Research Article

Comparative analysis of smear testing, in-house PCR and commercial real-time PCR for genital chlamydia detection

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Abstract

Introduction: Genital infection caused by *Chlamydia trachomatis* serovars D-K is the commonest bacterial sexually transmitted disease (STD) with the highest prevalence seen among youth. The objectives of the study were to determine the effectiveness of smear testing in diagnosing chlamydia infection, provide an alternative diagnostic test (in-house conventional polymerase chain reaction) for the detection of chlamydia, and compare it with a commercial rt-PCR as the reference test.

Methods: The study was a cross-sectional, descriptive study enrolling 216 female and 252 male consecutive adult attendees of the Central STD Clinic, Colombo. Gram stain of cervical smear, urethral smear, and urine was carried out and compared with the reference standard of COBAS® TaqMan® CT v2.0 rt-PCR on endocervical swabs from females and urine from males. In addition, a comparative study was conducted on 50 endocervical swabs to compare the commercial real-time (rt)PCR with the conventional in-house PCR.

Results: Cervical smears , urethral smears in females and males, and urine deposits in males had sensitivity of 10-50% and specificity of 0-99.4% compared to the reference test. The in-house PCR had sensitivity, specificity, positive and negative predictive values, diagnostic accuracy, positive likelihood ratio, and negative likelihood ratio of 91.3%, 96.3%, 95.4%, 92.8%, 94%, 24.6 and 0.09, respectively, when compared to the reference test.

Conclusion: This study concluded that smear tests are not effective in diagnosing genital chlamydia infection. The PCR assay evaluated in this study can significantly improve the sensitivity and specificity of diagnosing genital chlamydia infection in the local setting.

Keywords: *Chlamydia trachomatis, sexually transmitted diseases, polymerase chain reaction*

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Introduction

Chlamydia trachomatis (serovars D-K) is the most prevalent sexually transmitted bacterial pathogen worldwide, with the highest prevalence among youth.[1,2] In 2020, the World Health Organization (WHO) estimated the global prevalence of chlamydia among women aged 15–49 years to be 4.0%. Among men, the estimated prevalence was 2.5%. According to the WHO, the estimated global incidence of chlamydia is 128.5 million cases among adults aged 15 to 49 years.[3]

C. trachomatis is a Gram-negative, obligatory intracellular bacterium with a single chromosome and an approximately 7500 base pair cryptic plasmid. In women, genital *C. trachomatis* infection causes a variety of clinical syndromes, including cervicitis, urethritis, endometritis, salpingitis, peri-appendicitis, and pelvic inflammatory disease, which, if left untreated, may lead to complications such as ectopic pregnancy and tubal factor infertility. In men, it causes urethritis and epididymitis. It is also a known cause of conjunctivitis, proctitis, and Reiter's syndrome in both males and females. Perinatal transmission of *C. trachomatis* during vaginal delivery results in ophthalmia neonatorum and neonatal pneumonia. The most typical clinical syndrome of genital infection is non-gonococcal urethritis (NGU) in males and females and non-gonococcal cervicitis (NGC) in females. Although many aetiological agents cause these syndromes, *C. trachomatis* is the most common, accounting for 30%–50% of cases of NGU.[5]

Many infected women do not seek medical care as 70% of genital infections in females are asymptomatic, and, even in men, asymptomatic infection accounts for 50% of cases[6], giving rise to a persistent chlamydia reservoir with sustained transmission. Undiagnosed and untreated chlamydial infections lead to individual health consequences and significant epidemiological, social, and economic problems for countries.

The goal of the study was to diagnose and treat lower genital tract infections by *C. trachomatis* early to prevent complications and to interrupt transmission. Early detection is of real value since effective treatment is available. The STD/AIDS clinics in Sri Lanka follow a modified version of the WHO syndromic approach[7], using conventional smear tests and gonococcal culture to differentiate gonococcal from NGU or NGC for management. This syndromic management approach explains both failures to diagnose a significant number of cases of *C. trachomatis* infection, including all asymptomatic patients, and over-treatment of uninfected patients.[7,8]

Nucleic acid amplification tests (NAATs) are the most sensitive diagnostic method for genital chlamydia infection. However, they have several limitations such as cost, availability, and the technical challenges of in-house polymerase chain reaction (PCR) tests. Many commercial NAATs for diagnosing genital chlamydia are available in the market[1] and many laboratories have also developed in-house PCR tests to reduce costs. This study utilised the *orf* 8 region in the cryptic plasmid to detect chlamydia. Several studies have found that the plasmid target is preferred over significant outer membrane protein complex genes (*omp*1, *omc*B) or 16S rRNA due to its higher sensitivity.[9,10,11] Although this target is absent in the plasmid-free variant of *C. trachomatis* (Swedish variant), this is not a significant limitation as this strain is exceptionally uncommon.

The study aimed to evaluate the effectiveness of cervical smear, urethral smear, and urine deposit microscopy in diagnosing genital chlamydia infection compared to a commercial NAAT test on

endocervical swabs in females and urine deposits in males. The second objective of the study was to provide an alternative diagnostic test, optimise an in-house polymerase chain reaction (PCR) assay for the detection of chlamydia in endocervical swabs and compare it with the commercial real-time PCR (rt-PCR) as the reference test. .

Methods

A cross-sectional, descriptive study was carried out on 216 adult female attendees and 252 adult male attendees of the Central STD Clinic, Colombo, recruiting consecutively from 1st December 2014 to 31st March 2015.

In females, endocervical swabs for the NAAT were collected using Dacron© swabs in a universal transport medium (UTM-RT, Copan, Italy) by inserting the swab 1–2 cm into the endocervix followed by 2-3 rotations of 360 degrees for 10-30 seconds. Endocervical and urethral swabs from females and urethral swabs from males were collected using sterile cotton swabs. In males, 30-40 mL of urine from the first part of the urinary stream was collected into a sterile container after refraining from urinating for at least two hours. Two mL of urine was centrifuged at 12,500 g for five minutes and the deposit used for Gram stain and rt-PCR.

Cervical smears (separate swabs) and urethral smears from females and urethral smears and urine deposits from males were stained by the Gram stain and interpreted according to the standard operating procedures of the Sri Lanka College of Microbiologists.[12] Aliquots of the transport medium of the endocervical swabs from females and the urine deposit from males were stored at -70 ºC for NAAT . The sensitivity, specificity, and likelihood ratio of the smear tests in diagnosing chlamydia infection were determined by taking the commercial rt-PCR as the reference standard.

In addition, we compared an in-house conventional PCR with the rt-PCR for the diagnosis of chlamydia in females using endocervical swabs. A systematic sampling technique of selecting every fourth swab was used until a total of 50 endocervical swab samples were achieved. Smear and urine deposit Gram stain microscopy and the commercial rt-PCR test were carried out at the laboratory of the Central STD Clinic. Simultaneously, the in-house conventional PCR was optimised and used at the Molecular Biology Laboratory of the Medical Research Institute (MRI).

Commercial real-time PCR (rt-PCR). Endocervical swabs from females collected into UTM-RT and urine deposits from males were tested using the COBAS® TaqMan® CT v2.0 rt-PCR (Roche Diagnostic Systems, Inc., Branchburg, N.J., USA), a commercial, validated, CE (Conformité Européene), IVD (*in-vitro* diagnostic) certified NAAT for the qualitative detection of *C. trachomatis* DNA from endocervical swabs in females and urine in males and females. The limit of detection of the kit for purified DNA is five copies of *C. trachomatis* DNA per PCR reaction. The primers used for amplification are directed at dual targets, the chromosomal *ompA* gene and the cryptic plasmid, common to all 15 *C. trachomatis* serovars, including the Swedish variant *C. trachomatis* (nvCT).

In-house PCR. Endocervical swabs were tested for chlamydial DNA by an in-house PCR targeted to amplify the cryptic plasmid gene (*orf* 8 region) of *C. trachomatis*. According to manufacturer's

instructions, DNA was extracted using a commercial DNA extraction kit (QIAamp DNA Mini Kit, Qiagen, Germany). Forward and reverse primers were adopted from a previously published study.[13] The primer sequences were aligned and compared with reference *C. trachomatis* sequences using primer-BLAST at the National Center for Biotechnology Information (NCBI) (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*).

Forward primer 5'-CTAGGCGTTTGTACTCCGTCA-3'
Reverse primer 5'- CTAGGCGTTTGTACTCCGTCA -3'

The protocol for the preparation of the master mix was adopted from the same study[13] and modified during optimisation. For optimisation of the PCR, positive control, negative control, nuclease-free deionised water, and five endocervical samples were used.

The master mix was prepared in a 1.5 mL micro-centrifuge tube within a cold box at 4 ºC. All reagents were thawed to 4 ºC before use. The final volume of the master mix was 12.5 µL, consisting of 5 µL of 5X Green Go Taq Flexi buffer, 2 µL of 25 mM $MgCl_2$, 1µlLof 10mM of each dNTP mix, 0.64 µL of each reconstituted forward and reverse primers, 2.97 µL of nuclease-free, deionised water and 0.25 µL of 5 U/µL Go Taq DNA polymerase which was added last. 12.5 µL of extracted DNA template was added to the master mix inside a PCR hood (Astec Microflow OMNI PCR workstation). Amplification was carried out in a thermal cycler (Mycycler, Bio-Rad, USA). The thermal profile was initial denaturation at 94 ºC for 5 minutes, followed by 37 cycles including denaturation at 94 ºC for 30 seconds, annealing at 57 ºC for 30 seconds, elongation at 72 ºC for 30 seconds, with a final extension step at 72 ºC for 5 minutes. The amplified products (10 µL) and a 50bp ladder (Cat. No. G4521, Promega, USA) were mixed with 2 µL of gel loading dye (Cat.No. G1881, Promega, USA) and loaded into wells in a 2% agarose gel in 1X TBE (Tris-Borate EDTA). Gel electrophoresis was carried out at 100V using a mini-gel electrophoresis apparatus using 1X TBE electrophoresis buffer. The gel was stained with ethidium bromide for 20 minutes under recommended precautions and visualised under a UV illuminator. The gel image was captured using a gel doc system (Molecular Image, Gel Doc XR System, Bio-Rad, USA). The gel was analysed and annotated using Quantity One quantification software version 4.3.0 (Bio-Rad, USA) to obtain data on lane and band detection and band labeling.

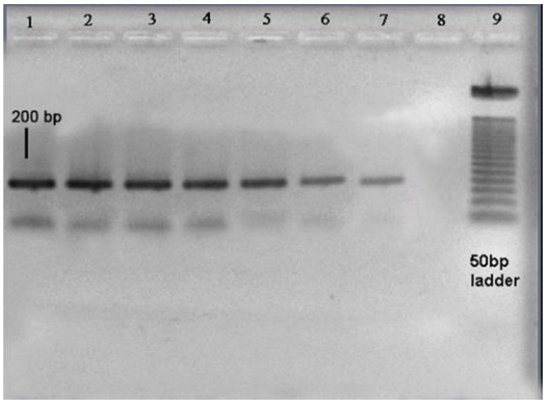


Figure 1. Gel photograph of the lower limit of detection.
Lane 1: 10^{-1} dilution of neat sample,
Lane 2, 3, 4, 5, 6, 7: 10^{-2} to10^{-7} dilution of neat sample,
Lane 9: 50bp DNA ladder

The lower limit of detection of the in-house PCR test was determined using serial 10–fold dilutions of the extracted DNA of the neat positive control (Figure 1).

The annealing temperature, the volume of extracted DNA, and the concentration of master mix ingredients were adjusted to obtain a sharp, clear amplicon band without smudging or primer dimers (Figure 1). A 10^5 dilution of a clinically corresponding, rt-PCR-confirmed positive specimen of endocervical swab with a CT value of 28.3, which was replicated three times to ensure its repeatability was used as the positive control. A rt-

PCR-confirmed negative sample which was replicated three times to ensure its repeatability was used as the negative control. Although the optimum annealing temperature was calculated as 58 ºC using http://tmcalculator.neb.com/#!/, it was adjusted to 57 ºC during optimisation.

Results

Comparison between commercial rt-PCR and microscopy

Analysis was done on 215 cervical swabs and 214 urethral swabs in females, as well as 81 urethral swabs, and 78 urine deposits in males. The sensitivity, specificity, and likelihood ratio (diagnostic odds ratio) of smear and urinary deposit microscopy, conventionally used for the diagnosis of chlamydia infection in Sri Lanka, were determined against commercial rt-PCR assay (Table 1).

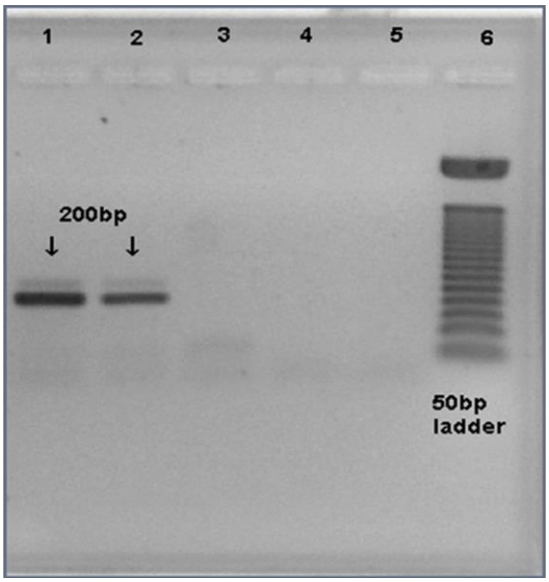
Table 1: Comparison of smear tests with commercial real-time PCR.

Test	No. positive (n/total)	Positivity (%)	Sensitivity (%)	Specificity (%)
Cervical smear (females) ≥30 pus cells/hpf	61/215[a]	28.4	35.1	73
Urethral smear (females) ≥10 pus cells/hpf	5/214[b]	2.3	10.8	99.4
Urethral smear (males) ≥10 pus cells/hpf	18/81[c]	22.2	25	78
Urine deposits (males) ≥15 pus cells/hpf	25/78[d]	32	50	70

[a]One participant out of 216 did not give a cervical smear specimen
[b]two of the female participants did not give urethral smears.
[c]Only 81 out of 252 male participants consented to carry out urethral smears
[d]Gram staining of urine deposits was done only from 78 of 252 specimens.

Detection of chlamydia by in-house PCR.

The appearance of a band of 200bp was considered positive for chlamydia[13] (Figure 2).



Lane 1:– positive control
Lane 2: positive sample
Lane 3: negative sample
Lane 4: negative control
Lane 5 : deionised water /extraction control
Lane 6:50bp ladder

Figure 2. Gel photograph of in-house PCR.

Comparison of in-house PCR assay with the commercial rt-PCR.

Table 2: Results of in-house PCR in comparison with commercial rt-PCR.

In-house PCR	rt-PCR		Total
	Positive	Negative	
Positive	21	1	22
Negative	2	26	28

The results of 50 endocervical swabs tested by the in-house and commercial rt-PCR were tabulated. When the swabs were tested using the in-house PCR, there were 21 true positives, 26 true negatives, one false positive, and two false negative results (Table 2).

The in-house PCR was compared with the rt-PCR as the reference standard, and test parameters were calculated (Table 3).

Table 3: Test parameters of the in-house PCR in comparison with commercial rt-PCR.

Test	Sensitivity	Specificity	Diagnostic accuracy	Predictive value	
				PPV	NPV
In-house PCR	91.3%	96.3%	94%	95.4%	92.8%

The positive likelihood ratio was 24.6, and the negative likelihood ratio was 0.09, indicating a statistically significant relationship between the results of in-house PCR and chlamydia infection. The high positive likelihood ratio found in this study showed a strong association between a positive test result and the presence of chlamydia, while a low negative likelihood ratio indicated a strong association between a negative test result and the absence of chlamydia.

Discussion

Genital infection with *C. trachomatis* is responsible for considerable pelvic inflammatory diseases, infertility, and ectopic pregnancy in women. However, since most infections are asymptomatic, they remain undetected and untreated, even in women obtaining healthcare in the community or hospital setting.[6]

This study aimed to clarify the effectiveness of conventional Gram stain smears in diagnosing *C. trachomatis* infection. Our study shows that none of these tests had adequate sensitivities, and the only test with an acceptable specificity of 99.4% was urethral smear microscopy in females. These values are very similar to those determined by a Canadian study done in 2001 which also showed that estimation of polymorphonuclear leucocytes per high power field (hpf) in endocervical swabs using a cut-off of >10 pus cells/hpf on its own, was unsuitable for the presumptive diagnosis of *C. trachomatis* due to low sensitivity (52%) and specificity (71%).[14] A syndromic approach to diagnosis, combined with microscopy, as recommended by the WHO, will result in misdiagnosis of a majority of infections due to the unacceptably low sensitivity of clinical diagnosis resulting from a high proportion of asymptomatic infection coupled with the poor sensitivity of the smear tests.[7,8] Therefore, it is prudent to switch to using a NAAT for the diagnosis of genital chlamydia as many experts recommend.[1]

The in-house conventional PCR developed by us had excellent performance parameters with sensitivity and specificity, positive and negative predictive values, and diagnostic accuracy of 91.3%, 96.3%, 95.4%, 92.8%, and 94%, respectively, a positive likelihood ratio of 24.6 and a negative likelihood ratio of 0.09 when compared to the commercial rt-PCR. The performance of this PCR is comparable to an in-house PCR developed in India with a sensitivity of 93.1%, specificity of 97.46 %, positive predictive value of 94.7%, and negative predictive value of 96.6 % when compared with the commercial Roche AMPLICOR MWP CT assay.[15]

Validation of the in-house PCR for urine samples in males is recommended in the future as it was not carried out due to time constraints. A specimen positive in the rt-PCR was used as the positive control for the in-house PCR as the ATCC culture stain of *C. trachomatis* was not available. .

Conclusion

Cervical and urethral smear and urine deposit microscopy are ineffective for diagnosing genital chlamydia infection. This accurate conventional PCR test may be a useful test to incorporate in diagnostic testing for genital chlamydia in the Sri Lankan context.

Declaration

Conflict of interest: The authors declare that there are no conflicts of interest associated with this manuscript.
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Ethics statement: Ethics approval (approval number: 23/2014) was obtained from the Ethics Review Committee, Medical Research Institute, Colombo. Written informed consent was obtained from the study participants before collecting the samples. The anonymity of the participants was maintained throughout the study. Permission to collect samples and carry out laboratory testing was obtained from the Director, the National STD/AIDS Control Programme (NSACP). The Director of MRI granted permission to use the Molecular Biology Laboratory to evaluate the in-house PCR.
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Author contributions:
RND De Silva: Conceptualization, Methodology, Project administration, Investigation, Software, Data curation, Validation, Writing-Original draft
EM Corea: Methodology, Writing -Review & Editing, Supervision
JP Elwitigala: Resources, Supervision, Funding acquisition
R Muthugala: Methodology, Validation, Writing -Review & Editing

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