Diagnosis of *Chlamydia trachomatis* Infection

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Important progress in the diagnosis of *Chlamydia trachomatis* (*C. trachomatis*) includes the development of nucleic acid amplification techniques such as polymerase chain reaction (PCR) and ligase chain reaction (LCR). Commercial kits are available, but they are costly, sporadic in availability, must be imported, and are economically beyond the reach of common people. To overcome this limitation, most research laboratories have standardized their in-house-developed PCR methods for diagnosing this infection. However, each laboratory has to spend a great deal of time and money to accomplish this. Published reports do not always elaborate the steps involved in standardizing a test so that it can immediately be reproduced in another setting. In the present study we attempted to elaborate the steps involved in standardizing a sensitive and specific PCR technique followed by hybridization with specific *C. trachomatis* probe to diagnose this infection in cervical, introital, and urine specimens, and used it to determine the infection rate in a clinical population. J. Clin. Lab. Anal. 20:8–14, 2006.   © 2006 Wiley-Liss, Inc.

Key words: *Chlamydia trachomatis*; diagnosis; PCR

INTRODUCTION

The incidence of sexually transmitted infections (STIs) continues to escalate and represents a major public health problem that can lead to significant morbidity and mortality in young individuals. Currently *Chlamydia trachomatis* (*C. trachomatis*) is the most common sexually transmitted bacterial pathogen (1). Long-term *C. trachomatis* infection may result in chronic salpingitis, and may eventually be complicated with tubal infertility and ectopic pregnancy (2,3). The detection of *C. trachomatis* genital infection to prevent transmission and its spread to the upper reproductive tract is challenging for both clinicians and laboratory workers (4). *C. trachomatis* genital infections are often asymptomatic, and this unique nature allows it to persist in relatively asymptomatic populations. Hence, early detection to avoid serious complications would be of real value because effective treatment exists. Confirmation of chlamydial infection usually depends on taking an appropriate specimen from the patient followed by direct detection of the organism using a suitable laboratory-based diagnostic test. The important progress in laboratory diagnosis of chlamydial infection includes the development of non-viability-dependent tests, which are gradually being superseded by methods to detect chlamydial nucleic acid by direct hybridization or nucleic acid amplification. The amplification reactions include polymerase chain reaction (PCR), ligase chain reaction (LCR), strand displacement amplification (SDA), and transcription mediated amplification (TMA). Thanks to these advances, cervical and urethral specimens collected by invasive genital sampling are no longer required. Noninvasive and self-collected specimens include male and female urine and various vulval-introital specimens. Although several PCR methods are available, not many laboratories are using these techniques because of the lack of detailed instructions to follow for immediate standardization and use. The objective of this study was to elaborate the steps involved in developing an in-house PCR with the use of specific primers from the conserved region of the major outer membrane protein (MOMP) gene of *C. trachomatis* followed by hybridization with a specific probe for diagnosing this infection with good sensitivity

and specificity. We also used this method to determine the infection rate in a clinic-based population.

MATERIALS AND METHODS

Standard

A standard of *C. trachomatis* (L2 strain, elementary bodies from the Scripps Organization, San Diego, CA) was used for standardization and determination of the minimum detection level of DNA for PCR amplification. For this purpose, various quantities of DNA (range = 0.001 ng to 5 μg) extracted from these elementary bodies were taken for amplification. The other source of the *C. trachomatis* standard is the Department of Pathology, School of Public Health and Community Medicine, University of Washington, Seattle, WA.

Specimens

Cervical (n = 503) and introital (n = 81) specimens were collected using a sterile swab (HiMedia Laboratories Pvt. Ltd., Mumbai) from women attending the Gynecology OPD of Seth G.S. Medical College and K.E.M. Hospital, and the Infertility Division of our institute. The first voided urine specimens were collected from males (n = 314) attending an STD clinic in Nagpada, Mumbai. These specimens were used for standardization of PCR. Each individual was informed about the study and provided consent before the specimens were collected. Two cervical specimens (one in 1 mL of PBS buffer, and one preserved as a dry swab for repeat testing) were collected. An introital specimen was collected in 1 mL of PBS.

Adequacy of the Specimens

Each specimen (10 μL) was checked for adequacy under a microscope. The presence of four to five epithelial cells per high-power field was accepted as an adequately collected (400–500 cells/mL) specimen. Specimens with fewer cells were not processed, and if possible a duplicate specimen or another specimen collected from that individual was used for screening. To evaluate the quality of specimens collected for use in PCR, endocervical and introital specimens (n = 81) were obtained from the same individuals (Infertility Department of our institute) and processed for PCR analysis. These results were compared with the adequacy of the specimen to predict sensitivity and specificity.

DNA Extraction

Several methods are available for DNA extraction. In the present study, a phenol chloroform and isoamyl alcohol (25.24:1, Sigma) extraction method was used.

In brief, each specimen in PBS was centrifuged at 10,000 rpm for 15 min to get a cell pellet. The pellet was lysed with 500 μL of lysis buffer (0.1 M Tris-HCl, pH 8.0, 0.10 M NaCl, 0.005 M EDTA, 1% SDS, and 9.5 μg proteinase K) and incubated overnight at 50°C in a water bath. An equal amount of phenol chloroform isoamyl reagent was added to this suspension, mixed, and centrifuged again to obtain an upper aqueous layer containing DNA. This layer was separated carefully and an equal amount of chilled absolute alcohol was added and kept overnight at –20°C to precipitate DNA. The precipitated DNA was collected after centrifugation at 10,000 rpm and washed twice with 75% alcohol to get rid of the salt. According to the size of the pellet, distilled water was added to elute the DNA.

Qualitative and Quantitative evaluation of DNA

An aliquot (10 μl) of the DNA specimens were run on a 0.8% agarose gel, stained with ethidium bromide (0.5 μg/mL), and observed on a transilluminator for the presence of DNA. Quantitation of DNA was done by measuring an aliquot of DNA (10 μL in 1 mL of distilled water) at 260 and 280 nm. The amount of DNA was calculated assuming that an O.D. of 1 corresponds to 50 μg of DNA at 260 nm.

Primers

A primer pair was selected from the conserved region of the major outer membrane protein (MOMP) gene of *C. trachomatis* (5). This primer pair amplifies a 180 bp DNA fragment, which is common to all serotypes of *C. trachomatis*. Primers were custom synthesized from Qiagen (Qiagen Operon, Cologne, Germany). The sequences from 5′ to 3′ of these oligonucleotide primers are as follows: sense: 5′ GCC GCT TTG AGT TCT GCT TCC 3′; antisense: 5′ GTC GAA AAC AAA GTC ACC ATA GTA 3′.

PCR Amplification

Standard precautions were taken to avoid contamination of the specimens and reaction mixtures. PCR was done in a total volume of 50 μL. The final mixture contained primers (0.5 μM each), 0.2 mM dNTPs, PCR buffer (10 mM Tris-HCl, pH 9, 50 mM KCl, 1.5 mM MgCl$_2$, 1.0% Triton X-100) and 1.25 U of Taq polymerase (Promega PCR Core System II). From each specimen 10 μL of neat DNA was used for amplification. For PCR control, plasmid DNA along with primers was used to amplify a 323 bp DNA fragment. In 50 μL of PCR mixture, at least 5 ng of positive chlamydia control was used, whereas autoclaved distilled water was used for the negative control. A quick

spin was given to each PCR tube before they were processed in the thermal cycler (Gene Amp PCR system 2400; Perkin Elmer) for 25–40 cycles of amplification. Initial denaturation was done at 95°C for 1 min followed by cyclic amplification. Different cycles consisted of sequential incubations at 94°C for 1 min for denaturation, 42–60°C for 1 min for annealing of primers, and 72°C for 2 min for DNA chain extension. At the end of the PCR cycles, the specimens were kept for 7 min at 72°C to complete the extension of the DNA chain. The PCR products were immediately frozen for further analysis or loaded onto 2% agarose gel for visualization with ethidium bromide stain after electrophoresis.

Specificity of the Amplified Product By EcoR1 Digestion

The amplified DNA product was extracted directly from 20 μL of the post-PCR solution. Extraction was carried out with a DNA binding resin, using a kit supplied by Boehringer Mannheim. Isolated DNA pellets were resuspended in 20 μL of TE buffer. A portion of the suspension was incubated with endonuclease EcoR1. For this purpose, a mixture of 25 μL was prepared containing 15 μL of isolated DNA product, 1 μL of EcoR1 endonuclease (40 U), and 2.5 μL of enzyme-specific buffer with 6.5 μL of autoclaved distilled water. The mixture of each specimen was incubated at 37°C for 3 hr or overnight and then frozen until used for detection. This digested mixture (10 μL) and the undigested PCR product (5 μL) from each specimen were subjected to 2% agarose gel electrophoresis. An appropriate DNA ladder was also run simultaneously to determine the size of the bands obtained.

Southern Hybridization

Southern hybridization was carried out to confirm each product using a *C. trachomatis*-specific probe, as described below.

Synthesis of Probe and Immunodetection

A PCR DIG Probe synthesis kit (Roche Diagnostics GMBH, Germany) was used to synthesize a *C. trachomatis*-specific probe for use in Southern hybridization. In this system, Digoxygenin (a steroid hapten) was used to label *C. trachomatis* DNA (extracted from the elementary bodies) with DIG-11-dUTP during its amplification. The PCR protocol followed the manufacturer's instructions. In brief, 50 μL PCR mixture for probe synthesis contained *C. trachomatis*-specific primers (0.5 μM each), 0.2 mM dNTP (equal parts of PCR DIG Probe labeling mix and dNTP stock solution), 1 × PCR buffer with $MgCl_2$,

Expand High Fidelity enzyme mix (2.6 U), and at least 5 ng of chlamydia DNA. The volume of this PCR mixture was adjusted with autoclaved double-distilled water and processed for amplification. This probe was used for hybridization without further purification. The concentration of this standard probe was determined using a DIG luminescent detection kit (Roche Diagnostics) by taking a serial dilution of the labeled DNA (0.1 pg/μL, 1 pg/μL, 10 pg/μL, 100 pg/μL, and 1 ng/μL) in the dilution buffer provided with the kit. The nylon membrane (Hybond-N+; Amersham Biosciences, UK) was marked with a pencil to identify each dilution. Then 1 μL from each of the diluted DIG-labeled probe was spotted on the nylon membrane, which was cross-linked in a UV cross-linker. After the membrane was washed with washing solution (100 mM maleic acid, 150 mM NaCl, 0.3% Tween 20, pH 7.5), it was incubated with blocking solution for 30 min. It was again washed and incubated with anti-DIG alkaline phosphatase diluted (1:10,000) in blocking solution for 30 min at room temperature. The membrane was washed twice (15 min per wash in washing buffer) and equilibrated with detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl). This was followed by incubation with CSPD substrate in the dark for 5 min in a hybridization bag. Excess substrate was squeezed off, and the hybridization bag was sealed and kept at 37°C for 15 min to enhance the reaction. The membrane was then exposed to X-ray film to capture the signals. Enzymatic dephosphorylation of CSPD by alkaline phosphatase leads to a light emission at a maximum wavelength of 477 nm, which was recorded on X-ray film. The spot intensities of the control and experimental dilutions were compared to estimate the concentration of the experimental probe to be used.

Transfer of the Amplified Product and Hybridization With the DIG-Labeled Probe

The PCR amplified product after visualization on 2% agarose gel was denatured in denaturing solution (0.5 N NaOH with 1.5 M NaCl; Qualigens Fine Chemicals) at room temperature for 15 min with constant agitation. The gel was rinsed with deionized water for 5 min and neutralized in neutralizing solution (0.5 M Tris with 3 M NaCl, pH 7.5; Qualigens Fine Chemicals) for 15 min with constant agitation. The gel was then processed for Southern blotting, for which a glass plate was placed on a tray with transfer solution (10 × SSC: 15 mM tris-Sodium Citrate with 150 mM NaCl; Qualigens Fine Chemicals). A Whatman paper wick was placed on the glass plate so that the ends were immersed in the buffer. The gel was then placed upside down on the plate. A nylon membrane was placed on the gel, followed by

Whatman paper and a stack of filter paper. This setup was allowed to stand overnight for capillary transfer of DNA from the gel to the nylon membrane at room temperature. The following morning the position of each well was marked on the nylon membrane. The membrane was cross-linked in the cross-linker using 700 J of UV to enhance the binding of the transferred products. The transferred products on the nylon membrane were hybridized with the DIG-labeled *C. trachomatis* probe as described above for immunodetection. Since the transferred DNA was in denatured form, a denatured DIG-labeled *C. trachomatis* probe could find its complementary sequence on amplified DNA and bind to it. Specimens that were positive for *C. trachomatis* could be detected with this system.

DNA Sequencing and BLAST Search for Sequence Analysis

The amplified products were sequenced (DNA sequencer model 377, version 3.0, AB1200; Bangalore Genei, Bangalore) and the results were matched with the published sequence for confirmation. A homology search for the amplified product was done using the site at www.ncbi.nlm.gov/blast.

Validation of PCR Results by Enzyme-Linked Immunosorbent Assay (ELISA), Direct Fluorescent Antibody (DFA), Pap, and Amplicor PCR

The efficacy of the PCR was evaluated by comparing its results with those obtained by PCR followed by hybridization with a specific probe. The PCR or PCR probe results were then evaluated with other nonculture methods such as ELISA (Chlamydiazyme; Abbott Laboratories Diagnostic Division), DFA (Chlamyset Antigen FA; Orion Diagnostic, Finland), Papanicolaou (Pap), and PCR (Amplicor CT/NG; Roche Diagnostics). Thirty selected endocervical swab specimens collected from women attending the Gynecology OPD of KEM Hospital and screened in the hospital laboratory (Microbiology Department of KEM Hospital) using the ELISA kit for *C. trachomatis* were analyzed by PCR only. Decoding of the supplied specimens for infection by the ELISA-based method was carried out after the PCR-based detection was performed.

Similarly, in our institute's Clinical Research Department a group of 16 specimens collected from known *C. trachomatis*-positive individuals were processed for PCR, DFA, and Pap stain. The order of specimen collection was taken into consideration and each technique had been used on the specimens, which were collected in three different sequences. DFA and Pap stain were carried out at the Department of Clinical Research, and the results were compared after the specimen numbers were decoded.

Another group of 76 endocervical specimens collected from women attending the Family Welfare Clinics of the institute were screened for *C. trachomatis* and *N.gonorrhoea* using Amplicor (CT/NG) and were also tested by our PCR for comparison with the results obtained. The duplicate specimens were sent to the Institute of Tropical Medicine, Antwerp, Belgium, as part of the quality control program.

C. trachomatis Infection in the Clinical Population

We analyzed *C. trachomatis* infections in this population by taking the confirmed positive and negative specimens from the 503 women and 314 men into consideration.

Statistical Analysis

The sensitivity and specificity of the PCR were calculated using 2×2 tables.

RESULTS

Standardization of PCR

Qualitative and quantitative analysis of extracted DNA

The presence of a visible pellet before the use of elution buffer indicated the presence of DNA. Each eluted DNA on 0.8% agarose gel had a bright illuminated band. The amount of extracted DNA in different specimens varied from a few nanograms (ng) to 20 µg of DNA per specimen.

Amplified Product of PCR and the PCR Protocol

An amplified product of 180 bp DNA fragment was detected using specific primers (Fig. 1). The standardized protocol uses a denaturation temperature of 94°C for 1 min, an annealing temperature of 52°C for 1 min, and an extension temperature of 72°C for 2 min for each cycle of PCR. The protocol standardized with different annealing temperatures had shown that 52°C was crucial and any change would result in nonamplification of the specific band. The cycle number was optimized to 40 cycles, since PCR with 25–35 cycles did not amplify the band of interest. The minimum detection level or sensitivity of PCR to detect a 180 bp DNA fragment was observed to be 1 ng, which corresponds to 140 epithelial cells.

Confirmation of the PCR Product

EcoR1 digestion of the PCR product yielded two fragments of 103 bp and 77 bp on 2% agarose gel

(Fig. 1). Sequence analysis of the amplified 180 bp DNA showed complete homology with the published sequence of the *MOMP* gene of *C. trachomatis*. A BLAST search
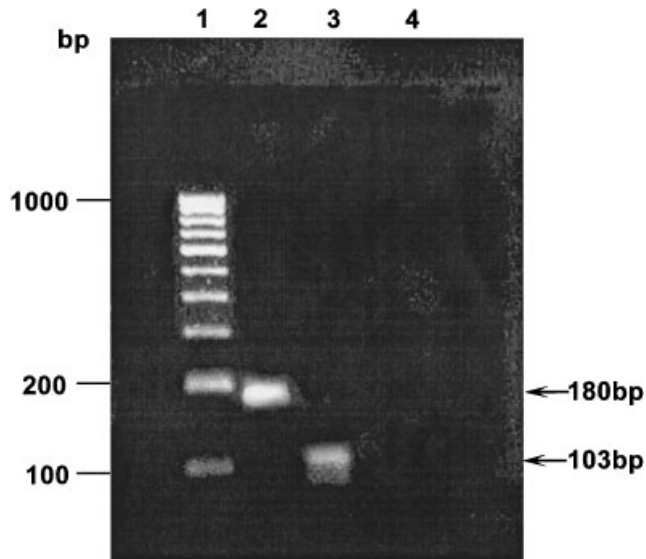

Fig. 1.   PCR amplified product of 180 bp DNA specific to the *C. trachomatis MOMP* gene, and its confirmation by enzyme digestion. Lane 1: 100 bp DNA ladder. Lane 2: Undigested 180 bp DNA fragment. Lane 3: EcoR1-digested 103 bp DNA fragment. Lane 4: Negative control.

(www.ncbi.nlm.nih.gov/blast) showed the amplified product was unique to the MOMP gene of *C. trachomatis*. In Southern hybridization, signals were detected on X-ray film corresponding to 180 bp amplified standard DNA of *C. trachomatis* for specimens (n = 57) that had a corresponding band on 2% agarose gel. However, some specimens (n = 33) that did not show an amplified band on agarose gel gave specific signals following Southern hybridization with the use of the *C. trachomatis* probe. This revealed the increased sensitivity of PCR followed by Southern hybridization (Table 1). Each specimen was processed for PCR and then hybridized with *C.trachomatis* probe and the result of hybridization (Southern blotting) was taken as the result of PCR.

Validation of the PCR Results

Hybridization with a specific probe increased the sensitivity and specificity of the detection, and hence each specimen was hybridized with the specific probe for reporting and evaluation. The evaluation results with other tests are presented in Table 1. The positivity and negativity of each specimen were confirmed by repeating the experiment and performing confirmatory tests, and were taken as the results of resolved specimens.

TABLE 1. Sensitivity, specificity, positive and negative predictive value of methods used for diagnosis of *C. trachomatis*

Tests		No. of resolved specimens			Sensitivity %	Specificity %	Positive predictive value %	Negative predictive value %
		+ve	−ve	Total				
N = 817								
Only PCR	+ve	57	0	57	63.3	100	100	95.7
	−ve	33	727	760				
PCR+Southern Hybridization	+ve	90	0	90	100	100	100	100
	−ve	0	727	727				
N = 30								
Only PCR	+ve	23	0	23	100	100	100	100
	−ve	0	7	7				
ELISA	+ve	20	0	20	86.96	100	100	70
	−ve	3	7	10				
N = 16								
PCR+Southern Hybridization	+ve	11	0	11				
	−ve	0	5	5	100	100	100	100
DFA	+ve	5	0	5				
	−ve	6	5	11	45.5	100	100	45.50
Pap	+ve	4 (5)[a]	3	12				
	−ve	1	1 (2)[b]	4	36.4 (81.8)	20 (60)	75	75
N = 76								
PCR+Southern Hybridization	+ve	0	0	0	100	100	100	100
	−ve	0	76	76				
Amplicor PCR	+ve	0	2	2	100	97.4	−	100
	−ve	0	74	74				

[a]Probably infected specimens on the basis of presence of intra-cytoplasmic vacuoles, increasing the specificity.
[b]Probably negative specimens on the basis of absence of intra-cytoplasmic vacuoles, increasing the sensitivity.

Of the 30 selected specimens, 20 specimens were positive for *C. trachomatis* by both PCR and ELISA, while PCR detected *C. trachomatis* in three additional specimens (Table 1). These positive specimens were further confirmed by analyzing the second specimen collected, as well as by the other confirmatory tests.

The presence of bright apple green elementary bodies in DFA-stained slides indicated that five of 16 tested specimens were positive for *C. trachomatis*. Pap smear detected intracellular organisms in vacuoles in the form of inclusion bodies in nine specimens. PCR results revealed 11 positive specimens, indicating a higher sensitivity and specificity for PCR (Table 1).

The commercially available Amplicor PCR kit was used to detect *C. trachomatis* in 76 samples, and it detected two positive specimens. These two positive specimens tested negative with our standardized PCR. The quality control specimen's result (from the Institute of Tropical Medicine, Antwerp, Belgium) matched with the result of our PCR, but not with the Amplicor PCR, suggesting 100% sensitivity and specificity of our PCR for the diagnosis of *C. trachomatis* (Table 1).

Specimens and Their Use in PCR

Of the 81 specimens received, 56.8% (n = 46) of introital specimens and only 43.2% (n = 35) of endocervical specimens had adequate cells (4–5 cells/10 μL/ high-power field). However, the sensitivity of the endocervical swab was higher than that of introital specimens (72.7%), detecting 90% of infected specimens that did not have sufficient cells. The overall sensitivity of endocervical specimens in diagnosing *C. trachomatis* was 92.9%, while that of introital specimens was 82.1%, when the specimens were tested without evaluating their adequacy for testing (Table 2).

Prevalence of *C. trachomatis* Infection

The results indicated an average 11.0% *C. trachomatis* rate in the clinic-based population, with 12.3% of women and 8.9% of males having this infection.

DISCUSSION

In the present study PCR followed by Southern blotting was taken as the result of PCR. Standardized PCR followed by Southern hybridization detected *C. trachomatis* in both cervical and introital specimens from women, as well as in urine sediments from males. The use of a DNA probe reduced the chance of false negativity, and increased the sensitivity and the negative predictive value of the technique. The sensitivity of an introital specimen was increased if adequate cells were procured for use in PCR. Care should be taken to collect at least two swabs in one sitting, so that if enough cells (at least four to five epithelial cells per high-power field) are not present in the first swab, the second swab can be used to confirm the result. In males, the noninvasive, self-collected approach was most acceptable. Urethral infection with *C. trachomatis* leads to the presence of copies of target DNA in urine, and is detectable by nucleic acid amplification tests. The standardized PCR can be used to diagnose *C. trachomatis* in the first morning void urine of males. Another advantage of this PCR is that it can be used to detect all of the serotypes of *C. trachomatis*, since the primer pair selected is from the conserved region of the published sequence of the *MOMP* gene of *C. trachomatis* (5). The specificity of the PCR was also improved with the use of 40 cycles of amplification when the number of infectious organisms was comparatively low, and the use of 25–35 cycles did not result in amplification of the visible band. Similarly, the annealing temperature is crucial and any deviation from a 52°C annealing temperature resulted in nonamplification of the specific sequence. The interpretation of results for any test with specificity of <100% should be made with caution, particularly for diagnosing infections such as *C. trachomatis*, which results in a high-risk pregnancy, infertility, and serious medical, legal, and social complications. The present study shows a 100% sensitivity and specificity for PCR followed by Southern hybridization, while ELISA had a low sensitivity. Although DFA is a rapid technique, microscopic evaluation of each specimen is laborious and requires highly trained and experienced personnel, as is evident from a survey of the College of American Pathologists (6). Our observations also suggest a low sensitivity for DFA, and indicate that experienced personnel are required for screening. Our own observations and earlier studies revealed that the Pap smear for diagnosing *C. trachomatis* infection

TABLE 2. Type of specimens (n = 81) and its adequacy for use in PCR

Specimens		Adequate		Inadequate		Total		Sensitivity %	Specificity %
		+ve	−ve	+ve	−ve	+ve	−ve		
Introital	+ve	15	–	8	–	23	–	82.1	100
	−ve	2	29	3	24	5	53		
Endocervical	+ve	8	–	18	–	26	–	92.9	100
	−ve	–	27	2	26	2	53		

was not sensitive (7–9) and cannot be recommended under any circumstances for that purpose (10). At present, no method of confirmation for PCR or LCR has been approved for use. The best method may be to confirm PCR by LCR or vice versa. In the present study we used the PCR test developed by Roche Diagnostics (Amplicor) to confirm our PCR results. Several studies have reported false-negative results with the Amplicor PCR (11–13); however, we observed false positivity with the Amplicor PCR, since our PCR results correlated well with the results from quality control specimens tested at the Institute of Tropical Medicine, Antwerp, Belgium. Our results indicated an average 11.0% infection rate in this clinic-based population (12.3% in women and 8.9% in men).

This study describes in detail the essential steps involved in standardizing an in-house-developed, sensitive, and specific PCR with Southern hybridization for diagnosing *C. trachomatis*. Cervical or introital specimens from women, and urine specimens from males can be used to detect this infection. The average infection rate was 11.0% in the clinic-based population of Mumbai.

REFERENCES

1. Piot P, Islam MQ. Sexually transmitted diseases in the 1990's: global epidemiology and challenges for control. Sex Transm Dis 1994;2:S7–S13.
2. Punnonen R, Terho P, Nikkanen V, Meurman O. Chlamydial serology in infertile women by immunofluorescence. Fertil Steril 1979;31:656–659.
3. DeMuylder X, Laga M, Tennstedt C, Van Dyck E, Alelbers GNM, Piot P. The role of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in pelvic inflammatory disease and its sequelae in Zimbabwe. J Infect Dis 1990;162:501–505.
4. Barnes RC. Laboratory diagnosis of human chlamydial infections. Clin Microbiol Rev 1989;2:119–136.
5. Stephens RS, Sanchez-Pescador R, Wagar EA, Inouye C, Urdea MS. Diversity of *Chlamydia trachomatis* major outer membrane protein genes. J Bacteriol 1987;169:3879–3885.
6. Woods GL, Bryan JA. Detection of *Chlamydia trachomatis* by direct fluorescent antibody staining. Results of the college of American Pathologists proficiency testing program 1986–1992. Arch Pathol Lab Med 1994;118:483–488.
7. Taylor-Robinson D. Evaluation and comparison of tests to diagnose *Chlamydia trachomatis* genital infections. Hum Reprod 12 (Natl Suppl JBFS 2(2)) 1997;12:113–120.
8. Fedorko DP, Smith TF. Chlamydial infections. In: Wentworth BB, Judson FN, Gilchrist MIR, editors. Laboratory methods or the diagnosis of sexually transmitted diseases. 2nd ed. Washington, DC: American Public Health Association; 1991. p 95–125.
9. Schachter J, Dawson CR. Human chlamydial infections. Littleton, MA: PGG Publishing Co.; 1978. p 187.
10. Schachter J, Stamm WE. Chlamydia. In: Murray PR, Pfaller MA, Tenover FC, Yolken RH, editors. Manual of clinical microbiology. 6th ed. Washington, DC: American Society for Microbiology; 1995. p 669–677.
11. Bauwens JE, Clark AM, Stamm WE. Diagnosis of *Chlamydia trachomatis* endocervical infections by a commercial polymerase chain reaction assay. J Clin Microbiol 1993;31:3023–3027.
12. Loeffelholz MJ, Lewinski CA, Silver SR, et al. Detection of *Chlamydia trachomatis* in endocervical specimens by polymerase chain reaction. J Clin Microbiol 1992;30:2847–2851.
13. Bass CA, Jungkind DL, Sliverman NS, Bondi JM. Clinical evaluation of a new polymerase chain reaction assay for detection of *Chlamydia trachomatis* in endocervical specimen. J Clin Microbiol 1993;31:2648–2653.