

Identification of *Trichomonas Vaginalis* Genotypes Using by Actin Gene and Molecular Based Methods in Southwest of Iran

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Abstract

Background: *Trichomonas vaginalis* (*T. vaginalis*) is a sexually transmitted protozoan parasite and the causative agent of trichomoniasis. The genetic characterization of *T. vaginalis* isolates shows notable genetic variation in this parasite. In the present study, we aimed to identify the *T. vaginalis* genotypes based on analyzing of actin gene in women specimens referred to health centers of Ilam city, southwest Iran.

Methods: A total of 1765 female samples were collected from gynecology clinics in the city of Ilam. DNA was extracted from positive samples and nested polymerase chain reaction (Nested PCR) was used to amplify the actin gene. Then, partial sequencing and genotyping of the actin gene was performed. A phylogenetic tree was drawn using the detected genotypes of *T. vaginalis* and reference sequences.

Results: Twenty-one of the 1765 urine and vaginal samples were positive for *T. vaginalis*. All infected individuals were married and their age in years was between 25 to 34. Further, the majority of infected women had cervical lesions, patchy erythema, and white color discharge. According to sequencing analysis, the isolates were identified as genotype G (n= 8) and genotype E (n= 2).

Conclusions: From the collected samples, we were able to distinguish at least two genotypes (G and E) of *T. vaginalis*. However, lesser is known about these genotypes in the city of Ilam. Further studies with a higher number of isolates should be performed in order to understand the implications of these results in this region.

Keywords: Actin gene, Genotypes, Ilam, Iran, *Trichomonas vaginalis*.

Introduction

Trichomonas vaginalis (*T. vaginalis*) is a flagellated protozoan or parasite that causes Trichomoniasis and exists primarily in the human urogenital tract (1). Trichomoniasis is a common sexually transmitted non-viral parasitic disease seen worldwide (2). The prevalence of *T. vaginalis* can vary based on

geographical location, sex, individual health status and sociocultural factors (3). Recent reports of different countries estimate the prevalence of *T. vaginalis* to be between 3.9 and 24.6% (4). Furthermore, *T. vaginalis* is ten times more prevalent in women than men, for example, in America and Africa, approximately 20% of women and 2% of men had *T. vaginalis* (5). In Iran, the prevalence

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was less than 1% in northern Iran and 39% in Tehran (6).

Due to antigenic variation and genetic diversity, different isolates of *T. vaginalis* may confer varying degrees of pathogenicity ranging from asymptomatic to severe vaginitis (7). Approximately 50% of individuals with trichomoniasis are asymptomatic. However, in women, *T. vaginalis* can cause vaginitis, abnormal vaginal discharge, vulvar irritation, inflammation, abnormal pregnancy outcomes, and cervical neoplasia, whereas in men, symptoms include prostatitis dysuria, inflammation, urethritis, epididymitis and reversible infertility (8, 9).

Given the advances in molecular genetics and biotechnology, various *T. vaginalis* genotypes have been documented and have provided various clinical manifestations associated with this disease (10). Though many studies have extensively evaluated the genetic diversity of *T. vaginalis* in certain countries, these investigations should be extended to regions where this data is less characterized (11, 12).

In the past, researchers have used several tools to study the genetic properties of *T. vaginalis*, including Restriction Fragment Length Polymorphism (RFLP) (13), internal transcribed spacer analysis (ITS) (14), quantitative real-time PCR (15) and nucleotide sequencing (PCR sequencing) (16).

Interestingly, the actin gene was found to induce morphological changes and play a major role in the pathogenesis of *T. vaginalis* (17) making it an ideal candidate for targeted investigations on the molecular level, and genotyping (including PCR sequencing) (18).

Considering the increase in prevalence and severe health consequences associated with a *T. vaginalis* infection, the objective of this study was to determine the genotypes of *T. vaginalis* that circulates amongst women in Ilam city using PCR sequencing of the actin gene. Finally, this study could have real-life implications in epidemiological research, public health, and parasitic management.

Materials and Methods

Ethical considerations

In this investigation, all procedures including the collection of vaginal secretions and urine samples were approved by the Ethics Committee of Ilam University of Medical Sciences (number: 2015-124).

Sample collection

This cross-sectional study was conducted on 1765 women that were referred to gynecology clinics across the city of Ilam (southwest Iran) between July 2018 and December 2019. The demographic and clinical data of patients were collected using a questionnaire. Vaginal secretions were sampled individually from the posterior vaginal fornix using a sterile cotton swab (3). To detect trophozoites of *T. vaginalis*, wet mount smears of samples were prepared using normal saline and surveyed immediately under a light microscope. Moreover, 25 ml of urine was collected for each patient in sterile urine containers and transported to the parasitology laboratory at the Ilam University of Medical Sciences. The samples were centrifuged at 2500 rpm for 3 min and the pellet were used to prepare glass slides, Giemsa-staining and finally, microscopic investigation. For molecular examination, the urine sediment was washed three times with 0.5 ml of phosphate buffered saline (PBS).

DNA extraction and PCR amplification assay

Total DNA extraction for each sample was performed using the DNG-plus™ kit according to the manufacturer's guidelines (Cinna Gene, Iran). The concentration and quality of extracted DNA was measured using a nanodrop instrument (Nano Drop Technologies, Wilmington, DE, USA). An 1100 bp fragment of actin gene was amplified by Nested PCR. External primer included Tv8s (5'-TCT GGA ATG GCT GAA GAA GAC G -3 ') and Tv9R (5'- CAG GGT ACA

TCG TAT TGG TC-3) and internal primers: Tv10s (5' -CAG ACA CTC GTT ATC G-3') and Tv11R (5' - CGG TGA ACG ATG GAT G-3') (19). In the first amplification round, the 25 µl reaction mixture contained 12.5 µl Master mix RED (Ampliqon), 2 µl template DNA, 10 pmol of each forward and reverse external primers (2 µl), and 8.5 µl double-distilled water. The cycling conditions were 95 °C for 5 min, 30 cycles of 95 °C for 30 sec, 57 °C for 45 sec, 72 °C for 45 sec and a final extension of 72 °C for 5 min.

Nested PCR was performed using 1 µl first-round PCR product as template DNA, 20 pmol of the internal primers (2 µl) and 25 µl Master mix in a 25 µl reaction mixture. The cycling conditions were 95 °C for 5 min, 35 cycles of 95 °C for 30 sec, 60 °C for 45 sec, 72 °C for 60 sec and a final extension of 72 °C for 10 min. The PCR products were analyzed by electrophoresis using 1.5% tris-borate-ethylenediaminetetraacetic acid (TBE) agarose gel containing SYBR Safe stain (Invitrogen, California, USA) and were visualized using a gel document system (Bio-Rad, USA).

Sequencing, phylogenetic analysis and genotyping

Ten samples of successful PCR products of second-round amplification (Nested PCR) were sequenced by the Sanger method in both directions using an ABI Prism™ 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) from the Macrogen Company (Seoul, South Korea). The sequence analysis was performed using the Bioedit Sequence Alignment Editor 7.1.3.0. Genotype identification was accomplished by conducting a nucleotide similarity search in Basic Local Alignment Search Tool (BLAST) to search for the most similar reference sequences to the *T. vaginalis* sequence. Strains of *T. vaginalis* were recognized at the species level, based on ≥99% identity with the reference sequences, deposited in the Gene Bank under the accession numbers MW355756-MW355765. Phylogenetic analysis was performed using the MEGA software (version 7). The phylogenetic tree, of obtained *Trichomonas* genotypes from the present study

together with sequences from Gene Bank, was constructed using the Maximum Likelihood method and Kimura 2-parameter model with bootstrap values (determined by 1000 replicates).

Results

Of the 1765 evaluated samples, 21 (1.18%) were positive for *T. vaginalis* infection upon microscopic and molecular examination. Both morphological and molecular analyses indicated that 2.62% (13/495) of secretions of posterior vaginal fornix samples and 0.62% (8/1270) of urine specimen samples were positive for *T. vaginalis*. The age of the infected women with *T. vaginalis* ranged from 15 to 50, however, the infection was more common in women between the ages of 25 and 34 (47.6%). Next, we determined that the most common clinical symptom was cervical lesions (28.6%), followed by patchy erythema (23.8%) and vaginal discharge (19%). Of the 21 infected women, one case (4.76%) had a history of taking metronidazole, one case (4.76%) had a history of taking cefixime, and two cases (9.52%) had a history of taking fluconazole. Also, no history of treatment was recorded in most of the patients (81%). Abortion and preterm birth were reported in 9.52% and 4.76% of patients, respectively. About 62% of patients consumed contraceptive pills, 19% of patients used IUD (Intrauterine Device) and the remaining 19% had no history of either forms of birth control (Table 1). Notably, all patients were married. The difference between trichomoniasis and age groups, clinical symptoms, history of abortion and prevention of pregnancy were not statistically significant (p value> 0.5) (Table 2).

We successfully detected *T. vaginalis* in 21 urine and vaginal swab samples using Nested PCR. A single 1100 bp product of the actin gene was amplified in all 21 samples, which was in agreement with the product size of *T. vaginalis* (Figure 1). Partial nucleotide sequences of *T. vaginalis* isolates using BLAST analysis with reference sequences in GenBank database showed that eight isolates

belonged to genotype G, and two isolates belonged to genotype E (Figure 2). According to the phylogenetic tree, *Tritrichomonas suis*

was considered the out-group branch (GenBank Accession no. AB4698092).

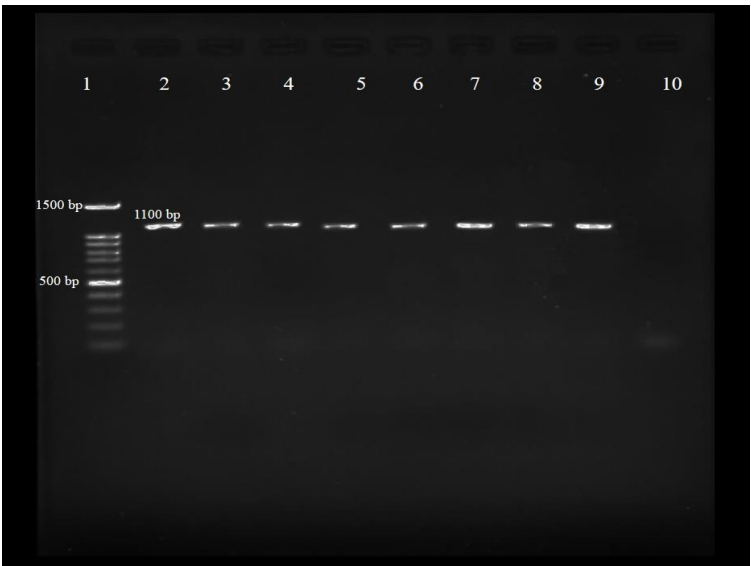


Fig. 1. Nested-PCR result of *T. vaginalis* isolates from women patients of Ilam city using actin gene. Line 1: 100 bp ladder marker, Lines 2–8: Sample of patients (current study), Line 9: Positive control and Line 10: Negative control.

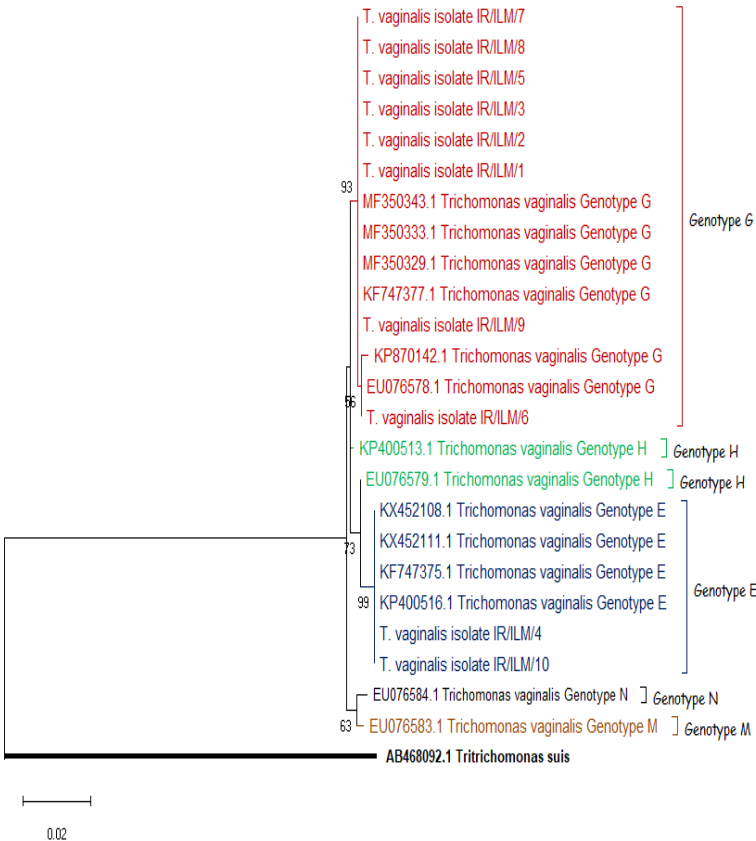


Fig. 2. Phylogenetic tree of actin gene sequences of *T. vaginalis* isolates constructed by the Maximum Likelihood method and Kimura 2-parameter model using Mega software (version 7). Isolate or strain names are as provided in Genbank as available, followed by accession numbers in parentheses. Sequences generated in this study named as *T. vaginalis* IR/ILM. *Trichomonas suis* (accession number AB4698092) used as the out-group. Bootstrap values (%) are indicated at the internal nodes (1000 replicates). Bootstrap values of less than 50% are not shown.

Table 1. Data regarding the frequency of *Trichomonas vaginalis* isolated from positive patients in Ilam city, southwest Iran.

Demographic data		Positive No. (%)
Age	< 15	0 (0)
	15-24	1 (4.76)
	25-34	10 (47.6)
	35-44	7 (33.33)
	44-49	2 (9.52)
	≥ 50	1 (4.76)
Clinical symptoms	Gray color discharge	1 (4.76)
	Erythema and vaginal edema	1 (4.76)
	Patchy erythema	5 (23.8)
	cervical lesions	6 (28.6)
	White color discharge	4(19)
	No symptoms	4 (19)
Drug consumption	Yes	4 (19)
	No	17 (81)
Pregnancy outcome	Abortion	2 (9.52)
	Death	0 (0)
	Preterm infant birth	1 (4.76)
	Normal infant birth	17 (81)
	No pregnancy	1 (4.76)
Prevention of pregnancy	Condom	0 (0)
	Consumption of contraceptive pills	13 (62)
	IUD	4 (19)
	TL	0 (0)
	No Prevention	4 (19)

Table 2. Data regarding the frequency of *Trichomonas vaginalis* isolated from women specimens referred to health centers of Ilam city, southwest Iran.

Demographic data	Total No.	Positive No.	%Positive	Chi square	p value
Age					
< 15	10	0	0	4.56	0.47
15-24	249	1	0.4		
25-34	593	10	1.69		
35-44	445	7	1.57		
44-49	363	2	0.55		
≥ 50	105	1	0.9		
Clinical symptoms					
Gray color discharge	184	1	0.54	5.11	0.41
Erythema and vaginal edema	310	1	0.32		
Patchy erythema	219	5	12.28		
cervical lesions	442	6	1.36		
White color discharge	330	4	1.21		
No symptoms	280	4	1.43		
Drug consumption					
Yes	144	4	2.78	3.36	0.06
No	1621	17	1.05		
Pregnancy outcome					
Abortion	105	2	1.9	7.52	0.11
Death	7	0	0		
Preterm infant birth	73	1	1.37		
Normal infant birth	1015	17	1.67		
No pregnancy	565	1	0.18		
Prevention of pregnancy					
Condom	207	0	0	5.06	0.28
Consumption of contraceptive pills	842	13	1.54		
IUD	256	4	1.56		
TL	113	0	0		
No Prevention	347	4	1.15		

Discussion

Trichomoniasis is considered one of the most prevalent non-viral sexually transmitted infections worldwide. Most infections are asymptomatic and highly communicable between sexual partners (20, 21).

In accordance with our findings, similar prevalence rates were observed in other regions of Iran. We found that 21 (1.18%) out of 1765 specimens were infected with *T. vaginalis*. A study that analyzed 1353 pap smears in women from Ahvaz city in southwest Iran, also reported similar findings where 1.4% of patients were infected with *T. vaginalis* (22). In Mazandaran, a province in northern Iran, PCR results showed that 0.48% of pregnant women referred to health centers were infected with *T. vaginalis* (23). The frequency distribution of *T. vaginalis* infection in Iran also varies (0.5% to about 39%) amongst different age groups of women and geographical location (24).

The incidence of *T. vaginalis* infection depends on several factors including socioeconomic status, poverty level, level of education and hygiene during sexual encounters, having multiple sexual partners, and high-risk sexual behaviors (25). Furthermore, selecting appropriate methods to evaluate the spread of this disease is important. One of the most sensitive diagnostic techniques for clinical samples taken from patients with trichomoniasis is Nested PCR (26).

We found the infection to be prevalent in women between the ages of 25 and 34 (47.6%). This may be due to increased sexual contact or changes in the genital tract due to predisposing factors such as increasing age (27).

As demonstrated in our results, cervical lesions were the most common clinical symptom followed by patchy erythema and vaginal discharge. Similarly, a previous study confirmed that there is a significantly higher risk of developing cervical lesions in women exposed to *T. vaginalis* compared to those who were not exposed (28).

Trichomoniasis is a ping-pong disease. Since all 21 patients were married, their sexual

partners may also be infected, and the prevalence of *T. vaginalis* should, therefore, be examined in these individuals (29). Subjects in the study who used condoms during sexual intercourse did not confer a *T. vaginalis* infection. Conversely, *T. vaginalis* is usually spread by having sex without a condom. Therefore, to prevent disease transmission, the use of condoms is highly recommended.

Drug resistance is a major concern for *T. vaginalis* infections and threatens current prevention treatments (30). Of the 21 positive trichomonas infections, four cases had a consumption history of metronidazole, cefixime, and fluconazole. With this in mind, drug resistance may play a role in the isolated samples, however, drug resistance and pathogenic aspects of *T. vaginalis* still remains to be fully elucidated. One factor that may contribute to *T. vaginalis* drug resistance is genetic diversity (30).

Genetic investigations can shed light on drug resistance, pathobiology, etiology and other features of epidemiology for *T. vaginalis* and the identification of critical genes (31). Various techniques have been used to identify and detect *T. vaginalis* including monoclonal antibody binding (32), antigenic characterization (33), PCR, random amplified polymorphic DNA (RAPD) (34), restriction fragment length polymorphisms (RFLP), Nested polymerase chain reaction (Nested PCR), and real-time PCR (35). The above-mentioned techniques have been described as more sensitive compared to direct and culture methods (36). PCR is the preferred method in the diagnosis of trichomoniasis. Moreover, the actin gene can be used as a unique indicator owing to its structural role in organisms (10). Following the amplification of the actin gene, we found the most common genotypes to be G and E. This is consistent with the results of Hezarjaribi et al (23) who also used the actin gene to show that the genotypes G, E, and I were the predominant genotypes in women and men in northern Iran. Lastly, using PCR-RFLP patterns, sequencing, and phylogenetic

analyses, Spotin et al (37) found the most predominant genotypes in asymptomatic patients of Tabriz in northwest Iran to be G (73.4%) and E (26.6%).

In 2015, the five predominant actin genotypes exposed using PCR-RFLP in Kenya were E, N, G, I and P (38). Comparatively, the results of this study differed from Matini et al (39), where the genotype A was the most prevalent in women referred to gynecology clinics in western Iran. The genome of *T. vaginalis* is 160 Mb consisting of approximately 60,000 protein-coding genes, where repeated and transposable nucleotide of the genome may explain the genetic diversity of *T. vaginalis* (40). The consequence of all investigations with regards to genotyping and phylogenetic examination of *T. vaginalis* shows that it is genetically diverse.

The prevalence of *T. vaginalis* infection

was low in the city of Ilam city in southwest Iran and based on molecular findings, the main genotypes were G and E. Here, we demonstrate that there is genotypic diversity in *T. vaginalis* clinical samples in women within this region. These results suggest that there may be genetic polymorphisms in the open reading frame (ORF) of the actin gene of *T. vaginalis*, thereby, making the actin gene a key genetic target for molecular epidemiology and genotyping of *T. vaginalis*.

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The authors declare that there is no conflict of interests.

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