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Full Length Research paper

Feasibility of a nested PCR for the diagnosis of vaginal trichomoniasis: study in Al-Madinah Al-Munwarrha, Saudi Arabia

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In the present study, three vaginal swabs were collected from 1469 females clinically suspected of having *Trichomonas vaginalis* (*T. v)* infection. All samples were screened by both wet mount and Diamond's culture media that was considered as the golden standard in this study. *T. vaginalis gene* detection by nested PCR using 4 primers targeting the Tv-E650 gene was performed on the preserved vaginal uncultivated samples corresponding to the culture positive vaginal specimens plus 30 randomly selected samples equaled to those obtained negative culture results. The prevalence of *T. vaginalis* infection among our patients was calculated according to the results of the golden standard culture method to be 1.43% (21 out of 1469). Wet preparation was positive for only 13 samples and missed 8 samples. PCR diagnosed 20 samples and missed one specimen that became positive after 4 days of cultivation. In this study, PCR for trichomonads does not appear to offer a diagnostic advantage and its sensitivity did not exceed that of culture. Successful culture of *T. vaginalis* requires only the multiplication of a single organism, the same as that needed for PCR. Therefore, the present work is highly recommending the use of Diamond's culture in the diagnosis of trichomoniasis in women.

Key words: *Trichomonas vaginalis*, nested PCR, wet mount, diamond's culture, vaginal swabs, Saudi Arabia, Al-Madina Al-Munawarah.

INTRODUCTION

Trichomonas vaginalis is one of the major pathogens responsible for vaginitis, cervicitis and urethritis in women (Lin et al., 1997). Infection during pregnancy is predispo-sing to premature rupture of the placental membranes, premature labour, and low-birth weight infants. Moreover, linked to this disease are cervical cancer, atypical pelvic inflammatory disease and infertility (Bakhtiari et al., 2008). However, the prevalence of *T. vaginalis* is likely to be underestimated in our region, because there are no guidelines for screening such infection, and clinicians often rely upon insensitive diagnostic, methods (Crucitti et al., 2003). Accurate diagnosis is ne-cessary for specific treatment that facilitates control of the *T. vaginalis* infection and prevents complications. It is es- timated that untreated *T. vaginalis* can persist for up to 5 years in women and is highly transmissible between part-ners (Klinge et al., 2006). The conventional methods for diagnosis involve the direct microscopic examination of wet-mount or culture-based systems. Despite their high specificity, the former is limited by poor sensitivity; the latter has the disadvantage of prolonged turnaround time. However, culture remains the most accurate single method for detecting trichomoniasis and it is considered to be the "gold standard" (Sakru et al., 2005). Unfortunately, the use of culture is almost non-existent in most of our laboratories. On the other hand, Food and Drug Admini-

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stration (FDA) approved nucleic acid amplification tests as a substitute of culture for *T. vaginalis* and they are currently available for researches (Center for Disease Control and Prevention, 1998; Mabey et al., 2006). Our objective in this study was to assess the feasibility of a nested PCR using 4 primers targeting the Tv-E650 gene in comparison to the conventional microscopic tests, wet mount and culture for the diagnosis of trichomoniasis from vaginal swab.

PATIENTS AND METHODS

Study group

Women of 18 years of age or above attending the outpatient clinic at Maternity and Children's Hospital, Al-Madina Al-Munawarah, Saudi Arabia and Medical unit in Taibah University presenting with signs and symptoms of vaginitis were eligibly enrolled in a prospective, cross-sectional study. Cases included in this study assured not to have used oral or topical metronidazole during the 4 weeks prior to specimens' collection. Women not meeting these criteria and those who refused to participate in the study were excluded. Enrollment occurred over a period of about 15 months.

Collection of specimens

Following history taking and examination, three vaginal samples were obtained from each female patient by swabbing the high vaginal vault. One swab was placed in a transport medium consisting of 1/10 nutrient agar [MDM, Medical disposable and Diagnostic Manufacturing] and used for wet-mount microscopy, the second one was used to immediately inoculate a Diamond's culture media. The third swab was placed in 1.5 ml of sterile (PBS), pH 7.2 and the resulting suspension was kept at -20°C to be served for *T. vaginalis* gene detection.

Microscopic examination

The Diamond's medium was prepared according to Diamond, 1986 and was used in the present work as the reference, gold standard method. Samples were observed at daily intervals for 5 days. Wet-mount and culture samples were examined microscopically using the low (x100) and high power (x400) magnification.

Polymerase chain reaction: nested- PCR

Nested PCR was done for the preserved uncultivated vaginal specimens corresponding to those proved positive by the culture method plus 30 samples were randomly chosen from preserved specimens that matched negative culture results. The positive control was collected from women who gave positive results for *T. vaginalis* by the Diamond's culture. One milliliter of the culture was mixed with 9 ml of Fuji medium [Remel, Lenexa, Kans.] and incubated for 24 h at 37°C. The culture was then centrifuged at 800 rpm for 10 min, and 9 ml of supernatant was removed from the culture. Of the remaining 1 ml of the concentrated culture, a 25 µl aliquot was diluted with Evans blue, and *T. vaginalis* organisms were counted in a haemocytometer to determine the organism concentration (number per milliliter). To stabilize the *T. vaginalis* DNA, an additional 50 µl aliquot was subjected to DNA extraction and was frozen before serial dilution and use as a PCR standard.

The PCR mixture with DNase-free water was used as negative control. DNA extraction was done using the Proteinase K – Phenol/Chloroform method according to Rawal et al. (1994). The dried pellet was then dissolved in 20 µl Tri- EDTA (TE) or/and kept at -20°C until PCR amplification would be done and the resulted DNA was examined on 0.8% agarose. Considering the different sample volumes and dilution factors during template preparation, nested DNA amplification, and agarose gel electrophoresis, the resulting detection limit per assay and the number of *T. vaginalis*/ DNA genome equivalents were calculated (Lin et al., 1997). For Semi-quantification, serial dilutions of the positive DNA samples were done to estimate the lowest volume of DNA to be amplified which represents amplified DNA of one *T. vaginalis,* and hence, semi quantification of the number of trophozoite/ml could be calculated.

According to Lin et al. (1997), DNA amplification was done using single tube nested PCR technique. The outer primers sequence was as follows: forward: 5' GTG AAA ATC TCA TTA GGG TAT TAA CTT 3', and the reverse: 5' GTT TTA TTT ATC ACT GGA AAA TAA CGC TT 3'. The inner primers sequences are as follow: forward: 5' AAC ATC CCC AAC ATC TT 3', and the reverse: 5' CCA TTC TTT TAG ACC CTT 3'. These primers were targeting the Tv-E650 gene. The PCR final volume of 20 µl was adjusted to contain: 30 nM of each outer primers and 500 nM of each of the inner primers, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM Mg Cl_2, 200 µM each dNTPs, 0.5 unit of Qiagen Taq polymerase, and 5 µl of the extracted DNA. PCR amplification and Visualization was performed in a Biometra thermal cycler in two stages with different annealing temperatures, an initial denaturation for 5 min at 95°C, then 30 cycles of denaturation at 95°C for 45 s, annealing at 62°C for 1 min, extension at 72°C for 1 min. This was followed by 20 cycles at annealing temperature of 45°C for 45 s, followed by final extension at 72°C for 10 min. 10 µL amplified PCR products was visualized on 3% agarose, in 1X TBE buffer (90 mM Tris-base, 90 mM boric acid and 2 mM EDTA, pH 8). The gel was stained by ethidium bromide (0.5 µg/ml) and viewed under UV illumination. In negative samples subsequent re-analysis was done after addition of external *T. vaginalis* DNA to test for inhibitors. In proved false negative samples, a trail was done to eliminate PCR inhibitors according to Lawing et al. (2000) in which a threefold increase in *Taq*. was added, then repeating the PCR cycle. [All chemicals were purchased from Sigma pharmaceuticals].

Statistical analysis

The Diamond's medium was used as the "gold standard" diagnostic method to study the vaginal swab specimens. The results of the wet mount for vaginal swabs were analyzed in relation to culture. The study evaluation was done by using Excel (Microsoft Corp., Redmond, Wash.). The sensitivities, confidence intervals were calculated. Comparisons of the difference in sensitivities of wet mount and PCR were done using McNemar's chi-square test for paired samples.

RESULTS

In the present study, sensitivity of the nested PCR was tested using the 4 primers targeting the T.v-E650 gene (on the control positive sample). Different amplified DNA fragments of *T.vaginalis* were obtained from the reactions between both forward primers with the reverse primers and the PCR products were visualized as ethidium bromide- stained bands of 521 and 448 pb resolved by agarose gel electrophoresis. Negative controls did not show any reactions. The reaction Semi quantification of *T. vaginalis* was done after serial dilution of the DNA samples (Fiure 1A). The minimal amount of DNA to be amplified was

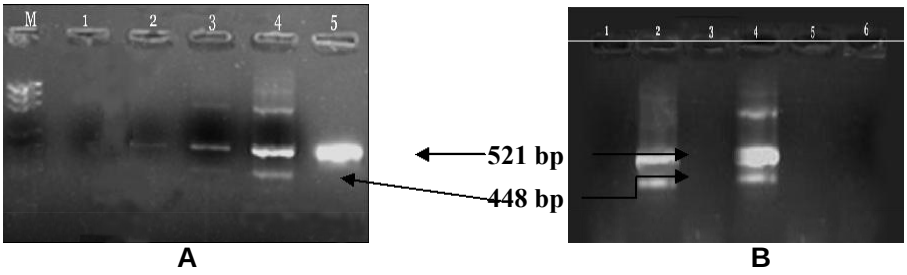

Figure 1. Agarose gel electrophoresis of PCR product of amplified *Trichomonas vaginalis* DNA visualized by ethedium bromide and UV transillumination.
(A) End point dilution (semi quantification) of a control sample of *T. vaginalis* , *lane* M is pGem DNA marker, while *lanes* 5 to 1 are serial dilution of DNA, amplified by PCR and visualized on 3% agarose by electrophoresis. *Lane* 1 is considered the end point where detection of *T.vaginalis* is below the 1 organism, so the *lane* 2 is considered as an amplified DNA of at least one *T.vaginalis* trophozoite, *Lane* 3, is 10 fold (10 trophozoites), *lane* 4, is amplified DNA of 100 trophozoites, while *lane* 5, is 1000 trophozoites.
(B) Optimization of single nested PCR amplification conditions, lane 2 and 4, PCR product of amplified positive *T. vaginalis* sample (vaginal swab), while *lanes* 1 and 3 and 5 are negative samples, *lane* 6 is a negative PCR control.
NB: Absence of 290 bp due to incorporation and amplification of most of *T. vaginalis* DNA by outer primers giving rise to 521 and 448 bp.

Table 1. Comparison between wet mount microscopy and PCR method in relation to the golden standard culture for the diagnosis of vaginal trichomoniasis.

Test method	Positive	False +ve	False -ve	True -ve	Total number	% Sensitivity (95% CI)**
*Culture	21	0	0	1448	1469	The golden standard
Wet mount	13	0	8	1448	1469	61.9% (43.4-80.4)
PCR1st run	18	0	3	0	21	85.7% (70.7-100)
2nd run	20	0	1	0	21	95.2% (86.1-100)

* Culture was used in the study as a reference test (golden standard).
**CI, Confidence interval.

containing DNA of 1-5 trichomonads-/assay. On the other hand, Wet mount and Diamond's culture were read for all the received samples from the total of 1469 women that were enrolled in this study with signs and symptoms of vaginitis, positive culture results were observed in 21 samples. Therefore the pre -valence of *T. vaginalis* infec-tion among our patient was calculated to be 1.43%. PCR test was performed only for 51 preserved samples (21 corresponding to that obtained positive culture and 30 randomly selected negative specimens).

Results of wet mount and PCR test were compared in relation to the "gold standard" culture results, as summa-rized in Table 1; sensitivities and confidence interval [CI] were calculated. Taking in consideration, the characteris-tic morphological features of the *T. vaginalis* organisms, no false positive results were observed with the micro-scopic methods used in this study. Out of 21 samples with positive cultures, only 13 (61, 95% CI = 43.4-80.4) were diagnosed by wet microscopy. Regarding nested PCR, positive reaction was observed in 18 samples, 2 of these 3 missed samples showed positive PCR results after removal of DNA inhibitors confirming that these 2 samples were initially false negatives. No PCR reaction was observed in the 30 randomly selected negative sam-ples. PCR test missed one specimen that became posi-tive after 4 days of culture. Therefore, the recorded sensi -tivity of PCR in detection of *T. vaginalis* infection in vagi-nal samples was calculated to be 85.7-95.2% with 95% CI = 70.7-100 and 86.1-100, respectively. PCR assay was statistically more sensitive than wet preparation mi-croscopy (85.7-95.2 versus 61.9%, $p< 0.05$).

DISCUSSION

Accurate diagnosis of *T. vaginalis* is affected by many variables, including patient factors, clinician's experience, specimen sampling, processing and test interpretation as well as the skill set and expertise of those doing micro-scopic assessments. On the other hand, sensitivity, spe-cificity, cost, ease of use, and time to results for the differ-rent methods for diagnosing *T. vaginalis* infection should be also considered (Harsstall and Carabian, 1998). In the present study, a large number of clinical samples (1469), were analyzed by wet mount and Diamond's culture that

was considered the "gold standard" whereas, 21 positive cases of trichomoniasis were detected. The culture was able to diagnose the 8 cases missed by wet vaginal mount. Despite that wet-mount microscopy is the most available and commonly used in clinical practice, as it is inexpensive, easy to perform. However, *T. vaginalis can* produce an over-whelming inflammatory response, concealing the parasites, or the number of organisms may be very low. Thus, even with skilled diagnosticians, the diagnostic sensitivity of wet-mount microscopy is only 60% compared with culture (Kaurth et al., 2004). Therefore, culture methods is recommended in the present study as has been advised by many authors who reported sensitivity approaches 100% as few as one parasite in the sample may be detected (Wilkerson, 2003; Mabey et al., 2006; Crucitti et al., 2008). Unfortunately, culture is not available in most clinical laboratories (Schwebke, 2002).

The prevalence of infection among women included in the present study was estimated to be 1.43%. A higher prevalence rate (4%) was recorded by Al Quaiz, (2000), a survey in a University Primary Care Clinic in Riyadh City. Some of the discrepancies in the findings of this and other studies probably result from the wide diversity of patterns of vaginal infections in different populations. However, the prevalence of trichomoniasis is among the lowest reported among Moslems and this could be explained by the strict religious and cultural beliefs which prohibit illegal sexual relationships. Internationally, trichomoniasis affects approximately 200 million women worldwide. The frequency in Europe is similar to that of the United States, ranging from 20 to 30% among women attending the STD clinics. In Africa, the prevalence may be much higher. In a study in South Africa, trichomoniasis was estimated to be 65% among pregnant women attending an antenatal clinic (Schwebke, 2002).

On the other hand, molecular diagnosis of *T. vaginalis* was first reported by Riley et al. (1992) in clinical samples using primers TVA5 and TVA6. Subsequently, many additional primer sets have been described. The sensitivity and specificity of these primers in clinical studies using vaginal swab specimens have varied, with sensitivities of 85 to 100% being reported. In the present study, the nested PCR was performed using the 4 primers targeting the T.v-E650 gene that previously studied and evaluated recording 100% sensitivity and specificity by Lin et al. (1997). In the present work, different amplified DNA fragments of *T. vaginalis* were obtained from the obtained from the reactions between both forward primers with the reverse primers and the PCR products were visualized as ethidium bromide-stained bands of 521 and 448 pb resolved by agarose gel electrophoresis (Figure 1). The minimal amount of DNA to be amplified was containing DNA of 1-5 trichomonads/assay (Figure 1A). The assay was as sensitive as to detect one to 100,000 *T. vaginalis* per PCR mixture; the same sensitivity was recorded by Men-Fang et al. (1997). The technique's sensitivity was such high as to be able to amplify and detect just one trichomonad and which contains as low as 0.15 pg of DNA, which is 100-fold less than DNA/host cell (Lin et al., 1997). One factor that helps increasing the sensitivity of this PCR is that the copy number of the Tv-E650 repeat has been estimated to be about 100 to 1000 repeats /genome (Paces et al., 1992) . The present results are in agreement with Gordan et al. (2001) , in which their PCR assay was able to detect as few as five *T. vaginalis* organisms per milliliter of medium. Despite this sensitivity, the techni-que failed to detect 3 vaginal samples and PCR was repeated after addition of three fold increase in *Taq* to exclude the possibility of DNA inhibitors that were proved in 2 samples. Therefore, in the present study, PCR-based detection of *T. vaginalis* from vaginal swabs was not equivalent to culture; its sensitivity was recorded to be 85.7-95.2%. More or less similar results (84% sensiti-vity of PCR) were obtained by Wendell et al. (2002). On the contrary, the work of Gordan et al. (2001) revealed a high level of agreement between PCR and culture for detecting *T. vaginalis*.

Unlike PCR for infections diseases such as gonorrhea and *Chlamydia,* which appears to have greater sensitivity than culture methods (Gaydes et al., 1998), PCR for trichomonads does not appear to offer a diagnostic advantage and the sensitivity of PCR did not exceed that of culture. This may be due to the fact that *T. vaginalis* is much less fastidious for culture than is *Neisseria gonorrhoeae* or *Chlamydia trachomatis*. Successful culture of *T. vaginalis* requires only the multiplication of a single organism, the same as that needed for PCR. Sakru et al. (2005) recommended the use of Diamond's culture and stated that it is the most reliable and gold standard among conventional diagnostic methods in the detection of *T. vaginalis.* Moreover, he recommended subculture of the samples to increase its diagnostic efficacy.

Conclusion and Recommendations

Sensitivity of PCR for *T. vaginalis* using vaginal swab specimens did not exceed culture. Moreover, the cost of PCR highly exceeds the wet preparation and culture method. Therefore, PCR seems to be not feasible for the diagnosis of such infection. The present work is highly recommending the use of Diamond's culture in the diagnosis of trichomoniasis in women; PCR may be superior to culture for the diagnosis of *T. vaginalis* in males.

Important areas for future investigations include further studies of rates of urethral colonization with *T. vaginalis* in females and males, enhancement of the sensitivity of PCR assays including comparison and refinement of *T. vaginalis*-specific primers, and suitability of PCR for de-tection of trichomonads in male urine specimens. More-over, the degree to which inhibitors influence the determi-nation of prevalence needs to be further studied.

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