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Original Article

Microsatellite-Based Genotyping, Analysis of Population Structure, Presence of *Trichomonas vaginalis* Virus (TVV) and *Mycoplasma hominis in T. vaginalis* Isolates from Southwest of Turkey

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Abstract

Background: The present study aimed to determine genetic diversity of *Trichomonas vaginalis (T. vaginalis)* isolates with microsatellite markers in Turkey (Nov 2015 to 2016) and to create a web-based microsatellite typing (MT) approach for the global interpretation of the data. In addition, the endosymbiosis of *Mycoplasma hominis (M. hominis)* and *T. vaginalis* virus (TVV) in the isolates was also examined.

Methods: The allele sizes for each locus were calculated and microsatellite types were determined according to the allele profiles. The population structure was examined with Bayesian clustering method. A website (http://mttype.adu.edu.tr) was created for collection and sharing of microsatellite data. Presence of TVV and *M. hominis* in *T. vaginalis* isolates were investigated with electrophoresis and PCR.

Results: Of 630 vaginal samples *T. vaginalis* was detected in 30 (4.7%) and those were used for further analysis. The structure produced by a clustering algorithm revealed eight genetic groups. The typing of isolates according to microsatellites revealed 23 different microsatellite types. Three clones were determined among isolates (MT10 16.7%; MT18 10% and MT3 6.7%). The frequency of TVV and *M. hominis* was 16.6% (n=5) and 20% (n=6), respectively.

Conclusion: Presence of three clones among 30 *T. vaginalis* isolates indicated that microsatellite-based genotyping was efficient to determine the clonal distribution of *T. vaginalis* isolates. Therefore, a promising tool might be developed further and adapted to the studies dealing with molecular epidemiology of *T. vaginalis*. Microsatellite data from forthcoming studies will be deposited and presented on the website. In addition, we also presented the frequency of two endosymbionts in *T. vaginalis* isolates for the first time in Turkey.

Introduction

Trichomonas vaginalis is a sexually transmitted protozoon that causes a self-limiting or mild genitourinary tract infection in humans. Although the infection is mostly asymptomatic in both women and men, it can lead to a variety of symptoms in women, such as vaginitis and cervicitis (1). The parasite may also cause some adverse pregnancy outcomes including preterm birth and early membrane rupture (2). In addition, an association with *T. vaginalis* infection and increased rate of human immunodeficiency virus (HIV) transmission was reported (3).

Despite being one of the most common sexually transmitted pathogens, there is limited information about the molecular epidemiology of *T. vaginalis*. The use of polymorphic markers with high discriminatory ability (microsatellite typing, etc.) has allowed a better understanding of genetic variation in *T. vaginalis* isolates (4). Among the sequenced protozoans, *T. vaginalis* has the largest genome: a haploid, 160 Mb in size and with almost 60.000 genes residing in six chromosomes (5, 6). Almost 45% of its genome is composed of non-coding regions and genome duplication events have been uncovered recently (5). The methodologies relying on microsatellites polymorphisms are widely used for detecting variation in eukaryote genomes. The number of microsatellites greatly varies from species to species and among individuals of the same species. The microsatellites of *T. vaginalis* were first identified by scanning the genome database resource TrichDB (http://trichdb.org) (7). No study in the literature determined the population structure of *T. vaginalis* using microsatellite loci.

The viruses from Totiviridae family infect protozoan parasites and the presence of a non-segmented, double-stranded RNA (dsRNA) virus in clinical isolates of *T. vaginalis* was reported at varying rates in different countries (8). Some biological and disease-related factors were attributed with virus infection of isolates, such as, expression of major surface antigen P270, growth or viability of parasite, and changes at cysteine protease level (8). However, there is also conflicting findings about TVV infection and the pathogenesis of trichomoniasis, probably due to the small sized number of previous studies (9). The endosymbiotic relationship between *T. vaginalis* and *Mycoplasma hominis (M. hominis)* was reported in many studies with a prevalence up to 94% (10). These bacteria may be related to the pathogenicity, immunology and metabolism of *T. vaginalis*, such as, pyruvate: ferredoxin oxidoreductase (PFOR) and ferredoxin related drug resistance (11, 12). There is no data about the presence of TVV in *T. vaginalis* isolates from Turkey.

The present study aimed to analyze the genetic diversity of *T. vaginalis* isolates from Turkey according to microsatellite polymorphisms and to create a microsatellite-based genotyping approach. Besides, we aimed to investigate the presence of TVV and *M. hominis* in *T. vaginalis* isolates.

Materials and Methods

Isolates and nucleic acid extraction

The vaginal swabs were collected from 630 symptomatic cases at two gynecological clinics in Aydin, Turkey from Nov 2015 to 2016. The swab samples from cervical posterior fornix were inoculated into Trypticase yeast medium (TYM) with 100 U/ml penicillin-streptomycin and 10 µg/ml gentamycin. The cultures were examined by direct microscopy every two days for one week and positives were cultured in TYM medium without agar. The trophozoites were pelleted by centrifugation and nucleic acid extraction was performed with a commercial kit (NucleoSpin®Tissue, Macherey Nagel). The kit was able to extract both RNA

and DNA simultaneously from the same sample. Symptoms of the cases (vaginal discharge, itching, dysuria, dyspareunia, etc.) and clinical findings (vaginal discharge, vaginal vulvar erythema) were recorded. In the case of at least one finding or symptom, the case was evaluated as symptomatically.

Determination of microsatellite markers

21 *T. vaginalis* specific microsatellite loci were selected as previously reported (7). The primers were labeled with FAM, HEX fluorescent dye, but taq sequence was not used. PCR amplifications were performed in 20-µL volumes containing 1X PCR buffer (ABM Inc.), 1.5 mM $MgCl_2$ (ABM Inc.), 2.5 mM dNTP, 0.5 mM each primer, 1.0 unit of *Taq* polymerase (ABM Inc.), and 50 ng of template DNA. Each locus was amplified in a single reaction: 95 °C for 5 min followed by 35 cycles (94 °C for 30 sec, 55-60 °C for 30 sec and 72 °C for 45 sec) with 72 °C final extensions for 5 minutes. Allele sizes were determined by Macrogen Inc. with 3730xl DNA Analyzer (Applied Biosystems).

Genetic variability

Microsatellite allele sizes were precisely determined using the software Genemarker 2.6.3 (SoftGenetics LLC, USA). The genetic variation in microsatellite loci was calculated using the program GenAlEx 6.5 (13). GENEPOP 3.3 (14) program was used to determine the genetic diversity of isolates for each locus. The allele numbers, effective alleles, allele frequencies, intra-population allele diversity, pairwise comparisons of isolates were determined. In addition, haplotype overlaps in multiple loci were determined with GenAlEx 6.5 (13). The expected heterozygosity (H_E) value was calculated with Arlequin 3.0 (15). The product sizes of amplified microsatellites were presented in Table 1.

Microsatellite typing and web page

Allele types for eight loci (MS1, MS17, MS20, MS77, MS135, MS153, MS168 and MS184) were defined according to lengths and each type was numbered. Microsatellite type of the first isolate was defined as MT1 and remaining isolates (which had at least one different allele type) were numerated with a new MT number. A website "http://mttype.adu.edu.tr" was created to share the data obtained from this study and to collect new microsatellite types from other researchers. It will be possible to add new allele and microsatellite types to website via contacting the curator.

Population structure

Population structure was examined using the software STRUCTURE ver. 2.3 (16), which employs a Bayesian clustering method. By using admixture model and correlated allele frequency parameters, number of genetic clusters (K) was estimated using 10 runs with K 1-10 at 100.000 MCMC repetitions combined with a 10,000 burn-in period. The ad hoc estimated likelihood of K (ΔK) (17) was used to determine the most likely number of populations (K) based on the rate of change in the log probability of the data [Ln Pr (X/K)]. Structure Harvester v0.6.94 (18) was used to infer the most likely number of genetic clusters (K) present using both the Evanno and Delta K methods. In addition, the evolutionary relationships among the microsatellite types were drawn employing median-joining algorithm in NETWORK 5.3 (www.fluxus-engineering.com) (19, 20).

Determination of T. vaginalis virus (TVV) and M

hominis. The previously isolated nucleic acid samples were used for determination of TVV and *M. hominis*. The presence of TVV in *T. vaginalis* isolates was investigated by agarose gel electrophoresis. In brief, 10 µl of the nucleic acid sample was run in 1% agarose gel, 90 V, 60 min. The gel images were analyzed; the expected (4.5-5 kb) bands were evaluated

as TVV positive (21). The positivity of *M. hominis* in culture samples was determined with PCR using RNAH1 and RNAH2 primers (22). The previous sample (confirmed with 16S rRNA sequence) was used a positive control and the sample with no template was used as negative control. A commercial company (Macrogen Inc., Korea) sequenced the amplicons and the presence of *M. hominis* was confirmed with Basic Local Alignment Search Tool (BLAST).

Ethics statment

T. vaginalis isolates were obtained from routine Diagnostic Laboratory in Aydin Adnan Menderes University Training and Research Hospital. The study was approved by the local Ethical Committee (2015/604).

Results

The isolates

Trophozoites of *T. vaginalis* were detected in 30 (4.7%) of 630 samples by in vitro culture and used in further analyses. All of the positive cases were in sexually active period (Mean: 34.6 ± 8.4 yr). Of the cases nine (30%) were pregnant and two (6.7%) had a history of premature birth. The most common symptom was vaginal discharge (43%), followed by itching (40%) and dysuria (16.7%). The typical sign of trichomoniasis, yellow-green foamy, was the most common type of discharge (53.3%). Another characteristic of trichomoniasis, strawberry-cervix, was detected in 5% of cases.

Genetic variability

Sixteen of 21 microsatellite loci for *T. vaginalis* were successfully amplified with PCR. Because of fragment analysis, only one locus (MS03) was monomorphic. The highest number of alleles for all polymorphic loci was found in the MS38 (nine alleles) and the least alleles were found in MS09, MS17 and MS100 loci (two alleles) (Table 1). The mean effective allele number for *T. vaginalis* in Aydın province was 2.788 (Min: 1.226, Max: 5.294). In addition, mean expected heterozygosity was 0.609 and this value was 0.839 at the MS38 locus of 0.191 in MS20 locus (Table 1).

Table 1: Genetic variability at 16 microsatellite loci in Aydin/Turkey population of *Trichomonas vaginalis*.

Locus	N_A	*Allele size (bp)*	*Repeat*	N_E	H_E
MS01	7	137-185	GAATAA	3.600	0.747
MS03	1	235	AAAATA	-	-
MS04	3	229-235	CAA	2.074	0.536
MS06	6	379-406	TTC	3.719	0.747
MS07	6	371-395	ATTAAT	3.982	0.789
MS08	6	222-264	TTC	3.629	0.749
MS09	2	408-412	AGAA	1.965	0.508
MS17	2	176-181	TTTTA	1.471	0.404
MS20	3	405-414	TGT	1.226	0.191
MS38	9	222-396	CTT	5.294	0.839
MS77	5	208-216	GA	2.601	0.637
MS100	2	171-173	TG	1.991	0.515
MS135	5	216-224	TA	2.922	0.681
MS153	5	215-223	AT	2.344	0.593
MS168	6	152-162	TG	3.082	0.699
MS184	4	223-232	TTG	1.915	0.494
Mean	4.67	-	-	2.788	0.609

N_A: the number of alleles per locus, N_E: effective number of alleles, H_E: expected heterozygosity

Microsatellite typing and web site

Microsatellite types (MT) were determined according to the microsatellite length polymorphisms and 23 different MT's were determined in *T. vaginalis* isolates from Aydin. Accordingly, two isolates were MT3, five were MT10 and three were MT18. The remaining isolates had different MT numbers, as they were different in at least one locus from the others. The findings were presented and deposited in the web site (http://mttype.adu.edu.tr). The webpage was divided into three sections: the general information about the parasite, methods for PCR and table of defined microsatellite types. The contact and necessary information of curator was also presented on the website.

Population structure

Analyses of microsatellite data by structure clustering algorithm suggested the presence of eight genetic groups (K = 1.727) (Fig. 1) (17).

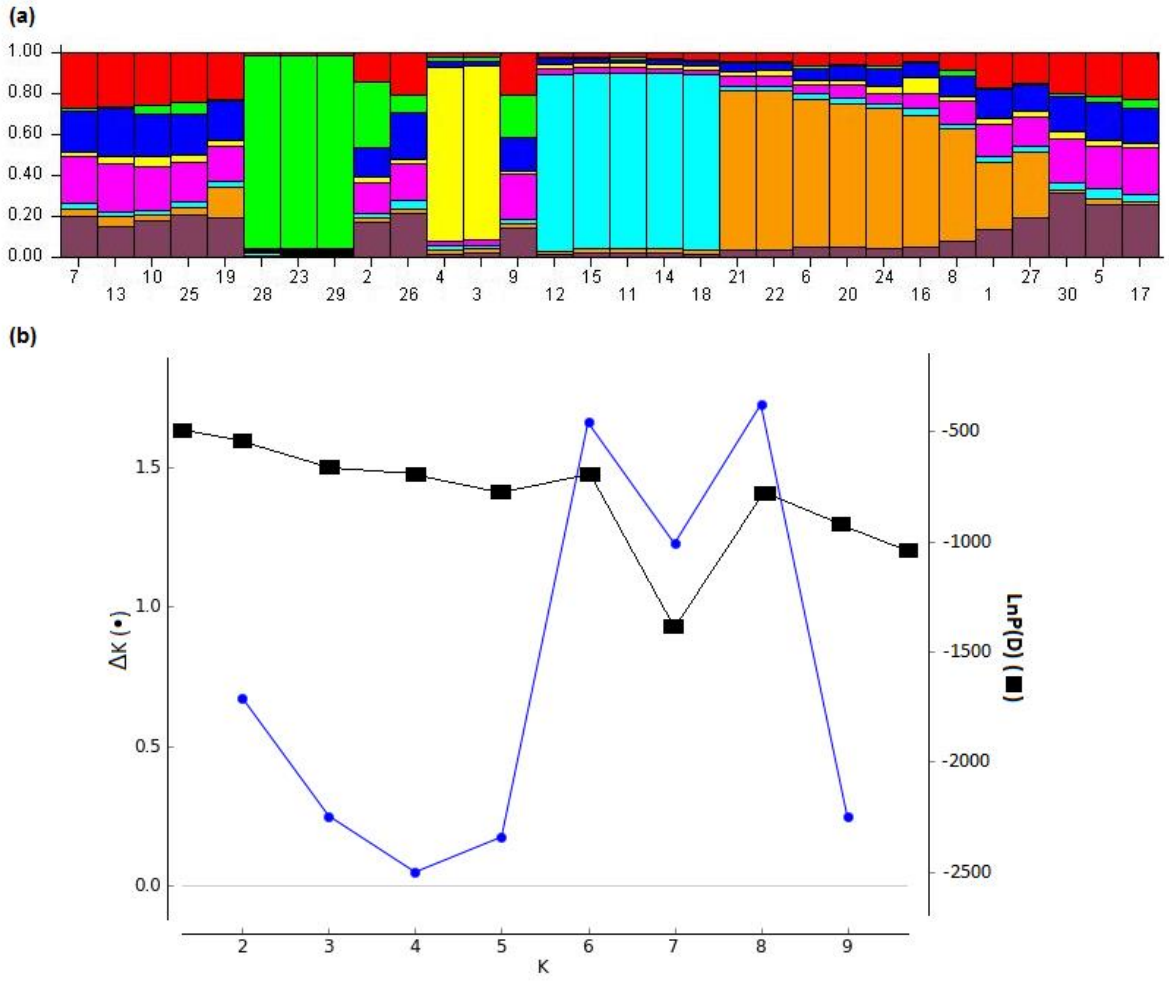

Fig. 1: (a) The graphical display of the STRUCTURE result for the entire data set analysis, representing the number of the genetic groups (ΔK = 8) of *Trichomonas vaginalis*. (b) Scatter plot of possible number of clusters (K; horizontal axis) against ad hoc estimated likelihood of ΔK (vertical axis) by changing the likelihood rate (circle) for *Trichomonas vaginalis* samples. The estimate of ln Pr[X|K] (square) which is the several independent runs for each K and the verification of estimates are consistent across runs

Besides, three microsatellite types (MT3, MT10 and MT18) were differentiated from others. In the network analysis, MT 20 was located in the center of the network diagram because of allele diversity. In other words, MT20 was the main body of the network diagram and the other originated from this MT (Fig. 2).

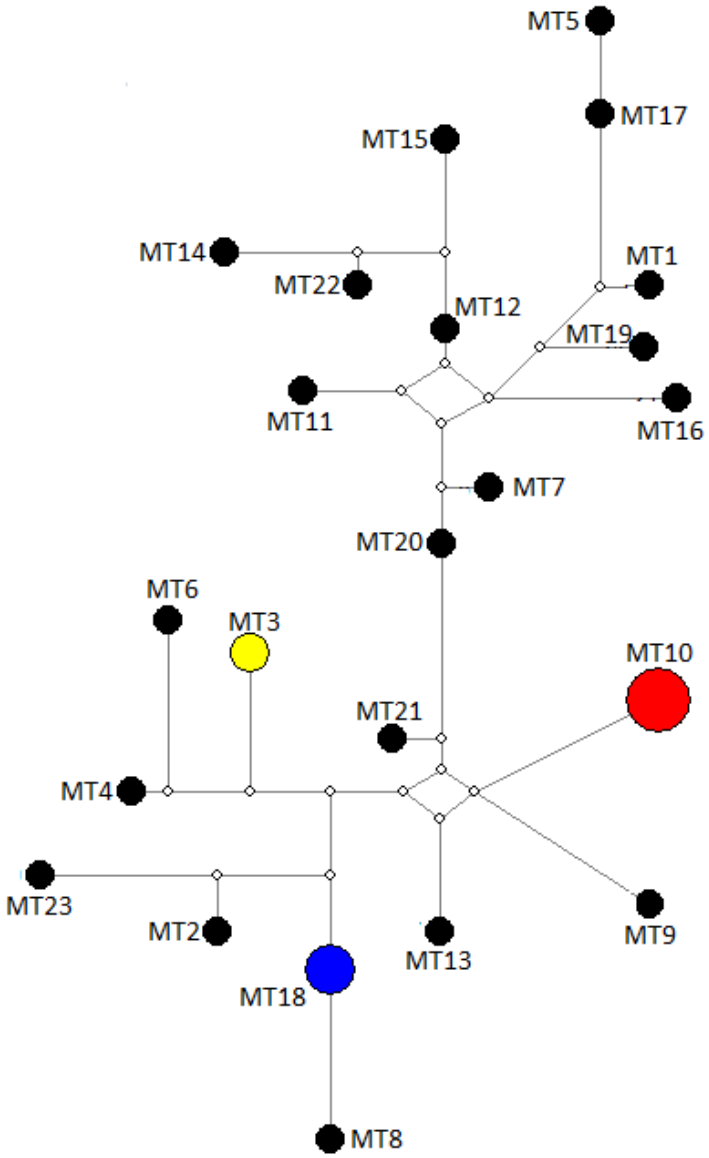

Fig. 2: Median-joining tree of *Trichomonas vaginalis* isolates based fifteen microsatellite loci

The positivity of TVV and M. hominis in T. vaginalis isolates

In our study, *T. vaginalis* virus positivity was detected in five samples (16.6%) and *M. hominis* in six (20%) of the samples. The partial sequence of 16S rRNA gene was submitted to Genbank with Accession number: MK780183. Agarose gel electrophoresis of nucleic acid samples for TVV show in Fig. 3.

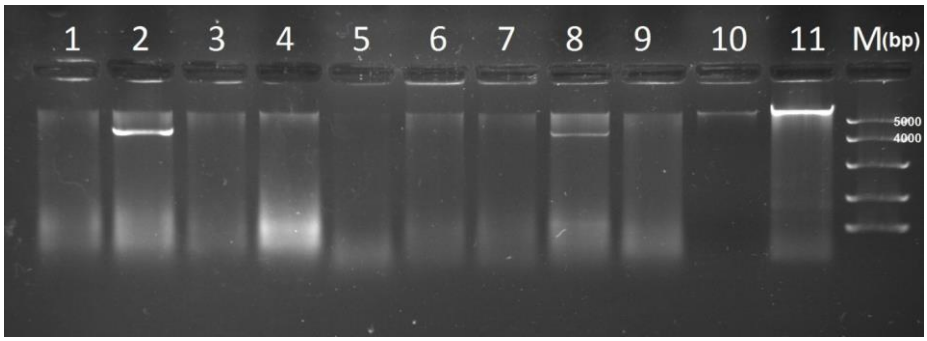

Fig. 3: Electrophoresis of nucleic acid samples in 1% agarose gel, 90 V for *T. vaginalis* virus. Lines 2 and 8 are positive (4.5-5 kb), the others are negative, M: marker (1000 bp)

Discussion

Trichomonasis is a common sexually transmitted infections (STI) with a global distribution. It is an important cause of vaginitis in women; however, it may lead to genital cancers, PID, premature birth in pregnancy, early membrane rupture, etc. and increase HIV transmission (23). The main factors that affect the prevalence of parasites are sexual intercourse, number of partners, low education level and age (24). In our study, the positive rate of *T. vaginalis* was 4.7%. Although the incidence of *T. vaginalis* in Turkey range from 0.3% to 42.8, methods and studied populations greatly varied from each other (25, 26).

Microsatellite polymorphisms were previously used to determine population structures of eukaryotic parasites such as *Leishmania* sp. (27), *Trypanosoma* sp. (28, 29), *Plasmodium* sp. (30, 31) and *Toxoplasma gondii* (32). Particularly, it was used to monitor the displacement and distribution of drug resistance of *P. falciparum* (33). Overall, 21 microsatellite loci were in the whole genome sequence of *T. vaginalis* (7). In our study, 16 of these loci were amplified and *T. vaginalis* genotypes were determined. The average number of alleles was 3.33 (7); however, it was 4.67 in our study. In addition, the expected heterozygosity was very close to each other in both of the studies (H_E: 0.609 this study; H_E: 0.66 (7)). The study of Conrad et al (7) included the samples from different countries: Australia, United States, England, Puerto Rico and Taiwan. The genetic diversity of *T. vaginalis* in Turkey was similar to those countries.

In this study, 8 of 15 polymorphic loci were selected for microsatellite typing. While determining these loci: we considered representations, reliability of microsatellite locus length, and easy amplification/reproducibility. In addition, the typing according to eight loci gave the same MT numbers as in 15 loci for the same strains. The identification of 23 different MT among 30 isolates may be explained by the high genetic diversity of parasite (mean H_E=0.609) in Aydin. Population structure analysis demonstrates the relationships between allele frequencies of individuals and it determines the genetic structure of microsatellite types (34). In our study, structure analyses in *T. vaginalis* suggested eight main genetic groups. The approach in our study demonstrated the clonality of isolates. Out of 30 isolates, five were the same clone, MT10. This result proved the ability of microsat ellite method for dissemination of clones among patients.

A limitation of our study was that we could not make a connection between demographical data about cases and

genotypes. However, we could make some assumptions that rely on the geographical and economical properties of the study area. The coastal and land-based touristic activities are very common in Aydin, in southwest Turkey. Therefore, the high genetic diversity of *T. vaginalis* in our study could be attributed to the intense human mobility in this region. Previously, actin and ITS gene sequences were used to determine genotypic diversity of *T. vaginalis* isolates in Aydin, similar to our study, high genetic diversity was reported (35, 36). Our study was restricted to the isolates only from the south-west of Turkey, so, microsatellite typing should be performed with the isolates from other regions. The website for microsatellite typing (http://mttype.adu.edu.tr) will be used for storage and comparison of MT data from different regions.

Some double-stranded RNA viruses of Totiviridae can infect *T. vaginalis* isolates and may increase the infectivity of the parasite (8, 37, 38). The first data about the frequency of TVV was acquired in the present study, of the isolated 20% were infected with TVV. The infection rate of TVV was 17.3% in Iran and 14% in Korea (39-41). However, another study from Iran reported a higher rate of TVV1 (50%) than the previous report (42). The prevalence of the virus differs between regions, whereas in Western societies, TVV rates are generally higher than in the East, however, the reasons are remain unclear.

There were few studies on the association of *T. vaginalis* and *Mycoplasma* spp. in the world and no data was available in our country. In recent years, the relationship between the two pathogens was linked to the human vaginal microbiome and immune-pathogenicity of the parasite. It could correlate and reproduce both on cell surface and intracellularly with *T. vaginalis* (43). In addition, *the* infection transmitted to human cervical cells by infected parasite (44). *T. vaginalis* might play a "Trojan horse" role for bacteria during parasitic infections, because the intracellular position of *M. hominis* may contribute to the protection of bacteria from the host immune system in *T. vaginalis* cells (45). In our study, 16.6% of *T. vaginalis* isolates were infected with *M. hominis*. The incidence of *Mycoplasma* spp. in *T. vaginalis* isolates in other countries was reported between 5% and 89.1%. The highest rate in these studies was reported in Cuba, the lowest rate in isolates from Italy, Mozambique and Angola, besides; the rate was reported as 56.7% in Brazil, 20% in the USA, 50% in China, The Netherlands 69% (11, 38, 45).

Conclusion

We presented a new genotyping approach according to the microsatellite polymorphisms of *T. vaginalis*, the website will allow us to collect and compare microsatellite types from all around the world. It will contribute to a better understanding of genetic diversity and population characteristics of the parasite. Our study also presented the first data from Turkey about the presence of TVV and *M. hominis* in *T. vaginalis* isolates.

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Conflict of interest

The authors declare that there is no conflict of interest.

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