A multiplex assay of *Trichomonas vaginalis*, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections in genital specimens

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Abstract

Introduction: A significant proportion of patients with Sexually Transmitted Infections (STIs) are coinfected with multiple pathogens. We report here development of a multiplex PCR for simultaneous detection of *Chlamydia trachomatis* (*C. trachomatis*), *Neisseria gonorrhoeae* (*N. gonorrhoeae*) and *Trichomonas vaginalis* (*T. vaginalis*) in genital specimens from women.

Methodology: After detection of the organisms by routine techniques including PCR, culture and direct smear, multiplex-PCR was optimized to detect *omp1* gene of CT, *parC* of NG, ITS ribosomal RNA of TV as target genes. The limit of detection (LOD) was determined using serially diluted genomic DNA from known number of each pathogen.

Results: Totally 300 volunteers with mean age of 36.5 ±7.03 years were included and 266 (88.7%) had genitourinary clinical manifestations. Of 300 women, 150 (50.0%) were infected. Of them, 17 (5.7%) had *N. gonorrhoeae*, 98 (32.7%) *T. vaginalis* and 35 (11.7%) *C. trachomatis*. Multiplex-PCR revealed a total of 10 coinfections (3.3%) including 2 specimens of *C. trachomatis*/ *N. gonorrhoeae*, 3 specimens of *C. trachomatis*/ *T. vaginalis* and 5 specimens of *N. gonorrhoeae*/ *T. vaginalis* coinfections. The sensitivity and specificity of multiplex-PCR were 100% and 98.59% (279 of 283) respectively and, for *C. trachomatis* and *T. vaginalis* were 100%. The LOD was 0.1 pg of DNA for *C. trachomatis* and *N. gonorrhoeae*, and 1.5 pg for *T. vaginalis*.

Conclusions: The performance of this multiplex-PCR makes it a sensitive, rapid and affordable technique in clinical laboratory for simultaneous detection of STIs.

Key words: Multiplex PCR; *Chlamydia trachomatis*; *Neisseria gonorrhoeae*; *Trichomonas vaginalis*.


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Introduction

According to the estimate of the World Health Organization [1], each year about 500 million people become affected by one of 4 sexually transmitted infections: chlamydirosis, gonorrhoea, syphilis and trichomonirosis. More than 1 million people acquire a STI every day, with the largest proportion in the region of south and southeast Asia [2]. More than 30 bacterial, viral and parasitic STI pathogens, *T. vaginalis* and *C. trachomatis* are the most common and curable sexually transmitted pathogens worldwide [3]. STIs can have serious complications beyond the usual presentations of the infection such as placental transmission of infection, facilitation of HIV transmission, ectopic pregnancy, stillbirth, and pelvic inflammatory disease (PID), one of the major causes of infertility [4,5]. However, most of the women with genital infections are asymptomatic and early detection of STIs would be useful to prevent serious complications. WHO supports development of rapid and low-cost point-of-care diagnostic tests as new approach for prevention of STIs [1]. The purpose of reporting STIs is to ensure that infected patients are quickly diagnosed and appropriately treated. Evidences show that in areas where STIs screening programs were implemented for sexually active women, the rate of infections and their complications were decreased [3]. Over the past years, the diagnosis of STIs has been largely dependent on traditional techniques such as culture, stained smear and enzyme immunoassay [6]. These methods are usually time consuming and labor-intensive and lack convincing sensitivity for definite identification of STIs
in symptomatic and asymptomatic patients. With the advent of technologies such as nucleic acid amplification (NAAT), major improvements in the ability to detect these STIs have occurred [7-10]. PCR-based tests have generally been more sensitive than traditional tests in diagnosis of C. trachomatis and N. gonorrhoeae [11-14]. Several studies have shown that a significant proportion of patients are coinfected with two STIs simultaneously [15,16]. With the annual incidence of about 500 million cases of STIs, significant rate of coinfections is conceivable which justify development of methods to diagnose multiple genital infections in simple steps. In addition, identification of more than one agent in one specimen is more cost-effective, more rapid, and more acceptable for the patients. Although multiplex PCR has been applied to the detection of multiple viruses and bacteria in clinical specimens [17,18] this approach has less been established for simultaneous detection of prokaryotic and eukaryotic STIs [19]. We report here the development of a multiplex PCR for the simultaneous detection of C. trachomatis, N. gonorrhoeae and T. vaginalis in genital swab specimens collected from women.

Methodology
Study population, ethical considerations and sampling

The study was approved by Ethical Committee of Tehran University of Medical Sciences (TUMS) Tehran, Iran. All procedures were in accordance with the ethical standards of human experimentation of 1964 Declaration of Helsinki and its later amendments. From May 2014 to April 2015 women who admitted to Gynecology Hospital and Health Clinics of Qom city, central Iran, because of genital complaints were recruited for the study after provision of written consent and willingness to participate. Inclusion criteria included age range between 18 to 60 years and no provision of systemic or topical antibiotics within last 1 month. After examination of each volunteer by a gynecologist, demographic data, history and clinical signs/symptoms were recorded in a questionnaire.

Four swab samples were collected from each volunteer under aseptic conditions. First endocervical swab was cultured on Thayer Martin (TM) agar (Merck, Darmstadt, Germany) for detection of Neisseria, second vaginal swab was used for smear preparation for detection of Trichomonas, and two swabs from vaginal and endocervical canals were transferred to sterile PBS, pH 7.2, mixed and used for DNA extraction.

DNA extraction

DNA extraction was carried out on the vaginal/endocervical samples by using AccuPrep Genomic DNA Extraction Kit (BIONEER, Daejeon, South Korea). Briefly, 1.5 ml sample tubes were centrifuged 5 minutes at 7000 rpm, then 20 µl of proteinase K was added to the pellet followed by adding 200 µl of GC binding buffer and tubes were incubated at 60°C water bath for 10 minutes. Then 100 µl of absolute isopropanol was added and the contents were transferred to binding tubes. Tubes were washed with 500 µl of W1 washing buffer at 8000 rpm for 1 minute then with W2 washing buffer at 12000 rpm for 1 minute. Finally, 50 µl of elution buffer was added to the tubes and centrifuged at 8000 rpm for 1 minute and elutes were collected as DNA and kept at -20°C until use. In some instances DNA extraction was done by phenol-chloroform method as described [20].

Detection of C. trachomatis infection

For molecular identification of C. trachomatis in genital specimens, ompI target gene encoding for chlamydial major outer membrane protein (MOMP) was amplified by nested PCR as previously described [21]. The sequence of external primers (Ex-p1/Ex-p2) and nested primers (In-p1/In-p2) are shown in Table 1 (CinnaGene, Tehran, Iran). C. trachomatis ATCC VR-878D DNA was used as standard.

Detection of N. gonorrhoeae infection

N. gonorrhoeae strain was cultured and identified by biochemical reactions [22]. For molecular detection of N. gonorrhoeae, NG.F/NG.R primer pair, which amplify ParC gene encoding for topoisomerase IV enzyme of the organism, was used (CinnaGene, Tehran, Iran) (Table 1). Amplification was carried out in a total volume of 25 µl containing 2.5 µl 10× PCR buffer, 1.5 mM MgCl2, 0.5 µl of each primer (25 pmol/ml), 0.2 mM of each dNTPs, and 1 U Taq DNA polymerase (Fermentas Life Sciences, York, UK). The conditions for N. gonorrhoeae PCR were as follows: initial denaturation of 94°C for 4 minutes, then 30 cycles of 94°C for 60 seconds, 53.8°C for 45 seconds, 72°C for 45 seconds, followed by final extension of 72°C for 10 minutes. Positive results were further confirmed by real-time PCR assay targeting the porA pseudogene as previously described [23]. Sequences of primer pair NIS.F and NIS.R were shown in Table 1 (CinnaGene, Tehran, Iran). N. gonorrhoeae ATCC 49226 was used as a standard reference strain.
Detection of T. vaginalis infection

Wet mount smears were examined for identification of motile flagellated trophozoite of T. vaginalis immediately after sampling. For molecular detection of T. vaginalis, TV.F/TV.R primer pair (CinnaGene, Tehran, Iran), which amplify ITS ribosomal RNA gene was used (Table 1) following the protocol as previously described [23]. For confirmation of the results, some T. vaginalis PCR products were submitted for sequencing.

Multiple PCR assay

Primers with acceptable physicochemical parameters as analyzed by GeneRunner program Ver. 3.05 (Hastings software Inc., NY, USA) were selected and aligned with the sequence from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) to test for possible nonspecific interactions with other organisms. BioEdit Sequence Alignment Editor software ver. 7.0.4.1 (Tom Hall, Carlsbad, USA) was used to align gene sequences from different strains to identify conserved regions for designing specific primers. The specific primers used to amplify the 3 target organisms were Ch.F/Ch.R, NG.F/NG.R and TV.F/TV.R pairs as shown in Table 1.

The specificity of the assay of each primer pair was tested with common eukaryotes and Gram-positive and Gram-negative bacteria which representing normal vaginal flora. Suspensions of bacteria adjusted to 0.5 McFarland standard (equal to $1.5 \times 10^8$ colony-forming units (CFU)/mL) were prepared. Genomic DNAs were extracted from: Candida albicans, Escherichia coli, Gardnerella vaginalis, Lactobacillus acidophilus, Pseudomonas aeruginosa, Staphylococcus aureus, Gardnerella vaginalis, Lactobacillus acidophilus, Staphylococcus epidermidis, Streptococcus agalactiae, and Enterobacter aerogenes.

To determine the limit of detection (LOD) for each pathogen, genomic DNA was extracted from known number of C. trachomatis, N. gonorrhoeae and T. vaginalis, then was 10-fold serially diluted and spiked into the reactions then multiplex PCR was performed.

Multiplex PCR conditions were established by using a mixture of DNA from all three organisms. The 25 µl PCR reaction mixture consisted of 67 mM Tris-HCl; pH 8.8, 0.01% Tween 20, 3 mM MgCl$_2$, 2.5 mM each dNTPs, 3 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 10 µM of each specific primer pair. The target DNA concentrations were adjusted to 1-3 µl (100 ng) depending on the organism. Annealing temperatures ranging from 50°C to 62°C were tested and after several adjustments it was optimized at 56°C. Once the conditions were optimized, genital swab samples from 300 women were subjected to multiplex PCR assay. The amplified products were electrophoresed on 1.5% agarose gels containing ethidium bromide in buffer.

For determining the performance of multiplex PCR, a combination of molecular and conventional methods was considered as gold standard for each pathogen.

Results

In this study, a total of 300 women volunteers were included. Mean ±SD age of the volunteers was 36.5±7.03 years. Of total 300 women, 266 (88.7%) had clinical signs/symptoms such as lower abdominal pain (182; 60.7%), malodorous discharge (123; 41.0%), genital itching (115; 38.3%), and painful intercourse (111; 37.0%). Pregnancy-related complications included history of curettage (45; 15.0%), abortion (66; 22.0%), ectopic pregnancy (3; 1.0%), premature neonate (10; 3.3%), still birth (8; 2.7%), infertility (23; 7.7%) and premature rupture of membranes (PROM) (32; 10.7%).

In evaluation of primer pairs, no significant similarities between primer sequences and any of the tested genomes, which could theoretically lead to non-

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Table 1. Characteristics of primer pairs used for identification of infections in genital specimens.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Target gene</th>
<th>Primers</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. trachomatis</td>
<td>MOMP PCR</td>
<td>Ch.F 5<code>-CCTGTGGGAATCCCTGCTGAA-3</code>, Ch.R 5<code>-GTCGAAACAAAGTCACCATAAGTA-3</code></td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>ompl</td>
<td>Ex-p1: 5<code>-ATGAAAAAATCTTGTAAATCCG-3</code>, Ex-p2: 5<code>-TTTCTAGATTTCATCTTGTG-3</code></td>
<td>1100</td>
</tr>
<tr>
<td></td>
<td>nested-PCR</td>
<td>In-p1 5<code>-TTTCTAGATTTCATCTTGTG-3</code>, 5<code>-CCTTGCAACGCTCGTGGGGATCCCT-3</code></td>
<td>1000</td>
</tr>
<tr>
<td>N. gonorrhoeae</td>
<td>ParC</td>
<td>NG.F: 5<code>-GTTTCAGACGGGAACAAAGCC-3</code>, NIS.F 5<code>-CGGTTCCTCGGTTACAGA-3</code></td>
<td>331</td>
</tr>
<tr>
<td></td>
<td>Prod</td>
<td>NIS.R 5<code>-CTGTTTTCACTCTGATTACCTTCA-3</code>, TV.F: 5<code>-TAGGTTGACCTCGGCGTG-3</code>, TV.R: 5<code>-AGTTCAGGGGTCTTCTCGG-3</code></td>
<td>131, 361</td>
</tr>
<tr>
<td>T. vaginalis</td>
<td>ITS ribosomal RNA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
specific amplification, were found. Results showed no cross reactivity between target sequences and common vaginal flora in multiplex PCR. The LOD was 0.1 pg of DNA (equivalent to approximately 20 copies) for C. trachomatis and N. gonorrhoeae, and 1.5 pg (equivalent to approximately 1-2 copies) for T. vaginalis.

In detection of genital infections, single or multiple infections were shown in 150 (50.0%) volunteers, and the rest had negative results. Totally N. gonorrhoeae infection was detected in 5. 7.0% (17/300) of the specimens. N. gonorrhoeae was identified in 21 specimens by PCR. However, in real-time PCR assay 17 specimens provided positive results for N. gonorrhoeae and 4 false PCR-positive specimens were negative in real-time PCR and identified as other Neisseria spp. In multiplex PCR 21 (7.0%) specimens showed positive results for N. gonorrhoeae including 2 (0.7%) mixed infection with C. trachomatis and 5 (1.7%) mixed infections with T. vaginalis (Table 2). Four false positive specimens had no mixed infections.

Totally T. vaginalis infection was detected in 32.7% (98/300) of the specimens. T. vaginalis was positive in 34 (11.3%) specimens in wet mount examination. T. vaginalis infection was identified in 96 (32.0%) specimens by using conventional PCR. In multiplex PCR 98 (32.7%) specimens showed positive results for T. vaginalis including 3 mixed infections with C. trachomatis (1.0%) and 5 mixed infections with N. gonorrhoeae (1.7%) (Table 2). Sequence data of PCR products of T. vaginalis ITS ribosomal RNA gene were submitted to GenBank under the accession number KP221674.1.

Totally 11.7% (35/300) of specimens showed positive results for C. trachomatis infection. C. trachomatis was identified in 35 (11.7%) specimens in ompI nested-PCR assay. By using multiplex-PCR, C. trachomatis was identified in 35 (11.7%) specimens including 2 mixed infection with N. gonorrhoeae (0.7%) and 3 mixed infections with T. vaginalis (1.0%) (Table 2).

In the next step, the multiplex PCR assay was established with the annealing temperature of 56°C, in which all three amplicons were efficiently generated. Multiplex PCR revealed a total of 10 mixed double coinfections comprising 3.34% of the total specimens (Table 2). None of genital specimens showed infection with all three organisms simultaneously.

All T. vaginalis positive specimens with conventional PCR as well as two other specimens were positive in multiplex PCR (98/98). Multiplex-PCR method showed 100% sensitivity and 100% specificity in detection of T. vaginalis infection in vaginal specimens (Table 3). Multiplex PCR detected all 17 N. gonorrhoeae true positive specimens. Four specimens of other Neisseria spp. were falsely positive with multiplex assay. Multiplex PCR method showed 100%

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### Table 2 Frequency of STIs in genital swab specimens from studied women.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Frequency</th>
<th>subtotal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydia trachomatis</td>
<td>Single infection</td>
<td>30 (10.0%)</td>
</tr>
<tr>
<td></td>
<td>Multiple infection</td>
<td>with NG 2 (0.67%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with TV 3 (1.0%)</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>Single infection</td>
<td>10 (3.34%)</td>
</tr>
<tr>
<td></td>
<td>Multiple infection</td>
<td>with CT 2 (0.67%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with TV 5 (1.67%)</td>
</tr>
<tr>
<td>Trichomonas vaginalis</td>
<td>Single infection</td>
<td>90 (30.0%)</td>
</tr>
<tr>
<td></td>
<td>Multiple infection</td>
<td>with CT 3 (1.0%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with NG 5 (1.67%)</td>
</tr>
<tr>
<td>Negative specimens</td>
<td></td>
<td>150 (50.0%)</td>
</tr>
</tbody>
</table>

### Table 3 Sensitivity and specificity of different methods used for detection of STIs.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Method</th>
<th>Sensitivity % (no. of true positive/no. of infected persons)</th>
<th>Specificity % (no. of true negative/no. of non-infected persons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydia trachomatis</td>
<td>nested-PCR</td>
<td>100% (35/35)</td>
<td>100% (265/265)</td>
</tr>
<tr>
<td></td>
<td>mPCR</td>
<td>100% (35/35)</td>
<td>100% (265/265)</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>Conventional PCR</td>
<td>100% (17/17)</td>
<td>98.59% (279/283)</td>
</tr>
<tr>
<td></td>
<td>Real time PCR</td>
<td>100% (17/17)</td>
<td>100% (283/283)</td>
</tr>
<tr>
<td></td>
<td>mPCR</td>
<td>100% (17/17)</td>
<td>98.59% (279/283)</td>
</tr>
<tr>
<td></td>
<td>Wet mount</td>
<td>34.69% (34/98)</td>
<td>100% (202/202)</td>
</tr>
<tr>
<td>Trichomonas vaginalis</td>
<td>Conventional PCR</td>
<td>97.96% (96/98)</td>
<td>100% (202/202)</td>
</tr>
<tr>
<td></td>
<td>mPCR</td>
<td>100% (98/98)</td>
<td>100% (202/202)</td>
</tr>
</tbody>
</table>
sensitivity and 98.59% specificity in detection of *N. gonorrhoae* infection in endocervical specimens. Multiplex PCR detected 35 of 35 endocervical specimens with *C. trachomatis* infection. Multiplex PCR method showed 100% sensitivity and 100% specificity in detection of *C. trachomatis* infection in endocervical specimens.

**Discussion**

Several reports have shown that coinfections of STIs are common in women [15, 16]. We have recently reported the significant prevalence of STIs in genital samples of the women of Qom, central Iran [23]. Application of conventional PCR is a common strategy for detection of STIs, which is adopted by many laboratories in Iran. However, collection of genital samples for diagnosis of each STI often causes discomfort for the female patients, the principal advantage of the multiplex PCR described here is simultaneous detection of 3 common infections in one reaction. In the current study, 10 specimens had DNA from two pathogens, with the highest rate for mixed infections of *N. gonorrhoae* with *T. vaginalis*. The sensitivities of multiplex PCR for detecting *C. trachomatis*, *N. gonorrhoae* and *T. vaginalis* were 100%. The specificities were between 98.6%-100% for all the three organisms.

The performance of multiplex PCR is affected by several factors such as the type of specimen and clinical characteristics of the patients. Although use of urine samples provides a noninvasive approach to the diagnosis of STIs in men, but in women genital swab is usually the preferred specimen for diagnosing STIs. Mahony and colleagues developed a multiplex PCR assay for the simultaneous detection of *C. trachomatis* and *N. gonorrhoae* in first void urine specimens. They reported the sensitivity of multiplex PCR for detecting *C. trachomatis* was 100%, compared with 81.8% for enzyme immunoassay and for detecting *N. gonorrhoae* was 92.3% compared with 84.6% for urethral culture and the specificity was 100% for both *C. trachomatis* and *N. gonorrhoae* [24]. In comparison, our multiplex PCR has a similar sensitivity/specificity for detection of *C. trachomatis* but higher sensitivity for detection of *N. gonorrhoae* on endocervical specimens. Several sensitive multiplex assays for the simultaneous detection of common STIs from various specimen sources have been introduced which utilize automated systems or real-time PCR platform [19,25,26]. However, our multiplex assay is not laborious and such an assay is an affordable method that can be performed on different laboratory settings in developing countries. In this study, coinfection rates of two pathogens were from 0.7% to 1.7%. Similar to our findings, another study from United States of America performed with a cohort of more than 7500 urogenital samples revealed prevalence of *T. vaginalis*, *C. trachomatis*, and *N. gonorrhoae* as 8.7%, 6.7%, and 1.7% respectively, coinfection rates 1.3% for *T. vaginalis/C. trachomatis, 0.61% for N. gonorrhoae/C. trachomatis*, and 1.3% for *T. vaginalis/N. gonorrhoae* [15]. We found two coinfections of *C. trachomatis* with *N. gonorrhoae* among 300 endocervical specimens. Higher rates of coinfection of *C. trachomatis* with *N. gonorrhoae* in urine samples of women or men with urethritis were reported [27,28]. In a study from Iran, by using a multiplex PCR, *N. gonorrhoae* was detected in 31 (46.0%), *C. trachomatis* in 15 (22.4%), and mixed two infections in 7 (10.4%) urine samples from patients with urethritis [16].

In this study the frequency of *T. vaginalis* infection was 32.7% in the studied population. This is higher than our previous report on the general population of the same region [23]. One explanation might be that this study included women with genital complaints. It is believed that in central provinces of Iran, including Qom and the capital Tehran, the prevalence of *T. vaginalis* infection is higher than other regions [29]. Current data shows that *T. vaginalis* infection was detected in significantly higher number of specimens by conventional PCR compared to wet mount examination (96/98 vs 34/98). Since usually tiny drops of liquid culture or vaginal discharge are examined under the microscope, the sensitivity of microscopy is low. We found that the sensitivity of multiplex PCR was more than uniplex for *T. vaginalis*, and higher number of specimens tested positive by multiplex PCR compared to conventional PCR (98/98 vs 96/98). One explanation might be that the enzyme used in multiplex PCR was more efficient with higher unit activity than enzyme used in conventional PCR. The higher sensitivity of nucleic acid amplification tests over smear or culture for genital infections has been evidenced in several publications [30]. In one investigation, the urine and vaginal specimens of women were rechecked for *T. vaginalis* detection by PCR and results showed that out of 161 negative samples by direct smear and culture, 7 samples (4.3%) turned positive by PCR [31].

For *C. trachomatis* infection, PCR assay is more sensitive than cell culture [32] and is able to detect all the serotypes of *C. trachomatis* [33]. It has been shown that culture and immunoassay have sensitivity of as low as 55% to 85% for *C. trachomatis* [34]. In a previous study we found that antigen based enzyme
immunoassay for *C. trachomatis* has 59.5% sensitivity [21]. In the present study, prevalence rate of *C. trachomatis* was 11.67% by PCR, while ienzume immunoassay demonstrates prevalence rate of 7.14% in women of the same region [21].

In this study, false detection of four non-gonorhea Neisseria species in multiplex-PCR reduced the specificity. Given the great similarity of nucleic acid sequences of bacteria of the genus Neisseria, it is very difficult to find specific primers for gonorrhea and many tests, even commercialized, are nonspecific. However, low frequency of successful culture gave PCR a priority for detection of gonorrhea [35].

**Conclusions**

Present multiplex PCR assay showed same specificity and equivalent or higher sensitivity compared to uniplex PCR and other routine assays. Multiple infections are difficult to detect, because a few clinics and laboratories perform diagnoses to *N. gonorrhoeae, C. trachomatis* and *T. vaginalis* and some centers may not look for a second STI after one is detected. The introduction of multiplex PCR offers a sensitive and more rapid method for the detection of multiple pathogens involved in STIs in a single specimen. Additional studies performed with larger numbers of women, symptomatic and asymptomatic men, and other specimens such as urine will be required to determine the true performance of this multiplex PCR for detection of *T. vaginalis, C. trachomatis* and *N. gonorrhoeae* simultaneously.

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**Authors’ contributions**

MNR designed and supervised the study, performed data analysis and wrote the paper. BHR helped in samples collection and volunteers’ examination. MNR helped in development of protocols and establishment of the methods. FA and AH performed the laboratory experiments.

**References**


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**Conflict of interests:** No conflict of interests is declared.