Original Article

Multiplex nested polymerase chain reaction targeting multiple genes for the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in genitourinary specimens

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Abstract

Objectives: The objective of this study was to design and evaluate a novel multiplex nested polymerase chain reaction (PCR) protocol for simultaneous detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in genitourinary specimens obtained from symptomatic patients clinically suspected of sexually transmitted infections (STIs), targeting two different genes each for these pathogens. **Materials and Methods:** A total of 116 genitourinary specimens were collected from men (n = 12) and women (n = 104). Direct microscopy, culture isolation, and antimicrobial susceptibility testing for *N. gonorrhoeae* were performed. Multiplex nested PCR was performed on clinical samples using novel designed primers targeting *porA* pseudogene and *opa* gene of *N. gonorrhoeae* and *momp gene and* cryptic plasmid of *C. trachomatis* simultaneously. DNA sequence analysis of nested PCR amplicons for each of four gene targets was carried out for the validation of in-house designed primers and PCR protocol. **Results:** A total of 51.72% (60/116) patients were detected to have either of the two STIs. About 35.35% (41/116) of patients were positive for *C. trachomatis* and 33.62% (39/116) for *N. gonorrhoeae* by employing multiplex nested PCR. Coinfection with *N. gonorrhoeae and C. trachomatis* was detected in 17.24% (20/116) patients. 31.5% endocervical swabs (n = 54), 64.4% speculum-assisted high vaginal swabs (n = 45), and 80% self-collected vaginal swabs (n = 5) were detected positive for either of two STIs. **Conclusions:** The multiplex nested PCR protocol designed and employed in the present study may be used in the diagnosis and management of both symptomatic as well as asymptomatic cases of *N. gonorrhoeae and C. trachomatis*, particularly among high-risk groups.

Key words: Cervicitis, *Chlamydia trachomatis*, multiplex nested polymerase chain reaction, *Neisseria gonorrhoeae*, urethritis

INTRODUCTION

Sexually transmitted infections (STIs) are major public health problem worldwide, with *Neisseria gonorrhoeae* and *Chlamydia trachomatis* being the most prevalent bacterial STI pathogens. Early and accurate diagnosis of these STIs is necessary as delay in the diagnosis and treatment may lead to severe complications. Culture isolation of both *C. trachomatis* and *N. gonorrhoeae* is

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technically demanding and relatively insensitive. Of the nonculture tests available, only nucleic acid amplification testing (NAAT) is recommended for routine use.[1]

For an effective and successful NAAT, two most important aspects are the proper selection of the target gene and use of specific primers amplifying the conserved region of target genes, which undergo minimal genetic changes or mutations and should have considerable differences from their commensal species.[1]

porA pseudogene of *N. gonorrhoeae* is highly conserved, with the low genetic polymorphism and found absent in commensal *Neisseria* species, and sufficiently divergent from *porA* gene of *Neisseria meningitidis*.[2] *opa* genes of *N. gonorrhoeae* are family of 11 genes contained in separate loci that harbor highly conserved regions and encode proteins with physiological functions.[3] The *momp* gene of *C. trachomatis* consists of five highly conserved constant domains separated by four evenly spaced variable regions.[4] The 7.4-kb cryptic plasmid is highly conserved and essential component of *C. trachomatis* genome with <1% nucleotide sequence variation. It occurs as 2–10 copies per genome and contains eight open reading frames (ORFs).[4,5]

Further, CDC, Australian PHLN, and UK's HPA guidelines recommend the use of two separate NAATs targeting different genes known to have the discriminatory capacity for the detection of *N. gonorrhoeae* and *C. trachomatis* when applied to the low-prevalence setting.[1,6,7] Therefore, a multiplex polymerase chain reaction (PCR) targeting two different genes simultaneously, for detection of each of these two pathogens, is the need of the hour.

This study was conducted to design and evaluate a multiplex nested PCR protocol for simultaneous detection of *N. gonorrhoeae* and *C. trachomatis* in genitourinary specimens obtained from patients clinically suspected of STIs, targeting two different genes each for these pathogens and considering the variations in these genes reported in PubMed database till date.

MATERIALS AND METHODS

The study was conducted on 116 patients in the age group of 15–45 years [Table 1] with 104 sexually active women attending obstetrics and gynecology outpatient department (OPD) clinics with the complaint of cervicovaginal discharge (n = 96; 92.31%), secondary infertility of undiagnosed etiology (n = 6; 5.77%), and history of recurrent spontaneous abortion (n = 2; 1.92%), and 12 promiscuous men attending Dermatology and Venereology OPD clinic with a complaint of urethral discharge and/or dysuria between April 2016 and March 2017. Women, who were pregnant or at menopause, were excluded from the study. The study was approved by the Institute Ethical Committee, IMS, BHU.

Table 1: Distribution of patients with respect to age in the study*

Age (years)	Total (n=116), n (%)	Women (n=104), n (%)	Men (n=12), n (%)
15-20	10 (8.62)	7 (6.73)	3 (25)
21-25	30 (25.86)	25 (24.03)	5 (41.67)
26-30	44 (37.93)	42 (40.38)	2 (16.66)
31-35	11 (9.48)	10 (9.62)	1 (8.33)
36-40	11 (9.48)	11 (10.58)	0
41-45	10 (8.62)	9 (8.65)	1 (8.33)

*Original

Informed consent was obtained from every patient. Anterior urethral swabs and/or expressed discharge was collected from men (n = 12). From women, either endocervical swab (n = 54; 51.92%) or clinician-collected high vaginal swab (n = 45; 43.27%) or patient's self-collected vaginal swab (n = 5; 4.81%) was collected. Samples were processed as early as possible. One of the swabs was put in an aliquot in phosphate buffered saline (PBS) (pH 7) for PCR analysis.

Direct microscopy by Gram staining was examined for the presence of pus cells and Gram-negative diplococci. For culture isolation of *N. gonorrhoeae*, chocolate agar plate and modified Thayer-Martin agar plate were inoculated and incubated at 37°C in 5% CO_2. Plates were examined for growth daily for 72 h. Gram staining, oxidase test, and superoxol test were performed from colonies suspected of gonococci. Colonies which are Gram-negative diplococci, and both oxidase and superoxol test positive were presumed to be *Neisseria*. Rapid carbohydrate utilization test along with acidometric test and chromogenic cephalosporin disk test for β-lactamase was performed.

Antimicrobial susceptibility testing by Kirby–Bauer disk diffusion method using both Clinical and Laboratory Standards Institute guidelines and Calibrated Dichotomous Susceptibility guidelines for penicillin, ceftriaxone, cefixime, cefpodoxime, azithromycin, spectinomycin, tetracycline, and ciprofloxacin, and minimum inhibitory concentration determination by E-test method for ceftriaxone and azithromycin was performed.

The novel primers were designed for multiplex nested PCR detection of *N. gonorrhoeae* and *C. trachomatis* by targeting two genes each of these bacteria. Primers for the first and second round of PCR targeting *porA* pseudogene of *N. gonorrhoeae* were designed considering *porA* gene sequences of all six *N. gonorrhoeae* strains which were enlisted in the PubMed search database [Table 2].

Further, primers targeting *opa* gene of *N. gonorrhoeae* were designed considering the gene sequences of ten *opa* genes (*opaB*– *opaK*) of strain MS11 enlisted in the PubMed search database [Table 2].

The primers targeting *momp gene* of *C. trachomatis* were designed considering the gene sequences of four variants of major outer membrane protein (*momp*) gene present in *C. trachomatis*, namely *omp1*, *ompA*, *ompB*, and *ompC* of 40 *C. trachomatis* strains submitted in the NCBI database, which are placed in the five highly conserved regions, with four evenly spaced variable domains in between [Table 2].

The primers targeting cryptic plasmid of *C. trachomatis* were designed using the gene sequences of 11 variants of cryptic plasmid[5] present in different serovars available in NCBI database [Table 2]. The primers were positioned in the ORF-8 region and ORF7– ORF8 interjunction.

DNA isolation was performed using clinical samples aliquot in PBS centrifuged at 10,000 g for 10 min. The pellet was suspended in 500 µl of Tris-EDTA buffer (10 mM Tris-Hcl and 1 mM ethylenediaminetetraacetic acid [EDTA] [pH 7.6]), 100 µl of 10% sodium dodecyl sulfate, and 5 µl of Proteinase K (20 mg/ml) and incubated for 2 h at 37°C. Subsequently, 100 µl of 5 M NaCl, 80 µl of 10% cetyltrimethylammonium bromide (CTAB) were added and incubated in water bath at 60°C for 10 min, then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform:isoamyl alcohol (24:1), and DNA precipitation using chilled isopropyl alcohol. After washing pellet with 70% ethanol, extracted DNA was suspended in 50 µl of TE buffer and is stored at −20°C. The DNA content and purity of the extracted DNA in Tris-EDTA buffer (i.e., 260/280 ratio and 260/230 ratio) were estimated using spectrophotometer (NanoDrop®).

About 25.0 µl of master mix for PCR was prepared using 2.5 µl Taq 10X buffer, 2 µl 10 mM dNTP mix, 0.33 µl Taq polymerase (3U/µl), 1 µl (10 pmol) each of four forward and reverse primers sets (Genei, Merck) along with 5 µl extracted DNA as template for the first cycle, and 1 µl of first-round PCR product for second-round nested PCR.

The annealing temperature for the first and second round of PCR protocol was optimized employing gradient PCR and observed to be 48°C and 51°C, respectively. Using thermal cycler (Bio-Rad, USA), the reaction mixture was subjected to 10 min of initial denaturation at 95°C, followed by 40 cycles consisting of 45 s of denaturation at 95°C, 45 s of annealing at 48°C and 51°C, respectively, for the first- and second-round PCR and 90 s of extension at 72°C, followed by 10 min of final extension at 72°C. The amplified PCR product (10 µl) was analyzed by electrophoresis in a 2% agarose gel (HiMedia,

Table 2: Details of strain (along with accession number) present in NCBI PubMed database whose nucleotide sequence is utilized for primer designing*

STI pathogens	Target genes	Strain (accession number) in PubMed database
N. gonorrhoeae	*porA* pseudogene	MS11 (Accession: AJ223446.1), FA1090 (AJ223447.1), NCTC8375 (AJ223448.1), NIBSC2120 (AJ223449.1), sample 1 (AJ010732.1), sample 2 (AJ010733.1)
	opa gene	*opaB* (X52373.1), *opaC* (X52370.1), *opaD* (X52372.1), *opaE* (X52369.1), *opaF* (X52368.1), *opaG* (X52367.1), *opaH* (X52366.1), *opaI* (X52365.1), *opaJ* (X52371.1), *opaK* (X52364.1)
C. trachomatis	*momp* gene	C/TW3/OT (AF352789.1), D/B-120 (X62918.1), D/B-185 (X62919.1), Da/TW-448 (X62921.1), D/LSU-PM12 (AF279588.1), D/IC-Cal-8 (X62920.1), D/LSU-EP212 (AF279587.1), I/UW12/ Ur (AF063200.2), J/UW36/Cx (AF063202.2), K/UW31/Cx (AF063204.2), omp1L2 (M14738.1), omp1F (X52080.1), omp1 (U80075.1), A/Har-1 (DQ064279.1), B/TW-5 (DQ064281.1), B/Har-36 (DQ064297.1), Ba/Apache-2 (DQ064282.1), B/Tunis-864 (DQ064280.1), C/TW-3 (DQ064283.1), C1 (EU040363.1), C3 (EU040365.1), C-Har32 (DQ064298.1), C2 (EU040364.1), D/UW-3 (DQ064284.1), D/IC-CAL8 (DQ064285.1), E/Bour (DQ064286.1), F/IC-CAL3 (DQ064287.1), G-UW57 (DQ064299.1), G/392 (DQ064288.1), H/580 (DQ064289.1), I/UW-12 (DQ064290.1), Ia/870 (DQ064291.1), J/ UW-36 (DQ064292.1), K/UW-31 (DQ064293.1), L2/434 (DQ064295.1), L1/440 (DQ064294.1), L3/404 (DQ064296.1), L1 (M36533.1), B/TW-5/OTompB (M17342.1), C/TW3/OTompC (M17343.1)]
	Cryptic plasmid gene[5]	pLGV440 (serovar L1), pUCH-1 (serovar L2b), pL2 (serovar L2), pCTA (serovar A), pCTB (serovar B), pJALI (serovar B), pCHL-1 (serovar D), pSW-2 (serovar E), pSW-3 (serovar E), pSW-4 (serovar F, pSW-5 (serovar F)

*Original. *Momp*=Major outer membrane protein; *N. gonorrhoeae=Neisseria gonorrhoeae*; *C. trachomatis=Chlamydia trachomatis*; STI=Sexually transmitted infection; NCBI=National Center for Biotechnology Information

RM273) stained with ethidium bromide with tris-borate-EDTA buffer.

The PCR amplification of human β-globin gene sequence was employed as an internal control to assess the extraction of adequate amplifiable DNA and absence of PCR inhibitory substances in the extracted DNA, yielding 92 bp amplicon [Tables 3 and 4].

The PCR protocol was optimized for the detection of *N. gonorrhoeae* using WHO *N. gonorrhoeae* reference strains (WHO-K, WHO-P, WHO-C, and WHO-O). As the reference strain of *C. trachomatis* or its DNA were not available in this study, the specificity of the designed primers targeting *C. trachomatis* was confirmed by analyzing the DNA sequencing results of ~438 bp and ~368 bp PCR amplicons of *C. trachomatis momp* and cryptic plasmid gene sequences, respectively, obtained from a clinical sample (Sample ID-47) and then used as in-house control.

DNA isolated from all clinical samples was subjected to multiplex nested PCR for simultaneous detection of *N. gonorrhoeae* and *C. trachomatis*. DNA sequence analysis was carried out at AgriGenome, for nested PCR amplicons obtained from clinical sample, each for *N. gonorrhoeae porA* pseudogene (Sample ID-50) and *opa* gene (Sample ID-59) and *C. trachomatis* cryptic plasmid gene (Sample ID-82) and *momp* gene (Sample ID-75) for the validation of in-house designed primers and PCR protocol.

RESULTS

On Gram staining, five of 12 urethral discharges obtained from men shown Gram-negative diplococci and also yielded *N. gonorrhoeae* on culture. None of the women samples shown Gram-negative diplococci on Gram staining nor yielded *N. gonorrhoeae* on culture. All five *N. gonorrhoeae* isolates obtained on culture were resistant to penicillin and ciprofloxacin, three were resistant to tetracycline, and all were susceptible to cefixime, ceftriaxone, cefpodoxime, azithromycin, and spectinomycin.

In the present study, PCR result was considered positive if it was able to yield amplicons specific for either of the two genes, *porA* pseudogene (~190 bp) or *opa* gene (~298 bp) for *N. gonorrhoeae*, and/or for either of the two genes, *momp* (~438 bp) or cryptic plasmid (~368 bp) for *C. trachomatis*.

Table 3: Primers for first/primary round of multiplex nested polymerase chain reaction*

Target organism	Target gene	Oligo primer	Sequence	Tm (°C)	Amplicon size
N. gonorrhoeae	*porA* pseudogene	Forward	5'-CGGCTCGTTTATCGGCTT-3'	57.9	567
		Reverse	5'-ATCGGTATCACTCGCTCTGC-3'	59.69	
	opa gene	Forward	5'-GCACGGTAAGCGATTATTTC-3'	55.28	559
		Reverse	5'-TGGGTTTTGAAGCGGGTG-3'	58.15	
C. trachomatis	*momp* gene	Forward	5'-TGACGCTATCAGCATGCG-3'	57.98	707
		Reverse	5'-CTCCAATGTARGGAGTGAACAT-3'	57.51	
	Cryptic plasmid gene	Forward	5'-GGGATTCCTGTAACAACAAGTC-3'	56.97	773
		Reverse	5'-ATCAATGCCCGGGATTGG-3'	57.39	
Internal control	Beta globin gene[8]	Forward	5'-CAACTTCATCCACGTTCACC-3'	55.1	268
		Reverse	5'-GAAGAGCCAAGGACAGGTAC-3'	55.6	

*Original. *Momp*=Major outer membrane protein; *N. gonorrhoeae=Neisseria gonorrhoeae*; *C. trachomatis=Chlamydia trachomatis*; Tm=Melting temperature

Table 4: Primers for second/nested round of multiplex nested polymerase chain reaction*

Target organism	Target gene	Oligo primer	Sequence	Tm (°C)	Amplicon size
N. gonorrhoeae	*porA* pseudogene	Forward	5'-GATCCTTGGGACAGCAAT-3'	54.17	190
		Reverse	5'-TCTGATTACTTTCCAGCGTG-3'	55.5	
	opa gene	Forward	5'-GAAACATCCGTACGCATTCC-3'	54.65	298
		Reverse	5'-TGTCTGACGTGTCCGTAG-3'	56.02	
C. trachomatis	*momp* gene	Forward	5'-GGGATCGYTTTGATGTATTYTG-3'	55.26	438
		Reverse	5'-AACTTGCTTGCCAYTCATGG-3'	57.25	
	Cryptic plasmid gene	Forward	5'-TTCTTATTGTTCTGGGGAAGAG-3'	55.44	368
		Reverse	5'-TCTTCGTAACTCGCTCCG-3'	56.52	
Internal control	Beta globin gene	Forward	5'-TGGTGTCTGTTTGAGGTTGC-3'	56.6	92
		Reverse	5'-AGGGCTGGGCATAAAAGTC-3'	55.6	

*Original. *Momp*=Major outer membrane protein; *N. gonorrhoeae=Neisseria gonorrhoeae*; *C. trachomatis=Chlamydia trachomatis*; Tm=Melting temperature

In this study, a total of 51.72% (60/116) patients were detected to have either of the two STIs. A total of 35.35% (41/116) patients were positive for *C. trachomatis* and 33.62% (39/116) for *N. gonorrhoeae* by employing multiplex nested PCR. Coinfection with *N. gonorrhoeae and C. trachomatis* was detected in a total of 17.24% (20/116) patients [Table 5].

About 31.5% endo-cervical swabs (n = 54), 64.4% speculum assisted high vaginal swabs (n = 45), and 80% self-collected vaginal swabs (n = 5) were detected positive for either of two STIs. All five men positive for gonococcal culture, were also positive for *N. gonorrhoeae* by PCR, with two of them having coinfection with *C. trachomatis*.

Of the 39 patients which were PCR positive for *N.* gonorrhoeae, 36 yielded specific amplicons for both *porA* pseudogene and *opa* gene sequences. However, three patients yielded specific amplicons for *opa* gene sequence only. Of the 41 patients, which were PCR positive for *C. trachomatis*, 39 yielded specific amplicons for *momp* gene, whereas 18 yielded specific amplicons for *cryptic plasmid* gene sequence. Overall, 16 patients yielded specific amplicons for both *momp* and *cryptic plasmid* genes.

Out of six women presenting with secondary infertility of undiagnosed etiology, three women were found to be infected with C. *trachomatis*. Further, both women who were enrolled in the present study with recurrent spontaneous abortion had an infection with *C. trachomatis*.

Table 5: Positivity for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* by multiplex nested polymerase chain reaction*

STI agent	Positivity for target gene	Total (n=116), n (%)	Women				Men (n=12) n (%)
			Total (n=104), n (%)	endocx swab (n=54), n (%)	HVS (n=45), n (%)	Self PV swab (n=5) n (%)	
N. gonorrhoeae	*porA* pseudogene and/or *opa* gene	39 (33.62)	31 (29.81)	12 (22.2)	16 (35.5)	3 (60)	8 (66.67)
	porA pseudogene	36 (31.04)	29 (27.88)	-	-	-	7 (58.33)
	Opa gene	39 (33.62)	31 (29.81)	-	-	-	8 (66.67)
	Both *porA* pseudogene and *opa* gene	36 (31.04)	29 (27.88)				7 (58.33)
C. trachomatis	*momp* and/or cryptic plasmid gene	41 (35.35)	37 (35.58)	12 (22.2)	23 (51.1)	2 (40)	4 (33.33)
	momp gene	39 (33.62)	35 (33.65)	-	-	-	4 (33.33)
	Cryptic plasmid gene	18 (15.52)	18 (17.31)	-	-	-	0
	Both *momp* gene and cryptic plasmid gene	16 (13.79)	16 (15.38)				0
Co-infection with *N. gonorrhoeae* and *C. trachomatis*		20 (17.24)	18 (17.31)	7 (12.9)	10 (22.2)	1 (20)	2 (16.67)
Either of 2 STIs		60 (51.72)	50 (48.08)	17 (31.5)	29 (64.4)	4 (80)	10 (83.33)

*Original. *Momp*=Major outer membrane protein; *N. gonorrhoeae=Neisseria gonorrhoeae*; *C. trachomatis=Chlamydia trachomatis*; Endocx=Endocervical; HVS=High vaginal swab; PV=Per vaginum; STIs=Sexually transmitted infections

DISCUSSION

Since 2003, the WHO recommended syndromic case management and diagnostic approach for the management of STIs in low-resource settings. Evaluations showed that the algorithm for vaginal discharge lacks both sensitivity and specificity for the identification of women with *C. trachomatis* and *N. gonorrhoeae* infection.[9] Furthermore, symptoms of both these STIs are overlapping and indistinguishable with each other and with other vaginal pathology, especially bacterial vaginosis.[10] This leads to need for a reliable and accurate diagnostic tool for prompt diagnosis of these STIs.

A study conducted at the WHO STI regional center, New Delhi, from 2002 to 2006, revealed that 68.6% specimens from men with acute urethritis (n = 563) and 0.6% from women with cervical/vaginal discharge (n = 4153) were smear positive for *N. gonorrhoeae,* and 76.8% specimens from men and 0.8% from women yielded gonococcus on culture

isolation.[11] Similarly, we also observed poor smear positivity and culture isolation of gonococci among women.

Intra- and inter-species genetic recombination and variations occur between members of the genus. Isolates which lack sequences or have variation in sequences have been reported. Clinical gonococcal isolate with *N. meningitidis porA* sequence giving false-negative results using *N. gonorrhoeae porA* pseudogene PCR have been reported.[12] Strains exhibiting variation in the multicopy *opa* gene have also been reported.[3] Further, *C. trachomatis* strains lacking cryptic plasmid have also been reported.[13]

The detection of a single-specific gene that might be acquired by a commensal isolate would lead to false-positive results, particularly in low-prevalence settings. However, simultaneous recombination and genetic variations in two genes targets that are located separately are unlikely and rare.[12] Multiplex PCR targeting two genes simultaneously may minimize the chance of false-negative PCR results. This has been observed in an earlier study, in which investigators designed Duplex PCR targeting gonococcal *porA* pseudogene and multicopy *opa* genes and was found to be superior to three monoplex real-time PCR assays.[14] In the current study, incorporation of two gene targets for PCR increased the sensitivity of detection of STIs.

Further, the nested PCR method has increased sensitivity and specificity as compared to single-round conventional PCR, because there is 2-step exponential amplification of target DNA present in clinical samples, preventing nonspecific amplification of DNA, and a reduced load of PCR inhibitors present in the clinical sample.[15]

Several investigators and studies have evaluated and commented about the merits of targeting porA pseudogene[2] and multicopy opa gene[3] for molecular detection of *N. gonorrhoeae*, and utility of major outer membrane protein gene[4] and cryptic plasmid gene[4,5] for molecular detection of *C. trachomatis*, which have been employed in this present study.

In women, the endocervical swab is the sample of choice for culture and NAAT, with clinician-collected high vaginal swabs and self-obtained low vaginal swabs as acceptable alternatives, all having better PCR positivity in hospital-based studies.[16-18] Various studies using urine samples for PCR detection of STIs yielded poor results in resource-limited settings.[18] In this study, all the three types of genital swab samples yielded positive results on PCR and found to be an appropriate sample for PCR detection of *C. trachomatis* and *N. gonorrhoeae*.

Various hospital-based studies in other parts of the country documented that 7.5% and 19.3% prevalence for gonococcal infection and 8%, 16.3%, 19.9%, and 23% prevalence for genital Chlamydiasis among symptomatic patients.[16,17] In this PCR-based study, high positivity rate of 39.66% for gonococcal infection and 35.35% for genital Chlamydiasis among symptomatic patients has been observed.

C. trachomatis has been associated with secondary infertility and recurrent spontaneous abortion.[19,20] Similar association has also been seen in the present study, but the number of samples included in the study is very small to reach any definite conclusion. However, it suggests investigating for bacterial STIs, especially *C. trachomatis* as a probable etiology or association with cases of secondary infertility or recurrent spontaneous abortion, where another etiological diagnosis cannot be established.

Out of 39 samples which were positive for *opa* gene, three were negative for *porA* pseudogene amplification. The most common cause of a false-negative result for *porA* pseudogene target may be due to the presence of meningococcal *porA* sequence in gonococci[12] which needs further evaluation.

Further, the syndromic case diagnosis and management approach, currently being practiced in most of the developing world need to be reevaluated. PCR-based diagnosis of STIs should be encouraged, and the empirical syndromic treatment if started should be modified after test results.

CONCLUSION

PCR based diagnosis of *N. gonorrhoeae and C. trachomatis* infections should be encouraged. The study concludes that novel multiplex nested PCR protocol designed and employed in the present study may be used in the management of both symptomatic as well as asymptomatic cases of *N. gonorrhoeae and C. trachomatis* infections, particularly among high-risk groups. Incorporation of two different gene targets for each STI pathogens improved detection.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Centers for Disease Control and Prevention. Recommendations for the laboratory-based detection of chlamydia trachomatis and *Neisseria gonorrhoeae*--2014. MMWR Recomm Rep 2014;63:1-9.
2. Whiley DM, Anderson TP, Barratt K, Beaman MH, Buda PJ, Carter M, *et al.* Evidence that the gonococcal porA pseudogene is present in a broad range of *Neisseria gonorrhoeae* strains; suitability as a diagnostic target. Pathology 2006;38:445-8.
3. Geraats-Peters CW, Brouwers M, Schneeberger PM, van der Zanden AG, Bruisten SM, Weers-Pothoff G, *et al.* Specific and sensitive detection of *Neisseria gonorrhoeae* in clinical specimens by real-time PCR. J Clin Microbiol 2005;43:5653-9.
4. Mahony JB, Luinstra KE, Sellors JW, Chernesky MA. Comparison of plasmid and chromosome-based polymerase chain reaction assays for detecting chlamydia trachomatis nucleic acids. J Clin Microbiol 1993;31:1753-8.
5. Seth-Smith HM, Harris SR, Persson K, Marsh P, Barron A, Bignell A, *et al.* Co-evolution of genomes and plasmids within chlamydia trachomatis and the emergence in Sweden of a new variant strain. BMC Genomics 2009;10:239.
6. Smith DW, Tapsall JW, Lum G. Guidelines for the use and interpretation of nucleic acid detection tests for *Neisseria gonorrhoeae* in Australia: A position paper on behalf of the public health laboratory network. Commun Dis Intell Q Rep 2005;29:358-65.
7. Health Protection Agency. Detection of *Neisseria Gonorrhoeae* Using Molecular Methods. National Standard Method QSOP 62 Issue 1 2010. Available From: http://www.hpastandardmethods.org.uk/pdf_sops.asp. [Last accessed on 2017 Jul 21].
8. Bauer HM, Ting Y, Greer CE, Chambers JC, Tashiro CJ, Chimera J, *et al.* Genital human papillomavirus infection in female university students as determined by a PCR-based method. JAMA 1991;265:472-7.
9. Hawkes S, Morison L, Foster S, Gausia K, Chakraborty J, Peeling RW, *et al.* Reproductive-tract infections in women in low-income, low-prevalence situations: Assessment of syndromic management in Matlab, Bangladesh. Lancet 1999;354:1776-81.
10. Patel V, Pednekar S, Weiss H, Rodrigues M, Barros P, Nayak B, *et al.* Why do women complain of vaginal discharge? A population survey of infectious and pyschosocial risk factors in a South Asian community. Int J Epidemiol 2005;34:853-62.
11. Bala M, Ray K, Gupta SM, Muralidhar S, Jain RK. Changing trends of antimicrobial susceptibility patterns of *Neisseria gonorrhoeae* in India and the emergence of ceftriaxone less susceptible *N. gonorrhoeae* strains. J Antimicrob Chemother 2007;60:582-6.
12. Trembizki E, Lahra M, Stevens K, Freeman K, Hogan T, Hogg G, *et al.* A national quality assurance survey of *Neisseria gonorrhoeae* testing. J Med Microbiol 2014;63:45-9.
13. Stevens MP, Tan SE, Horvath L, Fairley CK, Garland SM, Tabrizi SN, *et al.* Absence of a chlamydia trachomatis variant, harbouring a deletion in the cryptic plasmid, in clients of a sexually transmissible infection clinic and antenatal patients in Melbourne. Commun Dis Intell Q Rep 2008;32:77-81.
14. Goire N, Nissen MD, LeCornec GM, Sloots TP, Whiley DM. A duplex *Neisseria gonorrhoeae* real-time polymerase chain reaction assay targeting the gonococcal porA pseudogene and multicopy opa genes. Diagn Microbiol Infect Dis 2008;61:6-12.
15. Sandin RL. Polymerase chain reaction and other amplification techniques in mycobacteriology. Clin Lab Med 1996;16:617-39.
16. Malhotra M, Bala M, Muralidhar S, Khunger N, Puri P. Prevalence of sexually transmitted infections in patients attending a tertiary care hospital in North India a retrospective study. Indian J Sex Transm Dis 2008;29:82-5.
17. Patel AL, Sachdev D, Nagpal P, Chaudhry U, Sonkar SC, Mendiratta SL, *et al.* Prevalence of chlamydia infection among women visiting a gynaecology outpatient department: Evaluation of an in-house PCR assay for detection of chlamydia trachomatis. Ann Clin Microbiol Antimicrob 2010;9:24.
18. Cook RL, Hutchison SL, Østergaard L, Braithwaite RS, Ness RB. Systematic review: Noninvasive testing for chlamydia trachomatis and *Neisseria gonorrhoeae*. Ann Intern Med 2005;142:914-25.
19. Baud D, Goy G, Jaton K, Osterheld MC, Blumer S, Borel N, *et al.* Role of chlamydia trachomatis in miscarriage. Emerg Infect Dis 2011;17:1630-5.
20. Mania-Pramanik J, Kerkar S, Sonawane S, Mehta P, Salvi V. Current chlamydia trachomatis infection, a major cause of infertility. J Reprod Infertil 2012;13:204-10.