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Original Article

Molecular Diagnosis of Trichomoniasis in Negative Samples Examined by Direct Smear and Culture

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Abstract

Background: Trichomoniasis is an extremely common sexually transmitted infection (STI) worldwide and is associated with important public health problems, including amplification of HIV transmission. This disease is in forms of symptomatic and asymptomatic in women and may depend on host as well as parasite variables. Most of the studies reported from females are based on examination of vaginal secretions and urine samples by direct smear and culture in modified Diamond's media. The aim of this study was checking the samples, which were negative by direct smear and culture, with PCR technique.
Methods: The urine samples and vaginal discharge of patients attending Gynecology Clinics of Mazandaran Province, Iran with different symptoms rechecked for *Trichomonas vaginalis* by PCR technique using primers targeting a conserved region of the beta-tubulin genes of the parasite. Data were analyzed by Epi Info software program
Results: Out of 161 negative samples by direct smear and culture, seven samples (4.3%) were positive by PCR technique.
Conclusion: Diagnosis of trichomoniasis by PCR is a sensitive and specific method that could play important role to help the physicians for properly treatment and control of infection.

Keywords: *Diagnosis, PCR,* Trichomonas vaginalis

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Introduction

Trichomoniasis is an extremely common sexually transmitted infection (STI) worldwide and is associated with important public health problems, including amplification of HIV transmission. Trichomoniasis may be asymptomatic or have signs or symptoms of infection, which include a frothy, yellow green vaginal discharge with a strong odor (1, 2). In rare cases, lower abdominal pain can occur (3). The complications of this infection include low birth weight infants, preterm labour, predisposition to cervical cancer, atypical pelvic inflammatory disease and infertility. However, serious aspect of this infection is the association between *T. vaginalis* and an increase risk of transmission and acquisition of other sexually transmitted diseases including human immunodeficiency virus (4).

In spite of the limited sensitivity, direct microscopy examination of vaginal secretion and urine samples remains the most widely utilized diagnostic test for this infection. Although culture media is not economy way for diagnosis but it is the current gold standard method (5). DNA amplification technique becomes more widely used for STIs, that similar technique for trichomoniasis would be highly desirable.

Thus, a PCR technique targeting the β-tubulin genes of *T. vaginalis* was used for detection of microorganism in vaginal swab and urine samples. The targeted genes encode the amino acid sequences of beta-tubutin protein, a major component of *T. vaginalis* cytoskeleton (6). The aim of this study was to compare the molecular way of detection of *T. vaginalis* from vaginal specimen and urine samples with other methods of diagnosis.

Materials and Methods

We surveyed by questionnaire 161 female who attended Gynecology Clinics of Mazandaran Province (north of Iran) over 12-month period. The vaginal discharge and urine samples of these subjects were negative for *T. vaginalis* by direct smear and culturing in Diamond's TYIS-33 medium (7).

Samples

One sterile cotton swab for collecting vaginal discharge from posterior vaginal fornix of each patient put in sterile tube containing 1 ml normal saline and 5ml urine samples in sterile tubes were used for detection of parasite by PCR. Those patients complaining vaginal discharge and/or pruritus, dysuria, and dyspareunia were considered as symptomatic patients (Sp). Samples obtained from patients with no complain of above mentioned symptoms were considered as asymptomatic patients isolates (Asp). Specimens for PCR were processed for freezing with 2-4 hrs according to the methods described by Lawing et al. (8). Vaginal swabs were vigorously agitated in 1 ml normal saline and then centrifuged at 2000 × g for 10 min. The supernatant was removed and the pellet was resuspended in 1 ml of sterile distilled water and then frozen at -20˚C. The urine samples were centrifuged at 2000 × g for 10 min, and its pellet was suspended in 1 ml of PBS, and then frozen at -20˚C.

DNA extraction

For extraction of DNA the DNG™ –Plus (CinnaGen Inc.) solution were used. Three hundred micro liter of pre-warmed DNG™ –Plus solution by placing in 37˚C for 20 min, were added to100 µl of thawed sample, and vortexed for 15 sec. Then 300 µl of isopropanol added to the sample, kept for 30 min in -20˚C and then centrifuged at 12000 rpm for 10 min. The supernatant discarded and 1ml ethanol 75% added to the pellet, vortexed by 3-5 sec, centrifuged at 12000 rpm for 5 min (twice). Poured off the ethanol completely and dried the pellet at room

temperature. In next step, DNA pellet dissolved in 50 µl of sterile distilled water by gentle shaking and placing at 65˚C for 5 min. The wall of tube washed for dissolving of any residual pellet by softly pippeting. The unsolved material pelleted by spin for 30 sec at 12000 rpm, and used supernatant that contains purified DNA for PCR.

PCR primers

A set of primers targeting a conserved region of the β-tubulin genes of *T. vaginalis* was used to amplify a 112 bp piece of the gene. The BTUB9/2 sequences were as follows: Forward 5' CAT TGA TAA CGA AGC TCT TTA CGA T3'; and Reverse: 5' GCA TGT TGT GCC GGA CAT AAC CAT 3'.

PCR protocol

This protocol was performed according to the method described by Kazemi et al. (9). The mixture of PCR reaction had 30 µl volumes and contained 0.1mM dNTP, 1 U Taq DNA polymerase, 20 ρmole each of the forward and reverse primers, 1x PCR buffer, 1.5 mM $MgCl_2$, 0.1 µg template DNA and distilled water up to30 µl. The temperature profile consisted of initial pre incubation at 94˚C for 5 min, and then incubated for 30 sec at 94˚C (denaturation), 52˚C for 30 sec (annealing) and 72˚C for 30 sec (extention) repeated for 30 cycles and then the final incubation at 72˚C for 5 min.

Agarose gel electrophoresis

A 3% agarose gel containing DNA Stain was used for electrophoresis of each sample. Ten µl of amplified product was electrophoresed at 80 V in Tris-Borate EDTA buffer. The sizes of amplified products were assessed by comparing with commercial 100 bp weight marker (Fermentase). All the samples were checked three times.

Statistical analysis

The data analyzed by the help of Epi Info software program.

Results

In this study, 161 patients that their vaginal discharge and urine samples were negative by direct smear and cultured in TYIS-33 media, subjected for PCR examination. Results showed that seven (4.3%) patients were positive for *T. vaginalis* by PCR method. Out of all positive patients, the urine samples of four patients and vaginal discharge of six patients were positive (Table 1). Results showed that vaginal discharge was much better than urine sample in order to check for trichomoniasis. According to geographical area, although Ramsar had least number of samples but had the most positive patients, i.e. more than 50% of the patients belonged to Ramsar City.

Detection of *T. vaginalis* from vaginal discharge and urine samples of the patients with primer set of BTUB 9/2 is shown in Fig. 1. The seven positive samples are recorded as number 1-7 in figures. Each set of test contained negative and positive control as well as DNA marker. Primer set amplified the predicted 112-bp product in all seven samples. The standard strain of *T. vaginalis* also was checked beside the positive samples as shown in Fig.1. In these figures (A&B) the urine sample of patient's number 2, 5 and 6 are negative, however only the discharge of one sample (3D) was negative.

The age of the infected women was between 20 to 40 years old. According to questionnaires, out of 161 women only 31 of them had the history of abortion which 3 out of 7(43%) infected ones had the abortion history, that differences were statistically significant (P<0.5). Among the women referred to Gynecology Clinics, 84.5% were house workers and 5 out of 7 patients belonged to this group. Based on using contraceptive, three patients were using oral contraceptive, 2 of them by condom, 1 by IUD and 1 patient did not use any contraceptive device.

Table 2 shows that most of the patients due to some clinical symptoms seeking health cares. Vaginal discharge is most common symptoms among them, and 5 out of 7 positive patients complained of that. The duration of the complained symptoms in 44.7 % of the women in this study was less than one month that 50% of infected ones belonged to this group. Based on vaginal speculum examination, reports in questionnaires showed that just one of the positive subjects had normal appearance of vagina and cervix.

Table 1: Comparison of the cities according to the number of positive samples for *T. vaginalis*

City	Samples n (%)	Positive Urine samples	Positive vaginal discharge samples	Total positive n(%)
Amol	89 (55.3)	1	2	2 (28.6)
Noor	32 (19.9)	1	0	1(14.3)
MahmoodAbad	23 (14.3)	0	0	0 (0)
Ramsar	17 (10.6)	2	4	4 (57.1)
Total	161	4	6	7

Table 2: Common reported symptoms of the women attended in gynecology clinics in this study

Symptoms	Discharge	Discharge and itching	Discharge, itching and burning	Itching and dysuria	Others	Total
No. of patients	64	25	41	7	24	161
No. of positive cases	2	2	1	2	0	**7**

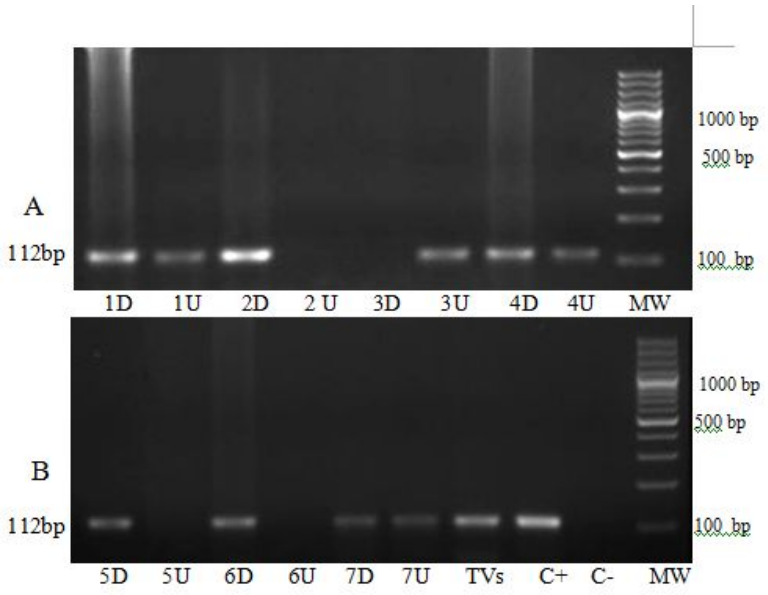

Fig.1: PCR reactions of positive samples (1-7) targeting BTUB genes in A&B. D,discharge. U,urine. MW, size marker of DNA. TVs, *Trichomonas vaginalis* standard strain. C+, positive control. C-, Negative control

Discussion

Trichomonas vaginalis is a flagellated parasite that infects urogenital tract of women. The association of increase risk of infection to HIV with trichomoniasis made the attention of researchers to study about this parasite (10). Asymptomatic disease is common in both men and women as carrier state, thus screening for disease is important. In addition, the women who are infected with *T. vaginalis* have a greater risk of suffering from complications, which consequently may cause serious problems such as infertility, cervical cancer and complications for the fetus and newborn (11). Diagnosis is usually made from wet mount microscopy and direct visualization, which is economy but not sensitive (12). DNA amplification techniques with good sensitivity are not yet approved for diagnostic purposes. In areas where diagnostic methods are limited, diagnosis of trichomoniasis is usually as part of clinical symptoms, yellow or greenish vaginal discharge, itching and burning sensation for women and urethral discharge for men. Different studies have been performed in Iran by various methods. The infection of trichomoniasis by using clinical parameters reported 26% between women referred to medical center of Shahroud City (13), however by using culture media no growth were seen and by direct smear only 0.3% were positive. Thus, clinical diagnosis by gynecology physician has low level of specificity. Kazemi et al. (9) reported 48% positivity out of 155 women suffering from vaginitis by PCR, which seems very high in our society. In one study by Rezaeian et al ,(14) reported 3.2% in vaginal discharge samples from women attended in STD clinic of Mirzakuchak Khan Hospital by wet-mount and culture methods. The vaginitis due to trichomoniasis in pregnant women causes premature rupture of membrane (8). Jamali et al. (15) studied the prevalence of trichomoniasis among women attending the health care centers in Tabriz. Out of 100 randomly selected negative samples, they found 3% positive patients by using PCR method that is near to our result. As previously reported (10,16), this study also showed that urine samples were not good samples for detection of *T. vaginalis* as compare to vaginal discharge even by PCR.

In conclusion, the results of this study show that PCR technique as a sensitive method can be used for diagnosis of patients. Sexually transmitted infections are very important as public health problems and a serious danger for the family because mostly are asymptomatic. Molecular study of many STIs such as *C. trachomatis* and *N. gonorrhoeae* infections is currently in use in many laboratories by PCR technique in Iran. Because the prescription of physicians in our community is mostly based on signs/symptoms of the patients refer to the health care centers so drug resistance, drug fee and drug side effects are main problems. So it is suggested that treatment of the patients should be performed after a definite diagnosis, Thus *Trichomonas* PCR also could easily be down as other diagnostic procedures in laboratories. In addition, a useful program is needed for STIs control and community health promotion.

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