PCR based diagnostic assay targeting the beta tubulin gene for the detection of *Trichomonas vaginalis* infection in vaginal swab samples of symptomatic and asymptomatic women in India

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ABSTRACT

Objective: To develop an in−house PCR based diagnostic assay for identification of strains isolated from symptomatic and asymptomatic subjects of India, targeting the β −tubulin gene using specific primers. **Methods:** In the present study a primer set is designed to target a well−conserved region in the beta−tubulin gene of *Trichomonas vaginalis (T. vaginalis)*. All strains of *T. vaginalis* were tested and successfully detected by PCR yielding a single predicted product of 198 bp in gel electrophoresis, while there was negative response with DNA from *Giardia lamblia, Toxoplasma gondii, Leishmania donovani* and *Entamoeba histolytica*. The sensitivity and specificity for a single *T. vaginalis* cell per PCR was achieved. Axenic Culture, performed with long term axenized *T. vaginalis* culture system, was routinely examined to identify *T. vaginalis*. **Results:** The PCR based investigations with 498 vaginal swab samples from women attending OPD clinics of Halberg Hospital Moradabad and Queen Mary′s Hospital, Lucknow, India and 17 long term axenic cultures maintained at PGIMER, Chandigarh, India using primer set BTUB 1 & BTUB 2 showed sensitivity and specificity response of 98% and 100%, respectively, while wet preparation in clinically isolated samples responded up to 62.5%. The PCR product sequencing result of symptomatic strains (SS1) of *T. vaginalis* (744 bp long) was submitted to NCBI (Accession No: JF513200). It shows maximum identity 98 % with XM_001284521 *Trichomonas vaginalis* G−3 beta−tubulin (btub) putative partial mRNA. **Conclusions:** The data gathered in the present study entail that the diagnosis of *T. vaginalis* infection by PCR may be established as a sensitive and specific protocol, to be incorporated into a joint strategy for the screening of multiple STDs by employing molecular amplification technique. The merits and precautions of the protocol have been discussed.

1. Introduction

Trichomonas vaginalis (T. vaginalis) is one of the most prevalent, non−viral protozoan pathogens and the most common sexually transmitted organism in females, yet it is one of the least investigated infectious agents. An estimate of around 180 million new infections is found worldwide every year[1,2]. The high *T. vaginalis* prevalence worldwide is concentrated in developing countries and socioeconomic disadvantaged groups with a dramatic decline in *T. vaginalis* rates in some developed countries in the past few decades[2]. Symptomatic women with trichomoniasis usually complain of vaginal discharge, vulvovaginal soreness, and/or irritation. Dysuria and dyspareunia are also common[3] *T. vaginalis* can be asymptomatic in 10 to 50% of women[4,5]. The organism can be recovered in 11% of men attending sexually transmitted disease (STD) clinics and from 30 to 60% of male sexual partners of infected women, usually as a self−limited mild urethritis[6]. It can be transmitted to neonates during passage through an infected birth canal (2 to 17%), but the infection is usually asymptomatic and self limited[7]. The incidence of trichomoniasis is highest in women with multiple partners and in groups with a high prevalence of other STDs[8]. Whether trichomoniasis is a risk factor for human immunodeficiency virus transmission or just a marker for high−risk heterosexual activity remains unclear[9]. An association of pelvic

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inflammatory disease, tubal infertility, and cervical cancer with previous episodes of trichomoniasis has been reported but may be explained by its association with other STDs[10–12]. Complications of trichomonal vaginitis that have been reported include premature rupture of membranes, premature labor, low birth weight, and post–abortion or post–hysterectomy infection[8, 13,14].

Traditionally physicians make the diagnosis based on clinical grounds, but in women, the characteristics of the vaginal discharge, including color and odor, are poor predictors of *T. vaginalis*[13–15]. Since no symptom alone or in combination is sufficient to diagnose *T. vaginalis* infection reliably, laboratory diagnosis is necessary[5]. *T. vaginalis* may be identified in vaginal secretions by using a wet preparation, but this method is only 35 to 80% sensitive compared with culture[16]. The sensitivity of wet preparation is highly dependent on the expertise of the microscopist[4] and the prompt transport and laboratory processing of samples before the organism becomes lysed or loses motility. Other diagnostic techniques, such as fluorescent antibody[17], enzyme–linked immunosorbent assay[17], and a hybridization test[19] have been used to detect *T. vaginalis* and have had reported sensitivities between 70 and 90%[11]. Culture in microaerophilic conditions is estimated to be 85 to 95% sensitive and has been considered the "gold standard" for diagnosis[4]. But it has lower sensitivity as it undergoes frequent contamination of cultures.

In this study, a PCR targeting the beta–tubulin genes of *T. vaginalis* was developed for the detection of the organism in vaginal washes samples. The targeted genes encode the amino acid sequence of beta–tubulin protein[19], a major component of the *T. vaginalis* cytoskeleton. *Trichomonas* PCR was validated on long term axenized *T. vaginalis* culture and compared with wet mount preparation. Since elevated pH of vaginal secretions in patients infected with *T. vaginalis* has been reported as a parameter of selection for symptomatic and asymptomatic women[3] its utility as a predictor was also explored in this study.

2. Materials and methods

2.1. Long term culture maintained T. vaginalis isolates and DNA extraction

T. vaginalis isolates maintained by *in vitro* cultures were taken as sensitivity assessment for PCR primer set targeting the β–tubulin gene. Seventeen long term culture maintained *T. vaginalis* isolates from symptomatic and asymptomatic patients attending OPD at Postgraduate Institute of Medical Education & Research (PGIMER) Chandigarh were considered for assessing the sensitivity of the PCR primer set. The specificity of the primer set was evaluated by using the DNA of other related *Trichomonas* spp., flagellates, amoebae, or cervicovaginal pathogens (*Giardia lamblia* (*G. lamblia*), *Toxoplasma gondii* (*T. gondii*), *Leishmania donovani* (*L. donovani*) and *Entamoeba histolytica* (*E. histolytica*) isolated from culture maintained at PGIMER, Chandigarh.

Genomic DNA was extracted from *T. vaginalis* or the other microorganisms mentioned above from mass–cultures by using phenol–chloroform extraction method[17]. The culture having $2-8\times10^7$ parasites in log phase of growth were obtained. Centrifuged at 1 500 *g* for 10 min, supernatant was discarded and pellet was obtained. To the cell pellet, lysis buffer was added 10 times its volume and gently mixed. The mixture was incubated for 30 minutes in a water bath at 65 ℃. The lysate thus obtained was centrifuged at 900 *g* for 5 min to check

for completion of lysis and cell debris was removed. To the supernatant, equal volume of Tris EDTA (pH 8.0) and saturated phenol was added and vortexed gently and centrifuged at 12 000 rpm for 15 min at 40C in cooling centrifuge. Upper viscous layer was pipette out in a fresh eppendorf and equal volume of phenol chloroform mixture (25:24) was added and again centrifuged at 12 000 rpm for 15 minutes at 4 ℃ in cooling centrifuge. Upper layer was pipetted out in a fresh eppendorf and mixed with an equal volume of chloroform isoamyl alcohol mixture (24:1) and again centrifuged at 12 000 rpm for 15 min at 4 ℃ in cooling centrifuge. The clear upper layer was pipetted out in a fresh tube. Add, chilled absolute ethanol (2.5 volume) and 5M Sodium acetate (1/10 volume). This solution was again mixed well and kept at −20 ℃ for overnight. The DNA was recovered by centrifugation at 12 000 rpm for 20 minutes at 0 ℃. The supernatant was discarded and the pellet washed with 70% ethanol and recentrifuged at 12 000 rpm for 10 min at 4 ℃. The supernatant was again discarded and the tube left open on the bench, at room temperature, until the fluid traces had evaporated. The DNA pellet was dissolved in 500 μL of TE buffer (pH 8.0) and stored at 4 ℃ till further use.

2.1.1. RNase treatment

RNase was added to DNA solution at the concentration of 20 μ g/mL and the solution was incubated for 30 min at 37 ℃. A repeated step was again followed by phenol, chloroform extraction and ethanol precipitation. The recovered DNA pellet was dissolved in 500 μL of TE buffer (pH 8.0) and stored at −20 ℃ until amplification.

2.1.2. DNA isolation from clinical isolates

DNA extraction from clinical isolates was extracted by QIAGEN–DNA extraction kit QI® Amp DNA mini 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 qualitated by agarose gel electrophoresis.

2.2. Clinical isolates of T. vaginalis

Informed consent was obtained from all 498 study participants. Self administrated vaginal specimens were collected by the study participants with sterile synthetic swabs or vaginal wash samples from women attending the OPD clinics of Queen Mary's Hospital, Lucknow, and Halberg Hospital, Moradabad from July 2008 to May 2011. The sampled population was ranging between 19 to 45 years. The median age of the sampled population was 22 years.

The women subjects were instructed to insert swab into the vaginal and to rotate the swab two times. Vaginal swab samples were placed in 2 mL of a collection vial with screwed cap and kept at 4 ℃ 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 ℃ 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 (×10) wet examination of *Trichomonas* in vaginal fluid. The pH of vaginal secretions was measured by using pH test strips (Sigma).

2.3. PCR primers

An alternative set of PCR primers targeted to conserved regions in beta–tubulin genes of *T. vaginalis* was designed,

synthesized, and tested. The sequences were as follows: for BTUB 1, 5′ GGA CAG TGC GGT AAC CAA AT T 3′ (positions 275 to 294); and for BTUB 2, 5′ GCA CTC ACT TGT GCC TGG TT 3′ (positions 472 to 491).

2.3.1. Alternate primer designing

An alternative set of PCR primer targeting β–tubulin gene of *T. vaginalis* genome by using conserved regions different from other related parasites and human β–tubulin gene. An automated software primer3 was used picking PCR primers and their properties. The primer set includes forward and reverse primer of 20 bp. Some minor modifications were made for optimizing the value of tm and GC%. The size of the PCR product amplified through this primer would be 198 bp.

2.4. PCR amplification

PCR amplification and optimization was performed in an automated gradient programmable thermocycler (Eppendroff, USA) with 20 μL of reaction mixture using thin–walled PCR tubes. PCR reagents were purchased from Fermentas, USA. PCR primers were synthesized and biotinylated by Sigma USA.

The final volume of the reaction mixture was 20 μL contained 250 ng genomic DNA, 10 pmol of forward and reverse primers at a concentration of 1×, 10 mmol of each dNTPs, and 1 unit of Taq polymerase. The PCR amplification was performed in thermocycler with the standard amplification programme. An attempt for optimization of PCR condition in gradient thermocycler was done using a temperature gradient of 0.5 ℃ annealing temperature. The best amplification results were obtained at PCR programme having 4 minutes of denaturation at 94 ℃, followed by 30 cycles each consisting of 45 seconds of denaturation at 94 ℃, 45 sec of annealing at 48.6 ℃ and 45 seconds of extension at 72 ℃. A final extension step at 72 ℃ for 10 min was also included. Each amplification cycle were included a DNA sample from *T. vaginalis* as positive control and DNA from other related species as negative control.

2.5. Agarose gel electrophoresis

Upon completion of PCR, an aliquot was analyzed by electrophoresis in a 3% agarose gel having 0.5 μg/mL ethidium bromide in TAE (TRIS–acetate–EDTA) buffer (pH–8.5). The primer set BTUB 1/2 was designed to amplify an amplified product of 198 bp from the three beta–tubulin genes. Two microliters of amplified product was electrophoresed at 60 V in 2% 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 photographed under short Ultraviolet light. in Gel documentation unit (BIOVIS, India).

2.6. Sequencing

The PCR products of symptomatic and asymptomatic axenic and clinical isolates of *T. vaginalis* were sequenced using an automated DNA sequencer (ABI prism genetic analyser 310, USA).

2.7. Sequence analysis

Closely related homologous sequence were identified through phylogenetic analysis by comparing partial sequence with non–redundant database of nucleotides sequences deposited at NCBI web server (www.ncbi.nlm.nih.gov.), using the standard nucleotide–nucleotide basic local alignment tool (BLAST)

program (http://www.ncbi.nlm.nih.gov/blast/).

3. Results

The DNA sequence for the primer set BTUB 1/2 were designed to target conserved regions of the three beta–tubulin genes of *T. vaginalis* to improve the analytical sensitivity (GenBank accession numbers: btub1, L05468; btub2, L05469; and btub3, L05470). Besides, to improve specificity, these DNA sequences were selected from the regions of the *T. vaginalis* beta–tubulin genes that differed substantially from the beta–tubulin gene sequences of humans and other microorganisms. (GenBank accession numbers: *G. lamblia*, X06748; *Trypanosoma brucei* subsp. *rhodesiense*, K02836; *Trypanosoma cruzi*, M97956; *Toxoplasma gondii*, M20025 and *Homo sapiens*, V00598 J00317). A well conserved sequence region of β–tubulin gene was identified by comparative sequence alignment of GenBank accession number btub1, L05468; btub2, L05469; and btub3, L05470 of *T. vaginalis* (Figure 1).

Figure 1. PCR primers for the detection of *T. vaginalis* with the beta–tubulin genes. Primers were selected from well–conserved and specific regions of the genes (btub1, –2, and –3). Sequences of the beta–tubulin genes of *T. vaginalis* were compared to the beta–tubulin gene sequences of humans and to those of other pathogens. A dot indicates the same base, and a letter indicates a different base compared to the *T. vaginalis* btub1 gene sequence.

Initial detection of specimens collected from wet–mount positive patients with suspected infection of *T. vaginalis* were tested to compare the sensitivities of PCR assay and wet–mount microscopy. In total, 498 patients were included after taking their informed consent. *T. vaginalis* infection was isolated from 17 (3.42%) out of 498 women screened (260 symptomatic and 238 asymptomatic). Of theses, 10 (3.85%) out of 260 were symptomatic and 7 (2.94%) out of 238 were asymptomatic women. The *T. vaginalis* infection rate in symptomatic and asymptomatic was not significantly different ($P>0.05$). Total seventeen patients were found positive by PCR using our designed set of primers. However twenty patients were conformed *Trichomonas*–positive by microscopic pap–smear test. Out of which eight patients were *Trichomonas*–negative by PCR. Five new cases were found *Trichomonas*–positive by PCR. A similar proportion of confirmed *Trichomonas*–positive patients was symptomatic (10 of 17, 58.87%) compared to patients who were negative for *Trichomonas* (481/498, 96.58%). Out of seventeen subjects, 64.7% (11 of 17) of confirmed *T. vaginalis*–positive patients had vaginal secretions of pH of >4.5 compared to 48% (239 of 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 of 17) of patients with confirmed *trichomonas* infections received appropriate treatment with metronidazole at the clinic site; twelve of four hundred ninety eight (12/498) were suggested for the diagnosis of bacterial vaginosis, as suspected from wet mount test.

Table 1.
BLAST N result (Sequence producing significant alignments).

Accession	Description	Max score	Total score	Query coverage	E value	Max identity
JF513200.1	Trichomonas vaginalis isolate SS1 beta−tubulin (btub) gene, partial sequence	1375	1375	100%	0.0	100%
XM_001311949.1	Trichomonas vaginalis G3 beta−tubulin, putative (TVAG_008680) partial mRNA	235	235	18%	3e−58	98%
XM_001300401.1	Trichomonas vaginalis G3 beta−tubulin, putative (TVAG_148390) partial mRNA	235	235	18%	3e−58	98%
XM_001582993.1	Trichomonas vaginalis G3 beta−tubulin, putative (TVAG_456920) partial mRNA	233	233	19%	1e−57	95%
XM_001284521.1	Trichomonas vaginalis G3 beta−tubulin, putative (TVAG_525430) partial mRNA	233	233	17%	1e−57	98%
L05468.1	Trichomonas vaginalis beta−tubulin (btub1) gene, complete cds	233	233	19%	1e−57	96%
XM_001321203.1	Trichomonas vaginalis G3 beta−tubulin, putative (TVAG_073810) partial mRNA	230	230	18%	2e−56	97%
XM_001579619.1	Trichomonas vaginalis G3 beta−tubulin, putative (TVAG_062880) partial mRNA	224	224	18%	7e−55	96%
AY277782.1	Tetratrichomonas gallinarum strain A6 beta−tubulin 2 gene, partial cds	207	207	19%	7e−50	93%
AY277786.1	Tritrichomonas foetus strain ATCC 30924 beta−tubulin 1 gene, partial cds	193	193	17%	2e−45	93%
AY277783.1	Tetratrichomonas gallinarum strain A6 beta−tubulin 3 gene, partial cds	187	187	17%	1e−43	92%
AY277781.1	Tetratrichomonas gallinarum strain A6 beta−tubulin 1 gene, partial cds	187	187	17%	1e−43	93%
AY277787.1	Tritrichomonas foetus strain ATCC 30924 beta−tubulin 2 gene, partial cds	182	182	17%	4e−42	92%
XM_001318453.1	Trichomonas vaginalis G3 beta−tubulin, putative (TVAG_034440) partial mRNA	159	159	17%	2e−35	89%
AB600307.1	Carpediemonas sp. NC gene for beta−tubulin, partial cds	84.2	84.2	11%	1e−12	84%

Primer set BTUB 1/2 had amplified the predicted 198−bp product in all seventeen axenic strains of *T. vaginalis* after the successful validation of PCR primers on axenic strain (Figure 2), seventeen *T. vaginalis* clinically isolated strains (ten symptomatic and seven asymptomatic) were tested and successfully amplified the 198 bp length of PCR product (Figure 3). The analytical sensitivity of PCR with primer set BTUB 1/2 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 BTUB 1/2 primer set.
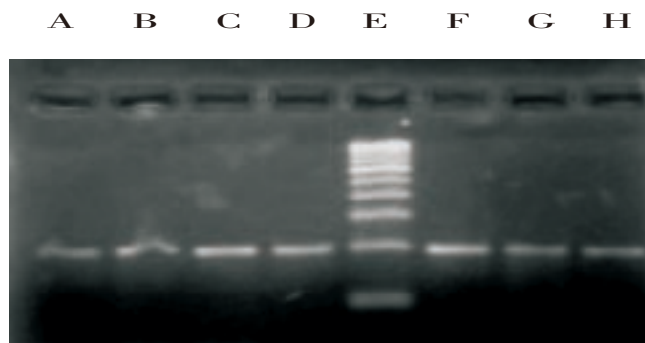
Figure 2. Detection of *T. vaginalis* axenic strains A to D and F to H by PCR with primer sets BTUB 1/2. Lane E has 100 bp ladders for sizing the amplified product. Strains A to D and F to H are representative of 17 strains of *T. vaginalis* isolated from long term axenized culture.
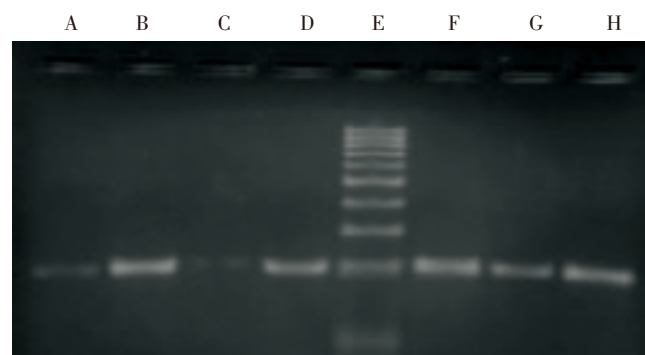
Figure 3. Detection of *T. vaginalis* clinical strains A to F by PCR with primer sets BTUB 1/2. Strains A to F are representative of 8 strains of *T. vaginalis* (designated A to G) isolated from vaginal secretions of patients attending the OPD.
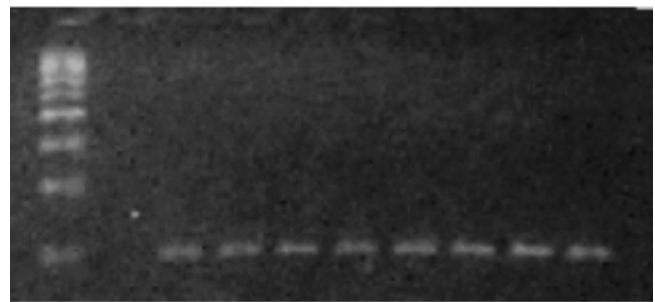
Figure 4. PCR sensitivity testing based on *T. vaginalis* genomic DNA quantity, Lanes A to H, 1 pg, 10 pg, 100 pg, 1 ng, 10 ng, 100 ng 1 μ g and 2 g with primer set BTUB 1/2. Lane M has 100 bp ladder for sizing the amplified product.

3.1. Sequencing

Sequencing result of β −tubulin gene of symptomatic and asymptomatic clinical isolates of *T. vaginalis* were similar. The sequencing result of symptomatic strains (SS1) of *T. vaginalis* (744 bp long) was submitted to NCBI (Accession No: JF513200). The sequence generated was aligned by BLAST N (Nucleotide− Nucleotide pair wise alignment) indicated that our query sequence (Accession No: JF513200) shows maximum identity 98% with XM_001284521 *T. vaginalis* G−3 beta−tubulin (btub) putative partial mRNA and minimum identity 84% with XM001318453.1 *T. vaginalis* G−3 beta−tubulin (btub) putative partial mRNA gene among the related *T. vaginalis* strain (Table 1).

3.2. Phylogenetic analysis

Similarity and differences among species can be used to infer evolutionary relationship (Phylogeny). This is because; if two species are very similar they are likely to have shared a common ancestor. In the present study, β −tubulin gene of *T. vaginalis* has been analyzed, to detect out the conservation among DNA nucleotides using multiple sequence Alignment technique. The resulting alignment indicates the regions of similar sequences at least 84% in all the sequences that define a conserved conscience pattern or domain. In this case

alignment is particularly strong, used to align position to try and drive the possible evolutionary relationship among the sequences. The presence of similar domain in several similar sequences implies a similar biochemical function or structure fold that may become the basis of further experimental investigations. A group of similar sequences may define a protein family, which may share a common biochemical function or evolutionary origin. A phylogenetic distance tree was generated by BLAST N result indicating the distance from related species of *T. vaginalis*, interpreting the phylogenetic relationship (Figure 5).
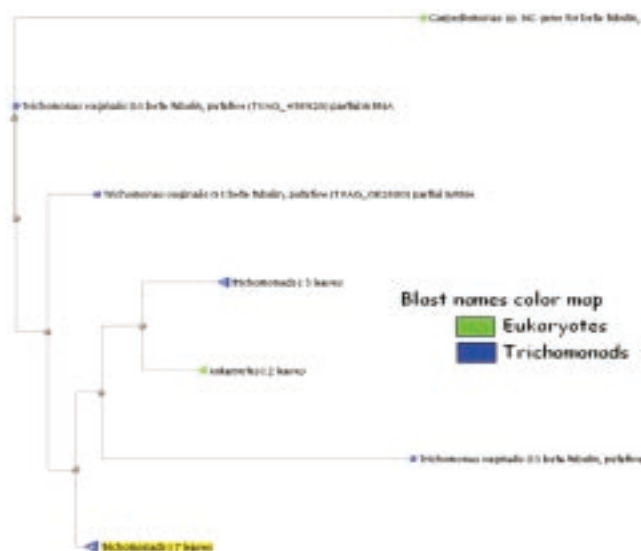

Figure 5. Phylogenetic tree showing relationship between trichomonads and eukaryotes.

4. Discussion

Rapid and sensitive diagnosis of *T. vaginalis* infections is important for appropriate treatment and to reduce the spread of the disease. It is important to reduce the prevalence of this disease, as the infection increases the risk of HIV transmission and complications during pregnancy. The infection of *T. vaginalis* was detected by PCR with primer set BTUB 1/2 was observed 100% (17 of 17) sensitive and specific for long term culture maintained axenic strains. It was found more sensitive for Indian population. All the axenic strains of *T. vaginalis* were 100% PCR positive from designed primer set. PCR with designed primer set BTUB 1/2 had good analytical sensitivity and was able to amplify even one pictogram of DNA per PCR. The predicted DNA product (198 bp) in the targeted beta–tubulin gene was amplified with all *T. vaginalis* strains tested and axenized (17 of 17). The analytical specificity of primer set BTUB 1/2 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 *E. histolytica*, *G. lamblia*, *L. donovani*, *N. gonorrhoeae* and *T. gondii*.

A combination of PCR based diagnostic assay targeting of two different genetic regions of *T. vaginalis* provides a more specific and sensitive PCR test[21]. A different set of primers were used for PCR RFLP pattern of 18S rDNA gene of *T. vaginalis* on same group of patients using Tv1/Tv2[22]. The combination of these two set of primers can increase the detection sensitivity of the PCR diagnostic assay in Indian symptomatic and asymptomatic women for trichomoniasis. The increased detection sensitivity of the PCR can be achieved by the combination of these two primers. This study could offer a useful rapid screening of trichomoniasis in asymptomatic patients of Asian continent.

The variability among the strains of *T. vaginalis* isolates from different geographical regions is because of transposable elements and repeats comprises about two–third of the ~160 megabase genome of *T. vaginalis*, reflecting a massive expansion of genetic material[23]. The genetic diversity in *T. vaginalis* isolates including multilocus gene family in *Trichomonas* provides a tool for genome analysis and variation in symptoms of human trichomoniasis in different populations[24]. In the *T. vaginalis* genome, there are several copies of the three genes encoding beta–tubulin proteins (beta–tubulin 1, 2, and 3)[18]. Primer set BTUB 1/2 was designed to target a well–conserved region in all three beta–tubulin genes, thus improving sensitivity because of increased number of DNA target copies available for amplification.

The annealing temperature plays a pivotal role for specificity of the PCR primers, with higher annealing temperatures favors high specificity. The use of a gradient annealing temperature increases 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 Taq polymerase used in PCR (Fermentas, USA) is inactive until the temperature raises up– to 95 ℃. In present study a hot–start PCR technique was used as simulating parameter. Very good amplification results were obtained using this hot–start technique in the present study. The use of this enzyme also avoided at lower temperatures for erroneous amplification of DNA products due to nonspecific annealing of primers.

In a comparative study for PCR sensitivity with culture and wet–mount microscopy for the detection of *T. vaginalis* infection in vaginal swabs obtained from 350 women attending a health clinic on a military base. The sensitivities of PCR, culture, and wet–mount microscopy were 97%, 70%, and 36%, respectively[25]. In a different 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[26]. In a most recently reported study using real– time PCR for 1 978 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%[27]. In our previous study using the same specimen for the sensitivity of PCR, culture and wet–mount microscopy were 94%, 82% and 70% respectively. In the present study the sensitivity of PCR with wet–mount microscopy were 100% and 78% respectively. The increased sensitivity of wet–mount microscopy depends on skill of the microscopy and the time of sample collection and wet–mount preparation. The low sensitivity of wet–mount microscopy makes it unstable as the only test for laboratory diagnosis of *T. vaginalis* infection.

In our study, PCR with designed primer set detected 100% (17 of 17) of long term axenized culture–positive specimens. In previous studies, when compared with culture, PCR with primer set TVA 5–1/6 gives 61% (14/23)[28] and TVA 5/6 detected 90% (44 of 49) of culture–positive distal vaginal swab samples from an STD clinic in Pittsburgh, Pa[29], and detected 91% (41 of 45) of culture–positive tampon specimens collected from a population of the Torres Strait Island[30].

The present study includes collection of vaginal swab samples by the clinicians and health–workers and sometimes self–administrated, but the sample collected self–administered vaginal washes samples, which was practically rapid and easy to obtain, in comparison to vaginal swab samples. Other studies have demonstrated that self–administered vaginal swab samples are comparable to physician–administered samples for the PCR detection of trichomonal infection[23].

The increased pH of the vaginal secretions of the patients infected with *T. vaginalis* has been reported previously[24]. In our study, more than 75% 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.

Conclusively the molecular diagnostic methods have been found to be very sensitive and more specific. Once the diagnosis of *T. vaginalis* has been documented, other sexual transmitted infections (STIs) can also be actively locked for the patients[28].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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