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ORIGINAL ARTICLE

Diagnosis of *Trichomonas vaginalis* infection by PCR

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ABSTRACT

Background: Trichomonas vaginalis infection is the most prevalent sexually transmitted disease in the world. It causes vaginitis, urethritis and preterm birth. It has been associated with nongonococcal urethritis in men. In this study, a polymerase chain reaction (PCR) targeting the beta-tubulin genes of T. vaginalis was developed for the detection of the organism in both vaginal swab and urine specimens from infected patients.

Materials and methods: Random urine samples were collected from 30 patients (23 females and 7 males) tested positive for T. vaginalis by wet preparation and the Inpouch T. vaginalis culture system. Two vaginal swabs were collected by each woman, first before insertion of the speculum and then after the insertion of the speculum. A previously published T.vaginalis specific primer set, (BTUB 9/2), BTUB 9 (5' CAT TGA TAA CGA AGC TCT TTA CGA T 3') and BTUB2 (5' GCA TGT TGT GCC GGA CAT AAC CAT 3') recognizing a 112-bp target within the β-tubulin gene of the T. vaginalis organism was used for this purpose.

Results: The positive result was reported 28.6% in male urine and 39.1% in female urine samples, first swab 65.2% and second swab 78.3% by wet preparation diagnosis. By the culture test, the male urine samples recorded 42.9% positive, female urine 69.6% while the first swab recorded 86.9% positive and the second swab 91.3% positive. All negative cases by culture in urine and vaginal samples were tested by PCR, which resulted as 2 cases positive in male urine samples and 5 cases were positive in female urine samples, but one case only gave positive with PCR in first swab of vaginal samples and 2 cases of second swab became positive by PCR. No statistical differences were observed in incidences among patients

Conclusion: On conclusion the PCR assay was even more sensitive than wet preparation and culture and afforded the practical advantages of providing results.

Keywords: *Trichomonas vaginalis, Diagnosis, Polymerase chain reaction*.
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INTRODUCTION

Trichomonas vaginalis is the agent of a highly prevalent sexually transmitted infection (STI) that can result in vaginitis, urethritis, and preterm birth. It has been associated with nongonococcal urethritis in men. Many individuals are asymptomatic. Traditional methods of diagnosis, including wet preparation, can be unreliable because of poor sensitivity, more over, clinicians mast obtain a vaginal specimen to perform standard tests for wet preparation and culture. Developing an assay for urine would be desirable. Such a method would confer the advantages of easy

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procurement, transport, and storage of patient samples (1). Other diagnostic techniques, such as fluorescent antibody (2), enzyme-linked immunosorbent assay (3), and a hybridization test (4) have been used to detect T. vaginalis.

In this study, a PCR targeting the beta—tubulin genes of T. vaginalis was developed for the detection of the organism in both vaginal swab and urine specimens from infected patients (5). Traditional PCR methods require postamplification detection of products, which could be error prone, laborious, and /or time consuming (6).The targeted genes encode the amino acid sequence of beta-tubulin protein (7), a major component of the T. vaginalis cytoskeleton. Trichomonas PCR was compared with culture and wet preparation. Discrepant results were adjudicated by PCR using a previously described set of primers and a set of primers targeting the adhesion genes of T. vaginalis (8).

The method of PCR allows the selective amplification from DNA, a particular fragment is selected from a complex genome by enzymatic amplification in vitro. The double-stranded genomic DNA template is denatured by heating and the temperature is then decreased to allow specific oligonucleotide primers to hybridize to their complementary sequences on opposite strands of the template. The template-directed DNA synthesis then proceeds in both orientations from the primer sites by enzymatic catalysis with a thermo-stable polymerase and results in double-stranded products (9).

This synthesis is repeated over 30-35 cycles in an automated thermocycler, and can take one to several hours to complete. During each cycle, each template is replicated by a factor of two, so that upon completion of the cycling, millions of copies of the original template have been produced and are available for subsequent manipulations or analysis.

PATIENTS and METHODS

Random urine samples were collected from 30 patients (23 females and 7 males), tested positive for T. vaginalis by wet preparation and the Inpouch T. vaginalis culture system. One milliliter of the Inpouch culture (Biomed Diagnostics) was mixed with 9ml of trichomonas medium and incubated for 24 hours at 37ºC. The culture was then centrifuged (800Xg) for 10 minutes and 9ml of supernatant was removed from the culture. Of the remaining 1ml of the concentrated culture a 25-µl aliquot was diluted with Evans blue, and T. vaginalis organisms were counted in a hemocytometer to determine the organism concentration (number per milliliter)(10). To stabilize the T. vaginalis, DNA was extracted by the Chelex method and then frozen. For this technique, mixtures of 50µl of cultures with 200µl of a 5% suspension of chelating resin (Chelex 100; Sigma, St. Louis, Mo) in tris buffer (0.01M,PH 8.0) were incubated at 56ºC for 15 to 30 minutes. Preparation was mixed gently and then boiled for 8 to 10 minutes, then centrifuged (12000Xg) for 1 minute in a microcentrifuge and stored at -70ºC.

Two vaginal swabs were collected by each woman (23), first before insertion of the speculum, and then after the insertion of the speculum. The first sample was placed in 1ml of a commercial PCR transport medium (Amplicor; Roche Diagnostic systems, Branchburg, NJ) and kept at 4ºC until arrival at the laboratory within 4 days of collection. An equal volume of specimen diluents (Amplicor) was added to sample, and the preparation was mixed, incubated at room temperature for 10 minutes, and stored at -70ºC until tested (6). A second vaginal swab sample was obtained after the insertion of the speculum. It was immediately touched to glass slide together with a drop of normal saline for microscopic (X100) wet examination for Trichomonas in vaginal fluid. After the wet preparation was made, the swab was immediately inoculated into the Inpouch T. vaginalis culture system (Biomed Diagnostic, San

Jose, California) (11), incubated at 30ºC and examined daily with a light microscope to identify T. vaginalis.

A set of primers targeting a conserved region of the beta-tubulin genes of T. vaginalis was designed, synthesized, and tested. It was based on FRET (Fluorescence Resonance Energy Transfer) probe chemistry. A previously published T. vaginalis-specific primer set, BTUB9 (5' CAT TGA TAA CGA AGC TCT TTA CGA T3') (positions 850 to 874) and BTUB2 (5' GCA TGT TGT GCC GGA CAT AAC CAT 3') (positions 961 to 938), recognizing a 112-bp target within the β-tubulin gene of the T. vaginalis organism (6). The TETR (Touchdown Enzyme Time Release) PCR and primer set have been utilized in additional studies involving male and female urine and female vaginal samples.

PCR was performed with 10µl of cultures processed by the Chelex method. PCRs were performed in an automated thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). The final reaction mixture (100µl) contained 25pmol of each primer, 2.5mM deoxynucleoside triphosphate 1XPCR buffer (10mM Tris HCl,PH=8.4), 50mM KCI, and 2µ of Ampli Taq Gold DNA polymerase overlaid with 2 drops of mineral oil. Since 1.0mM $MgCl_2$ is a component of the AMPLICOR specimen buffers, 1.5mM $MgCl_2$ was added to the reaction mixture, otherwise, 2.5mM $MgCl_2$ was used when cultures of microorganisms processed with Chelex were tested. The cycling conditions used were as follows: 95ºC for 10 minutes, followed by 50 cycles of 95ºC for 10s, 55ºC for 10s, and 72ºC for 5s. A final cooling step of 3 minutes at 40ºC was included for handling of the samples, because the light Cycler has no cooling block. Twenty microliters of amplified product was electrophoresed at 120V in 12% polyacrylamid gels in Tris-borate-EDTA buffer and stained with ethidium bromide (0.01mM). The sizes of the amplified products were assessed by comparison with a commercial 50-bp weight marker.

Negative controls, including uninoculated transport media, were used throughout the specimen preparation and PCR process. A low copy number of Trichomonas as positive control was included in every PCR run.

RESULTS

Male urine samples reported by wet preparation were positive in 2 and negative in 5 while female urine samples were positive in 9 and negative in 14. First swab of vaginal samples recorded positive in 15 and negative in 8 while second swab resulted in 18 positive and 5 negative. The positive urine cultures were 3 and 16 for male and female groups, respectively. Moreover, first and second swab of vaginal samples were recorded positive in 20 and 21 cases, respectively (tables 1 and 2).

Table 1. *PCR, wet preparation and culture results for urine samples tested for T. vaginalis*

		Wet preparation	Culture	PCR*
Male	Positive	2(28.6)	3(42.9)	2(50)
(n=7)	Negative	5(71.4)	4(57.1)	2(50)
Female	Positive	9(39.1)	16(69.6)	5(71.4)
(n=23)	negative	14(60.9)	7(30.4)	2(28.6)
Total	Positive	11(36.7)	19(63.3)	7(63.6)
(n=30)	Negative	19(63.3)	11(36.7)	4(36.4)

* Samples negative by culture were tested by PCR

Table 2. *PCR, wet preparation and culture results for vaginal samples tested for T. vaginalis*

		Wet preparation	Culture	PCR*
1st swab	Positive	15(65.2)	20(86.9)	1(33.3)
(n=23)	Negative	8(34.8)	3(13.1)	2(66.7)
2nd swab	Positive	18(78.3)	21(91.3)	2(100)
(n=23)	negative	5(21.7)	2(8.7)	0

* Samples negative by culture were tested by PCR

All negative urine and vaginal cultures were tested by PCR, resulted in 2 and 5 positive cases in male and female urine samples, respectively, while

one and 2 cases were positive in first and second swab of vaginal samples, respectively. There was no statistically significant difference between cases.

DISCUSSION

Trichomonas PCR with primer set BTUB 9/2 revealed a sensitivity and specificity of 100%. It was more sensitive than wet preparation or culture. A recent study utilizing TETR PCR for analysis of male urine specimens found that the sensitivity of TETR PCR was superior to that of culture (12). Jordan et al (13) reported a real-time PCR assay tested with vaginal samples, at 40 cycles. Their Taq Man PE thermocycler-based system took about 2.5 hours to complete. By using light cycler technology, runs of the BTUB FRET PCR assay were completed in less than 30 minutes at 50 cycles. Additionally, assay time was also improved by utilizing the Roche Magna Pure LC robot for DNA extraction. The robot is capable of extracting 32 samples, including standards and negative controls. In 90 minutes, yielding a total assay time of approximately 2 hours, a vast improvement over traditional method was achieved. Medico et al. detected amplifications from three sets of PCR primers with standard numbers of replicates (6). The detection limit of the system was found to be consistently between one and four T. vaginalis organisms per PCR, which is comparable to the limit of another reported real-time method for T. vaginalis detection (11). Justin et al. recorded that the sensitivity and specificity of the BTUB FRET PCR in urine approach the sensitivity (97.8%) and specificity (97.4%) of the other real-time assay utilizing vaginal swabs for sample collection (10). Although the sensitivity of the BTUB FRET PCR was lower (90.4%), the specificity was higher (100%). Ultimately, a quick, accurate assay for urine sample is needed to make high-throughput screening for T. vaginalis possible. Culture may be the gold standard, but it is inherently limited because it relies on the organism to be viable for proper detection. Furthermore, culture results can be subject to interpretation by the viewer, whereas PCR offers a more definitive result. Traditional gel-based PCR assays have been found to be less accurate with urine samples than with vaginal swabs (14). In Madico et al study, positive samples were increased by 39% by PCR compared to culture (32 of 350; 9.1%) versus (23 of 350; 6.6%) (6). PCR with primer set BTUB9/2 for the detection of T. vaginalis had good analytical sensitivity and was able to amplify one Trichomonas organism per PCR. The predicted DNA product (112bp) in the targeted beta-tublin gene was amplified with all T. Vaginalis strains tested (15 to 15) (6). The analytical specificity of primer set BTUB 9/2 was optimal, since no targeted DNA products were detected with other protozoa or vaginal pathogens. In the T. vaginalis genome, there are several copies of the three genes encoding beta-tubulin proteins (beta-tubulin 1,2 and 3) (7).

Primer set BTUB9/2 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 (15). Although wet preparation had minimal cost, its sensitivity is highly dependent on the expertise of the microscopist, prompt transport and laboratory processing before the organism lyses or loses motility (16). Even when the wet preparation was performed at the collection site, its sensitivity (36%) was suboptimal compared to PCR (6). Culture had a better sensitivity (70%) in their study 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. Although Trichomanas PCR requires more technical skill, molecular amplification techniques are currently in use in many laboratories for the detection of C.

trachomatis and N. gonorrhoeae infections, thus, Trichomonas PCR could easily by incorporated into the work flow of other diagnostic amplification procedures (6).

On other hand, Hardick et al explained that the performance of the real-time PCR was compared with wet mount or culture (17), but some potentially important details are lost in this type of comparison, for example, they surveyed only vaginal swabs, whereas the current study used urine specimens, and the earlier validated TETR PCR employed agarose gel electrophoresis and visualization of ethidium bromide-stained DNA bands for detection of amplified products, which is inherently less sensitive than the fluorescence resonance energy transfer technology of the newly developed real-time PCR. Thus, some TETR PCR negative/real-time PCR positive specimens were probably true positive.

In conclusion the incidence of T. vaginalis infection was quite high. In our study, there were only 6 cases negative for T. vaginalis, while 24 were positive. PCR assay was even more sensitive than wet preparation and culture and afforded the practical advantages of providing results.

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