Jpn. J. Infect. Dis., 56, 88-92, 2003

Original Article

Evaluation of Diagnostic Efficacy of PCR Methods for *Chlamydia trachomatis* Infection in Genital and Urine Specimens of Symptomatic Men and Women in India

Joyee A. George, Thyagarajan S. Panchatcharam*, Rajendran Paramasivam, Sowmya Balasubramanian, Venkatesan Chakrapani[1] and Ganapathy Murugan[1]

Department of Microbiology, Dr. ALM Postgraduate Institute of Basic Medical Sciences, University of Madras and
[1]Institute of Sexually Transmitted Diseases, Chennai Medical College, Chennai, India

(Received February 28, 2003. Accepted June 16, 2003)

SUMMARY: In India, given the scarce availability of sensitive and specific methods, *Chlamydia trachomatis* genital infections may lead to severe clinical complications when left undiagnosed or underdiagnosed. The present study was conducted to evaluate the diagnostic efficiency and feasibility of polymerase chain reaction (PCR) assays using genital and urine specimens from men and women in India. Genital swabs and urine specimens collected from 143 patients attending the sexually transmitted disease (STD) clinic, Government General Hospital, Chennai, were tested by culture and a plasmid based PCR. Culture was positive in 27 (18.9%) patients. PCR gave positive results for 46 (32.2%) cases using genital specimens, and the positivity rate in urine was 25.2%. Once the discordant results between culture and PCR had been resolved by using a major outer membrane protein PCR, the overall sensitivity, specificity, and positive and negative predictive values for the plasmid PCR in genital specimens were 100%, 98%, 95.7%, and 100%, respectively. Corresponding values for urine PCR were 81.8%, 100%, 100%, and 92.5%, respectively. The prevalence of confirmed *C. trachomatis* infection was 30.8% in this STD population. The results confirmed the need to use sensitive and specific molecular assays like PCR to prevent underdiagnosis of genital chlamydial infections and to facilitate better clinical management of this infection in India.

INTRODUCTION

Chlamydia trachomatis infections are known to manifest in a variety of syndromes in both men and women when left undiagnosed and untreated. While the clinical presentations in men include urethritis, epididymitis, etc., women suffer more serious complications such as mucopurulent cervicitis, pelvic inflammatory disease (PID), ectopic pregnancy, and tubal infertility (1). Cell culture was long the gold standard for diagnosis of *C. trachomatis* infections because of its absolute specificity. However, due to its labor-intensive methodology, turnaround time, cost, and requirements for infrastructure and technical expertise, cell culture facilities were limited to specialized research laboratories only. Other nonculture tests, such as enzyme immunoassays (EIAs), serological tests, etc., lack the high sensitivity and specificity needed for accurate diagnosis of ongoing infection (2). Recently, nucleic acid amplification methods like polymerase chain reaction (PCR) and ligase chain reaction (LCR) have been reported to offer improved performance over those of culture and other non-culture tests (2,3).

Despite the wide availability of commercial nucleic acid amplification tests (NAAT) and their routine use in most of the industrialized countries in recent years, budgetary constraints have impeded the use of these techniques in developing countries like India. Reliable estimates of the rate of genital chlamydial infections in these countries using sensitive and specific methods like PCR are scarcely available in the literature. Few available Indian reports highlight the increased prevalence of genital chlamydial infection in women (4-8). The present study was conducted to evaluate the diagnostic efficiency of a PCR assay using genital and urine specimens of symptomatic sexually transmitted disease (STD) patients, both male and female, and to determine whether PCR could improve the reliability of diagnosis of genital chlamydial infection.

*Corresponding author: Mailing address: Department of Microbiology, Dr. ALM Postgraduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai-600 113, India. Tel: +91-44-24480785, 24925861, Fax: +91-44-24926709, E-mail: sptrajan@md4.vsnl.net.in

MATERIALS AND METHODS

Patients and specimens: A total of 143 symptomatic patients (80 women and 63 men) attending the STD outpatient clinic at the Institute of STDs, Government General Hospital, Chennai, during the period of September 1998 to August 2000, were enrolled in this study. In compliance with the Helsinki declaration, written informed consent to participate was obtained from each patient after explaining to him or her the study protocol and possible outcomes besides specific treatment. Detailed history, demographical and clinical features were recorded. Both genital swab specimens and first-voided urine specimens were collected from each patient. Thus, study materials consisted of 246 samples, including 80 endocervical specimens, 63 male urethral specimens, and 143 corresponding urine specimens. For culture, the genital swab specimens were placed in 0.2 M sucrose phosphate (2SP) buffer with 5% fetal bovine serum and antibiotics and transported on ice to the Microbiology laboratory. They were

either cultured on the same day or stored at −85°C for 12 to 24 h until being process for culture. Aliquots of the 2SP genital samples were used for PCR testing. The first-voided urine samples (15 ml) from the enrolled patients were collected in sterile containers, transported on ice, and processed. Separate aliquots of the genital swab specimens and urine, to be used for confirmatory testing, were stored at –85°C.

Isolation of *C. trachomatis* in cell culture: The culture for *C. trachomatis* was performed as described previously (9) with some modifications. The specimens in the 2SP transport medium were vortexed with 2 - 3 sterile glass beads, then 0.2 ml was inoculated onto 80% confluent McCoy cell monolayers in 24-well tissue culture plates. The inoculation was done in quadruplicate, in wells containing coverslips. The plates after centrifugation at 1000 × g for 1 h were subsequently incubated for 2 h at 37°C in 5% CO_2. The inoculum was further replaced with 1 ml of chlamydial growth medium containing 1 μg/ml cycloheximide. The plates were incubated for 48 - 72 h. After removal of the medium, the monolayers were fixed with 95% methanol for 10 min and subsequently stained with Lugol's iodine to detect intracytoplasmic inclusion bodies. The coverslips were parellelly stained with fluorescein-labeled species-specific monoclonal antibodies against the major outer membrane protein (MOMP) of *C. trachomatis* (Chlamyset Antigen FA; Orion Diagnostika, Espoo, Finland) and examined for chlamydial elementary bodies using a fluorescence microscope (Optiphot-2; Nikon Corp., Tokyo).

PCR: The urethral/endocervical specimens in the 2SP transport medium were freeze-thawed, and 400 μl of each sample was centrifuged at 16,000 × g for 30 min. Forty microliters of lysis buffer (1 mM EDTA, 1% Triton X-100, and 50 mM Tris-HCl pH 7.5, and 400 μg/ml proteinase K) was added to the pellet, and the suspension was incubated for 60 min at 37°C. The lysate was then boiled for 10 min to inactivate proteinase K, centrifuged briefly, and 5 μl of the supernatant was directly used for the amplification reaction. In the case of urine specimens, 500 μl of each urine sample was centrifuged at 16,000×g for 15 min, then the pellet was washed with 1 ml of PBS. After the supernatant had been discarded, 500 μl of the lysis buffer was added and the solution was incubated for 60 min at 37°C. The preparation was then extracted with phenol:chloroform and ethanol-precipitated. The final pellet was dissolved in 50 μl of sterile nuclease free water. Ten microliters of this preparation was used for PCR testing. Oligonucleotide primer sequences (T1: 5′-CTA GGC GTT TGT ACT CCG TCA-3′ and T2: 5′-TCC TCA GGA GTT TAT GCA CT-3′) derived from the common endogenous plasmid of *C. trachomatis* generating a species-specific 200 bp amplified product with all the known serovars of *C. trachomatis* was used (10). A 50 μl amplification reaction was performed. The reaction mixture contained Tris-HCl (10 mM), KCl (50 mM), $MgCl_2$ (2.5 mM), nuclease free BSA (100 μg/ml), 200 μM (each) of the dATP, dTTP, dGTP, and dCTP, 0.1 μM of each of the primer; and 1 unit of Taq DNA polymerase (Thermoprime plus DNA polymerase; AB Gene, Epsom, Surrey, UK). The amplification was performed in a PCR thermal cycler (PTC-100, MJ Research, Watertown, Mass., USA) for 40 cycles. Each cycle contained a denaturation step at 95°C for 1 min, an annealing step at 55°C for 2 min, and an elongation step at 72°C for 3 min. The amplified product was analysed by 2% agarose gel electrophoresis.

Restriction digestion analysis of the PCR product: This was performed for confirmation of the 200 bp PCR product, based on the fact that the product possessed a restriction cleavage site for the restriction enzyme *Hpa*II. Ten microliters of the PCR product was subjected for restriction endonuclease digestion. A 20 μl cleavage reaction was performed using a reaction buffer containing 5 mM $MgCl_2$ and 5 U of the restriction enzyme *Hpa*II (Gibco BRL Life Technologies Inc., Grand Island, N.Y., USA). The reaction mixture was incubated at 37°C, and the reaction was terminated at 65°C after 2 h. The digested product was separated by electrophoresis on a 3% high resolution agarose gel (Midi-B agarose; AB Gene). DNA fragments of 74 bp and 126 bp were observed under UV light and compared with the molecular weight marker, ϕ 174 *Hae*III digest (Gibco BRL Life Technologies Inc.).

The sensitivity limit of the PCR assay, determined by serial dilutions of purified *C. trachomatis* L2 DNA, was found to correspond to 0.1 i.f.u. Specificity of the PCR was confirmed by testing control DNA samples of *C. psittaci* and *C. pneumoniae*.

Confirmatory PCR testing: Samples giving positive results by PCR were tested by a nested MOMP PCR assay which is as sensitive as the plasmid PCR (sensitivity limit determined corresponded to 0.1 i.f.u.), as described previously (11). The primers used were Sero 1A (5′-ATG AAA AAA CTC TTG AAA TCG G-3′) and Sero 2A (5′-TTT CTA GAT/C TTC ATT/C TTG TT-3′) for primary PCR, and PCTM3 (5′-TCC TTG CAA GCT CTG CCT GTG GGG AAT CCT-3′) and Sero 2A for the nested PCR.

All the culture positive samples and discordant samples (both genital specimens and urine) were subjected to confirmatory testing. A patient was considered to be truly infected if culture was positive and/ or if the MOMP PCR confirmed the initial PCR positive results. Based on this expanded spectrum of positivity, the performance value of the PCR assay for different types of specimen was calculated.

Statistical methods: The data analysis was performed by using the statistical software SPSS for Windows version 8.0. and Epi Info version 6.04 (12).

RESULTS

There were 143 patients (63 males and 80 females) enrolled in this study. The age distribution of the cases showed the mean age to be 29.7 ± 6.1 years (the mean age of males was 30.1 ± 6.7 years and that of females was 29.3 ± 5.7 years). *C. trachomatis* was isolated using cell culture in 27 cases (18.9%); 11 (17.5%) of 63 men and 16 (20.0%) of 80 women with no significant difference in isolation rate between males and females ($P > 0.05$). PCR was done for both genital swabs and urine specimens. Genital swab PCR detected 46 positive cases out of 143 patients using aliquots of the same specimens in 2SP medium that had been used for culture. PCR was positive in 24 (38.1%) of 63 male urethral samples and 22 (27.5%) of 80 female endocervical samples. The urine PCR was positive in 36 urine samples, 17 of which were from men and 19 of which were from women. A comparison of cell culture and PCR results relative to gender is shown in Table 1. The 63 male patients included 58 urethritis cases and five epididymitis cases, and the 80 female patients included 53 cervicitis cases, 22 PID cases, and five infertility cases. *C. trachomatis* positivity corresponding to these clinical manifestations by cell culture and plasmid PCR is shown in Table 2.

All the positive specimens of culture and PCR were

Table 1. Comparison of cell culture and PCR for the detection of *Chlamydia trachomatis* in samples from men and women

	n	Culture No. positive (%)	Genital swab PCR No. positive (%)	Urine PCR No. positive (%)
Males	63	11 (17.5)	24 (38.1)	17 (27.0)
Females	80	16 (20.0)	22 (27.5)	19 (23.8)
Total	143	27 (18.9)	46 (32.2)	36 (25.2)

subjected to confirmatory MOMP nested PCR assay. Culture positive specimens were positive by both plasmid and MOMP PCR assays. The initial PCR testing raised the *C. trachomatis* infection rate from 18.9% (as determined by culture) to 32.2%. Nineteen discrepant genital samples (13 male urethral and six female endocervical) were tested by MOMP PCR, and 17 were reconfirmed; two male urethral specimens were negative by MOMP PCR. Similarly, of nine urine samples (six male and three female urine specimens) from the culture negative PCR positive group, six positives (five male and one female urine specimens) were confirmed by MOMP PCR.

Patients who were positive by culture and/or by confirmatory PCR on genital specimens were considered 'truly infected'. By this definition, there were 44 truly infected patients (22 males and 22 females). Two plasmid PCR positive cases that turned out to be MOMP PCR negative (males) were scored as false positives in this analysis; these cases could not be confirmed because the samples were insufficient for retesting by either of the assays. Follow-up specimens could not be collected because the patients had received syndromic treatment by that time. Based on this expanded spectrum of positivity, the sensitivity, specificity, and predictive values for the plasmid PCR assay on different types of specimens were calculated. The prevalence of confirmed *C. trachomatis* infection was determined as 30.8% (44/143). The resolved sensitivity of the genital swab PCR was 100% (44 out of 44); and specificity was 98% (Table 3). The positive predictive value (PPV) and negative predictive value (NPV) were 95.7% and 100%, respectively. The overall sensitivity of culture was 61.4% based on the expanded spectrum. In both men and women, genital swab PCR exhibited 100% sensitivity. The resolved sensitivity, specificity, PPV, and NPV for the urine PCR were 81.8%, 100%, 100%, and 92.5%, respectively. Both genital and urine PCR assays were significantly more sensitive than culture ($P < 0.05$). Table 3 summarizes the performance characteristics of plasmid PCR and compares them to those of the diagnosis using culture after expanding the spectrum of positivity with MOMP PCR testing. Out of the 44 truly infected patients based on genital swab positivity, urine specimens of eight patients did not show positivity by either of the PCR assays. The difference in sensitivity between genital swab PCR and urine PCR was statistically significant ($P < 0.05$), suggesting that the genital swab obtains a better clinical specimen for PCR testing in genital chlamydial infection.

DISCUSSION

The performance of the PCR technique used for diagnosis of *C. trachomatis* may vary among different settings and

Table 2. *Chlamydia trachomatis* positivity by cell culture and PCR associated with different clinical manifestations in men and women

	n	Clinical manifestations		Cell culture No. positive (%)	Genital swab PCR No. positive (%)
Males	63	Urethritis	58	10 (17.2)	23 (39.7)
		Epididymitis	5	1 (20.0)	1 (20.0)
Females	80	Cervicitis	53	12 (22.6)	16 (30.2)
		PID*	22	3 (13.6)	4 (18.2)
		Infertility	5	1 (20.0)	2 (40.0)

*PID: pelvic inflammatory disease

Table 3. Performance characteristics of the plasmid PCR assay in genital and urine specimens from males and females compared to those of culture based on expanded spectrum of positivity after confirmatory PCR testing

Assay	Sensitivity	Specificity	PPV*	NPV**
Cell culture	61.4% (27/44)	100% (99/99)	100% (27/27)	85.3%(99/116)
PCR (genital swabs)	100% (44/44)	98.0% (97/99)	95.7% (44/46)	100% (97 /97)
PCR (urine)	81.8% (36/44)	100% (99/99)	100% (36/36)	92.5%(99/107)
Cell culture				
Males	50.0% (11/22)	100% (41/41)	100% (11/11)	78.8% (41/52)
Females	72.7% (16/22)	100% (58/58)	100% (16/16)	90.6% (58/64)
Genital PCR				
Males	100% (22/22)	95.1% (39/41)	91.7% (22/24)	100% (39/39)
Females	100% (22/22)	100% (58/58)	100% (22/22)	100% (58/58)
Urine PCR				
Males	77.3% (17/22)	100% (41/41)	100% (17/17)	89.1% (41/46)
Females	86.4% (19/22)	100% (58/58)	100% (19/19)	95.1% (58/61)

* PPV: Positive predictive value.
**NPV: Negative predictive value.

different types of clinical specimens. Many studies have evaluated different PCR systems, commercial as well as in-house PCR assays, and many of these have been shown to be highly sensitive for the detection of *C. trachomatis* in clinical specimens (13-17). However, hindering factors like cost and specialized infrastructure have precluded the use of PCR for diagnostic purposes in developing countries like India. To our knowledge, the present study is the first to evaluate the diagnostic efficacy of PCR in both urine and genital swab specimens of symptomatic male and female patients in India.

Some early studies reported the sensitivity of PCR to be less than or similar to that of culture or antigen detection assays (18-20). In contrast, several recent studies, including the present one, demonstrated that PCR has higher sensitivity than culture for the detection of *C. trachomatis* in clinical specimens. The sensitivity of culture has been estimated to range from 50-85% in different laboratory settings when compared to that of molecular amplification assays (14-16,21). The overall sensitivity of culture in our study was 61.4% (50% in males and 72.7% in females). It was observed that PCR on urethral swab specimens, compared to culture, detected twice as many cases. In addition to rapidity of diagnosis, PCR has specificity and sensitivity superior to those of culture methods.

The results of the present study emphasize the diagnostic value of PCR, which showed increased detection of male and female truly infected patients. For example, by the confirmed PCR results, the number of *C. trachomatis* positive cases among our cases increased from 27 (18.9%) to 44 (30.8%). Nearly 39% of the infected patients would have been left undiagnosed if PCR testing had not been performed. In both men and women, PCR testing, compared to culture, increased the rate of detection. Two male patients (plasmid PCR- positive and MOMP PCR-negative) could not be confirmed because their samples were insufficient for retesting. Follow-up specimens from these patients would not have been useful, given that they had by then received treatment on a syndromic basis. Even though these patients were scored as false positives for analytical purposes, it can be speculated that the likelihood of *C. trachomatis* infection cannot be excluded in such cases of high-risk symptomatic patients. In symptomatic patients, PCR sensitivity appeared very high in genital swab specimens. Even though urine PCR testing is a useful alternative to genital swabs, it must be emphasized that the presence of PCR inhibitory substances in urine, which can affect the sensitivity of PCR, should be monitored to avoid false negative PCR results.

In the current study, a DNA spiking method (*C. trachomatis* L2 DNA corresponding to 1 i.f.u. was spiked onto the samples) was used to identify the presence of PCR inhibitors in urine as well as in swab specimens. Although none of the genital swab specimens showed PCR inhibition, a high rate (6.3%) of inhibition was observed in the urine specimens. PCR inhibitory substances were present in 8.6% of female urine samples and in 3.2% of male urine samples, which rates are comparable to those reported by others for urine specimens (22,23). In our study, urine samples of three culture positive female patients were found to be PCR inhibitory. Extraction with phenol and chloroform after 1:10 dilution of the specimens and boiling of the extracted DNA samples for 10 min removed inhibitory activity from these urine specimens, facilitating successful amplification with the PCR assay.

Our findings showed a high prevalence of *C. trachomatis* infection (35%) among male patients attending the STD clinic. It is important to consider that failure to identify low level infections in men presumably allows for the maintenance of a large untreated reservoir of infection in the general population, and, if these cases are left undiagnosed, they may lead to transmission of the disease to female partners. It is therefore emphasized that efforts to control chlamydial infection that ignore males harboring the infection will ultimately fail (24). Most of the previous studies in India have focused on the impact of *C. trachomatis* infections in females only. This is the first PCR-based report from India on genital chlamydial infections in symptomatic men and women. Although financial constraints are projected as impediments to the routine use of molecular diagnostic methods in developing countries like India, it should be borne in mind that the cost arising from the clinical sequelae of leaving genital *C. trachomatis* infections undiagnosed and untreated may exceed the cost of these diagnostic methods.

ACKNOWLEDGMENTS

We are thankful to Dr. W.H.M. Vogels, Laboratory for Medical Microbiology, University Hospital, EZ Groningen, the Netherlands, for the kind gift of plasmid primers for the study. We thank Dr. Fay Betsou, Biobanque de Picardie and Department of Bacteriology, University Hospital, Amiens, France, for kindly sending the purified DNA samples of *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*.

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