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# Genotyping of *Trichomonas vaginalis* isolates in Iran by using single stranded conformational polymorphism-PCR technique and internal transcribed spacer regions

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**Abstract.** Infection with *Trichomonas vaginalis*, the causative agent of human urogenital infection, is the most prevalent nonviral sexually transmitted disease worldwide. In spite of the high prevalence and medical importance of trichomoniasis, there is little knowledge about genetic epidemiology and genetic characterisation of this parasite. For this purpose, a Single Stranded Conformation Polymorphism-PCR (SSCP-PCR) typing method was conducted for Iranian *T. vaginalis* isolates using 5.8s ribosomal gene (rRNA gene) and the flanking internal transcribed spacer (ITS) regions. Nine hundred and fifty vaginal swab samples were examined in which 50 (5.3%) samples were parasitologically positive and used for molecular identification based on SSCP-PCR and nucleotide sequence analyses. Results of the SSCP analysis showed two distinct reproducible banding patterns (I, II) which were confirmed by nucleotide sequence analysis in the ITS1 regions. Frequencies of the SSCP banding patterns I and II were 84% (42/50) and 16% (8/50), respectively. In conclusion, SSCP-PCR analysis provided a reliable and sensitive method for strain genotyping of *T. vaginalis* based on the ITS1/5.8s/ITS2 region. This finding may help us gain more information about correlation between genetic properties and biological features of this parasite.

## INTRODUCTION

*Trichomonas vaginalis* is a flagellated protozoan that causes human trichomoniasis, a sexually transmitted disease (STD) (Schwebke & Burgess, 2004). Trichomoniasis is associated with clinically adverse pregnancy outcomes, pelvic inflammatory disease, infertility, incidence of cervical neoplasia, *Mycoplasma hominis* infection, as well as increased risk of Human Immunodeficiency Virus (HIV) transmission (Zhang *et al.*, 1995; Sorvillo *et al.*, 2001; Soper, 2004; Dessi *et al.*, 2005).

Fairly comprehensive investigations have been conducted on different aspects of trichomoniasis such as epidemiology, diagnosis and clinical features, *however, little information is available about genetic diversity of T. vaginalis*. A number of investigations have been performed in this field and yielded variable results due to the use of different genetic markers as well as different molecular techniques such as

antigen characterization, isoenzyme profile analysis, random amplified polymorphic DNA (RAPD), genomic DNA hybridization, ribosomal gene sequencing, restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE) and microsatellite technique (Krieger *et al*., 1985; Proctor *et al*., 1988; Vanacova *et al*., 1997; Ryu *et al*., 1998; Snipes *et al*., 2000; Stiles *et al*., 2000; Hampl *et al*., 2001; Kaul *et al*., 2004; Simões-Barbosa *et al*., 2005; Upcroft *et al*., 2006; Crucitti *et al*., 2008; Meade *et al*., 2009; Conrad *et al*., 2011).

Results of the above mentioned studies are unsatisfactory and none of them has been widely used for typing and classification of *T. vaginalis*. Therefore, a reliable and relatively simple method is required for investigating the genetic characteristics and molecular typing of *T. vaginalis strains*.

Ribosomal genes (rRNA genes), as a genetic marker, one generally used to study genetic diversity in organisms. The rRNA gene family in eukaryotic organisms consists of tandemly repetitive units of the 18S, 5.8S, and 28S structural genes. Two noncoding internal transcribed spacer (ITS) regions are located on either side of the 5.8S rRNA gene, and are known as ITS1 and ITS2 (Commar *et al*., 2007). The ITS regions are much less conserved than rRNA genes. Therefore, they are appropriate regions for studies of intraspecies variations in organisms (Hillis & Dixon, 1991).

*SSCP-PCR* is a simple and reliable tool to identify mutations by the effect of a single nucleotide change on DNA secondary structure. The purpose of this study was to develop a simple and efficient method for strain typing of Iranian *T. vaginalis* isolates based on SSCP-PCR analysis of the ITS1/5.8s/ ITS2 region.

## MATERIALS AND METHODS

**T. vaginalis isolates and DNA extraction**

The present study was conducted on 950 women referred to gynecology clinics, in two provinces of Iran; Hamadan in the west and Tehran in the north-central parts, from November 2010 to July 2011. After obtaining informed consent, two vaginal swab specimens were collected from each participant. The swab samples were subjected to wet mount examination and culture techniques by using light microscope and Dorset medium (Bashirybod, 1988), respectively. Axenisation of isolates was achieved after cultivation and several subcultures in TYI-S-33 (Diamond's) medium (Clark & Diamond, 2002). Total genomic DNA was extracted from all isolates *using phenol/ chloroform/isoamyl alcohol* method according to published protocol (Rezaie *et al*., 2000).

**PCR amplification and SSCP analysis**

A pair *of oligonucleotide PCR primers* was designed according to the conserved ITS1/ 5.8s/ITS2 region of *T. vaginalis* (GenBank Accession No. TVU86613): ITS-S (5' CGG TAG GTG AAC CTG CCG TTG G 3') and ITS-As (5' AGT TCA GCG GGT CTT CCT GCG 3'). Each PCR reaction mixture contained 10 µl of 10x PCR amplification buffer (Roche), 1 µl template DNA, 20 pmol of sense and antisense primers, 0.2 mM dNTP mix, and 1 unit of *Taq* DNA polymerase (Advance Biotechnologies, UK). Sterile distilled water was added to the reaction mixture to make up a final volume of 50 µl. The *PCR amplification was performed* (PeqLab Biotechnologie GmbH, Germany) according to the following profile: *initial denaturation* at 94°C, 5 min, followed by 35 cycles in series of denaturation at 94°C, 30 sec, annealing at 65°C, 30 sec, extension at 72°C, 45 sec, and one final extension step at 72°C for 10 min. The expected single band was confirmed by electrophoresis in 2% (w/v) agarose gel in 1X TBE buffer containing ethidium bromide (0.5 µg/ml) and visualized under a UV transilluminator (UVP/Upland, USA).

Five µl of PCR product was mixed with 10 µl SSCP loading buffer including, 95% (v/v) deionized formamide, 20 mM EDTA (PH 8.0), 10 mM NaOH, 0.05% (w/v) xylene cyanol FF, and 0.05% (w/v) bromophenol blue. The reaction mixture was incubated at 94°C for 10 min and snap chilled in a bath of crushed ice to prevent re-annealing of the separated *strands*. The total sample volume was subjected to *vertical slab* polyacrylamide gel

electrophoresis. Optimal band resolution was achieved empirically as follows: electrophoresis in a 8% polyacrylamide gel (29:1 ratio of acrylamide to bis-acrylamide) containing 5% glycerol, using 0.5X TBE running buffer, for 20 hours at room temperature and 160 V. After electrophoresis, the gel was stained by silver staining method. The gel was fixed in 10% ethanol solution supplemented with 0.5% acetic acid for 8 min, incubated in 0.1% (w/v) silver nitrate solution for 15 min, rinsed twice with distilled water, developed in 1.5% sodium hydroxide solution supplemented with 0.4% formaldehyde, and then in 7.5% sodium carbonate solution for 10 min. Finally, the gel was rinsed with distilled water, dried and placed between two sheets of cellophane membrane and photographed for permanent record (Robinson, 2005).

**Sequencing and Statistical analysis**

Representative PCR products that demonstrated different electrophoretic banding patterns in the SSCP analysis were chosen and subjected to direct sequencing. PCR product sequencing was carried out using the Applied Biosystems Automated *3730xl DNA Analyzer* (Bioneer Inc., Korea) with the same primers as those utilized for the initial PCR amplification. The 5' and 3' end of the ITS1/5.8s/ITS2 nucleotides were defined by comparison with previous sequence data GenBank (Accession Nos. JN007004, L29561, TVU86613) with the help of the BLASTn program (http://www.ncbi.nlm.nih.gov/BLASTn). Sequence editing and multiple sequence alignments were then performed by Chromas software, version 2.33, (http:// www.technelysium.com.au/chromas.html) and MultAlin program (Corpet 1988).

Statistical analysis was carried out using SPSS statistical software (version 13.5). Fisher's exact test was used to determine the relationship between frequency distribution of the obtained SSCP banding patterns and their geographic origin. A P-value of less than 0.05 was considered as statistically significant.

## RESULTS

A total of 950 vaginal specimens were isolated from patients in two provinces of Iran, Hamadan and Tehran. The isolates were tested for the presence of *T. vaginalis* by culture and wet mount methods. Fifty samples of *T. vaginalis* were identified, among which 43 isolates were from Hamadan and 7 from Tehran. *Trichomonas vaginalis* was detected by culture in 5.3% of the isolates (50/950), however, wet mount method could detect the positive isolates in 4.2% of cases (40/950). Therefore, sensitivity of wet mount method in this study was estimated to be 80% for diagnosis of *T. vaginalis versus Dorset's culture*. Six hundred and ninety (72.6%) of the participants were symptomatic. The most frequent symptom encountered was vaginal discharge (506/690). However, some of the clinical manifestations of trichomoniasis are common in other sexually transmitted diseases (STDs).

The PCR amplification of the ITS1/5.8s/ITS2 region and flanking sequences resulted in the expected single product of 362 bp in all isolates (Figure 1). Agarose gel electrophoresis showed no variation in the length of all 50 amplicons. SSCP analysis of the amplicons yielded two distinct, reproducible banding patterns (I, II) (Figure 2). Each pattern showed three single stranded bands located at different positions. The frequencies of the two banding patterns as according to geographic areas in Iran are presented in Table 1. Pattern I (42/50) has a higher frequency than pattern II (8/50). None of the isolates displayed mixed SSCP pattern. No significant relationship was observed between the percentage of the patterns in the two provinces of Iran (P=0.31).

Three representative amplicons of each banding pattern were selected for sequencing. Nucleotide sequences of these isolates revealed 96-100% similarity with other available sequences of *T. vaginalis* in the GenBank Database. The obtained sequences of the ITS1/5.8s/ITS2 region of these six *T. vaginalis* isolates were submitted to GenBank database under Accession Numbers JQ768330-JQ768335.

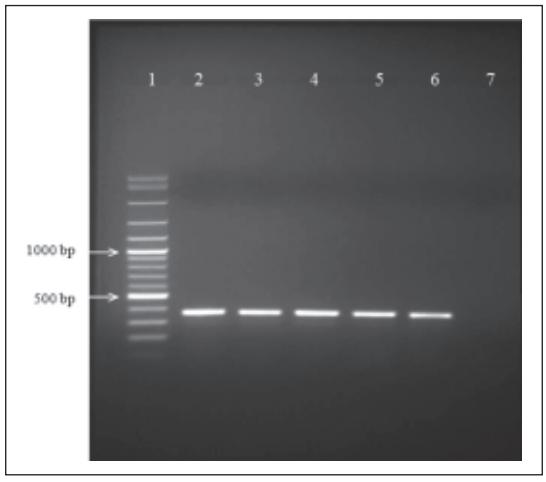


Figure 1. Agarose gel electrophoresis of PCR product amplification (362 bp) from *T. vaginalis* isolates. Lane 1: DNA marker (100 bp), Lane 2-6: some of the Iranian *T. vaginalis* isolates and Lane 7: control

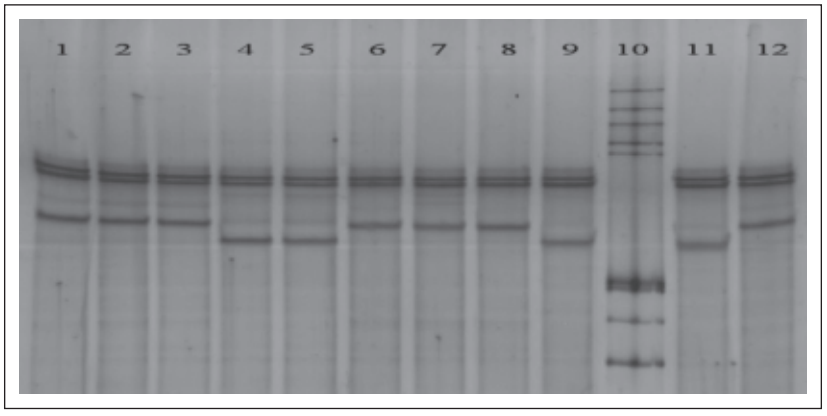


Figure 2. Representative SSCP analysis of the amplified ITS1/5.8s/ITS2 region of *T. vaginalis* isolates. Polyacrylamide gel electrophoresis shows *mobility shift of* single-stranded DNA, leading to two types of SSCP banding patterns (I, II). Lane 10: DNA marker (100 bp); Lanes 1-3, 6-8 and 12 represent SSCP banding pattern I; Lanes 4-5, 9 and 11 represent SSCP banding pattern II

Table 1. Frequency of the SSCP banding patterns of *Trichomonas vaginalis* isolates from two provinces of Iran

| SSCP banding patterns type | Frequencies (%) | | |
|---|---|---|---|
| | Hamadan | Tehran | Total |
| I | 37 (86) | 5 (71.4) | 42 (84) |
| II | 6 (14) | 2 (28.6) | 8 (16) |
| Total | 43 (100) | 7 (100) | 50 (100) |

No statistical correlation was observed between the frequency distribution of the SSCP pattern and their geographic origins (P=0.31)

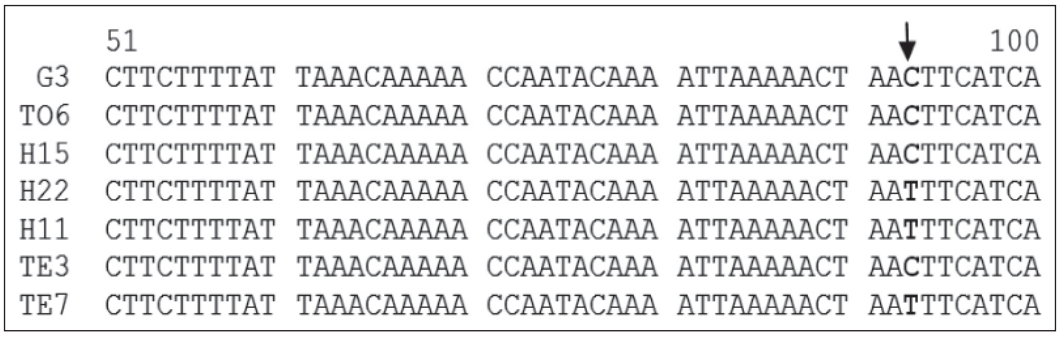


Figure 3. Partial nucleotide sequence alignment of the ITS1 region in the two sequence types of Iranian *T. vaginalis* isolates, compared with *T. vaginalis* reference strain (G3 strain, GenBank Accession No. JN007004). Difference in nucleotide sequences revealed in sequence type I: (TO6, H15 and TE3) as well as sequence type II: (H22, H11 and TE7) is pointed by arrow

Two different types of sequence were observed within the six mentioned nucleotide sequences; sequence type I (3/6) and II (3/6) (Figure 3). One point mutation is observed when compared with the reference sequence (G3 strain). The single point mutation was found in sequence type II at nucleotide position 66 (C/T) of the ITS1 region.

## DISCUSSION

Variation in phenotypic behavior of *T. vaginalis* such as pathogenicity, metronidazole sensitivity and outcome of infection has been previously reported. It is unclear that different manifestations of trichomoniasis are due to strain variation or host factors. Genetic characterization and strain typing are useful to answer these questions. To address this issue, a number of molecular approaches has been used to clarify relationship between genetic as well as phenotypic differences of *T. vaginalis*.

The RAPD technique was first employed for strain typing of *T. vaginalis* by researchers (Vancova *et al.*, 1997). Their research findings revealed *statistically significant* association between genetic relationships and metronidazole resistance in *T. vaginalis*. Snipes *et al.* (2000) demonstrated the relationships between metronidazole resistance and ITS1 C66T mutation. Besides, a genetic marker of 490 bp was exclusively detected in symptomatic patient populations by Rojas *et al.* (2004). RFLP technique has been also used in the genetic characterization of *T. vaginalis* (Stiles *et al.*, 2000; Simões-Barbosa *et al.*, 2005; Crucitti *et al.*, 2008; Meade *et al.*, 2009).

Application of microsatellite marker analysis has been considered recently as a molecular typing system for *T. vaginalis*. This method, like other methods, has shown genetic diversity in *T. vaginalis* (Conrad *et al.*, 2011).

PCR coupled with SSCP provides a sensitive and cost effective method for investigating genetic polymorphism in eukaryotes and prokaryotes (Gasser, 1997). In the present study, the SSCP-PCR analysis represented a suitable tool for strain typing of *T. vaginalis*. This technique was able to identify two different *T. vaginalis* strain types, according to distinct SSCP banding patterns (I, II). These two patterns were then confirmed by sequence analysis and yielded two distinct nucleotide sequences, type I and II. Sequence of type I, related to the SSCP pattern I, displayed 100% similarity with the reference sequence (G3 strain, wild type). Sequence of type II, related to the SSCP pattern II, had single point mutation C66T which was first reported as a mutant type by Snipes *et al.* (2000). Results of our study revealed that majority of our isolates (84%, 42/50) belong to the SSCP pattern I. Snipes *et al.* (2000) sequenced the ITS region of 109 *T. vaginalis* isolates. Majority of the sequences (85%, 93/109) were identical to those

previously reported by Katiyar *et al.* (1995) and identified as wild type. However, the remaining isolates (15%, 16/109) displayed a point mutation (C/T substitution) at nucleotide position 66 of the ITS1 region which is classified as mutant type.

In another study, Xiao *et al.* (2006) found two types of sequences and mentioned them as wild (82%, 23/28) and mutant (14%, 4/28) types. Furthermore, strain typing revealed 6 sequence types (H1- H6) in the Philippines (Rivera *et al.*, 2009). The H1 sequence type, with prevalence rates of approximately 81% (46/57), was identical to sequence type I *(wild-type)* obtained in our study. BLAST search determined that sequence type H2, with prevalence rates of approximately 12% (7/57), is identical with sequence type II in the present study.

All reported mutations in the ITS1/5.8s/ ITS2 region of *T. vaginalis* were located at the ITS region which is much less conserved than the functional rRNA gene. The C66T mutation in ITS1 region observed in this and previous studies indicates that it is not a random mutation. In this study, correlation between clinical manifestations and genetic variation was not investigated. Future studies can be designed to determine relationship between genetic characterization and biological features.

In conclusion, this study has provided a sensitive but simple method for molecular strain typing of *T. vaginalis* based on SSCP-PCR analysis of the ITS region. *However*, future studies are required to demonstrate the application of these sequence types as *genetic markers* in molecular epidemiology and genetic studies of *T. vaginalis*.

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