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18S ribosomal DNA based PCR diagnostic assay for *Trichomonas vaginalis* infection in symptomatic and asymptomatic women in India

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ABSTRACT

Objective: To identify the cases of trichomoniasis in symptomatic and asymptomatic *Trichomonas vaginalis* (*T. vaginalis*) infected patients by PCR amplification of hypervariable 18S rRNA gene and to assess the sensitivity of restriction fragment length polymorphism (RFLP) technique for their diagnosis. **Methods:** We enrolled 498 women of child bearing age groups, with their pre-informed consent, attending OPD for their routine checkups and STI related problems. Trichomoniasis was diagnosed on the basis of wet mount preparations and PCR with a primer set targeting a well-conserved region in the 18S rRNA genes of *T. vaginalis*, respectively. Sequencing was done for differentiating the symptomatic and asymptomatic strains of axenic and clinical isolates. **Results:** After PCR diagnosis *T. vaginalis* infection was detected in 17 (3.42%) out of 498 clinical isolates. Seventeen axenic and sixteen clinical strains of *T. vaginalis* tested were successfully detected by PCR yielding a single predicted product of 312 bp in gel electrophoresis followed by restriction digestion with restriction endonuclease HaeIII. After restriction digestion they gave two bands, one of 101 and the other of 211 bp, while there was negative response with DNA from *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Giardia lamblia*, *Toxoplasma gondii*, *Leishmania donovani* and *Entamoeba histolytica*. An optimal analytical sensitivity and specificity of one *T. vaginalis* organism per PCR was achieved. Sequence of symptomatic and asymptomatic strains of axenic and clinical isolates are somewhat differentiated on the basis of point mutations in their 18S rRNA gene. **Conclusions:** Only few factors are known to predict symptoms of *T. vaginalis* infection, although the majority of women are infected with trichomoniasis are reported. Therefore the application of sensitive PCR based diagnosis may be quite useful for routine diagnosis of *T. vaginalis* strains.

1. Introduction

Infection of *Trichomonas vaginalis* (*T. vaginalis*) is one of the most prevalent nonviral sexually transmitted infections (STI) in developing countries. It has been estimated that about 170 million to 190 million cases of vaginal trichomoniasis occur annually worldwide[1,2]. A significant association has also been observed with HIV, cervical cancer, atypical pelvic inflammatory disease, infertility, low birth weight and respiratory-tract infection in neonates[2,3]. Infection of *T. vaginalis* increases the risk of HIV transmission. Both the diseases have similar mode of acquisition. The patients with trichomoniasis

having increased risk of HIV could be due to presence of inflammatory cells harboring the virus and the minor abrasions on the genital mucosa due to STI[4,5]. The cases of trichomoniasis is about 50% asymptomatic, having no clinical signs in women or the patients[5]. The symptoms of trichomoniasis is not specific enough for their diagnosis. Routine clinical diagnosis depends on wet mount preparation but it detects only about 60% of positive culture samples[6]. Culture is more reliable for diagnosis but it needs frequent microscopic observation for 2–7 days. The probability of contamination is another demerit of culture. Although sensitivity of culture is about 90%, small numbers and nonviable parasites could make it give false negative culture test[6,7]. Cytological preparations and other diagnostic techniques, such as monoclonal antibodies, in situ hybridization, and immunological assays are expensive, time-consuming and lack sensitivity and specificity[6–8].

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After the complete draft of genome sequence of *T. vaginalis*, diagnosis by PCR based molecular methods is considered superior to wet mount and culture. The sensitivity and specificity of PCR ranges from 91–99% and 96–100%, respectively^[9,10]. Most of the PCR based diagnoses target repetitive DNA sequence. β -tubulin gene is one of the most common target gene sequence for PCR diagnosis but it fails to detect strain level variation in different geographical areas^[11].

Ribosomal genes have highly conserved hypervariable region in their genome sequence. However coding regions like 5.8S, 18S and 28S genes of ribosomal DNA are more conserved. The 18S rRNA gene shows hypervariable region and used for the differentiation of the strains. Mayta *et al*^[12] using 18S rRNA as a gene target, have reported high specificity than culture as *T. vaginalis* was detected by PCR in 8.3% of the sample compared to 6.5% detected by culture. A recent study by Meade *et al*^[13] on genetic diversity of *T. vaginalis* clinical isolates determined by EcoRI restriction fragment length polymorphism (RFLP) of heat-shock protein 70 genes reveals that *Trichomonas* organisms exhibit considerable polymorphism in their Hsp70 RFLP patterns. The phenotypic and genotypic variation in *T. vaginalis* is well documented^[14,15]. Till date reports from India in this direction are scarce. Earlier, Kaul *et al*^[16] have reported the randomly amplified polymorphic DNA analysis of *T. vaginalis* isolates from symptomatic and asymptomatic women in Indian infected subjects.

Highly repetitive nature in the genome of organism make these genes could be a good target for PCR diagnosis and to differentiate among the strains isolated from symptomatic and asymptomatic patients. Our aim was to develop a PCR assay, based on *Trichomonas* strains prevalent in the Indian population. This would be the basis for a new test to be devised and to differentiate the strain variations in symptomatic and asymptomatic infected women. Sequencing was also performed for identification of strain variation and their regulatory genomic studies. A phylogenetic relationship was also studied among the symptomatic and asymptomatic strains of *T. vaginalis*.

2. Materials and methods

2.1. Clinical specimens

Informed consent was obtained from all study participants. Before insertion of the speculum, clinicians collected vaginal wash samples ($n=498$) from women attending the OPD clinics of Queen Mary's Hospital, Lucknow, and Halberg Hospital, Moradabad from July 2008 to May 2011. The median age of the sampled population was 22 years old (ranging between 19 to 45 years).

Vaginal swab samples were placed in 2 mL of a collection vial with screwed cap and kept at 4 °C until arrival at the laboratory within the same day or 1 day of collection. An equal volume of PBS buffer (pH=7.4) was added to the sample, and the preparation was mixed, incubated at room temperature for 10 min, and stored at -70 °C until DNA extraction.

A second vaginal swab sample was obtained after the insertion of the speculum, and immediately touched to a

glass slide together with a drop of normal saline for the microscopic ($\times 10$) wet examination of *Trichomonas* in vaginal fluid. The pH of vaginal secretions was measured by using pH test strips (Sigma).

2.2. Axenic *T. vaginalis* strains and DNA extraction

Seventeen long term axenized strains of *T. vaginalis* isolated from culture of vaginal secretions from symptomatic and asymptomatic patients attending an OPD at Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh were considered for assessing the sensitivity of the PCR primer sets. The specificity was evaluated by testing other related *Trichomonas* spp., flagellates, amoebae, or cervicovaginal pathogens like *Chlamydia trachomatis* (*C. trachomatis*), *Neisseria gonorrhoeae* (*N. gonorrhoeae*), *Giardia lamblia* (*G. lamblia*), *Toxoplasma gondii* (*T. gondii*), *Leishmania donovani* (*L. donovani*) and *Entamoeba histolytica* (*E. histolytica*) isolated from culture maintained at PGIMER, Chandigarh.

DNA extraction from *T. vaginalis* or the other microorganisms mentioned above was prepared from cultures by using the Phenol-chloroform extraction method for cell^[17]. After thawing the vaginal washes at room temperature for 30 minutes, 500 mL of 1:10 diluted phosphate buffer (PBS) (pH 7.4) (1 part of PBS and 9 parts of saline) were added and mixed by gently vortexing for 5–10 seconds; 250 μ L of the well mixed specimens were added to a 2 mL propylene tube containing 500 μ L of 1:10 diluted PBS. The specimen suspension was vortexed at maximum speed for 20 sec and incubated at room temperature for 10 min. After centrifugation at 6500 \times g for 5 min, the supernatant was discarded.

To cell pallet (1×10^9 trophozoites/mL) lysis buffer containing 1 \times saline sodium citrate (150 mM NaCl, 15 mM sodium citrate, 300 mM NaCl), 0.2% sodium dodecyl sulfate and 100 μ g/mL proteinase K was added and incubated at 65 °C water bath for 30 min. Phenol chloroform extraction of DNA was performed according to previously described method^[17]. The DNA obtained was precipitated with ethanol (-20 °C overnight). The DNA was recovered by centrifugation for 20–30 min at 0 °C. The supernatant was discarded and pellet was washed with 70 percent ethanol and recentrifuged at 12000 g for 10 min at 4 °C. After drying DNA pellet was dissolved in 500 μ L of TE buffer (pH=8.0) and stored at 4 °C till further use.

Since the amount of clinical specimen is in very less amount, DNA extraction from clinical isolates were extracted by QIAGEN-DNA extraction kit, according to the slight modifications in instructions provided by supplier. DNA concentration for each isolate was quantitated on optical density readings at a wavelength of 260 nm and the quality of DNA was checked in 1% agarose gel by agarose gel electrophoresis.

2.3. Culture

Tryptone yeast extract and serum containing media (TYS) culture media was used for axenic cultures. After culturing and sub-culturing, culture tubes were incubated at 37 °C for 4 days. The cultures were examined microscopically daily till 7th day after inoculation. A positive result will be

defined as the presence of motile trichomonads at any time; a negative result will be defined as the absence of motile trichomonads till the 7th day post inoculation.

2.4. PCR primers

A set of primers targeting conserved regions of the 18S ribosomal DNA of *T. vaginalis*, as designed earlier by Mayta *et al*[12] was used. The primers are picked from uncommon regions of the 18S ribosomal gene of *Trichomonas tenax* (accession No. U37711) *Tritrichomonas foetus* (U17509) *Entamoeba gingivalis* (D28490), *Trypanosoma brucei* (AJ009149), *Candida albicans* (M60302), *Giardia lamblia* (U09492), and *Homo sapiens* (U13369)[12]. The sequences of the primers were as follows: Tv1, 5' TAA TGG CAG AAT CTT TGG AG 3', and Tv2, 5' GAA CTT TAA CCG AAG GAC TTC 3'.

2.5. PCR amplification

PCR was performed in an automated gradient thermocycler (Eppendroff, USA) with 20 μ L of reaction mixture using thin-walled PCR tubes. Designed PCR primers were synthesized from Sigma USA.

The final volume of 20 μ L of PCR reaction mixture contained 250 ng genomic DNA of *T. vaginalis*, 10 pmol of forward and reverse primers at a concentration of 1×10^{-4} M of each dNTPs, and 1 unit of taq polymerase. The tubes were kept in thermocycler with the programme: 4 min of denaturation at 94 $^{\circ}$ C, followed by 40 cycles each consisting of 10 seconds of denaturation at 94 $^{\circ}$ C, 45 seconds of annealing at 55.8 $^{\circ}$ C, and 15 seconds of extension at 72 $^{\circ}$ C. A final extension step at 72 $^{\circ}$ C for 5 min was included. Each amplification cycle were included a sample with DNA extracted from *T. vaginalis* for positive control and a blank containing water as a negative control.

The annealing temperature was lowered half degree every four cycles until reaching 62 $^{\circ}$ C, and then kept constant till the end of the cycling process.

2.6. Detection of amplified targets

The primer set Tv1/Tv2 taken was designed to amplify a DNA product of 312 bp. Ten microliters of amplified product was electrophoresed at 60 V in 3% agarose gels in Tris-borate-EDTA buffer. The sizes of the amplified products were assessed by comparison with a commercial 100-bp weight marker (Fermentas, USA). The gel was observed under UV rays in gel documentation unit (BIOVIS, India).

2.7. Sequencing

Sequencing of 18S rDNA gene PCR product of symptomatic and asymptomatic axenic and clinical isolates of *T. vaginalis* was done using an automated DNA sequencer (ABI prism genetic analyzer 310, USA). The sequencing results of symptomatic and asymptomatic axenic (SS1 and SA1) and clinical strains (SS2 and SA2) of *T. vaginalis* (respectively 476 bp, 266 bp, 282 bp and 411 bp long) were submitted to NCBI (Accession No. JF513196–99).

2.8. Sequence analysis

Sequence alignments were obtained by using the program CLUSTAL W EBI ClustalW2 program method developed by Higgins and Sharp[18]. Computation parameters were set to a K-tuple value of 5, a gap penalty of 5, a window size of 10, and a filtering level of 2.5.

2.9. RFLP analysis

For conformity of the amplified PCR product targeting 18S ribosomal gene of *T. vaginalis*, PCR products of all positive cases were digested with restriction endonuclease *Hae*III (10 U/ μ l) provided by Bangalore Genei, India. The volume of reaction mixture was setup for 20 μ L, containing 4 μ L of RE buffer (provided with enzyme), 400 ng amplified PCR product and 4 U (0.4 μ L) *Hae*III restriction enzyme. Final volume was set up by HPLC grade water. The complete reaction mixture was incubated at 37 $^{\circ}$ C for 60 minutes for restriction digestion. 10 μ L of digested products were tested on 3% agarose gel electrophoresed at 50 V. The sizes of the digested products were assessed by comparing with a commercial 100-bp weight marker (Fermentas, USA). Gels were stained with ethidium bromide (0.5 μ g/mL; HiMedia). The gel was documented under UV rays in gel documentation unit (BIOVIS, India).

3. Results

All axenic strains used for standardization of PCR were found positive amplification of 312 bp long sequence using Tv1/Tv2 set of primers (Figure 1). Primer set Tv1/Tv2 had amplified the predicted 312-bp product in sixteen *T. vaginalis* clinically isolated strains tested (Figure 2). In all the axenic reference strains, the origin of PCR products was derived from 18S ribosomal gene. The PCR products, after digestion of *Hae*III restriction endonuclease yielded two fragments of 211 bp and 101 bp (Figure 3). Among the clinically isolated *T. vaginalis* strains in patients, sixteen patients were found positive by PCR. However twenty patients were conformed trichomonas-positive by microscopic PAP-smear test, out of which six patients were trichomonas-negative by PCR. Three new cases were found trichomonas-positive by PCR. Ten out of sixteen (62.5%) of confirmed trichomonas-positive patients was symptomatic. Six patients were found positive from asymptomatic subjects. The percentage of patients having no significant symptoms for trichomoniasis compared to patients who were negative for trichomonas from PCR assay using Tv1/Tv2 primer sets were 96.78% (482/498). Out of sixteen subjects, 62.5% (10/16) of confirmed *T. vaginalis*-positive patients had vaginal secretions of pH of more than 4.5 compared to 48% (239/498) of negative patients. The odds ratio of having a trichomonas infection was 2.8 (95% CI, 1.3 to 5.6) when pH was >4.5. In this study, a retrospective analysis, 76.5% (13/17) of patients with confirmed trichomonas infections received appropriate treatment with metronidazole at the clinic site; twelve out of four hundred ninety eight (12/498) were suggested for the diagnosis of bacterial vaginosis, as suspected from wet mount test.

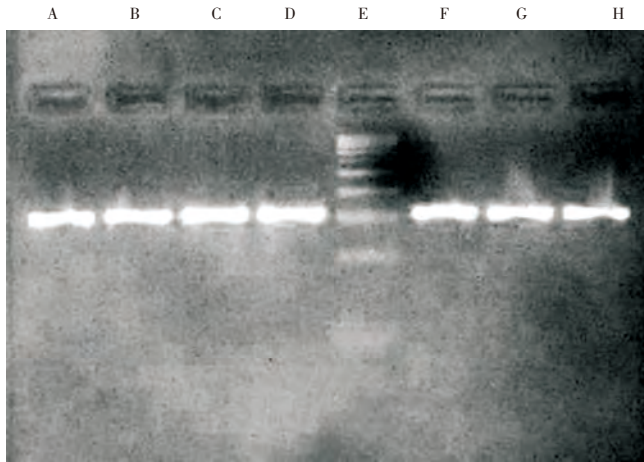


Figure 1. Detection of *T. vaginalis* axenic strains A to D and F to H by PCR with primer sets Tv1/Tv2. Lane E have 100 bp ladder for sizing the amplified product. Strains A to D and F to H are representative of 17 strains of *T. vaginalis* isolated from long term axenized culture.

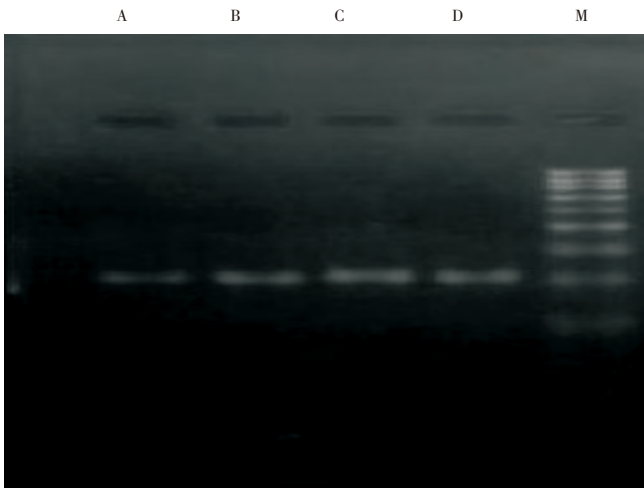


Figure 2. Detection of *T. vaginalis* clinical strains A to F by PCR with primer sets Tv1/Tv2. Strains A to F are representative of 8 strains of *T. vaginalis* (designated A to G) isolated from vaginal secretions of patients attending the OPD.

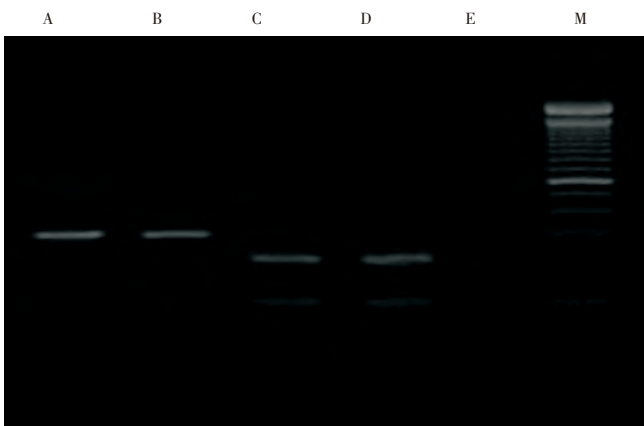


Figure 3. Agarose gel electrophoresis of undigested PCR products and restriction enzyme *HaeIII* treated PCR product of *T. vaginalis*. Lane A and B are undigested PCR products of asymptomatic and symptomatic strain respectively. Lane C and D are digested PCR products by *HaeIII* restriction enzyme of asymptomatic and symptomatic strain respectively with Lane M with 100 bp marker. Lane E digested PCR product of negative control.

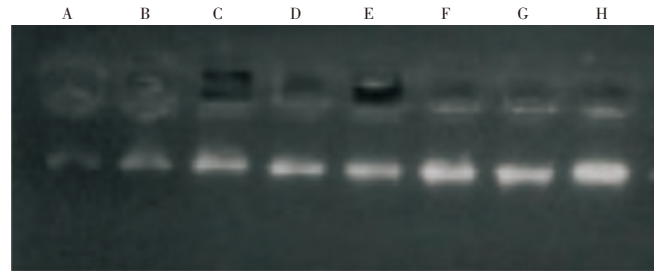


Figure 4. PCR sensitivity testing based on *T. vaginalis* genomic DNA quantity. Lanes A to H, 1 pg, 10 pg, 100 pg, 1 ng, 10 ng, 100 ng, 1 µg and 2 µg with primer set Tv1/Tv2.

The analytical sensitivity of PCR with primer set Tv1/Tv2 on the twofold dilutions of *T. vaginalis* DNA was the amplification of the DNA of per PCR (Figure 4). No targeted PCR products were amplified when DNAs from other vaginal pathogens or protozoa were tested with the Tv1/Tv2 primer set.

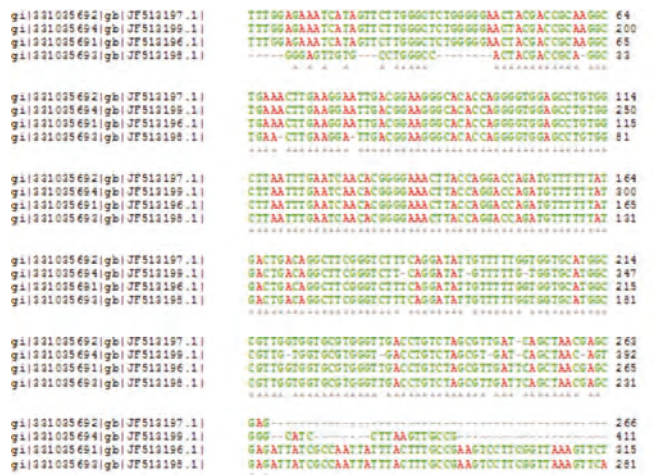


Figure 5. Alignment of 18S rDNA gene using primer Tv1/2 and the start of the 18S rRNA coding region from *T. vaginalis* SA1 (JF513196), *T. vaginalis* SA2 (JF513197), *T. vaginalis* SS1 (JF513198) and *T. vaginalis* SS1 (JF513199). Hyphens indicate alignment gaps; asterisks identify conserved positions.

Table 1

Pairwise and multiple sequence alignment scores table.

Alignment pattern	Sequences aligned	Alignment score
Pairwise alignments	1:2	100
	1:3	97
	1:4	66
	2:3	85
	2:4	96
	3:4	81
Multiple alignments	Group 1: Sequences: 2	4760
	Group 2: Sequences: 2	5160
	Group 3: Sequences: 2	4744
Total alignment score		9952

The sequencing alignment of 18S rDNA gene PCR product coding region from symptomatic and asymptomatic axenic (SS1 and SA1) and clinical strains (SS2 and SA2) shows a total alignment score of 9952 among the three groups of multiple

sequence alignment (Table 1). The coding region of all the four sequence shows a maximum, 100 percent alignment between the sequence 1:2 and a minimum, 66 percent alignment between the sequence 1:4 in pair-wise alignment (Figure 5).

4. Discussion

Trichomonas PCR with primer set Tv1/Tv2 was observed 100% (17 of 17) sensitive and specific for axenic strains. It was more sensitive than wet preparation or culture. PCR conditions developed with primer set Tv1/Tv2 for the detection of symptomatic and asymptomatic strains of *T. vaginalis* had found good analytical sensitivity and was able to amplify even one pictogram of DNA per PCR. The predicted DNA product (312 bp) in the targeted 18S rDNA gene was amplified with all *T. vaginalis* strains tested and axenized (17 of 17). The analytical specificity of primer set Tv1/Tv2 was optimal, since no targeted DNA products were detected with other protozoa or vaginal pathogens. As no such targeted product was amplified with DNA from *C. trachomatis*, *N. gonorrhoeae*, *G. lamblia*, *T. gondii*, *L. donovani* and *E. histolytica*.

Repeats and transposable elements comprise about two-third of the ~160 megabase genome of *T. vaginalis*, reflecting a massive expansion of genetic material and causing variability among the strains of *T. vaginalis* isolates from different geographical regions[9]. The use of a multilocus gene family for *Trichomonas* genome analysis provides a tool with considerable discrimination power to analyze genetic diversity in *T. vaginalis* isolates responsible for human trichomoniasis in different populations[13]. Primer set Tv1/Tv2 was found suitable to target a well-conserved region of 18S rDNA genes for Indian symptomatic and asymptomatic women, thus improving sensitivity because of increased number of DNA target copies available for amplification. This region appears to be moderately conserved across other *Trichomonas* spp. isolated from symptomatic and asymptomatic women, as shown after sequence alignment.

The specificity of the primers depends on the annealing temperature, with higher temperatures favoring higher specificity. The use of a touchdown protocol increased the specificity of the PCR by favoring the amplification of targeted copies of DNA amplified during early cycles at higher annealing temperatures and eliminating spurious products. The DNA polymerase used (Fermentas, USA) is not active until heated to 95 °C, thus simulating a hot-start PCR technique. The use of this enzyme also avoided erroneous amplification of DNA products due to nonspecific annealing of primers at lower temperatures.

A comparative study for PCR sensitivity was compared to culture and wet mount for the detection of *T. vaginalis* in vaginal swabs obtained from 350 women attending a health clinic on a military base. The sensitivities of PCR, culture, and wet mount were 97%, 70%, and 36%, respectively[11]. In an observational study of 337 women, the diagnostic sensitivity of PCR for the detection of *T. vaginalis* was 84%, while 78% for culture and 52% for wet mount[19]. It was reported that real-time PCR were used to analyze specimens from 1978 women and 98 men who were suspected of having

trichomoniasis. The sensitivity of PCR was 100%, and the specificity was 99.9%. The positive and negative predictive values were 95% and 100%. By comparison, culture and microscopy had a combined sensitivity of 71%[20,21]. In previous studies, when compared with culture, PCR with primer set TVA 5–1/6 gives 61% (14/23)[22], TVA 5/6 detected 90% (44 of 49) of culture-positive distal vaginal swab samples from an STD clinic in Pittsburgh Pa[26], and detected 91% (41 of 45) of culture-positive tampon specimens collected from a population of the Torres Strait Island[22].

In the present study, the vaginal swab samples were collected by the clinicians, but self-administered vaginal washes samples, which was practical, rapid, and easy to obtain. Other studies have demonstrated that self-administered vaginal swab samples are comparable to physician-administered samples for the PCR detection of trichomonal infection[23].

An increased pH of the vaginal secretions in patients infected with *T. vaginalis* has been previously reported[24]. In our study, three fourth of the patients with confirmed positive *T. vaginalis* infection had vaginal secretion pHs of >4.5. Nevertheless, these patients represented only 12% of the total number of patients with elevated pHs, making pH an inadequate predictor for trichomonas infection.

Retrospectively, only 75% of patients with confirmed *T. vaginalis* infection were diagnosed by wet preparation, and they received appropriate treatment with metronidazole at the clinic, and no clinical diagnosis or treatment was given to 25% of the infected patients. These findings suggest the need for a more accurate diagnostic test for trichomonas, such as PCR, to prevent complications, transmission to sexual partners, and asymptomatic carriers, and to decrease possible transmission of HIV[25–27]. Molecular diagnostic methods have been found to be very sensitive and more specific. Once the presence of *T. vaginalis* has been documented, other STIs should also be actively looked for the patients[28].

Although wet preparation had minimal cost, its sensitivity is highly dependent on the expertise of the microscopy[28], prompt transport, and laboratory processing before the organism lyses or loses motility. In our study, even when the wet preparation was performed at the collection site, its sensitivity (75%) was suboptimal compared to PCR. Culture had a better sensitivity than wet mount examination but required more time for laboratory turnaround since cultures are held for 1 week. PCR results are available in 2 to 3 days and provided the highest sensitivity. The cost of PCR testing comes mainly from the cost of reagents, which is about two dollar per specimen in India, approximately equivalent to the cost of an InPouch TV culture bag. The technician time is probably equivalent since cultures require daily examination by light microscopy, although it needs an expertise technical skills in molecular amplification techniques. Currently in use, many laboratories are dealt with PCR based diagnosis of *C. trachomatis* and *N. gonorrhoeae* infections in routine. Thus, a more appropriate trichomonas PCR for identifying both the strains of *T. vaginalis* could be easily incorporated into the work flow of other diagnostic amplification procedures.

Vaginal washes samples for trichomonas PCR can be easily transported and processed with PBS buffers. Incorporating

PCR with Tv1/Tv2 for *T. vaginalis* into the routine laboratory diagnostic methods and using the same processed sample for the PCR detection of *C. trachomatis* and *N. gonorrhoeae* could be a cost-effective strategy and more appropriate test for identifying strains of *T. vaginalis* when screening programs for multiple STDs are implemented.

Conflict of interest statement

We declare that we have no conflict of interest.

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