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Original Article

Genetic Identification of *Trichomonas vaginalis* by Using the Actin Gene and Molecular Based Methods

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Abstract

Background: *Trichomonas vaginalis* is the agent of urogenital tract infection that causes human trichomoniasis with some serious health complications. More understanding about genetic features of the parasite can be helpful in the study of the pathogenesis, drug susceptibility and epidemiology of the infection. For this end, we conducted analysis of the actin gene of *T. vaginalis* by applying the PCR-SSCP (PCR-Single Stranded Conformational Polymorphism) and nucleotide sequencing method.

Methods: Fifty *T. vaginalis* samples were collected from 950 women attending gynecology clinics in two cities of Iran, Hamadan and Tehran, from November 2010 to July 2011. After axenisation of isolates, all samples subjected to PCR-SSCP and nucleotide sequencing.

Results: According to the SSCP banding patterns and nucleotide sequencing, seven sequence types were detected among the isolates. Alignment of the nucleotide sequences showed five polymorphic sites in the different strain types. Amino acid substitution was not observed in the nucleotide sequence translation of the all sequences.

Conclusion: The actin gene analysis represents genetic diversity of *T. vaginalis* and it suggests that various strains can be responsible for clinically different trichomoniasis in infected individuals. It is expected that further studies will be conducted to increase our knowledge about relationship between the actin gene polymorphism and different biological behavior of the parasite.

Introduction

Trichomonas vaginalis, an anaerobic flagellated protozoan, is the causative agent of human urogenital trichomoniasis with worldwide distribution. This infection is associated with the increased risk of adverse pregnancy outcomes, incidence of cervical neoplasia, infertility and a number of infections, significantly Human Immunodeficiency Virus (HIV) (1-4). The various biological characteristics are one of the most remarkable points of the parasite so that infection with *T. vaginalis* can be commonly observed with diverse clinical features. Infected individuals show clinical patterns in a variety of forms from severe symptomatic to asymptomatic infection (5). Thus, clinical trichomoniasis can be divided into three distinct subgroups, including acute, chronic and latent infection (6). Drug resistance and treatment failure with metronidazole, the only drug of choice for therapy of trichomoniasis, has been reported since 1962 (7). *The centers for Disease Control* and Prevention (CDC) have reported that 5% of *T. vaginalis* isolates have some level of resistance to metronidazole (8).

Despite the clinical significance of trichomoniasis, there are the number of questions about various aspects of epidemiology and biology of the parasite that have not been answered. The use of reliable classification and genetic characterization methods can help to clarify the ambiguities in this field. Commonly, PCR and its related methods, as a sensitive and reliable technique, were extensively applied for genetic studies in organisms. Actin protein is a main component of the cytoskeleton of organisms that has an essential role in cellular mobility and cell interaction. Morphological changes of *T. vaginalis* such as the transformation from flagellate to amoeboid form and surface adhesion are declared to be affected by it. Therefore, actin protein is apparently involves in the biological traits variation of *T. vaginalis* (9-11).

To achieve more information about genetic properties of *T. vaginalis*, we carried out this study to evaluate actin gene, as a genetic marker, for molecular investigation of the parasite by using PCR-SSCP (PCR-Single Stranded Conformational Polymorphism) and DNA sequencing methods.

Materials and Methods

T. vaginalis isolates

Fifty *T. vaginalis* specimens were isolated from 950 women attending gynecology clinics in Hamadan and Tehran, two cities of Iran, from November 2010 to July 2011. Sampling was performed according to the following procedure: two vaginal cotton swabs were taken from participants and examined by wet mount method and Dorset's culture. Then, positive samples subjected to axenic culture using Diamond's medium (TYI-S-33), previously described by Clark and Diamond (12).

DNA extraction and PCR amplification

DNA purification of all isolates was performed by using phenol/chloroform/isoamyl alcohol method (13). A 894 bp nucleotide segment of actin gene (type 6), with the most polymorphism, was selected as a target for PCR amplification by two pairs of primers. A pair of primers amplifying 425 bp nucleotide segment was Actin-S1 (5' GTT GGT GGC AAC GCC AAG GA 3') and Actin-As1 (5' GTG AGA TCA CGG CCA GCG AG 3') and the other pair of primers amplifying 492 bp nucleotide segment was Actin-S2 (5' CCT CGC TGG CCG TGA TCT CA 3') and Actin-As2 (5' CCA CCG ACC CAA ACG GCG TA 3').

The PCR reaction was performed (PeqLab Biotechnologie GmbH,Germany) with this manner: 10 µl of 10x PCR amplification buffer (Roche), 20 pmol of sense and antisense primers, 0.2 mM dNTP mix, 1 µl template

DNA and 1 unit of *Taq* DNA polymerase (Advance Biotechnologies, UK) and made final volume up to 50 µl by adding sterile distilled water. PCR amplification started with primary denaturation at 94ºC, 5 min, and then continued by 35 repetitive cycles in a set of denaturation at 94ºC, 30 sec, annealing at 65ºC, 30 sec, extension at 72ºC, 45 sec, and finally one extension stage at 72ºC for 10 min.

Two PCR amplifications produced anticipated single bands and evaluated by 2% (w/v) agarose gel electrophoresis in 1X TBE buffer, containing ethidium bromide (0.5 µg/ml) under a UV transilluminator (UVP/Upland, USA).

SSCP analysis

SSCP analysis and optimized conditions have been described in more details (14). Briefly, 10 µl SSCP loading buffer was added to 5 µl of PCR product in one microtube and mixed gently. Separation of two strands PCR product was conducted by incubation at 94ºC for 10 min and immediately cooled in a bath of freezing water to avoid of re-annealing of the detached strands.

Electrophoresis of the whole sample size was performed by vertical slab polyacrylamide gel. The samples were run on 8% polyacrylamide gel, a blend of acrylamide and bis-acrylamide (29:1 ratio), glycerol (5%) and using TBE running buffer solution (0.5X) at temperature room and 160 V for at least 20 hours.

Visualization of gel electrophoresis carried out by silver staining method.

After removing the gel from electrophoresis system, it was fixed for 8 min in ethanol solution (10%) completed with acetic acid (0.5%), placed for 15 min in 0.1% silver nitrate solution (w/v), washed two times in distilled water and then used sodium hydroxide solution (1.5%) completed with formaldehyde (0.4%) for developing of gel and finally stopped silver development by sodium carbonate solution (7.5%) for 10 min. After rinsing with distilled water, the gel was dried, covered with cellophane membrane (to avoid drying) and scanned (15).

PCR Amplicon Sequencing

Nucleotide sequencing of two PCR products (425 & 492 bp) was carried out by using the Applied Biosystems Automated 3730xl DNA Analyzer (Bioneer Inc., Korea). Homology survey of sequences was performed by the use of the BLASTn program (http://www.ncbi.nlm.nih.gov/BLASTn) and compared with sequence data of G3 strain, available at GenBank (Accession No XM-001281985). MultAlin program (16) and Chromas software, version 2.33, (http:// www.technelysium.com.au/ chromas.html) also were utilized for multiple sequence alignments and sequence editing, respectively.

Results

The 425 and 492 bp nucleotide fragments of actin gene were amplified separately from *T. vaginalis* genomic DNA belonged to 50 isolates. The PCR amplification of the two fragments led to produce the anticipated single products and agarose gel electrophoresis did not indicate variation in size among all of the amplicons (Fig. 1). Sequence analysis of the both 425 and 492 bp amplicons by SSCP individually demonstrated 4 and 2 different banding patterns, respectively (Fig. 2).

To specify the whole of nucleotide sequence of the 894 bp fragment of actin gene, the number of representative complementary amplicons of the two fragments (425 & 492 bp), with different SSCP banding patterns, were chosen and subjected to sequencing. Nucleotide sequence analysis of the sum of the two fragments, totally 894 bp, determined seven type sequences that have been submitted to GenBank database under accession numbers KC112364 - KC112370. Homology between them and other sequences of *T. vaginalis*, previously submitted in GenBank database, was 99 percent. In the multiple sequence alignment, five polymorphic nucleo-

tide positions were observed among themselves and the sequence of G3 strain (Fig. 3).

Polymorphic nucleotide sites caused silent mutations and no amino acid substitution was observed. Given the SSCP banding patterns and related nucleotide sequences, maximum and minimum frequencies of the sequence types were type I (44%, 22) and VII (2%, 1), respectively. The frequencies of the other types were included type II 34% (17), III 8% (4), IV 4% (2), V 4% (2) and VI 4% (2).

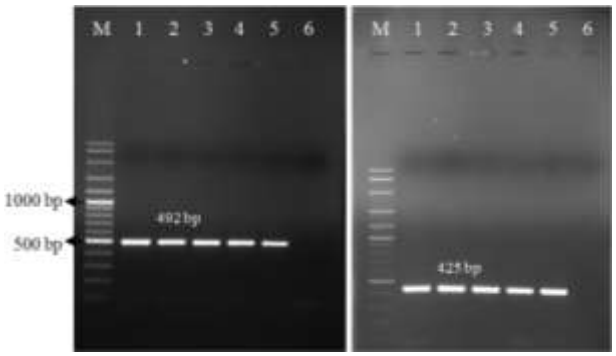

Fig. 1: Agarose gel electrophoresis of PCR product amplification
(425 & 492bp) from *T. vaginalis* isolates.
Lane M: DNA ladder (100 bp), Lane 1-5: some of the *T. vaginalis* isolates
Lane 6: negative control

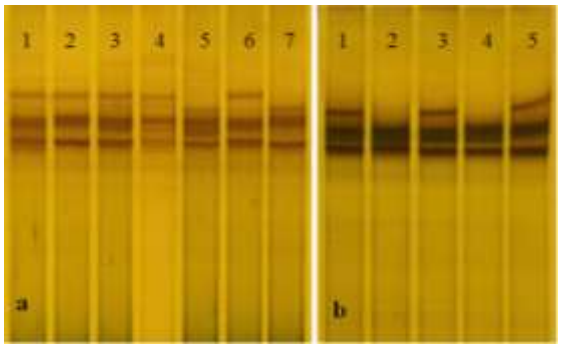

Fig. 2: Representative SSCP analysis of the two amplified segments of actin gene prepared from *T. vaginalis* isolates **a**: SSCP gel analysis belongs to 425 bp segment with 4 banding patterns including: type I (Lane 1 & 4), type II (Lane 2, 3 & 6), type III (Lane 5) & type IV (Lane 7). /**b**: SSCP gel analysis belongs to 492 bp segment with 2 banding patterns including: type I (Lane 1, 3 & 5) & type II (Lane 2 & 4).

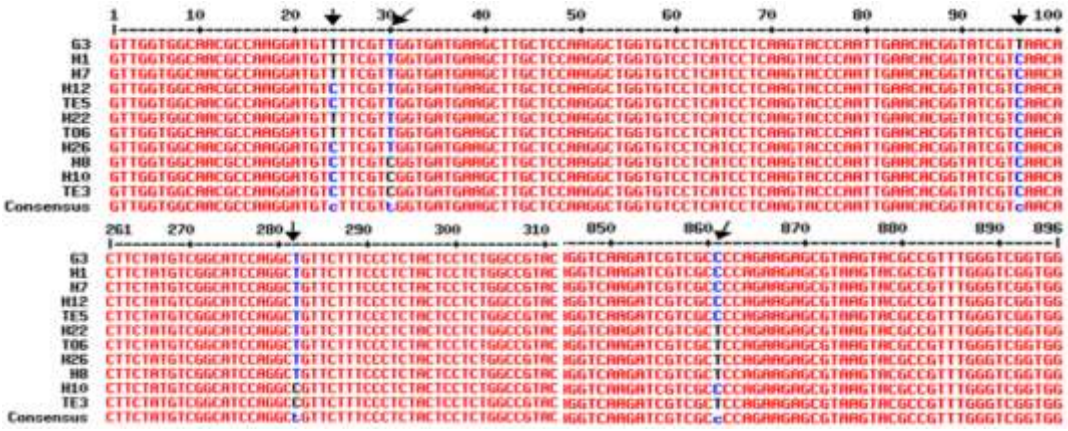

Fig. 3: Partial nucleotide sequence alignment of the actin gene in the seven sequence types of *T. vaginalis*, compared with reference strain (G3 strain, GenBank accession no XM-001281985). Variation in nucleotide sequences is pointed by arrow observed in sequence types: I (H1 & H7), II (H12 & TE5), III (H22 & TO6), IV: (H26), V (H8), VI (H10) & VII (TE3)

Discussion

Diversity of biological properties of *T. vaginalis* including drug resistance, variation of pathogenicity and broad range of clinical manifestations indicates the possibility of different strains or types of *T. vaginalis*, although the phenotypic variations of trichomoniasis can be

due to the condition of hosts. Molecular and genetic studies can facilitate to elucidate the uncertainties about etiology, pathobiology and other aspects of epidemiology of the ambiguous parasite and in this regard, identification of critical genes would be useful. To date, few studies have been conducted to establish a reliable strain typing methods and attempted to determine the correlation between genetic variation and different phenotypic behavior of the parasite. But approximately**,** they have been inconclusive because of probably using different techniques and genetic markers.

In addition to other molecular studies, genetic characterization is the most method utilized for typing of *T. vaginalis*. RAPD (Random Amplification of Polymorphic DNA), RFLP (Restriction Fragment Length Polymorphism) and SSCP (Single Strand Conformation Polymorphism) are three methods to produce the promising results. However, the results of RAPD technique are controversial due to the existence of internal symbiont in the parasite such as *Mycoplasma* species (17).

For the first time in 1997, Vancova and colleagues applied RAPD technique by using five random primers (OPD1-OPD5) and two supplementary primers, for phylogenetic analysis study of *T. vaginalis* (18). Their findings showed correlation between genetic diversity and geographical origin and also metronidazole drug resistance in the studied samples. In 2000, concomitance of ITS1 C66T mutation and metronidazole resistance firstly was introduced by Snipes et al, supported by a few studies (18,24,25), as well as they declared correlation between drug resistance and some RAPD profiles (19). RAPD analysis of symptomatic and asymptomatic isolates by Kaul et al. showed two discrete lineages so that all of the asymptomatic isolates were clustered in one branch (20). Rojas et al introduced a genetic marker of 490 bp in all isolates acquired from light, moderate and severe symptomatic patients whereas the genetic marker was not found in RAPD patterns of asymptomatic isolates (21). In a recent RAPD analysis of Iranian *T. vaginalis*, variation in the genome of the parasites was confirmed, similar to other RAPD studies, but there was no strong association between genetic variability and geographic origin of the isolates (22).

RFLP analysis of HSP70 gene of *T. vaginalis* was conducted and the RFLP profiles indicated considerable genomic diversity in the specimens gathered from in the United States (23). Intergenic spacer (IGS) of the ribosomal DNA gene was analyzed by RFLP technique and no correlation was seen between IGS polymorphism and clinical characteristics of trichomoniasis (24).

So far, some researchers have utilized sequence analysis of the internal transcribed spacer (ITS) region of the rDNA and all of the studies showed at least two major sequence types, wild and mutant (19, 25, 26). SSCP analysis of ITS1/5.8s/ITS2 and flanking regions was applied by Matini et al. and resulted two distinct banding patterns confirmed by sequence nucleotide analysis (14). Recently, microsatellite marker analysis has been used in order to study the genetic polymorphism and phylogenetic inference of the obscure flagellated parasite and the results demonstrated genetic variation among seven commonly used laboratory strains (27).

The outcomes of the all researches about genotyping and phylogenetic analysis of *T. vaginalis* point out that the parasite is a genetically diverse organism.

In biological studies, actin can be considerable as a distinguishing marker because of its structural role in organisms. Crucitti and colleagues conducted genotyping of *T. vaginalis* by using actin gene and PCR-RFLP. In their study, three ATCC reference strains and 151 isolates, collected from Democratic Republic of Congo and Zambia, were used for amplifying a 1100 bp fragment of the actin gene. PCR products were digested with three restriction enzyme including *Hid*II, *Mse*II and *Rsa*I so that the combinations of three restriction patterns represented several different genotypes of the parasite. In addition, they reported mix

infection in the samples belonged to Zambia (28).

In the present study, seven types of *T. vaginalis* were detected according to the SSCP banding patterns and their nucleotide sequences and no mixed infection was observed in the all samples. Nucleotide sequences analysis showed five polymorphic sites that none of them caused amino acid substitution. In the study that conducted by Crucitti et al., eight strains types and 15 different single nucleotide changes were demonstrated, with regard to RFLP patterns and nucleotide sequences, and amino acid substitution detected in translation of the actin gene because the three polymorphic nucleotides altered the amino acid sequences.

Analysis of the longer fragment of the gene, the use of a large number of samples and collected isolates from the geographically more widespread areas with high prevalence by Crucitti et al., may be responsible for the differences between the results of the two studies performed on the actin gene of the parasite.

Conclusion

Highly polymorphism in the open reading frame of the actin gene of *T. vaginalis* suggests that this target gene can be considered as a potential genetic marker for molecular epidemiology and genotyping of *T. vaginalis*. Further investigations need to evaluate the efficiency of the marker in epidemiological and biological studies and also correlation between the gene polymorphism and phenotypic behavior of the parasite must be determined.

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References

1. Schwebke JR, Burgess D. Trichomoniasis. Clin Microbiol Rev. 2004; 17(4): 794-803.
2. Zhang ZF, Graham S, Yu SZ, Marshall J, Zielezny M, Chen YX, Sun M, Tang SL, Liao CS, Xu JL, Yang XZ. *Trichomonas vaginalis* and cervical cancer. A prospective study in China. Ann Epidemiol. 1995; 5(4):325–32.
3. Soper D. Trichomoniasis: under control or undercontrolled? Am J Obstet Gynecol. 2004; 190(1):281-90.
4. Sorvillo F, Smith L, Kerndt P, Ash L. *Trichomonas vaginalis*, HIV, and African-Americans. Emerg Infect Dis. 2001; 7(6):927-32.
5. Matini M, Rezaie S, Mohebali M, Maghsood A, Rabiee S, Fallah M, Rezaeian M. Prevalence of *Trichomonas vaginalis* Infection in Hamadan City, Western Iran. Iran J Parasitol. 2012; 7(2):67-72.
6. Petrin D, Delgaty K, Bhatt R, Garber G. Clinical and microbiological aspects of *Trichomonas vaginalis*. Clin Microbiol Rev. 1998; 11(2):300-17.
7. Robinson SC. *Trichomonal vaginitis* resistant to metronidazole. Can Med Assoc J. 1962; 86(14):665.
8. Narcisi EM, Secor WE. In vitro effect of tinidazole and furazolidone on metronidazole-resistant *Trichomonas vaginalis*. Antimicrob Agents Chemother. 1996; 40(5):1121-5.
9. Bricheux G, Brucherolle G. Molecular cloning of actin genes in *Trichomonas vaginalis* and phylogeny inferred from actin sequences. FEMS Microbiol Lett. 1997; 153(1): 205–13.
10. Benchimol M. Trichomonads under microscopy. Microsc Microanal. 2004; 10(50: 528–50.
11. Crucitti T, Abdellati S, Van Dyck E, Buve A. Molecular typing of the actin gene of *Trichomonas vaginalis* isolates by PCR-restriction fragment length polymorphism. Clin Microbiol Infect. 2008; 14(9): 844-52.
12. Clark CG, Diamond LS. Methods for cultivation of luminal parasitic protists of clinical importance. Clin Microbiol Rev. 2002; *15*(3): 329-41.
13. Rezaie S, Ban J, Mildner M, Poitschek C, Brna C, Tschachler E. Characterization of a cDNA clone, encoding a 70 kDa heat shock protein from the dermatophyte pathogen *Trichophyton rubrum*. Gene. 2000; 241(1): 27-33.

14. Matini M, Rezaeian M, Mohebali M, Maghsood AH, Rabiee S, Rahimi-Foroushani A, Fallah M, Miahipour A, Rezaie S. Genotyping of *Trichomonas vaginalis* isolates in Iran by using single stranded conformational polymorphism-PCR technique and internal transcribed spacer regions. Trop Biomed. 2012; 29(4):605-12.
15. Robinson MD. Manual SSCP and Heteroduplex analysis gels. In: Guide to mutation detection, Taylor, G.R. & Day, I.N.M. (editors). New Jersey: Wiley, pp.202-207; 2005.
16. Corpet F. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res. 1988; 16(22): 10881-90.
17. Dessi D, Delogu G, Emonte E, Catania MR, Fiori PL. Rappelli P. Long-term survival and intracellular replication of *Mycoplasma hominis* in *Trichomonas vaginalis* cells: potential role of the protozoon in transmitting bacterial infection. Infect Immun. 2005; 73(2): 1180-86.
18. Vanacova S, Tachezy J, Kulda J, Flegr J. Characterization of trichomonad species and strains by PCR fingerprinting. J Eukaryot Microbiol. 1997; 44(6): 545-52.
19. 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(8): 3004-9.
20. Kaul P, Gupta I, Sehgal R, Malla N. *Trichomonas vaginalis*: random amplified polymorphic DNA analysis of isolates from symptomatic and asymptomatic women in India. Parasitol Int. 2004; 53(3): 255-62.
21. Rojas L, Fraga J, Sariego I. Genetic variability between *Trichomonas vaginalis* isolates and correlation with clinical presentation. Infect Genetic Evol. 2004; 4(1): 53-8.
22. Valadkhani Z, Kazemi F, Hassan N, Aghighi Z, Esmaili I, Talebi M. Gene Diversity of *Trichomonas vaginalis* Isolates. Iran J Parasitol. 2011; 6(3):101-6.
23. Meade JC, Mestral J, Stiles JK, Secor WE, Finley RW, Cleary JD, Lushbaugh WB. Genetic diversity of *Trichomonas vaginalis* clinical isolates determined by EcoRI restriction fragment length polymorphism of heat-shock protein 70 genes. Am J Trop Med Hyg. 2009; 80(2): 245-51.
24. Simões-Barbosa A, Lobo TT, Xavier J, Carvalho SE, Leornadecz E. *Trichomonas vaginalis*: intrastrain polymorphisms within the ribosomal intergenic spacer do not correlate with clinical presentation. Exp Parasitol. 2005; 110 (2): 108-13.
25. Rivera WL, Ong VA, Masalunga MC. Molecular characterization of *Trichomonas vaginalis* isolates from the Philippines. Parasitol Res. 2009; 106(1): 105-10.
26. Xiao JC, Xie LF, Fang SL, Gao MY, Zhu Y, Song LY, Zhong HM,Lun ZR. Symbiosis of *Mycoplasma hominis* in *Trichomonas vaginalis* may link metronidazole resistance in vitro. Parasitol Res. 2006; 100(1): 123-30.
27. Conrad M, Zubacova Z, Dunn LA, Upcroft J, Sullivan SA,Tachezy J, Carlton JM. Microsatellite polymorphism in the sexually transmitted human pathogen *Trichomonas vaginalis* indicates a genetically diverse parasite. Mol Biochem Parasitol. 2011; 175(1): 30-8.
28. Crucitti T, Abdellati S, Van Dyck E, Buve. Molecular typing of the actin gene of *Trichomonas vaginalis* isolates by PCR-restriction fragment length polymorphism. Clin Microbiol Infect. 2008; 14(9): 844-52.