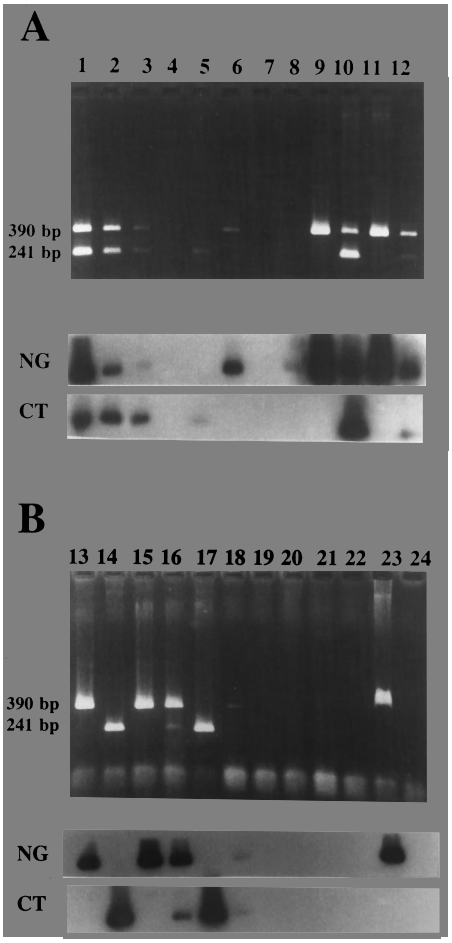


FIG. 3. Detection of *C. trachomatis* and *N. gonorrhoeae* in clinical specimens by M-PCR. Gels were transblotted and probed separately with a *C. trachomatis* probe and an *N. gonorrhoeae* probe. The positions of both amplicons are indicated. Lanes 1 to 4 contain 1 pg, 0.1 pg, 10 fg, and 1 fg of *C. trachomatis* and *N. gonorrhoeae* DNA, respectively, tested by M-PCR (lanes 1 to 3 were positive for both, while lane 4 was negative); lanes 5 and 7 contain 10 fg (positive) and 1 fg (negative), respectively, of *C. trachomatis* DNA tested by M-PCR; lanes 6 and 8 contain 10 and 1 fg, respectively, of *N. gonorrhoeae* DNA tested by M-PCR; and lanes 9 to 24 contain 3 endocervical specimens (lanes 9 to 11) and 13 FVU specimens (lanes 12 to 24), of which 6 specimens were positive for *C. trachomatis* (lanes 10, 12, 14, and 16 to 18), 9 were positive for *N. gonorrhoeae* (lanes 9 to 13, 15, 16, 18, and 23), and 4 were positive for both *C. trachomatis* and *N. gonorrhoeae* (lanes 10, 12, 16, and 18).

Kenyan urine samples analyzed were positive for both *C. trachomatis* and *N. gonorrhoeae.*

We have developed an M-PCR for the detection of *C. trachomatis* and *N. gonorrhoeae* in genitourinary specimens that correctly detected *C. trachomatis* or *N. gonorrhoeae* in 61 of 62 positive specimens. The overall sensitivity of M-PCR was 97.3% (36 of 37 specimens) for *N. gonorrhoeae* and 100% (31 of 31 specimens) for *C. trachomatis*. The specificity of M-PCR was 100% for both *C. trachomatis* and *N. gonorrhoeae*. These results indicate that M-PCR can detect *C. trachomatis* and *N. gonorrhoeae* in genitourinary specimens with sensitivities exceeding that achieved by conventional tests.

The ability of M-PCR to detect *C. trachomatis* and *N. gonorrhoeae* simultaneously in urine specimens with high sensitivity provides a new noninvasive approach to the diagnosis of urethritis in men. Other approaches for the detection in urine of *C. trachomatis* by culture or *N. gonorrhoeae* by EIA have

TABLE 1. Comparison of single and M-PCR results for the detection of *C. trachomatis* and *N. gonorrhoeae* in 27 selected specimens from a private laboratory[a]

| Type of specimen | No. of specimens | Results[b] | | | |
|---|---|---|---|---|---|
| | | *C. trachomatis* PCR | *N. gonorrhoeae* PCR | M-PCR | |
| | | | | *C. trachomatis* | *N. gonorrhoeae* |
| Male, urethral swab | 13 | − | + | − | + |
| | 2 | + | + | + | + |
| Female, endocervical swab | 5 | − | + | − | + |
| | 3 | + | − | + | − |
| | 4 | + | + | + | + |

[a] All swabs were collected in the appropriate male (STD-Pen) or female (STD-EZE) Chlamydiazyme collection tube and processed according to the manufacturer's instructions. An aliquot of the specimen in specimen diluent buffer (10 μl) was used for PCR analysis.
[b] All PCR products were hybridized with an oligonucleotide probe as described in the text.

lacked sensitivity (5, 6, 24). Recent studies of nucleic acid amplification technologies such as PCR and ligase chain reaction have indicated that FVU specimens may be the preferred specimens for diagnosing *C. trachomatis* infections in both men and women. Several laboratories have shown that urine PCR testing has a higher sensitivity than urethral culture or EIA testing of FVU specimens for *C. trachomatis* (2, 7, 14, 19, 20, 22, 23). These results confirm that PCR and, more recently, ligase chain reaction testing of urine specimens detected more *C. trachomatis* infections in both men and women than either urethral or endocervical culture or EIA or urine EIA testing (7, 19, 20). Additional studies including larger numbers of symptomatic and asymptomatic men will be required to determine the true performance of M-PCR for detection of *C. trachomatis* and *N. gonorrhoeae* in urine specimens and to determine if M-PCR testing of FVU specimens should be widely recommended.

M-PCR testing of FVU specimens should provide an improved method for diagnosis of both *C. trachomatis* and *N. gonorrhoeae* infections occurring either singly or as coinfections. Dual infections have in the past been difficult to detect, because some small clinic and hospital laboratories may not perform both *N. gonorrhoeae* and *C. trachomatis* diagnosis and some centers do not collect two specimens or may not look for a second STD after either *C. trachomatis* or *N. gonorrhoeae* has been detected, especially if treatment includes antimicrobial agents effective against both pathogens. The introduction of M-PCR may offer a sensitive and more rapid method for the detection of both of these STDs in a single specimen. In our hands M-PCR can be completed in 8 h and has a turnaround time of 1 working day versus 2 days on average for a confirmed *N. gonorrhoeae* culture result. In this study we detected six specimens that were positive for both *C. trachomatis* and *N. gonorrhoeae* (Table 1). These specimens were selected and did not permit us to determine the coinfection rate for *C. trachomatis* and *N. gonorrhoeae* in the patient population sampled. In the United States *N. gonorrhoeae* coinfection rates for *C. trachomatis*-infected individuals have ranged from less than 10% in one study to between 10 and 20% in another study (26, 30).

If the testing of FVU by PCR proves to be as sensitive for *N. gonorrhoeae* as it appears to be for *C. trachomatis*, then M-PCR may facilitate studies to determine the carriage rates for *C. trachomatis* and *N. gonorrhoeae* and the rates of coinfection in various populations of men. Commercial applications of M-PCR are presently under development, and provided that routine clinical laboratories can successfully adapt to PCR methodology and manufacturers can adequately address technical issues such as detection of *Taq* polymerase inhibitors that result in false-negative PCR results and carryover contamination, multiplex coamplification tests should provide powerful new tools for diagnosing STDs including *C. trachomatis* and *N. gonorrhoeae.*

TABLE 2. Performance of M-PCR compared with single PCR for detecting *C. trachomatis* and *N. gonorrhoeae* in 200 FVU specimens from symptomatic men attending an STD clinic[a]

| Specimen | Test | % sensitivity (no. of specimens positive/total) | % specificity (no. of specimens positive/total) |
|---|---|---|---|
| *C. trachomatis* | | | |
| Urethral | EIA | 81.8 (18/22) | 100 (178/178) |
| FVU | EIA | 81.8 (18/22) | 100 (178/178) |
| | *C. trachomatis* PCR | 100 (22/22) | 100 (178/178) |
| | M-PCR | 100 (22/22) | 100 (178/178) |
| *N. gonorrhoeae* | | | |
| Urethral | Culture | 84.6 (11/13) | 100 (187/187) |
| FVU | *N. gonorrhoeae* PCR | 92.3 (12/13) | 100 (187/187) |
| | M-PCR | 92.3 (12/13) | 100 (187/187) |

[a] All PCR products were hybridized with an oligonucleotide probe as described in the text.

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