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Molecular Identification and DNA Sequencing of *Trichomonas vaginalis* Strains from Agean region of Turkey

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Abstract. The aim of this study is to compare the presence of *Trichomonas vaginalis* (*T. vaginalis*) in symptomatic and asymptomatic women through microscopic examination, culture in Trypticase-Yeast Maltose (TYM) medium and PCR methods. In addition, *T. vaginalis* strains were analysed for genotyping with 18S rRNA-DNA and phylogenetic analysis. Axenized strains of *T. vaginalis* isolated from urine culture samples taken from symptomatic and asymptomatic women with clinical signs. Molecular characterization of the isolated strains of *T. vaginalis* was performed by using PCR. To evaluate molecular diagnosis and genotypic identification of *T. vaginalis* strains, 14 samples were analysed. Of the 14 samples, *T. vaginalis* was positive in 14 samples by microscopy, 6 in culture(TYM medium) and 14 by PCR, respectively. Although the sample size is very small, PCR was shown to be high sensitivity and specificity, and seems to be a promising diagnostic tool. 18S rRNA-DNA PCR results also confirmed with real time PCR method. In conclusion, it is considered that two strains of *T. vaginalis* isolated from samples, 5-TV1G and 13-TV1G, are subtypes of *T. vaginalis* as a result of 18S rRNA-DNA sequencing analysis. To best of our knowledge this is the first analysis of phylogenetic positions on *T. vaginalis* from Turkey.

INTRODUCTION

Trichomonas vaginalis (*T. vaginalis*) infection is the most prevalent sexually transmitted disease worldwide. It is a protozoan parasite that afflicts an estimated 180 million women per year worldwide. Symptomatic women with trichomoniasis usually complain of vaginal discharge, vulvovaginal soreness, itching and/or irritation (Uneke *et al*., 2007). *T. vaginalis* can survive in urine for several hours. Given the growing importance of *T. vaginalis*, information on the viability of this organism in diagnostic specimens is important, because current diagnostic methods rely on the continued viability of the organism (Shafir & Sorvillo, 2006).

In addition to reproductive tract symptoms, infection with *T. vaginalis* is increasingly being recognized as having an association with reproductive complications including premature rupture of membranes, premature birth, low birth weight, and infections occurring after abortion and caesarean delivery. It has an important role as a risk factor for the transmission of the human immunodeficiency virus. As with

other sexually transmitted diseases, symptoms and signs of trichomoniasis are not adequately sensitive or specific for diagnosis. Thus, diagnostic laboratory testing is usually required to confirm the presence of the organism. Routine clinical diagnosis usually depends on microscopic observation of motile parasites in vaginal smear samples. But this may not be sufficiently sensitive, because it may detect only about 60% of culture positive samples (Mayta *et al.*, 2000). Culture techniques have been considered as the gold standard for *T. vaginalis* detection for the past decades. The main disadvantages of culture include the need for viable organisms, suboptimal sensitivity compared to molecular diagnostics, and longer time until reporting the diagnostic result. Due to the suboptimal sensitivity of sample preparation and the disadvantages with culture methods, nucleic acid amplification is being introduced in the diagnosis of trichomoniasis (Shipitsyna *et al.*, 2012).

Ribosomal genes are highly conserved in *T. vaginalis*. This characteristic and their highly repetitive nature in the genome of most organisms make these genes good targets for detection by PCR (Madico *et al.*, 1998).

In this study, the detection of organism in samples was provided by a PCR targeting 18S rRNA-DNA genes of *T. vaginalis*. The targeted genes encode the amino acid sequence of 18S rRNA-DNA, a major component of the *T. vaginalis* cytoskeleton. Sequencing of phylogenetic positions on *T. vaginalis* from Turkey has not been previously reported.

MATERIALS AND METHODS

1. Clinical Samples

Trichomonas vaginalis strains were obtained from the previous study from the patients complaining itchy vaginal discharge (Inceboz *et al.*, 2012). The patients lived in Aegean region located in western of Turkey. The study was approved by the Clinic and Laboratory Research Ethics Committees of Dokuz Eylul University, School of Medicine. These isolates (14 numbers) were collected between 2011 and 2012 from patients.

2. Culture of Trichomonas vaginalis

T. vaginalis strains isolated from 14 different samples were incubated in 10-ml glass tubes containing 5 ml of Trypticase-yeast extract-maltose (TYM) medium complemented with 10% Foetal Calf Serum (FCS) (Biochrom Ag, Berlin, Germany) a combination of 100 IU of streptomycin per ml and penicillin at 37°C as previously described. *T. vaginalis* strains were incubated in the TYM culture medium at 37°C in incubator and allowed to reproduce (Diamond, 1957; Sugarman & Mummaw, 1990; Singh *et al.*, 1999).

The samples from the tube were evaluated under the microscope and viability, moving trophozoites were counted by using Thoma counting chamber. It was checked the density of the trophozoites in the culture media by using light microscope (x40) to make sure that the quantification was sufficient.

The samples were cryopreserved after completing 30 days in culture. Personal identifiers were removed and the source of isolates was kept anonymous to protect patient confidentiality.

3. Reference strain, DNA extraction and concentration

Trichomonas vaginalis strain (Accession number: XM 001279254.1) was used as reference. All of the DNA extractions were employed using DNA isolation kit (QIAmp DNA Mini Kit, Qiagen, Cat num: 51104, 14 Hilden, Germany). The measurement of the concentration of DNA of 14 cultured isolates using the nanodrop spectrophotometers (Thermo Scientific, Wilmington, 16 USA).

4. PCR method

PCR targeting18S rRNA-DNA gene of *T. vaginalis* was developed for the detection of organism in culture samples. DNA was extracted from the samples of culture by transferred to a microcentrifuge tube with a sterile Pasteur pipette. A pellet fraction was obtained and washed twice with phosphate buffered saline (PBS). Lysis of *T. vaginalis*

and exposure of its DNA were accomplished by resuspending the pellet in 0.25 ml Tris-HC1, pH 8.3, containing 50 mM KC1, 2.5 mM $MgCI_2$, 1% Brij 35 detergent, and 200 Ixg/ml proteinase K. The DNA amplification reaction was performed using a set of primers TV1G (5´-TGTCGAACATTGGTCTTACCCTCAG TT-3´) and TV2G (5´ CCAGTACTTACGCTT GGAGAGGACATG-3´) to amplify a 237 bp fragment of 18S rRNA-DNA of *Trichomonas vaginalis* (Shipitsyna *et al*., 2012). A total of 50 µL volume was prepared to containing 10 ng DNA template, and the thermal-cycler programme was an initial denaturation at 94°C for 5 min and 60 cycles of denaturation at 94°C for 10 s, annealing at 65°C for 10 s, extension at 72°C for 10 s and final extension at 72°C for 60 s. Amplification products were analysed by 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. A search for inhibitors was performed in samples that showed PCR-negative results to assess possible PCR failures to detect *T. vaginalis*. In the same tube, 1 µl DNA preparation from a negative sample and 1 µl *T. vaginalis* DNA standard sample were added. A positive result means no presence of inhibitors in the tested sample. In addition *T. vaginalis* beta-tubulin gene region amplification was carried out with real-time PCR assay kit (Genesig, Primerdesign Ltd, The Mill Yard, Nursling Street, Rownhams, Southampton, United Kingdom).

5. Sequencing and phylogenetic analysis

All of the PCR products were purified using a Sentro Pure DNA purification kit (Sentromer DNA, Istanbul, Turkey), and they were sequenced with DNA sequencing kit Big Dye Terminator™ (Applied Biosystems, California, USA) according to the manufacturer's instructions. 18S rRNA-DNA gene sequencing of *Trichomonas vaginalis* was performed by ABI Prism 310TM Genetic Analyser (Applied Biosystems, California, USA). The DNA sequences obtained were processed using GenBank and checked with basic local alignment search tool (BLAST) analysis software (www.ncbi.nlm.nih.gov/BLAST).

RESULTS

1. Culture and PCR

The results of microscopy, culture and PCR methods are seen in Table 1. Fourteen (100%) of the microscopy of urine samples were positive; 6 (42.9%) of the TYM were positive and 8 (57.1%) were negative; 14 (100%) of the PCR were positive. There were no inhibitors and no false negative result in PCR methods in this study. Also, 18S rRNA-DNA PCR results confirmed with putative beta-tubulin genes of *T. vaginalis* real time PCR method.

2. 18S rRNA-DNA sequencing method and phylogenetic analysis

As compared to the previous records in GenBank, the similarity between sequences of *Trichomonas* isolates 98% similarity was found. According to the results of 18S rRNA-DNA sequencing, phylogenetic analysis revealed that the *T. vaginalis* isolates were different from nucleotide sequencing. *T. vaginalis* isolates revealed different clusters as 5-TV1G, 13-TV1G. These strains were evaluated from symptomatic

Table 1. Microscopy, TYM and PCR results

Samples Number	Microscopy	TYM	PCR
1	+	–	+
2	+	+	+
3	+	–	+
4	+	–	+
5	+	+	+
6	+	–	+
7	+	–	+
8	+	+	+
9	+	–	+
10	+	–	+
11	+	+	+
12	+	+	+
13	+	–	+
14	+	+	+

+, positive; –, negative; TYM, Trypticase-Yeast Maltose (TYM) medium; PCR, Polymerase Chain Reaction

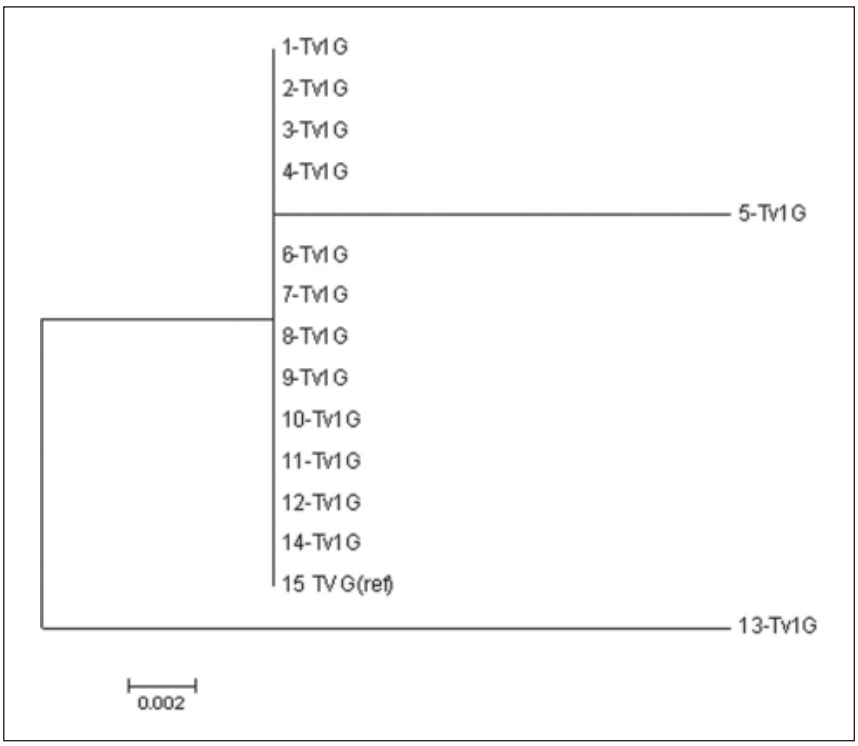

Figure 1: 18S rRNA-DNA sequence neighbor-joining tree with *Trichomonas vaginalis* Strains from Turkey, produced in MEGA6.05.

cases. In Figure 1, is seen 18S rRNA-DNA sequence neighbor-joining gene tree with *Trichomonas vaginalis* strains from Turkey. Bootstrap values are based on 1000 replications, produced in MEGA6.05 (Figure 1).

DISCUSSION

The *T. vaginalis* genome sequence, generated using whole-genome shotgun method, contains 1.4 million shotgun reads. At least 65% of the *T. vaginalis* genome is repetitive. Although many methods developed to improve the assembly, the superabundance of repeats resulted in sequence fragmentations. This makes investigation of *T. vaginalis* genome architecture difficult (Carlton *et al.*, 2007). Previously, Katiyar *et al.*, reported that majority of the nucleotide sequences have revealed in sequence type I: (TO6, H15 and TE3) as well as sequence type II: (H22, H11 and TE7) and identified as wild type (Katiyar *et al.*, 1995). In another study, Xiao *et al.*, found two types of sequences and mentioned them as wild (82%, 23/28) and mutant (14%, 4/28) types (Xiao *et al.*, 2006). Further studies on strain typing revealed 6 sequence types (H1–H6) in the Philippines (Rivera *et al.*, 2009). A core set of ~60,000 protein-coding genes was identified, endowing *T. vaginalis* with one of the highest coding capacities among eukaryotes (Vanacova *et al.*, 2005). All researchers are in agreement with the classification proposed by International Committee on Taxonomy of Viruses (ICTV) (Gerbase *et al.*, 1998; Jancovich *et al.*, 2012). GLV-like comprises viruses of the genus Giardiavirus and ScV-like comprises viruses of the genus Totivirus. The genus Victorivirus includes two groups, MoV-like and GaRV-like. The genera Leishmaniavirus and Trichomonasvirus include groups LRV-like and TVV-like respectively (Snipes *et al.*, 2000; Liu *et al.*, 2012). TVV has no known lytic cycle, and

attempts to infect isolates that are uninfected have been unsuccessful (Wang *et al.*, 1986). Therefore, it is probable that the virus is acquired exclusively through vertical transmission, making its presence or absence a useful genetic marker.

Although, eight strains were found negative in culture, the presence of deceased *T. vaginalis* strains in culture were diagnosed by PCR method. This was confirmed with second diagnostic method using real time PCR assay.

The growing awareness and appreciation on the serious health sequelae that are associated with *T. vaginalis* infection, and the widespread prevalence of trichomoniasis worldwide, have brought about the need to understand the genetic diversity of this species (Cornelius *et al.*, 2012).

In this study, sequencing was performed for the identification of strain variation, and phylogenetic relationship among the symptomatic cases. When compared with the previous studies in GenBank, the similarity between sequences of *T. vaginalis* isolates taken from urine samples were 98% and it was found to be similar to *T. vaginalis* G3 surface protein. In addition, Dwivedi *et al.* found the differentiation of sequencing of symptomatic and asymptomatic strains of axenic and clinical isolates (Dwivedi *et al.*, 2012). *T. vaginalis* surface proteins are involved in attachment to host cells and structures. It is considered that 5-TV1G and 13-TV1G are subtype of *T. vaginalis* as a result of 18S rRNA-DNA sequencing analysis. Although sequence similarity in strain of *T. vaginalis*, differences of the subtypes can be resulted in genetic differences and diverse population structure.

In conclusion, although conducted at a small scale, present study shows the possibility of genotyping *T. vaginalis* and making a concordance between strains and clinical states. We showed the presence 5-TV1G, 13-TV1G sequence types in the Turkey. To best of our knowledge this is the first analysis of phylogenetic positions on *T. vaginalis* from the Turkey. More studies at molecular level will provide more information on *T. vaginalis*.

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