Genetic Relatedness of *Trichomonas vaginalis* Isolates to the Clinical Variability

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Abstract

**Background:** Trichomoniasis manifestation shows an extensive clinical variability ranging from no symptoms to severe presentation and sequel. An association is suggestive between the high genetic diversity of *Trichomonas vaginalis* isolates and its clinical variability. The aim of our study was to identify *T. vaginalis* isolates in symptomatic and asymptomatic females besides, studying their possible relation to different genotypes in our locality.

**Methods and findings:** Two hundred female were included in the study and screened for *T. vaginalis* infection using the wet mount, culture in Diamond’s media and PCR amplification which targeted actin gene. For genotyping by RFLP, products of PCR amplification of actin gene were digested by 3 restriction enzymes (HindII, Msel and Rsal). *Trichomonas vaginalis* was detected in 12% (12/100) and 1% (1/100) in symptomatic and asymptomatic females respectively. But, by using modified Diamond culture and PCR, 22% (22/100) symptomatic and 5% (5/100) asymptomatic females were positive for *T. vaginalis*. After digestion of PCR products using restriction enzymes, different electrophoretic band patterns were demonstrated. Interpretation of these patterns, only one distinct electrophoretic pattern of actin genotype H in all studied isolates was demonstrated.

**Conclusion:** Our results concluded that genetic variation does not correlate with the clinical variability of *T. vaginalis* as one genotype band pattern of actin gene was detected in both symptomatic and asymptomatic groups. Further studies in a large scale of isolates are needed to elucidate this finding.

Introduction

*Trichomonas vaginalis* is the most common preventable sexually transmitted infection and considered as one of the neglected tropical parasitic infections [1]. Trichomoniasis is presented with various clinical manifestations. More than 80% of patients are asymptomatic [2]. Common manifestations of acute infection include: vaginitis, vulval irritation and inflammation, malodorous vaginal discharge and punctate microhemorrhages on the cervix known as ‘strawberry cervix’ [3]. Infection with *T. vaginalis* frequently leads to inflammatory pelvic disease and infertility [4]. Moreover, infection with *T. vaginalis* predisposes to prostate cancer and acquisition of other infections like the human immunodeficiency virus (HIV) and Human papillomavirus (HPV) [5-7].

There is evidence regarding the correlation between *T. vaginalis* genotypes and clinical manifestations of trichomoniasis but, it is still not definite. Studies suggesting this correlation based on the matching between the extensive clinical variability in trichomoniasis and its sequel, and extensive clinical variability in trichomoniasis [5,8-10]. Studies have shown concordance between *T. vaginalis* genotype and the clinical presentation of trichomoniasis, like susceptibility to metronidazole, presence of *T. vaginalis* virus, or concomitant Mycoplasma infection [11,12]. On the other hand, other studies based on RAPD and RFLP did not demonstrate any concordance between *T. vaginalis* genotypes and its symptoms [11,13].

*Trichomonas vaginalis* has a large genome of 160 Mb with approximately 60000 protein-coding genes. Most of its genome has repeats and transposable elements [14]. Actin protein of *T. vaginalis* is the main component of its cytoskeleton and has an essential role in cellular mobility and cell interaction [15]. This structural protein has a well-
conserved ubiquitous nature, making it a feasible choice for intra-species molecular identification [16].

Commonly, PCR and its related methods were widely used for genetic studies in organisms. Random amplified polymorphic DNA (RAPD) method was used to reveal an association between T. vaginalis genotype and metronidazole resistance [8]. Also, restriction fragment length polymorphism (RFLP) and PCR-RFLP methods were applied to demonstrate the genetic diversity in clinical isolates of T. vaginalis [15,17]. PCR-RFLP has advantages of both the sensitivity of PCR and reliability of RFLP and so, it is considered as the best method for isolates genotyping [18,19].

The aim of this study was to identify T. vaginalis infection in both symptomatic and asymptomatic women and to study the possible correlation between T. vaginalis genotypes and clinical variability based on PCR-RFLP of actin gene.

Materials and Methods

Ethics statements

This study was approved by the Institutional Review Board of the faculty of medicine, Mansoura University, Egypt. Prior to enrollment, a signed consent was gained from each participant. Females with confirmed trichomoniasis were all treated and followed up.

Study participants

This study was carried out in Gynecology out-patients clinic of Mansoura University Hospital, Biochemistry and Parasitology department, faculty of medicine, Mansoura University, Egypt. Vaginal swabs were collected from 100 female with symptoms suggestive of vaginitis and another 100 asymptomatic female. The study was conducted in the period from December 2013 to February 2016. The age of the study participants ranged from 18-48 years. Females who were pregnant, received anti-parasitic or anti-trichomonal therapy, used vaginal wash within the previous two weeks were excluded from the study. Sociodemographic and clinical data were recorded.

Specimen collection, processing and culture

Enrolled females were examined in accordance with a standard clinical protocol. Three speculum-assisted vaginal swabs samples were collected from the posterior fornix of the vagina of each participant. The first swab was kept in a tube containing 3 ml sterile phosphate Buffered Saline (PBS) for wet mounts microscopy which is done immediately according to McCann JS [20]. Other two were transferred to a 10 ml pre-wormed screw-cap tube with sterile modified Diamond’s medium for culture. The medium was supplemented with streptomycin-penicillin at 50 µg/mL and 10% heat-inactivated bovine serum. Cultures were incubated at 37°C and examined for the presence of T. vaginalis by microscopy every day for 5 to 7 days post-inoculation.

DNA extraction and PCR amplification

Positive culture media was centrifuged and the pellet was washed twice in PBS at 4000 rpm for 10 min. and stored at -20°C. Genomic DNA was extracted from pellets by Thermos Scientific Gene JET Genomic DNA Purification Kit (USA) according to the manufacturer’s instructions. Quantity and purity of the extracted DNA was measured with Nanodrop (Thermo scientific NanoDrop 2000 spectrophotometer, USA) while, the quality of extracted DNA was tested by 1.5% agarose gel electrophoresis. The actin gene fragments were amplified.

Actin gene amplification: Nested PCR was employed and two sets of primers were used to amplify the (1100 bp) of T. vaginalis actin gene [21]. The outer and inner primers were selected from Crucitti T et al. [15]. The outer set primers were Tv8S Forward: 5′-TCTGGATGGCTGAAGAAGAG-3′ and Tv9 Reverse: 5′-CAGGGTACATCGTATTGGTC-3′, the inner primers were Tv10S Forward: 5′-CAGACACTGTAGTATCG-3′ and Tv11 Reverse: 5′-CGGGTACATCGTTATCG-3′.

Reaction with outer primers: Each PCR reaction mixture contain 40 µl (20 µl of master mix, 10 µl of DNA template, 2 µl of forward outer primers, 2 µl of reverse outer primers and 6 µl sterile distilled water). PCR amplification was performed in two stages using thermocycler (TECHEN TC-312, FTC3102D, Barloworld Scientific Ltd. Stone, Stafford Shire, and UK). The first stage consist of 10 cycle, the first cycle was preceded by 5 min of denaturation at 95°C , then each cycle consisted of denaturation at 94°C for 30 sec, of annealing at 55°C for 30 sec, and extension at 72°C for 3 min. The second stage consisted of 25 cycles with the same denaturation and annealing steps. The extension step was extended by 5 seconds per cycle. The last cycle was followed by a 7 min final extension.

Reaction with inner primers: Using the same amplification program as outer primers with total reaction of 50 µl (25 µl master mix, 2 µl forward inner primers, 2 µl reverse inner primers, 4 µl PCR product and 17 µl DW).

The expected PCR products 1100 bp of amplified actin gene were confirmed by electrophoresis 2% (w/v) agarose gel, stained by ethidium bromide and visualized under an UV trans-illuminator.

Restriction fragment length polymorphism (RFLP)

For RFLP technique, amplicons of actin gene were digested by HindIII, MseI and Rsal restriction enzymes. In brief, in each PCR tube, 17 µl of sterile distilled water, 2 µl (10X) of Fast digest buffer, 10 µl of amplified PCR product and 1µl of the restriction endonuclease are added. The reaction mixture was incubated at 37°C for 15 minutes then the enzyme was inactivated by heating for 5 min at 65°C in heat block. Finally, for visualization of product after digestion, the reaction was run on 2% agarose gel, run with 50 bp DNA markers at 100 volts for 45 minutes and then visualized via light UV transilluminator.
Statistical analysis

The data was plotted and analyzed using the statistical software package SPSS version 22.

Results

The total number of females involved in this study was 200, of whom 100 symptomatic and 100 were asymptomatic. The mean age was (35.09 ± 9.82) for symptomatic female and (33.2 ± 11.08) for asymptomatic ones. Significant high prevalence was detected in age group 28-37 years (45.5%). The overall prevalence of *T. vaginalis* in both was 27/200 (13.5%), in symptomatic (22%) and (5%) in asymptomatic ones. The main reported complaint of positive symptomatic cases was vaginal discharge (81.8%), lower abdominal pain and itching (63.6%), dyspareunia (50%) and dysuria (40.9%). No statistically significant difference between positive symptomatic and asymptomatic cases regarding age, marital status and residence (Table 1).

Thirteen positive *T. vaginalis* samples were detected (6.5%) by using wet mount method, while both culture and PCR methods detected 27 (13.5%) positive samples. All positive samples by culture were also positive by nested PCR and actin gene (1100 bp) was amplified from extracted DNA of all positive cases (Figure 1).

The amplified products of actin gene by nested PCR were digested by three restriction endonucleases including, HindII, Msel and Rsal. As shown in Figures 2 and 3 digestions of the amplified products with HindII restriction enzyme yielded four DNA bands at 426, 401, 213, and 60 bp. While, the restriction enzyme Rsal digested the PCR products into five bands at 568, 236, 106, 103, and 87 bp. Using Msel, two bands were detected at 519 and 581bp. On Crucitti et al. [15] as shown in Table 2, genotype H was defined as a representative of pattern 2 after HindII and Msel digestion and pattern 1 after Rsal digestion. Accordingly, actin genotype H is the only genotype detected in all positive 27 symptomatic and asymptomatic *T. vaginalis* isolates with no statistical difference between both groups.

### Table 1 Sociodemographics of positive symptomatic and asymptomatic cases.

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic Positive <em>T. vaginalis</em> n=22</th>
<th>Asymptomatic positive <em>T. vaginalis</em> n=5</th>
<th>Test of significance P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>35.09 ± 9.82 (20.0-58.0)</td>
<td>33.2 ± 11.08 (19.0-50.0)</td>
<td><em>t</em>=0.381 P=0.71</td>
</tr>
<tr>
<td>Age groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-27</td>
<td>4 (18.2)</td>
<td>1 (20.0)</td>
<td>MC P=0.82</td>
</tr>
<tr>
<td>28-37</td>
<td>10 (45.5)</td>
<td>3 (60.0)</td>
<td></td>
</tr>
<tr>
<td>38-47</td>
<td>5 (22.7)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>48-58</td>
<td>3 (13.6)</td>
<td>1 (20.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Marital status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Married</td>
<td>21 (95.5)</td>
<td>5 (100.0)</td>
<td>FET P=1.0</td>
</tr>
<tr>
<td>Divorced</td>
<td>1 (4.5)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Residence</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>9 (40.9)</td>
<td>2 (40.0)</td>
<td>FET P=1.0</td>
</tr>
<tr>
<td>Rural</td>
<td>13 (59.1)</td>
<td>3 (60.0)</td>
<td></td>
</tr>
</tbody>
</table>

Z=Mann whitney test, FET: Fischer exact test, χ²= Chis-square test, MC:Monte Carlo test, *P* value significant <0.05, SD= standard deviation
Figure 1 Ethidium bromide- stained 2% agarose gel electrophoresis of nested PCR product of actin gene from T. vaginalis positive samples (1100-bp). Lane 1, 2 T. vaginalis positive symptomatic samples; lane 3, 50 bp DNA ladder; Lane 4, 5, T. vaginalis positive asymptomatic samples.

Table 2 Size of fragments, pattern groups and actin genotypes of the T. vaginalis [15].

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Restriction with HindII (bp)</th>
<th>Restriction with RsaI (bp)</th>
<th>Restriction with MseI (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>827, 213, 60</td>
<td>568, 236, 190, 106</td>
<td>581, 519</td>
</tr>
<tr>
<td>E</td>
<td>827, 213, 60</td>
<td>568, 236, 106, 103, 87</td>
<td>581, 315, 204</td>
</tr>
<tr>
<td>G</td>
<td>426, 401, 213, 60</td>
<td>568, 236, 190, 106</td>
<td>581, 519</td>
</tr>
<tr>
<td>H</td>
<td>426, 401, 213, 60</td>
<td>568, 236, 106, 103, 87</td>
<td>581, 519</td>
</tr>
<tr>
<td>I</td>
<td>426, 401, 213, 60</td>
<td>452, 236, 190, 116, 106</td>
<td>581, 519</td>
</tr>
<tr>
<td>M</td>
<td>426, 401, 213, 60</td>
<td>568, 236, 190, 106</td>
<td>581, 333, 186</td>
</tr>
<tr>
<td>N</td>
<td>426, 401, 213, 60</td>
<td>568, 236, 106, 103, 87</td>
<td>581, 333, 186</td>
</tr>
<tr>
<td>P</td>
<td>426, 401, 213, 60</td>
<td>452, 236, 116, 106, 103, 87</td>
<td>581, 333, 186</td>
</tr>
</tbody>
</table>

Discussion

Trichomoniasis is highly prevalent, frequently asymptomatic, and simply communicable among sex partners [22,23]. Trichomoniasis is characterized by significant heterogeneity in its clinical presentation as pathogenicity, metronidazole sensitivity, sequence to infection, and susceptibility to acquisition of other infectious agents [24]. Whether this clinical variability is caused by host factors or to differences in the phenotypic expression of individual T. vaginalis isolates is not clear. However, recent advances in genetic characterization of T. vaginalis isolates showed that the extensive clinical variability in trichomoniasis and its disease consequences are matched by significant genetic diversity in the organism itself, suggesting a correlation between the genetic identity of isolates and their clinical manifestations [10]. A number of investigations using different genetic markers as well as different molecular techniques such as RFLP and microsatellite technique have been done [25]. But, this association is still not proven and so, we studied this association among females in our locality.

In the present study, the overall prevalence of T. vaginalis infection was 13.5%. Globally, prevalence estimates vary from 0.9%-80% [26,27]. In some countries, it had wide variation: 1.2% in Libya and Jordan [28], 3.2% in Turkey [29] and 28.1% in Saudi Arabia [30]. In Egypt, several studies were carried out and revealed variant prevalence among symptomatic women with T. vaginalis, 10.16% in Cairo [31], 11% in Benha [32] and 12.7% in Minia [33]. However, higher prevalence was recorded in Cairo (23%) [34], 38.37% in Mansoura [35] and 79.9% in Alexandria [36]. The discrepancy between our results and those detected by Hegazy MM et al. [35] may be due to large number of examined females and using other diagnostic methods.

Strain typing techniques are valuable methods to study the epidemiology and diversity of parasitic infections. PCR-RFLP technique based on the actin gene amplification was considered as a sensitive and discriminative method for strain typing of T. vaginalis clinical isolates. In previous studies, digestive patterns of the actin gene with each of restriction
enzyme HindII, Rsal and Msel, detected 8 different genotypes as the major genotypes in Zambia and Kinshasa, with G and E as the most prevalent genotypes, respectively Crucitti T et al. [15]. Studying genetic diversity using PCR-RFLP technique targeting actin gene in our study, showed that genotype H is the only detected type in both symptomatic and asymptomatic groups with no difference between symptomatic or asymptomatic groups. This is in agreement with other studies which revealed no difference between symptomatic and asymptomatic groups using PCR-RFLP [37] and RAPD technique [9,13,26]. Also, in a study carried out by Rojas et al. [38], a genetic band of 490 bps was detected from T. vaginalis isolates collected from symptomatic cases of trichomoniasis and was absent in isolates from asymptomatic studied participant. On the other hand, genotyping of T. vaginalis, using PCR-RFLP by Crucitti et al. [15] and Momeni et al. [39], found eight different types (A, E, G, H, I, M, N, and P) and five types (G, E, H, I and mixed genotypes of G and E) respectively. The variation of results may be due to large sample size from wide regions with high prevalence rate.

**Conclusion**

In conclusion, PCR-RFLP technique is a dependable method to study different T. vaginalis genotypes in a population. Genotype H is the only genotype in the studied symptomatic and asymptomatic group concluding that no association is present between T. vaginalis genotypes and its clinical variability in our locality. Further studies on large scale are needed to prove this finding.

**References**


**Figure 3** Gel electrophoresis of DNA fragments obtained after digestion of T. vaginalis PCR products of actin gene by Msel restriction enzyme. Lane 1 is a 100 bp DNA ladder; Lane 2, shows 2 bands pattern after digestion with Msel enzyme at 519 and 581.


