Use of Internal Transcribed Spacer Sequence Polymorphisms as a Method for *Trichomonas vaginalis* Genotyping

Internal Transcribed Spacer (ITS) Sekans Polimorfizmlerinin *Trichomonas vaginalis* Genotiplendirmesinde Yöntem Olarak Kullanılması

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ABSTRACT

Objective: *Trichomonas vaginalis* is the most common non-viral, sexually transmitted pathogen with a worldwide distribution. The aim of the present study was to design a new genotyping tool for *T. vaginalis* isolates using internal transcribed spacer (ITS) sequences.
Methods: First, a total of 20 cryopreserved *T. vaginalis* isolates were thawed and genomic DNA was isolated from fresh cultures. A polymerase chain reaction (PCR) was performed to amplify the ITS regions and the amplicons were sequenced. These sequences were aligned with others from Genbank and polymorphisms were detected. At last, each ITS sequence was given a different sequence type.
Results: More than 99% homology was observed among sequences. Of 20 isolates, five had identical ITS sequence to reference (L29561) defined as ITST1. Moreover, 13 had A58 deletion (ITST10), one had C203T mutation (ITST2), and one had both A58 deletion and C203T mutation (ITST11). ITS typing of *T. vaginalis* sequences on Genbank revealed a total of 11 ITS types with the predominance of ITST1 (44.4%) globally.
Conclusions: ITS typing seems to be an applicable and useful tool for a better understanding of molecular epidemiology as well as for the dissemination of *T. vaginalis* clones.
Keywords: ITS, *Trichomonas vaginalis*, genotypes
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ÖZ

Amaç: *Trichomonas vaginalis* (*T. vaginalis*) cinsel yolla bulaşan hastalık etkenleri arasında viral patojenlerden sonra en sık görülen tür olup küresel bir dağılım göstermektedir. Bu çalışmanın amacı *T. vaginalis* internal transcribed spacer (ITS) dizilerini kullanarak parazitin genotiplendirilmesinde yeni bir yöntemin ortaya konulmasıdır.
Yöntemler: Çalışmamızda öncelikle farklı olgulardan izole edilen ve kriyoprezervasyon yapılarak saklanan 20 *T. vaginalis* izolatı canlandırılarak DNA izolasyonları yapılmıştır. Bu örneklerin ITS bölgeleri polimeraz zincir reaksiyonu (PZR) ile çoğaltılmış ve sekanslanmıştır. Genbank'da yer alan diğer ITS sekansları ile bu çalışmada elde edilenler sıralanarak polimorfizmler belirlenmiştir. Son olarak her bir farklı sekans için sekans tipi tanımlanmıştır.
Bulgular: *Trichomonas vaginalis* ITS sekansları arasında %99'a varan bir homoloji saptanmış olup bunlardan beşinin (n=20,%40), referans olarak seçilen L29561 Genbank numaralı ITST1 tipi *T. vaginalis* sekansı ile aynı olduğu görülmüştür. Bunun yanı sıra, izolatların 13'ünde A58 delesyonu (ITST10) ve birinde C203T mutasyonu (ITST2), birinde A58 delesyonu ve C203T mutasyonu (ITST11) tespit edilmiştir. Diğer ülkelerden izole edilen T. vaginalis izolatlarının ITS sekansları bizim çalışmamızdakilerle birlikte değerlendirildiğinde 11 farklı ITS sekans tipi tanımlanmış olup en sık ITST1 (%44,4) saptanmıştır.
Sonuç: Çalışmamızda ITS sekanslarına göre geliştirilen bu genotiplendirme yaklaşımının *T. vaginalis*'in moleküler epidemiyolojisinin daha iyi anlaşılmasına ve izolatların birbirinden genetik olarak ayırt edilmesinde faydalı olduğu düşünülmektedir.
Anahtar sözcükler: ITS, *Trichomonas vaginalis*, genotip
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INTRODUCTION

Trichomoniasis is the most common non-viral sexually transmitted infection (STI) worldwide and is caused by *Trichomonas vaginalis*, a flagellated, unicellular protozoon parasite of humans. However, very little public health attention has been provided to the disease (1). The World Health Organization reported an increase of 11.3% in global new cases of *T. vaginalis* infections from 248 to 276 million from 2005 to 2008 (2). It was reported that the frequency of infection in the USA was 1.3% among white Americans, 13.3% among African Americans, and 1.8% among Mexicans. Additionally, 2%-50% of people in Africa were thought to be infected with *T. vaginalis* (3, 4).

T. vaginalis infection is usually characterized by vaginitis, urethritis, and prostatitis; however, it may increase the risk of pelvic inflammatory disease and tubal infertility (5, 6). Recent reports have supported the idea that *T. vaginalis* may facilitate the transmission of HIV, particularly Type I (7-9). Trichomoniasis may cause profound health consequences, such as low birth weight infant, and preterm birth, in pregnancy (10, 11). Metronidazole, a 5-nitro imidazole derivative, is the primary drug of choice for trichomoniosis treatment; however, resistant cases to this drug have been reported in the USA, Russia, Africa, and Europe (12-15).

Although most studies have mainly focused on the prevalence of *T. vaginalis* and clinical characteristics of infection, little is known about the genetic diversity of *T. vaginalis* to date. Currently, a number of methods have been developed to discriminate *T. vaginalis* isolates; these methods include multisequence typing, microsatellite genotyping, and analyzing internal transcribed spacer (ITS) sequences (16, 17). It was reported that sequence analysis of ITS regions flanking the 5.8S subunit of the ribosomal DNA gene was a powerful tool for discriminating different genotypes (18).

The aim of the present study was to determine the genetic diversity of *T. vaginalis* isolates from a routine laboratory technique based on ITS sequences and also on a global scale.

METHODS

Isolates and Culture

In the study, we used cryopreserved *T. vaginalis* isolates that were isolated from 20 patients at Adnan Menderes University, Research and Training Hospital, Parasitology Laboratory between 2010 and 2014. The isolates were thawed, inoculated in trypticase yeast extract maltose medium, and incubated at 37°C. The cultures were observed after 48 h with direct microscopic examination, and positive cultures were utilized for DNA isolation.

PCR Amplification and Sequencing

The parasites in the logarithmic phase were pelleted by centrifugation for 5 min at 1500g. Genomic DNA was extracted from pellets using the High Pure PCR Template Preparation (Roche Applied Science, Berlin, Germany) according to the manufacturer's instructions and stored at −20°C.

The ITS region was amplified using primers as previously described by Snipes et al. (18): TVITSF (5'-ACCGCCCGTCGCTCCTACCGA-3') and TVITSR (5'-CTCCGCTTAATGAG ATGCTTC-3'). The reaction was set in a 50-µL volume containing 0.4 pmol of each of the primers, 1.5 U of Taq DNA polymerase, 0.2 mM of each dexynucleotide triphosphate (dNTP), 2 mM magnesium chloride ($MgCl_2$), and 1× Taq buffer with ammonium sulfate $(NH_4)_2SO_4$. The amplification was performed using a thermal cycler (TC-312, Techne, Minneapolis, USA) following the following protocol: initial denaturation step at 94°C for 5 min and 35 cycles (30 s at 94°C, 30 s at 54°C, and 60 s at 72°C) with a final extension step at 72°C for 5 min. A 5-8 µL of PCR product was run on a 2% agarose gel using SYBR Safe (Invitrogen, California, USA) and 50-base pair ladder marker (Thermo Scientific, Bartlesville, USA). The gels were visualized using a gel documentation system (VilberLourmat, Basel, Switzerland). PCR amplifications were purified and sequenced by a commercial facility (Macrogen Inc., South Korea). The sequences were aligned with references using CLUSTAL X (19).

ITS Sequences of *T. vaginalis* from Genbank

In total, 36 ITS sequences were found at Genbank. The accession numbers of these sequences were as follows: L29561, TVU86613, AY957955, AY871048, AY871047, AY871046, AY871045, AY871044, FJ813603, FJ813602, FJ813601, FJ813600, FJ813599, FJ813598, JN007004, AY245136, FJ376711, EU816897, AY349186, AY349185, AY349184, AY349183, KC513779, KC513778, KC513777, KC513776, KC513775, KC513774, JQ768335, JQ768334, JQ768333, JQ768332, JQ768331, JQ768330, KP221674, and KF164606. However, 2 of these sequences, AY957955, and KP221674, were excluded from the study. The sequence AY957955 had many undefined nucleotides, (symbolized as N) and the beginning as well as the end of the sequence KP221674 had multiple mismatches with the remaining sequences.

RESULTS

In total, 20 *T. vaginalis* isolates from patients at Adnan Menderes University, Research and Training Hospital were included in the present study. The ages of patients varied from 26 to 42 years (mean±standard deviation: 33.3±4.1). Total DNA was extracted from samples, and *T. vaginalis* ITS region was amplified and sequenced in all of the 20 culture-positive samples. The ITS sequences of these 20 samples were aligned, which showed >99 similarity. Sequences were deposited to Genbank (Accession Numbers: KP861811, KP861812, KP987798, and KP987799). The longest *T. vaginalis* ITS sequence in Genbank (Accession Number: L29561) was accepted as the reference ITS sequence. The comparative analysis of our sequences against the reference revealed that 5 of them (40%) were identical to the reference, 13 isolates had an "Adenine" deletion at the 58th position, one isolate had a single substation (C203T), and one isolate had both a deletion at the 58th position and a single substation (C203T).

In addition to our sequences, other *T. vaginalis* ITS sequences from Genbank were downloaded and each different ITS sequence was numerated. In total, eleven ITS types were defined by comparing sequences. Among them, ITST10 and ITST11 were from the present study. These sequences were different from the existing sequences in Genbank (Table 1). Based on the nomenclature described in this study, ITST10 was the most common ITS type among our samples (13 isolates, 65%), followed by ITST1 (5 isolates, 25%), ITST2 (one isolate, 5%), and

Table 1. ITS Typing; Distinct ITS types of sequences in Genbank and ITS sequences of the isolates*

	Base number (accession no. L29561)															
ITS Type	**58**	**68**	**69**	**127**	**128**	**135**	**170**	**180**	**203**	**352**	**359**	**424**	**435**	**439**	**Reference**	**Origin of isolate**
ITST1	A	A	A	T	T	G	T	A	C			A	A	A	L29561	USA
ITST2									T						AY349186	Brazil
ITST3		T	C						T						AY871046	China
ITST4												T			FJ813603	Philippines
ITST5							C								FJ813602	Philippines
ITST6													C		FJ813600	Philippines
ITST7														T	FJ813599	Philippines
ITST8				A	A	T				#C	#C				FJ376711	Brazil
ITST9								C							AY349185	Brazil
ITST10	‡Del														KP987798	Turkey (Present Study)
ITST11	‡Del								T						KP987799	Turkey (Present Study)

*Starting points: ITST2 and 9 at 137, ITST3 at 61, ITST4-7 at 131, ITST8 at 126, ITST10 and 11 at 46. #Insertion, ‡Del: deletion.

Table 2. Dissemination of *T. vaginalis* ITS types by country of 25 ITS sequences in Genbank and isolates from Turkey

Origin of country	**ITS Types**											
	ITST1	**ITST2**	**ITST3**	**ITST4**	**ITST5**	**ITST6**	**ITST7**	**ITST8**	**ITST9**	**ITST10**	**ITST11**	**Total**
USA	1	-	-	-	-	-	-	-	-	-	-	1
Brazil	3	1	-	-	-	-	-	1	1	-	-	6
China	1	3	1	-	-	-	-	-	-	-	-	5
Czech	1	-	-	-	-	-	-	-	-	-	-	1
Iran	3	3	-	-	-	-	-	-	-	-	-	6
Mexico	1	-	-	-	-	-	-	-	-	-	-	1
Philippines	1	1	-	1	1	1	1	-	-	-	-	6
Spain	6	-	-	-	-	-	-	-	-	-	-	6
Switzerland	2	-	-	-	-	-	-	-	-	-	-	2
Turkey	5	1	-	-	-	-	-	-	-	13	1	20
Total	24	9	1	1	1	1	1	1	1	13	1	54

ITST11 (one isolate, 5%). *T. vaginalis* ITS types were disseminated by country for 34 ITS sequences in Genbank and 20 isolates from Turkey (Table 2).

DISCUSSION

T. vaginalis infection is among the most common STIs all over the world. A systematic understanding of the variation in genotypes and outcomes are still lacking because of the low number of molecular studies on *T. vaginalis*. In previous studies, Random Amplified Polymorphic DNA analyses and ribosomal gene sequences were used to determine the genetic polymorphism of *T. vaginalis* isolates. The studies primarily focused on the relation between genotypes and possible outcomes such as clinical picture, resistance to drugs, and host specificity (20, 21). Hampl et al. (20) suggested that the clinical picture and resistance to metronidazole are related to the virulence and biological characteristics of isolates. It was reported that the strains from asymptomatic and symptomatic cases were different in terms of zymodeme patterns (22, 23). Another genotyping method for *T. vaginalis* isolates is sequencing the ITS region. ITS sequences are non-functional, neutrally evolving phylogenetic markers that are found in all eukaryotic cells. They are located between ribosomal genes and are less conserved than active genes. Snipes et al. (18) amplified the ITS region in 109 *T. vaginalis* isolates and found a C66 mutation in 16 isolates. They also found a high resistance in this group and concluded that it might be a result of this mutation.

In our study, 5 of the 20 isolates had identical ITS sequence to the reference sequence (ITST1), 13 had only an "Adenine" deletion (ITST10), one had C203T point mutation (ITST2), and one had both deletion at the 58th position and C203T point mutation (ITST11). Additionally, ITST10 was the most common (13 out of 20, 65%) ITS type in our study population. Although 2 ITS types

(ITST10 and 11) have not been reported yet in other parts of the world, we think that it is because of the absence of the data and lack of genotyping studies of *T. vaginalis*. ITS typing of more isolates from different regions in Turkey and the world will help us better understand the molecular epidemiology of the parasite, the relations among genotypes, and the virulence as well as resistance. In accordance with our study, Ibanez-Escribano et al. (24) reported a stable mutation in 26% of isolates and 99.7% ITS nucleotide sequence identity.

The analysis of ITS sequences in Genbank showed that ITST1 was the most common ITS type (24 out of 54, 44.4%) on a global scale. These ITST1 isolates were reported from a variety of countries such as USA, Brazil, Philippines, Spain, China, Switzerland, Czech Republic, Iran, Mexico, and Turkey. The second most common genotype ITST2 (9 out of 54, 16.6%) was reported from Philippines, China, Brazil, Iran, and Turkey. ITST3 was reported from China. ITST4, 5, 6, and 7 were reported from Philippines. ITST8 and 9 were reported from Brazil. Finally, ITST10 and 11 were reported from Turkey. The only available sequence from the USA was ITST1. There were 6 sequences from Brazil, 3 of them were ITST1 (50%) and the remaining were singleton ITST2, ITST8, and ITST9. In total, 5 sequences were reported from China and 3 (60%) were ITST2 and one (20%) was ITST1 and ITST3 each. From Philippines, 6 sequences were reported, one from each ITS types 1, 2, 4, 5, 6, and 7. One sequence reported from Czech Republic was ITST1. All of the 6 sequences from Spain and 2 from Switzerland were ITST1. There were 6 sequences from Iran, 3 of them were ITST1 (50%), and the remaining sequences were ITST2.

CONCLUSION

In the present study, we have genotyped *T. vaginalis* isolates by ITS sequencing in Turkey as well as we contributed a new approach for sequence-based genotyping; thus, the current findings have a potential of being a base for future studies. Further studies should be performed to show a relation between genotype and clinical outcome, virulence, resistance, and pathogenicity.

Ethics Committee Approval: Authors declared that the research was conducted according to the principles of the World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects", (amended in October 2013).

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REFERENCES

1. Van der Pol B. *Trichomonas vaginalis* infection: the most prevalent nonviral sexually transmitted infection receives the least public health attention. Clin Infect Dis 2007; 44: 23-5. [CrossRef]
2. WHO. Global incidence and prevalence of selected curable sexually transmitted infections – 2008: World Health Organization, 2012. ISBN: 978 92 4 150383 9.
3. Mairiga AG, Balla HJ, Ahmad MI. Prevalence of *Trichomonas vaginalis* infections among antenatal clients in Maiduguri Nigeria. Int J Biol Med Res 2011; 2: 998-1002.
4. Sutton M, Sternberg M, Koumans E, McQuillan G, Berman S, Markowitz L. The prevalence of *Trichomonas vaginalis* infection among reproductive-age women in the United States, 2001-2004. Clin Infect Dis 2007; 45: 1319-26. [CrossRef]
5. Cates W, Joesoef MR, Goldman MB. Atypical pelvic inflammatory disease: can we identify clinical predictors? Am J Obstet Gynecol 1993; 169: 341-6. [CrossRef]
6. Wilkinson D, Abdool Karim SS, Harrison A, Lurie M, Colvin M, Connolly C, et al. Unrecognized sexually transmitted infections in rural South African women: a hidden epidemic. Bull World Health Organ 1999; 77: 22-8.
7. Bersoff-Matcha SJ, Horgan MM, Fraser VJ, Mundy LM, Stoner BP. Sexually transmitted disease acquisition among women infected with human immunodeficiency virus type 1. J Infect Dis 1998; 178: 1174-7. [CrossRef]
8. McClelland RS, Sangare L, Hassan WM, Lavreys L, Mandaliya K, Kiarie J, et al. Infection with *Trichomonas vaginalis* increases the risk of HIV-1 acquisition. J Infect Dis 2007; 195: 698-702. [CrossRef]
9. Sorvillo F, Smith L, Kerndt P, Ash L. *Trichomonas vaginalis*, HIV, and African-Americans. Emerg Infect Dis 2001; 7: 927-32. [CrossRef]
10. Cotch MF, Hillier SL, Gibbs RS, Eschenbach DA. Epidemiology and outcomes associated with moderate to heavy Candida colonization during pregnancy. Vaginal Infections and Prematurity Study Group. Am J Obstet Gynecol 1998; 178: 374-80. [CrossRef]
11. Pastorek JG, Cotch MF, Martin DH, Eschenbach DA. Clinical and microbiological correlates of vaginal trichomoniasis during pregnancy. The Vaginal Infections and Prematurity Study Group. Clin Infect Dis 1996; 23: 1075-80. [CrossRef]
12. Dombrowski MP, Sokol RJ, Brown WJ, Bronsteen RA. Intravenous therapy of metronidazole-resistant *Trichomonas vaginalis*. Obstet Gynecol 1987; 69: 524-5.
13. Edwards D. Nitroimidazole drugs-action and resistance mechanisms. I. Mechanisms of action. J Antimicrob Chemother. 1993; 31: 9-20. [CrossRef]
14. Lossick JG, Muller M, Gorrell TE. In vitro drug susceptibility and doses of metronidazole required for cure in cases of refractory vaginal trichomoniasis. J Infect Dis 1986; 153: 948-55.[CrossRef]
15. Poppe WA. Nitroimidazole-resistant vaginal trichomoniasis treated with paromomycin. Eur J Obstet Gynecol Reprod Biol 2001; 96: 119-20. [CrossRef]

16. Conrad M, Zubacova Z, Dunn LA, Upcroft J, Sullivan SA, Tachezy J, et al. Microsatellite polymorphism in the sexually transmitted human pathogen Trichomonas vaginalis indicates a genetically diverse parasite. Mol Biochem Parasitol 2011; 175: 30-8. [CrossRef]
17. Carrillo-Ávila JA, Serrano-García ML, Fernández-Parra J, Sorlózano-Puerto A, Navarro-Marí JM, Stensvold CR, et al. Prevalence and genetic diversity of *Trichomonas vaginalis* in the general population of Granada and co-infections with *Gardnerella vaginalis* and Candida species. J Med Microbiol 2017; 66: 1436-42. [CrossRef]
18. Snipes LJ, Gamard PM, Narcisi EM, Beard CB, Lehmann T, Secor WE. Molecular epidemiology of metronidazole resistance in a population of *Trichomonas vaginalis* clinical isolates. J Clin Microbiol 2000; 38: 3004-9.
19. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25: 4876-82. [CrossRef]
20. Hampl V, Vanacova S, Kulda J, Flegr J. Concordance between genetic relatedness and phenotypic similarities of *Trichomonas vaginalis* strains. BMC Evol Biol 2001; 1: 11. [CrossRef]
21. Stiles JK, Shah PH, Xue L, Meade JC, Lushbaugh WB, Cleary JD, et al. Molecular typing of *Trichomonas vaginalis* isolates by HSP70 restriction fragment length polymorphism. Am J Trop Med Hyg 2000; 62: 441-5. [CrossRef]
22. Proctor EM, Naaykens W, Wong Q, Bowie WR. Isoenzyme patterns of isolates of *Trichomonas vaginalis* from Vancouver. Sex Transm Dis 1988; 15: 181-5. [CrossRef]
23. Vohra H, Sharma P, Sofi BA, Gupta I, Ganguly NK, Mahajan RC, et al. Correlation of zymodeme patterns, virulence & drug sensitivity of *Trichomonas vaginalis* isolates from women. Indian J Med Res 1991; 93: 37-9.
24. Ibanez-Escribano A, Nogal-Ruiz JJ, Aran VJ, Escario JA, Gomez-Barrio A, Alderete JF. Determination of internal transcribed spacer regions (ITS) in *Trichomonas vaginalis* isolates and differentiation among *Trichomonas* species. Parasitol Int 2014; 63: 427-31. [CrossRef]