Genotyping of *Trichomonas vaginalis* isolates from Egypt

Original Article

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ABSTRACT

Background: Trichomoniasis is the most prevalent non-viral sexually transmitted infection worldwide. The outcome of infections is governed by several parasite and host factors. Molecular typing methods revealed two-type population structures for *T. vaginalis*, type I and type II that may differ in pathogenicity, drug resistance, and clinical presentation.

Objective: To elucidate the genotype of the Egyptian isolates of *T. vaginalis* using multilocus sequence typing (MLST). In part, this study aims to evaluate possible relationship between genotypes and growth kinetics, metronidazole (MTZ) susceptibility and clinical presentation.

Patients and Methods: Three hundred vaginal washouts were collected from Egyptian women patients aged 20-45 years suspected of having trichomoniasis. Microscopically, positive samples were cultured on modified trypticase yeast extract maltose (TYM) medium and isolates were submitted to MLST targeting three housekeeping genes namely tryptophanase (*p1*), alanyl tRNA synthetase (*p8*) and DNA mismatch repair protein (*p13*).

Results: Of the 300 samples collected, 12 (4%) proved positive for *T. vaginalis* by wet mount examination and culture. MLST proved that Egyptian isolates comprised two types, genotype I, constituting 70 % of the isolates and genotype II, 20 %. Mixed infection was detected in 10% of cases. No correlation was found between genotype and growth kinetics, MTZ susceptibility and clinical presentation.

Conclusion: MLST is important in investigating the genetic diversity of *T. vaginalis* Egyptian isolates. It is recommended that a future larger multicenter study is carried out, whereby a larger number of isolates obtained from both females and males is investigated for a complete picture of genetic diversity and epidemiology.

Keywords: genotyping, growth kinetics, metronidazole resistance, MLST, *T. vaginalis.*

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INTRODUCTION

Trichomoniasis is the most prevalent non-viral sexually transmitted infection worldwide. It has been estimated that 276 million new infections occurred in 2008 with 11.5% increase over the 2005 incidence rate[1]. Worldwide, the estimated prevalence rates vary with geographic location, age, race, community and the method used for diagnosis. In 2016, the WHO global estimate for trichomoniasis in women was estimated at 5.3% and 0.6% in men with a total incidence of 156 million cases[2]. In Egypt, a prevalence rate of 8.0% was recently documented among symptomatic women in Beni Suef Governorate[3]; in addition to 11% in Benha, Qalyubia Governorate[4], and 5% in Cairo[5]. Another older study recorded prevalence rate of 27-57% using different techniques for diagnosing trichomoniasis in Shebin El-Kom, Menoufia Governorate[6].

While infections are mostly asymptomatic in men, women usually present with vaginal discharge, pruritus and dysuria[7,8]. Trichomoniasis is considered an important risk factor for herpes simplex virus type II infections and HIV transmission and acquisition[9,10]. Data from studies in Africa recorded an increase in HIV transmission associated with trichomoniasis[11-13]. In an American study, the investigators detected *T. vaginalis* in 20% of HIV-infected pregnant women using PCR assay[14]. Moreover, it may be associated with cervical cytological abnormalities and cervical cancer[15-18]. Such a difference in clinical traits as virulence, pathogenicity and drug resistance, signifies the need to link phenotypic variation to genotype[19].

In Egypt, former attempts to investigate the diversity of *T. vaginalis* included isoenzyme patterns[20], serotyping[21], immunoblotting[22,23], biological variability[24,25] and HSP70-RFLP[26]. These studies concluded that the different clinical isolates have different and common patterns at the levels of antigens, immunogens, pathogenicity and MTZ resistance.

Molecular typing methods revealed a two-type population structure for *T. vaginalis*, type I and type II[27-31]. Genotype I infections were found to have lower probability of associated discolored discharge, especially bloody discharge, bleeding during physical examination, or presence of greater than 20% clue cells. Infections with genotype II were significantly associated with these pathological findings. The increase in clue cells seen with genotype II are indicative of bacterial vaginosis, providing a change in the vaginal microenvironment that is favorable for infection with other microbial pathogens. Genetic diversity for *T.*

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gallinae was reported in Australia[32], and Europe[33]. Utilizing PCR-RFLP targeting actin gene, three studies were carried out in Turkey[34], China[31] and Iran[35]. Among twenty *T. vaginalis* isolates from symptomatic females, genotype E was the most common, followed by G, with only one isolate assigned for H, and two mixed isolates of genotypes E and H[34]. In another report, out of 68 *T. vaginalis* isolates, two E and H genotypes were found in addition to mixed genotypes in three isolates[31]. Recently, investigators detected three genotypes (E, G and I) in Iranian isolates of *T. vaginalis* infected with dsRNA viruses[35]. Moreover, the majority of isolates obtained from HIV positive women were parasites from genotype II[28]. Conrad *et al.*[36] found that type I parasites are more likely to be detected by wet-mount (microscopic) diagnosis than are infections with type II parasites and were less frequently MTZ resistant than type II. Furthermore, they found that type I was more frequently infected with the pathogenic *T. vaginalis* virus (TVV)[36].

In bacteria and eukaryotic pathogens, MLST has been used successfully to describe population diversity, delimit species, identify genetic components of important clinical phenotypes, and track the spread of epidemics[31,37-39] Moreover, the portability of its data allows comparison of results from different laboratories[38,40]. However, so far, MLST was not previously used on Egyptian *T. vaginalis* isolates. Hence, the aim of the present study is to elucidate the genotype of the Egyptian isolates of *T. vaginalis* using MLST targeting three housekeeping genes namely tryptophanase (*p1*), alanyl tRNA synthetase (*p8*) and DNA mismatch repair protein (*p13*)[28]. This would help in better understanding of the molecular epidemiology, phylogenetic relationship and population genetics of Egyptian isolates of *T. vaginalis*. Also, in part, this study aims to evaluate possible relationship between genotypes and clinical presentation of trichomoniasis, growth kinetics and MTZ susceptibility.

PATIENTS AND METHODS

Study design: A cross-sectional study was done during the period from May 2015 to July 2017 on vaginal washout samples collected from women suspected for trichomoniasis, attending the Early Cancer Detection Unit of Gynecology Obstetrics Hospital, Ain Shams University, and Outpatient Clinics of Cairo University and Ministry of Health Hospitals. Vaginal washout specimens were subjected to direct wet mount examination and culture on TYM medium. Positive cultures were kept frozen at -20°C until further subjected to MLST at the Molecular Department, Animal Health Research Center, Cairo University.

Selection of patients and history taking: Women aged from 20-45 years, complaining of vaginal discharge and other symptoms as pruritus, burning micturition and dyspareunia were included. A predesigned questionnaire was used for history taking from all cases. It included: demographic data: age, residence, parity, any gynecological problems as infertility, presenting complaints and treatment received (if any).

Vaginal examination: Vaginal examination was done by an experienced gynecologist. Findings including, character of discharge, congested cervix, erythema of vaginal wall, cervical erosions or ulceration, bleeding on examination, pain on moving cervix or adnexa, were recorded.

Collection of vaginal washout specimens: Three hundred sterile saline washout specimens were collected aseptically into sterile screw-capped tubes, and labeled with the patient's name, number and date of collection.

Wet mount examination and culture: One drop of the freshly collected vaginal washouts was examined with 10x and 40x of an inverted microscope, searching for the motile flagellate *T. vaginalis* trophozoites. Each washout specimen was cultured on modified TYM medium supplemented with 10% bovine serum albumin, 100 U penicillin, 100 µg of streptomycin and 50 µg amphotericin B/ ml of culture medium[41]. Examination started after 24 h incubation at 37°C for the presence of viable motile *T. vaginalis* trophozoites. Microscopic examination was repeated every 24 h for seven days. Positive cultures were maintained by sub-culturing every 24-48 h into a new culture tube.

Growth characteristics of *T. vaginalis* isolates: The growth kinetics for each isolate was estimated by inoculating 10^5 trophozoites into 2 tubes containing 10 ml of sterile culture medium followed by counting the trophozoites every 24 h over seven days using a hemocytometer. Two counts were made from each tube, i.e. 4 counts for each isolate inoculated into 2 culture tubes. Growth curves were drawn for each isolate by plotting the parasites numbers against time. Length of log phase, mean of growth peak, generation time (GT), and number of divisions were calculated for each isolate and compared as previously described[42].

Metronidazole susceptibility testing: Susceptibility for MTZ was conducted in 96-well microtitration plates by exposing 10^5 parasites/ml to serial twofold dilutions of MTZ ranging from 400 to 0.4 µg/ml according to Matini *et al.*[43]. The minimum inhibitory concentration (MIC) for each isolate was carried out in triplicate under aerobic and anaerobic incubation for 48 h at 37 °C.

DNA Extraction: DNA was extracted using the QIAamp® DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The purity and concentration of the extracted DNA was assessed spectrophotometrically, before storage at 4°C for further analysis.

PCR amplification targeting *tvk3/7* repetitive gene: Conventional PCR targeting *tvk3/7* repetitive gene, according to Abdolali *et al.*[44], was done to confirm diagnosis in positive samples and to detect other positive samples that might have given negative results by microscopy and culture.

PCR amplification of *p1, p8,* and *p13* genes: Samples proved positive by direct microscopy and culture, and confirmed by repetitive *tvk3/7* gene amplification were subjected to PCR targeting three house-keeping genes, *p1*, *p8* and *p13*, according to the technique described by Cornelius *et al.*[28] in such a way to amplify 489-bp, 494-bp and 491-bp, respectively. Briefly, PCR was performed in 50 µl PCR final volumes containing 0.2 µM concentrations of forward primer, 0.2 µM concentrations of reverse primer, 0.2 mM concentrations of each dNTPs, 2.5 mM $MgCl_2$, 1.25U of Taq DNA polymerase and 5 µl of template DNA.

Genotyping of *T. vaginalis* isolates: For genotyping of the isolates by MLST of the three house-keeping genes, the amplification products were purified, sequenced in both directions using BigDye® 3.1 sequencing reagents (Applied Biosystems, USA). The obtained sequences were first edited to create consensus sequences. This was followed by multiple alignments with previously published reference strains retrieved from GenBank using Bioedit software, version 7.1 to determine *Trichomonas* genotype. Cladograms of the sequences at the level of the individual three loci, was constructed by Geneious version 10.2.3 software.

Statistical analysis: Data were tabulated and analyzed using Statistical Package for Social Science (SPSS version 16, Chicago, USA). Fisher exact test was used to examine the relationship between two quantitative variables. *P* value <0.05 was considered statistically significant.

Ethical considerations: The study design was reviewed and approved by the Research Ethics Committee, Faculty of Medicine Ain Shams University, according to the regulation of Egyptian Ministry of Higher Education in accordance to declaration of Helsinki. Informed consent was obtained from each participant in the study after clear explanation of the study objectives. Cases proved positive for trichomoniasis were prescribed appropriate therapy by the gynecologist.

RESULTS

Clinical characteristics of females enrolled in the study: Three hundred females aged 20-45 were enrolled in the study based on complains suggestive of trichomoniasis. Of these, 12 (4%) proved positive for *T. vaginalis* by wet mount examination and culture. The clinical characteristics including complaint, character of discharge and findings on vaginal examination of all cases including those proved positive are correlated in table (1). The spectrum of clinical manifestations of the participants was variable. Mild to profuse (50% in each, respectively) watery (60% and 66.6%, respectively) or mucoid discharge (40% and 33.3%, respectively) and pruritus (50% and 75%, respectively) were the commonest complaints. Congested cervix and erythema of the vagina (33.3% and 1%, respectively), cervical erosions (16.6% and 8.3%, respectively) and bleeding on examination (66.6% and 16.6%, respectively) were the commonest clinical findings on vaginal examination.

Growth characteristics of *T. vaginalis* isolates: The twelve isolates exhibited different growth

Table 1. Clinical Characteristics of the 300 females enrolled in the study including those of the 12 proved positive for *T. vaginalis* by wet mount and culture.

Complaints and clinical findings on vaginal examination	No. (%)	No. (%) among *T. vaginalis* positive females by wet mount and culture
Pruritus	150 (50%)	9 (75%)
Burning micturition	50 (16.6%)	3 (25%)
Dyspareunia	40 (13.3%)	2 (16.6%)
Vaginal discharge	300 (100%)	12 (100%)
Mild	150 (50%)	6 (50%)
Profuse	150 (50%)	6 (50%)
Watery	180 (60%)	8 (66.6%)
Mucoid	120 (40%)	4 (33.3%)
Clear	120 (40%)	2 (16.6%)
White	60 (20%)	4 (33.3%)
Yellow	100 (33.3%)	5 (41.6%)
Bloody	20 (6.6%)	1 (8.3%)
Bleeding during examination	200 (66.6%)	2 (16.6%)
Cervical erosion	50 (16.6%)	1 (8.3%)
Congested cervix and erythema of vaginal mucosa	100 (33.3%)	1 (8.3%)
Ulcerative lesions on vaginal wall	50 (16.6%)	1 (8.3%)
Pain on palpation of pelvis or abdomen	40 (14.3%)	1 (8.3%)

characteristics in terms of duration of log phase and growth peaks reached, division rate, number of divisions, and generation time (Table 2). Comparison of the peak number of isolates at the end of log phases showed that the greatest significant yield was observed in isolate no. 10 (150.25 ± 3.13) after 48 h and the least significant yield was in isolate no. 3 (40.5 ± 21) after 96 h. The division rate, generation time and number of divisions exhibited variable parameters ranging from 0.09 to 0.18 mean division/h and generation time ranging between means of 5 h:26 min to 11 h:16 min. The number of divisions during the logarithmic phase ranged between means of 6.39-12.22.

Metronidazole susceptibility testing: Under aerobic condition, mean MIC varied between 0.6-25.0 µg/ml for isolates 8 and 3, respectively, while under anaerobic condition, mean MIC varied between 0.4-6.3 µg/ml for isolates 6, 8, 9 and isolate 3, respectively (Table 3). Isolate 3 had the highest mean MIC of 25.0 µg/ml and of 6.3 µg/ml under aerobic and anaerobic conditions, respectively.

Confirmation of *T. vaginalis* infection by PCR amplification of *tvk3/7* repetitive gene: Only the 12 samples that gave positive results by microscopy and culture reacted positively by PCR targeting *tvk3/7* repetitive gene, giving the expected 300 bp amplification products (Fig. 1A).

PCR amplification of *p1, p8* and *p13* genes: Results of PCR amplification of the 12 positive *T. vaginalis* samples are shown in table (4) and figures (1B-D). Eleven isolates (91.7%) were successfully amplified at least at one locus, of these 9 (75%), 8 (66.6%), and 8 (66.6%) reacted positively when tested by PCR targeting *p1, p8, p13,* respectively. Five isolates (41.7%) gave positive result at the levels of the three loci. Three isolates (25%) gave positive result at the levels of *p1* and *p13* and only one (8.3%) was positive at the levels of *p1* and *p8*. Two samples (16.7%) gave positive result at the level of *p8* alone. It is noteworthy that only isolate no. 7 (8.3%) gave negative result at the three loci, although it was successfully amplified by *tvk3/7* PCR at 300 bp.

Table 2. Growth kinetics of the 12 *T. vaginalis* isolates cultured on modified TYM.

Isolate	Log phase (duration/h)	No. x 10^4/ml at the end of log phase	Division rate (division/h) Mean ± SD	No. of divisions (during log phase) Mean ± SD	Generation time (h:min) Mean ± SD
1	48	120.5 ± 2.4	0.16 ± 0.02	8.00 ± 0.18	6:00 ± 00:27
2	48	81.0 ± 2.3	0.18 ± 0. 001	8.84 ± 0.09	5:26 ± 00:03
3	96	40.5 ± 21.0	0.09 ± 0.004	8.51 ± 0.14	11:16 ±00:07
4	72	80.0 ± 3.45	0.13 ± 0.02	9.27 ± 0.16	8.16 ±00:12
5	72	110.0 ± 2.9	0.17 ± 0.011	12.2 ±0. 16	5:55 ± 00:12
6	48	95.45 ± 45.0	0.18 ± 0.002	8.69 ± 0. 06	5:34 ± 00:08
7	72	139.8 ± 0.97	0.17 ± 0.03	12.22 ± 0.09	5:54 ± 00:19
8	48	100.0 ± 1.2	0.13 ± 0.01	6.39 ± 0.13	7: 30 ± 00:15
9	96	63.5 ± 1.7	0.09 ± 0.002	9.15 ± 0.02	10:31 ±00:04
10	48	150.3 ± 3.1	0.18± 0.01	8.73 ± 0.13	5:30 ± 00:14
11	72	81.3 ± 2.4	0.13± 0.01	9.47 ± 0.01	7:36 ± 00:07
12	72	115.5 ± 1.8	0.12± 0.03	8.78 ± 0.12	8:12 ± 00:08

Data shown in the table for: mean peak of growth, division rate, number of divisions and generation time are mean±SD from 4 counts, 2 from each culture tube.

Table 3. Assessment of MIC*of MTZ** for *T. vaginalis* isolates.

Isolate	Mean MIC (µg/ml) ± SD	
	Aerobic	Anaerobic
1	6.3 ± 0.0	1.6 ± 0.0
2	9.4 ± 4.4	4.7 ± 2.3
3	25.0 ± 0.0	6.3 ± 0.0
4	4.7 ± 2.3	3.1 ± 0.0
5	2.3 ± 1.1	1.2 ± 0.6
6	1.6 ± 0.0	0.4 ± 0.0
7	1.6 ± 0.0	0.8 ± 0.0
8	0.6 ± 0.3	0.4 ± 0.0
9	2.3 ± 1.1	0.4 ± 0.1
10	1.2 ± 0.6	0.6 ± 0.3
11	6.3 ± 0.0	1.2 ± 0.6
12	4.7 ± 2.3	3.1 ± 0.0

***MIC:** Minimal inhibitory concentration, ****MTZ:** Metronidazole.

Table 4. Summary of the results of multilocus PCR amplification of *p1, p8*, and *p13*, from the 12 collected *T. vaginalis* isolates.

Target gene	No.	~%
***p1, p8, p13* (Isolates No. 1, 2, 3, 4, 6)**	5	41.7
***p1, p8* (Isolate No. 8)**	1	8.3
***p1, p13* (Isolates No.5, 9, 10)**	3	25.0
***p8* (Isolates No. 11, 12)**	2	16.7
Negative (Isolate No. 7)	1	8.3
Total	**12***	**100**

*Of the 12 microscopically positive samples, 9 (75%), 8(66.6%), and 8 (66.6%) reacted positively when tested by PCR targeting *p1, p8, p13*, respectively.

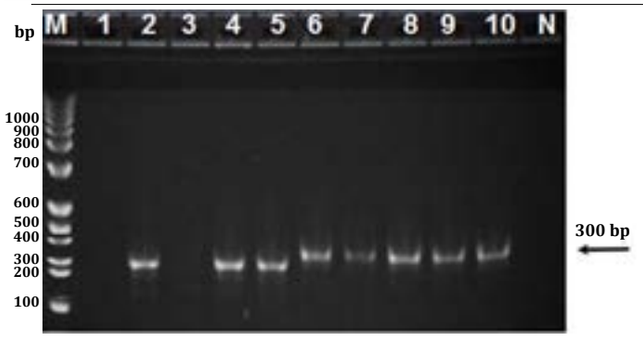

Fig. 1A. Agarose gel electrophoresis showing PCR product of *T. vaginalis* repetitive *tvk3/7* gene.
Lane M: DNA marker (100 bp).
Lanes 1, 3: Negative reactions.
lanes 2, 4-10: Positive reactions showing 300 bp expected amplification product.
lane N: Negative controls showing no PCR product.

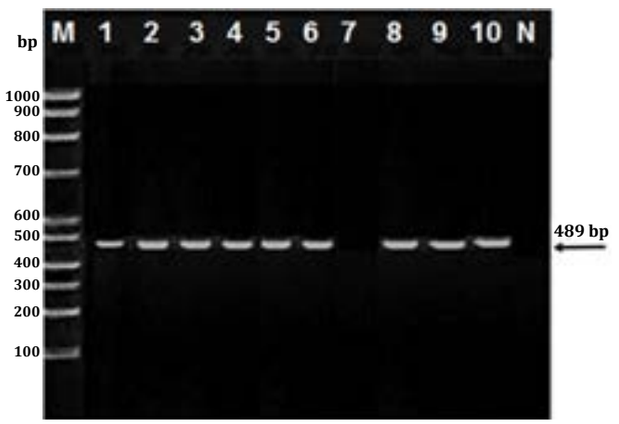

Fig. 1B. Agarose gel electrophoresis showing PCR product of *T. vaginalis p1* gene (489 bp).
Lane M: DNA marker (100 bp).
lanes 1-6 and 8-10: Positive reactions.
Lane 7: Negative reaction.
Lane N: Negative control showing no PCR product.

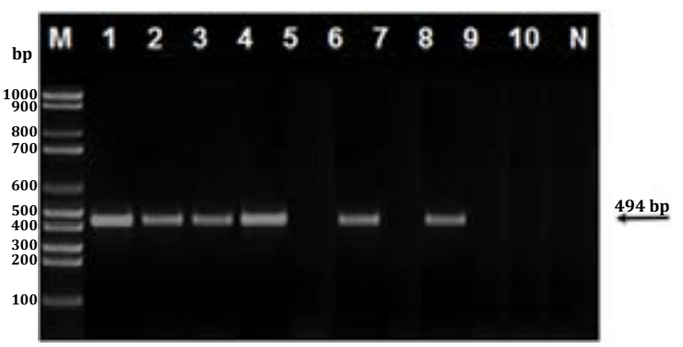

Fig. 1C. Agarose gel electrophoresis showing PCR product of *T. vaginalis p8* gene (494 bp).
Lane M: DNA marker (100 bp).
lanes 1-4 and 6-8: Positive reactions.
Lanes 5,7,9,10: Negative reaction.
Lane N: Negative control showing no PCR product.

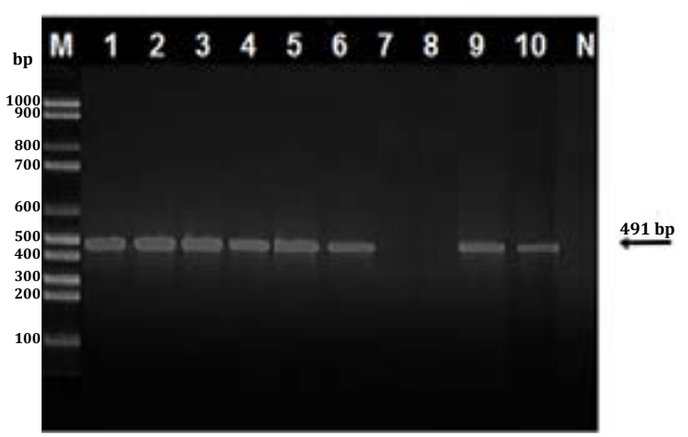

Fig. 1D. Agarose gel electrophoresis showing PCR product of *T. vaginalis p13* gene (491 bp).
Lane M: DNA marker (100 bp).
lanes 1-6 and 9,10: Positive reactions.
Lanes 7,8: Negative reaction.
Lane N: Negative control showing no PCR product.

Genotyping of *T. vaginalis* isolates by MLST: Sequencing of the amplification products was successful at the level of one or more loci in 10 out of 12 isolates (83.3%). Two isolates were discarded from sequence analysis, no. 6 due to failure of PCR amplification and no. 7 due to short sequences. Comparative analysis of the sequences of the 10 isolates that gave positive results at one or more of the three molecular markers, with reference strains revealed that at the level *p1*, 4 isolates (80%) belonged to type I and one (20%) belonged to type II. At the level of *p8*, 5 (71.5%) belonged to type I and 2 (28.5 %) belonged to type II. At the level of *p13,* 4 (80 %) belonged to type I and one (20%) belonged to type II (Table 5). At the level of one or more genes, combined level, 70% belonged to type I, 20% belonged to type II and 10% exhibited mixed type (one isolate, no. 8) (Table 6). Comparison of the concatenated sequence was not possible because of failure of sequencing of all isolates at the level of the three loci, except for isolate no. one.

The cladograms of the sequences at the level of the individual three loci as constructed by Geneious version 10.2.3 software is shown in figure (2). The cladograms show that the isolates were distributed into two phylogenetic clades; clade A designated genotype I comprising isolates numbers 1, 2, 4, 5, 8, 10, 11, 12; and clade B designated genotype II comprising isolates numbers 3, 8, 9.

Relation of *Trichomonas* genotype to clinical presentations: Correlation of age of patients with *Trichomonas* genotype revealed that in older age group, 35-45 years, the number of patients infected with genotype I was five times (83.3 %) more than that of those infected with genotype II (16.7%). In the younger age group of 20-34 years, the number of patients infected with genotype I was twice (50%) that of those infected with genotype II (25%). However, such differences were statistically insignificant either at genotype or age levels (P=0.588) (Table 7). Regarding the correlation between genotype and character of vaginal discharge, no consistent character of discharge could be associated with either genotype as such differences were statistically insignificant (P>0.05). Also, no statistical differences were found between

Table 5. Multilocus sequence typing of *T. vaginalis* isolates.

Isolate	*p1*	*p8*	*p13*
1	I	**I**	**I**
2	-	**I**	**I**
3	-	**II**	–
4	-	**I**	–
5	I	–	**I**
8	I	**II**	–
9	II	–	**II**
10	I	–	**I**
11	-	**I**	–
12	-	**I**	–

Table 6. Distribution of genotypes of *T. vaginalis* based on the different loci among the 10 successfully sequenced isolates.

Type	Marker gene			Combined typing*
	p1	*p8*	*p13*	
	No. (%)	No. (%)	No. (%)	No. (%)
I	4 (80)	5 (71.5)	4 (80)	7 (70)
II	1 (20)	2 (28.5)	1 (20)	2 (20)
Mixed#	-	-	-	1 (10)
Total	**5**	**7**	**5**	**10 (100)**

* Typing at one and/or more of the three loci.

\# Isolate 8 was identified as type I at the level of *p1* and as type II at the level of *p8.*

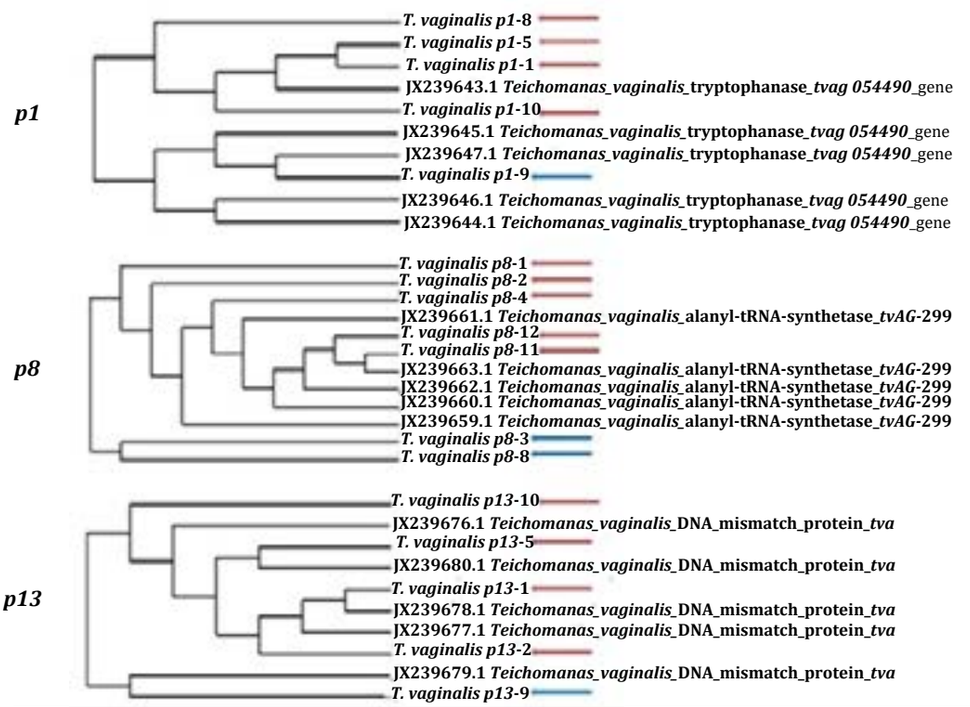

Fig. 2. *T. vaginalis* cladogram analysis of the sequences at the level of the individual three loci with reference strains (Clade A in red, Clade B in blue).

the genotype and findings on vaginal examination. It is worth noting that one of the 2 cases with genotype II had bloody discharge and bled during vaginal examination.

Relation of *Trichomonas* genotype to growth kinetics: Comparison of growth kinetics of the 10 isolates with their genotypes (Table 7) revealed that genotype I parasites grew faster with average shorter log phase, generation time and higher number of divisions than those of type II. However, such differences were statistically insignificant. Comparison of the growth peaks of the 10 isolates at the end of log phase, revealed statistically significant difference between genotype I (105.3±26.3) and genotype II (67.7±30.2) with a *P* value of 0.018.

Relation of *Trichomonas* genotype to MTZ susceptibility: Although all isolates were sensitive to MTZ, type I isolates exhibited non-significantly higher susceptibility than type II with lower MIC under aerobic (*P*=0.07) and anaerobic (*P*=0.3) conditions, respectively. Isolate 3 that exhibited the least susceptibility to metronidazole with MIC of 25.0 µg/ml and of 6.3 µg/ml under aerobic and anaerobic conditions, respectively belonged to genotype II.

Table 7. Association of *T. vaginalis* genotype with growth kinetics, MTZ susceptibility and clinical presentation.

	Genotype I	**Genotype II**	**Mixed**	***P* value***
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	
Growth kinetics				
Log phase duration	61.7±12.8	80.0±27.7	80.0±0.0	**0.17**
No. x 10^4/ml at the end of log phase	105.3±26.3	67.7±30.2	100.0±1.2	**0.018**
Number of divisions	9.57±1.45	8.83±0.32	6.39±0.13	**0.53**
Generation time (h:min)	6:29±1:16	10:73±0:42	0:4±0:0	**0.74**
MIC of MTZ@				
Aerobic	4.2±2.6	13.6±11.3	0.6±0.3	**0.07**
Anaerobic	1.8±1.3	3.3±2.9	0.4±0.0	**0.3**
Clinical presentations	**Genotype I**	**Genotype II**	**Mixed**	***P* value#**
	No. (%)	**No. (%)**	**No. (%)**	
Years of age				
20-34 Y	2 (50)	1 (25)	1 (25)	**0.583**
35-45 Y	5 (83.3)	1 (16.7)	0 (0)	
Amount of vaginal discharge				
Mile	5 (71.5)	1 (50)	0 (0)	**0.58**
Profuse	2 (28.5)	1 (50)	1 (100)	
Color of vaginal discharge				
Clear	0 (0)	1 (50)	1 (100)	
White	3 (42.8)	0 (0)	0 (0)	**0.43**
Yellow	4 (57.2)	0 (0)	0 (0)	
Bloody	0 (0)	1 (50)	0 (0)	
Congested cervix	4 (57.1)	1 (100)	0 (0)	**0.12**
Bleeding during exam	1 (14.3)	1 (100)	0 (0)	**1**
Cervical erosions	1 (14.3)	0 (0)	0 (0)	**-**
Congested cervix and erythema of vaginal mucosa	1 (14.3)	0 (0)	0 (0)	**-**
Ulcerative lesions at the vaginal wall	0 (0)	0 (0)	1 (100)	**-**

Data shown are for the 10 successfully sequenced isolates. *P* value < 0.05 indicates insignificant association.
* As calculated by Anova test, # As calculated by Fisher exact test, **MIC of MTZ: Minimal inhibitory concentration of metronidazole.

DISCUSSION

The outcome of trichomoniasis is governed by many factors including virulence, drug resistance, antigenic and genetic diversity of the parasite, beside host factors including age and race. Molecular typing methods revealed a two-type population structure for *T. vaginalis*, type I and type II that may differ in pathogenicity, drug resistance, and clinical presentation[27-30]. MLST of bacteria and eukaryotic pathogens has been used successfully to describe population diversity, delimit species, identify genetic components of important clinical phenotypes, and track the spread of epidemics[37-39]. MLST was used to characterize the genetic diversity and population structure of *T. vaginalis* using internal sequence fragments of seven housekeeping genes. Fragments of 450-500 base pairs were amplified by PCR and sequenced[28].

The aim of the present study was to elucidate the genotype of Egyptian isolates of *T. vaginalis* using MLST targeting three housekeeping genes, from the seven described by Cornelius *et al.*[28] namely tryptophanase (*p1*), alanyl tRNA synthetase (*p8*) and DNA mismatch repair protein (*p13*) genes. This would help in better understanding of the molecular epidemiology and phylogenetic relationship of Egyptian isolates of *T. vaginalis*. In part this study

aimed to evaluate possible relationship between genotypes and growth kinetics, MTZ susceptibility of the isolates as well as the clinical presentation of trichomoniasis.

Three hundred-sterile saline washout specimens were collected aseptically from Egyptian women patients aged 20-45 years suspected of having trichomoniasis. Of these 12 (4%) proved positive for *T. vaginalis* by wet mount examination and culture. The spectrum of clinical manifestations of the patients participated in the study including those proved positive for trichomoniasis was variable. The commonest complaints were mild to profuse watery or mucoid discharge and pruritus. Congested cervix, erythema of the vagina, cervical erosions and bleeding on examination were the commonest clinical findings on vaginal examination. The recorded clinical data are in accordance with those recorded by other authors ranging from absence of symptoms to mild or severe symptoms in the form of vaginal discharge, pruritus, dyspareunia, burning micturition, lower abdominal pain, congested cervix and erythema of the vaginal wall[7,8,45].

Comparison of the growth kinetics of the twelve *T. vaginalis* isolates revealed a salient difference among all isolates in terms of duration of log phase, growth peaks reached, division rate, number of divisions, and generation time. The fast-growing isolates reached maximum growth after 48 h with rapid division ranging from 0.16-0.18 division/h and short generation time ranging from a mean of 5:26-6:00. The greatest yield was observed in isolate 10 (150.25±3.13). Slow growing isolates reached maximum after 96 h with slow division rates of 0.09-0.1 division/h and generation time above 10 h. The least yield was observed in isolate 3 (40.5±21). Similar findings were previously recorded by other investigator who worked on clinical and laboratory-maintained isolates[42]. The relation between growth kinetics of the Egyptian isolates and clinical presentation was previously published[46].

In the present study, none of the clinical isolates exhibited resistance to MTZ under aerobic or anaerobic conditions with MIC <25.0 µg/ml and <6.3 µg/ml, respectively. These results conform with most reports from Egypt and other countries, where majority of clinical isolates were susceptible to MTZ with few resistant strains from USA, Europe and Africa[25,43,47,48]. It is worth mentioning that, in the present study, isolate 3 which exhibited the highest MIC of 25.0 µg/ml and 6.3 µg/ml under aerobic and anaerobic conditions, respectively, had the lowest significant growth peak of 40.5±21 after 96 h. Another study proposed different threshold concentrations of the 5-nitroimidazole drugs[47]. In this aspect isolate number 3 would be considered resistant under anaerobic conditions according to Matini *et al.*[43] who proposed a threshold concentration of 3.2 µg/ml under anaerobic conditions. On the other hand, Meri *et al.*[47] proposed a threshold concentration of 15µg/ml under anerobic conditions.

Of the 12 isolates proved positive by microcopy, culture and *tvk3/7* PCR, 11 gave positive results at least at one locus when tested by PCR amplification of *p1*, *p8* and *p13* genes. The failure of amplification of the three housekeeping genes at all loci for all isolates may be due a problem in the sensitivity of the PCR or due to the nature of the genes being single-copy genes, although these genes were previously selected[28] based on being readily amplified and sequenced on both DNA strands of all *T. vaginalis* isolates. Dos Santos *et al.*[49] found variable stability in the expression of nine housekeeping reference genes under different culture conditions. Genes encoding α-tubulin, actin and DNA TopII were the most stable and conversely, the widely used *T. vaginalis* reference genes encoding GAPDH and β-tubulin were less stable[49].

Comparative analysis of the sequences of the 10 isolates that gave positive results at one or more of the three molecular markers, with reference strains revealed that at the level of one or more genes, combined level, 70 % belonged to type I, 20 % belonged to type II and one isolate number 8 (10 %) exhibited mixed type (Table 6). The cladograms of the sequences at the level of the individual three loci showed that the isolates were distributed into two phylogenetic clades, clade A designated genotype I and clade B designated genotype II.

These results agree with those previously obtained using RFLP[50,51]. Using microsatellite genotyping targeting 21 loci, it was found that 188 global isolates clustered into 2 types in equal proportions, with two exceptions of isolates from Southern Africa and Mexico which are significantly biased towards type I and type II, respectively[27]. Likewise, Cornelius *et al.*[28] using MLST targeting 7 loci, found that 68 isolates clustered into 2 clades in almost equal proportions mainly at the level of 6 loci with highest bootstrapping support at the levels of *p3* and *p6*, modest support at the levels of *p14* and *p16*, least support at the levels of *p1*, *p8* and *p13*[28]. In Bristol, UK, MLST of the same 7 housekeeping genes revealed the predominance of isolates falling within type II (78%) rather than type I (22%)[19]. van der Veer *et al.*[30] described a MLST tool for *T. vaginalis* that used nested PCR targeting the same 7 housekeeping genes and revealed that infections with *T. vaginalis* type I (59.7%) occurred more frequently than type II (40.3%)[30]. More recently, Jehee *et al.*[52] using MLST on *T. vaginalis* positive clinical samples collected from Amsterdam, found that the isolates were segregated into two-genotype populations, type I (53.7%) and type II (46.2%).

Regarding mixed infection, our results agree with those of Conrad *et al.*[27], who identified mixed infection in 23 out of 211 (10.6 %) global isolates from five

continents, analyzed by microsatellite genotyping. Also, Tavakoli-Oliaee *et al.*[53], using PCR-RFLP typing of the gene encoding actin, found 50% (4 out 8) mixed infections in only one of the locations examined in Shiraz, Iran, which was relatively high. It was argued that the presence of mixed types in Shiraz may probably be related to sex behavior in drug addicts or HIV subjects in that region. However, no mixed infections were recorded among 71 clinical isolates from Amsterdam, Netherland[30]. Actually, demonstration of mixed infections requires prompt analysis of the initial sample, as even small differences in *in vitro* growth rates can result in the elimination of one *T. vaginalis* genotype during extended cultivation[29].

Few studies researched *T. vaginalis* genetic diversity in relation to patient demographic and clinical characteristics. Studies, based mainly on RAPD, have demonstrated concordance between *T. vaginalis* genotype and phenotype, including MTZ susceptibility, the presence of TVV and concurrent *Mycoplasma* infection[54-56]. However, other reports based on RAPD and RFLP patterns of a PCR-generated 28S-18S rRNA intergenic spacer failed to demonstrate concordance between genotype and symptoms[29,50,52].

In the preset study no statistically significant difference was found between genotype and age of the patients, character of vaginal discharge and clinical findings on vaginal examination. It was found that patients infected with genotype I were 5 times older than patients infected with genotype II. This agrees with Conrad *et al.*[27] who found that the mean age of women infected with type I parasites was older than of women infected with type II parasites, but the difference was not statistically significant. It is unlikely that this difference is influenced by hormonal changes caused by the onset of menopause, which affects older populations of women[53]. Alternative explanations for this difference, as explained by Conrad[57] may include: "(i) type I parasites may be less susceptible to self-clearance by the host than type II parasites; (ii) type I parasites may compete with type II parasites in instances of co-infections; and (iii) type I parasites may remain asymptomatic for longer periods of time than type II parasites, reducing the infections cleared through drug treatment".

No significant correlation was found between genotype and character of vaginal discharge or findings on vaginal examination. Severe pathology in the form of bleeding on vaginal examination was detected in both genotypes. Again, these findings were statistically insignificant between both genotypes. Cornelius[58] found that infection with genotype I was significantly associated with cervical erosions indicating affection of the upper portion of vagina and associated bacterial vaginosis. She also found that the pathological outcome of infections with genotype II was significantly associated with bleeding during vaginal examination.

No significantly statistical difference was found between growth kinetics of parasites belonging to genotype I and genotype II, although the former grew faster and had shorter log phase, shorter generation time and higher number of divisions. Comparison of the growth peaks of the isolates, number of parasites at the end of log phase, revealed that parasites belonging to genotype I had significantly higher numbers ($P = 0.018$) than genotype II. This characteristic is likely to provide an advantage in transmission. However, faster growth rates are also frequently associated with increased damage to the host. This conforms with Conrad[57] who compared the growth rates of 17 strains, representative of type I and type II, and found that type I parasites grew significantly faster than type II parasites.

Regarding MTZ sensitivity, although all our isolates were sensitive to MTZ, isolates belonging to type I exhibited lower non-statistically significant mean MIC than those belonging to type II. Isolate 3 which was found to be type II exhibited the least sensitivity to MTZ with MIC of 25.0 µg/ml under aerobic condition; one lower dilution level than the published cutoff for calling an isolate "MTZ resistant" in *in vitro* assays[55]. Under anaerobic condition, the same isolate exhibited MIC of 6.3 µg/ml. This too indicates resistance to MTZ taking into consideration the estimation of Matini *et al.*[43]. In contrast, Conrad *et al.*[27] recorded a statistically significant difference between the 2 types in their measured MTZ minimal lethal concentration (MLC). The study found that type II parasites are more resistant to MTZ than type I parasites with MLC greater than or equal to 50 µg/ml. Also, while studying the genetic diversity of MTZ susceptibility in 49 *T. vaginalis* clinical isolates of Egyptian females in Mansoura, mild resistance was observed in two isolates with MLCs of 64 µg/ml, and mild to moderate resistance in an additional two isolates with MLCs of 128 µg/ml. The four isolates displayed a unique genotype band pattern by RFLP compared to the other 45 samples that were MTZ sensitive at MIC of 1.0 µg/ml[48]. Other studies that employed RAPD technique showed a correlation between genetic relatedness of strains and similarity in their susceptibility to MTZ *in vitro*[54-56]. While Hussein *et al.*[26] using RFLP analysis reported that there was no relation between *T. vaginalis* isolates and MTZ susceptibility and RFLP subtypes[26].

Paulish-Miller *et al.*[59] identified SNPs in two nitroreductase genes (*tvnt4* and *tvntr6*) associated with MTZ resistance in 64 % of type II and 33 % of type I parasites; yet one SNP (*tvntr6* A238T), that results in a premature stop codon, was associated with resistance independent of population structure and may be of diagnostic value.

Conclusion and recommendation: The present study has shown that, using MLST, the Egyptian isolates comprised two types, genotype I, constituting 70% of the isolates and genotype II, 20%. Mixed infection was

detected in 10% of cases. No correlation was found between genotype and growth kinetics, MTZ sensitivity and clinical presentation. Although genotype I parasites appeared to be more common among older age females and showed significantly higher numbers of parasites at the end of log phase. Only one isolate of genotype II had a MIC of 25.0 µg/ml and of 6.3 µg/ml approaching the cut-off level specified for MTZ resistance under aerobic and anaerobic conditions. A limitation of the present study is that the sample size of patients with available clinical and demographic data was small and consequently the number of isolates was small. However, the study has supported the importance of MLST in investigating the genetic diversity of the Egyptian isolates of parasites for better understanding of their molecular epidemiology, phylogenetic relationship and population genetics. It is recommended that a future larger multicenter study should be done, whereby a larger number of isolates should be obtained from both females and males for a complete picture of genetic diversity and epidemiology.

Authors contribution: Mohamed BA collected the samples, performed wet mount examination and culture, PCR amplification and genotyping of the parasite; Elleboudy NA helped Mohamed BA all through the practical work of the study; Hussein HM supervised the study and revised the results; Khalifa KE planned and supervised the study, analyzed the results and wrote the manuscript; Azab ME revised the study plan, supervised the study, wrote and revised the manuscript.

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REFERENCES

1. WHO. Global incidence and prevalence of selected curable sexually transmitted infections-2008. Published by WHO Department of Reproductive Health and Research in 2012. Accessed at www.who.int/reproductivehealth/public/publications/rtis/ stisestimates/en/.
2. Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, *et al. Chlamydia*, gonorrhoea, trichomonasiasis and syphilis: global prevalence and incidence estimates, 2016. Bull World Health Organ 2019; 97(8): 548-562.
3. Hamdy DA, Hamdy HG. Prevalence, sociodemographic factors and clinical criteria of *Trichomonas vaginalis* infection among symptomatic women in Beni-Suef governorate, Egypt. J Egypt Soc Parasitol 2018; 48(1):109-117.
4. Hussein AH, Saleh MH, Nagaty IM, Gheith KA, El-Azab NM. Prevalence, clinical criteria and sociodemographic predictors of *Trichomonas vaginalis* infection in suspected Egyptian women, using direct diagnostic techniques. Iranian J Parsitol 2015; 10(3):432-440.
5. Mahmoud A, Sherif NA, Abdella R, El-Genedy AR, El Kateb AY, Askalani ANH. Prevalence of *Trichomonas vaginalis* infection among Egyptian women using culture and latex agglutination: cross sectional study. BMC Womens Health 2015; 15:7.
6. Nassef NE, Amera F. Afif AF, Basuni AA, Abo El-Nasr MF, Atia AF. Evaluation of microscopy and polymerase chain reaction for diagnosis of symptomatic and asymptomatic female trichomoniasis. PUJ 2014; 7:37-46.
7. Swygard H, SenaAC, Hobbs MM, Cohen MS. Trichomoniasis: Clinical manifestations, diagnosis and management. Sex Transm Infect 2004; 80(2): 91-95.
8. Kissinger P. *Trichomonas vaginalis*: a review of epidemiologic, clinical and treatment issues. BMC Infect Dis 2015; 15(1)307.
9. Alcaide ML, Feaster DJ, Duan R, Cohen S, Diaz C, Castro JG, *et al.* The incidence of *Trichomonas vaginalis* infection in women attending nine sexually transmitted diseases clinics in the USA. Sex Transm Infect 2016; 92(1):58-62.
10. Chemaitelly H, Weiss HA, Smolak A, Majed E, Abu-Raddad LJ. Epidemiology of *Treponema pallidum, Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis*, and herpes simplex virus type 2 among female sex workers in the Middle East and North Africa: systematic review and meta-analytics. J Glob Health. 2019; 9(2):020408.
11. McClelland RS, Sangaré 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(5):698-702.
12. Ijasan O, Okunade KS, Oluwole AA. The prevalence and risk factors for *Trichomonas vaginalis* infection amongst human immunodeficiency virus-infected pregnant women attending the antenatal clinics of a university teaching hospital in Lagos, South-Western, Nigeria. Niger Postgrad Med J. 2018: 25(1):21-26
13. Masha SC , Cools P , Sanders EJ , Vaneechoutte M, Crucitti T. *Trichomonas vaginalis* and HIV infection acquisition: a systematic review and meta-analysis. Sex Transm Infect. 2019; 95(1):36-42.
14. Price CM, Peters RPH, Steyn J, Mudau M, Olivier D, De Vos L, *et al.* Prevalence and detection of *Trichomonas vaginalis* in HIV-Infected pregnant women. Sex Transm Dis 2018;45(5):332-336.
15. Sayed el-Ahl SA, El-Wakil HS, Kamel NM, Mahmoud MS. A preliminary study on the relationship between *Trichomonas vaginalis* and cervical cancer in Egyptian women. J Egypt Soc Parasitol 2002; 32(1):167-178.
16. Donders GG, Depuydt C. Increased prevalence of *Trichomonas vaginalis* in mid-aged women is linked to sexual activity and not to hormonal changes. J Low Genit Tract Dis 2013; 17(4): e31-e32.
17. Muñoz-Ramírez A, López-Monteon A, Ramos-Ligonio A, Méndez-Bolaina E, Guapillo-Vargas MRB. Prevalence of *Trichomonas vaginalis* and human

papilloma virus in female sex workers in Central Veracruz, Mexico. Rev Argent Microbiol 2018; 50(4):351-358.
18. Shaw MK, Porterfield HS, Favaloro S, Dehon PM, Van Der Pol B, Quayle AJ, *et al.* Prevalence and cervical organism burden among Louisiana women with *Trichomonas vaginalis* infections. PLoS One. 2019; 20:14(6):e0217041.
19. Hawksworth J, Levy M, Smale C, Cheung D, Whittle A, Longhurst D, *et al.* Population structure and genetic diversity of the parasite *Trichomonas vaginalis* in Bristol, UK. Infect Genet Evol. 2015; 34: 36-43.
20. Salem SA, Azab ME, Abd el Ghaffar FM, El Sherif EA, Makled KA, Habib FS. Characterization of Egyptian isolates of *Trichomonas vaginalis*: I. isoenzymes patterns. J Egypt Soc Parasitol 1992; 22(3): 675-682.
21. Azab ME, Salem SA, Abd El Ghaffar FM, El Sherif EA, Habib KS, Habib FS. Characterization of Egyptian isolates of *Trichomonas vaginalis*: I. serotyping. J Egypt Soc Parasitol 1992; 22(3), 775-782.
22. Khalifa KE, El-Wakil HS, Habib FS, Abdel Baki MH, Azab ME. Immunoblot analysis of *Trichomonas vaginalis* antigens recognized by rabbit hyperimmune serum raised against exo-antignes. Parasitol Res 2004; 92(1):48-49.
23. El-Okbi LM, Khalifa KE, El Wakil HS, Mohamed AA, Abdel-Hameed DM, Tawfik RA. Characterization of specific *Trichomonas vaginalis* target antigens from different isolates by immunoblotting against hyperimmune rabbit serum. J Egypt Soc Parasitol 2005; 35(3):891-898.
24. Hussien EM, El-Sayed HZ, Shaban MM, Salm AM, Rashwan M. Biological variability of *Trichomonas vaginalis* clinical isolates from symptomatic and asymptomatic patients. J Egypt Soc Parasitol 2004; 34(3):979-988.
25. Boulos LM, El-Temsahy MM, Aly SM, El- Agamy EI, Amer EI. Biological and biochemical studies for characterization of some Egyptian *Trichomonas vaginalis* isolates. PUJ 2012; 5(2): 175-188.
26. Hussien EM, El-Sayed HZ, El-Moamly AA, Helmy MM, Shaban MM. Molecular characterization of Egyptian *Trichomonas vaginalis* clinical isolates by HSP70 restriction fragment length polymorphism. J Egypt Soc Parasitol 2005; 35(2):699-710.
27. Conrad MD, Gorman AW, Schillinger JA, Fiori PL, Arroyo R, Mall N, *et al.* Extensive genetic diversity, unique population structure and evidence of genetic exchange in the sexually transmitted parasite *Trichomonas vaginalis*. PLoS Negl Trop Dis 2012; 6(3): e1573.
28. Cornelius DC, Robinson DA, Muzny CA, Mena LA, Aanensen DM, Lushbaugh WB, *et al.* Genetic characterization of *Trichomonas vaginalis* isolates by use of multilocus sequence typing. J Clin Microbiol 2012; 50(10): 3293-3300.
29. Meade JC, Carlton JM. Genetic diversity in *Trichomonas vaginalis*. Sex Transm Infect 2013; 89:444-448.
30. van der Veer C, Himschoot M, Bruisten SM. Multilocus sequence typing of *Trichomonas vaginalis* clinical samples from Amsterdam, the Netherlands. BMJ open 2016; 6(10): e013997.
31. Zhang Z, Kang L, Wang W, Zhao X, Li Y, Xie Q, *et al.* Prevalence and genetic diversity of *Trichomonas vaginalis* clinical isolates in a targeted population in Xinxiang City, Henan Province, China. Parasit Vectors 2018;11(1):124.
32. Peters A, Das S, Raidal SR. Diverse *Trichomonas* lineages in Australasian pigeons and doves support a columbid origin for the genus *Trichomonas*. Mol Phylogenet Evol. 2019; 143:106674.
33. Martínez-Herrero MDC, Garijo-Toledo MM, González F, Bilic I, Liebhart D, Ganas P, *et al.* Membrane associated proteins of two *Trichomonas gallinae* clones vary with the virulence. PLoS One. 2019;14(10):e0224032.
34. Demirağ S, Malatyalı E, Ertuğ S, Ertabaklar H. Determination of *Trichomonas vaginalis* genotypes using PCR-restriction fragment length polymorphism (RFLP). Turkiye Parazitol Derg 2017;41(4):188-191.
35. Orujzadeh F, Tabatabaie F, Khanaliha K, Akhlaghi L, Bokharaei-Salim F, Fallah S, *et al.* Prevalence and genotyping of *Trichomonas vaginalis* infected to dsRNA virus by PCR-restriction fragment length polymorphism (RFLP). Iran J Parasitol 2019;14(2):250-257.
36. Conrad MD, 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(1):30-38.
37. Barker GC. Microsatellite DNA: a tool for population genetic analysis. Trans R Soc Trop Med Hyg 2002; 96(Suppl. 1), S21–S24.
38. Maiden MC. Multilocus sequence typing of bacteria. Annu Rev Microbiol 2006; 60(1):561-588.
39. Tibayrenc M. Multilocus enzyme electrophoresis for parasites and other pathogens. In Caugant D (eds) Molecular Epidemiology of Microorganisms. Humana Press, Totowa, NJ, Methods Mol Biol 2009; 551: 13-25.
40. Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, *et al.* Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Pro Natl Acad Sci USA 1998; 95: 3140 -3145.
41. Diamond LS. The establishment of various trichomonads of animals and man in axenic cultures. J Parasitol 1957; 43(4):488-490.
42. El-Okbi LM, Arafa M, Salama, MS, Abou El-Seoud SM, Mohamad AA, Tawfik RA. Growth patterns and antigenic analysis of Egyptian *Trichomonas vaginalis* isolates. J Egypt Soc Parasitol 2004; 34(3),841-55.
43. Matini M, Maghsood AH, Mohebali M, Rabiee S, Fallah M, Rezaie S, *et al. In vitro* susceptibility

of Iranian isolates of *Trichomonas vaginalis* to metronidazole. Iran J Parasitol 2016; 11(1),46-51.
44. Abdolali M, Zohreh KA, Shahintaj A, Parvin G, Jamshidi A. Comparison of three methods of clinical diagnosis, microscopic and PCR techniques for detection of trichomoniasis in women in the Yasuj City. Sci J Clin Med 2016; 5(1):12-15.
45. Schwebke JR, Burgess D. Trichomoniasis. Clin Microbiol Rev 2004; 17(4):794-803.
46. Mohamed BO, Khalifa KE, Elleboudy NA, Hussein HM, Azab ME. Growth kinetics of Egyptian isolates of *Trichomonas vaginalis*: Possible correlation to clinical presentation. Egyp J Hosp Med 2018; 72 (5) 4428-4433.
47. Meri T, Jokiranta TS, Suhonen L, Meri S. Resistance of *Trichomonas vaginalis* to metronidazole: report of the first three cases from Finland and optimization of *in vitro* susceptibility testing under various oxygen concentrations. J Clin Microbiol 2000; 38(2),763-767.
48. Abdel-Magied AA, El-Kholya EI, Abou El-Khair SM, Abdelmegeed ES, Hamoudaa MM, Mohamed SA, *et al.* The genetic diversity of metronidazole susceptibility in *Trichomonas vaginalis* clinical isolates in an Egyptian population. Parasitol Res 2017; 116(11): 3125-3130.
49. Dos Santos O, de Vargas Rigo G, Frasson AP, Macedo AJ, Tasca, T. Optimal reference genes for gene expression normalization in *Trichomonas vaginalis*. PloS One 2015; 10(9), e0138331.
50. Meade JC, de Mestral J, Stiles JK, Secor WE, Finley RW, Cleary JD, *et al.* 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):245-251.
51. Cornelius DC, Mena L, Lushbaugh WB, Meade JC. Genetic relatedness of *Trichomonas vaginalis* reference and clinical isolates. Am J Trop Med Hyg 2010; 83(6):1283-1286.
52. Jehee I, van der Veer C, Himschoot M, Hermans M, Bruisten S. Direct detection of *Trichomonas vaginalis* virus in *Trichomonas vaginalis* positive clinical samples from the Netherlands. J Virol Methods 2017; 250: 1-5.
53. Tavakoli-Oliaee R, Babaei Z, Hatam GR, Tavakoli Kareshk A, Mahmoudv H, Vafafar A, *et al.* Considerable genetic diversity of *Trichomonas vaginalis* clinical isolates in a targeted population in South of Iran. Iran J Parasitol 2017;12(2):251-259.
54. Snipes LJ, Gamard PM, Narcisi EM, Beard CB, Lehman T, Secor WE. Molecular epidemiology of metronidazole resistance in a population of *Trichomonas vaginalis* clinical isolates. J Clin Microbiol 2000;38(8): 3004-3009.
55. Hampl V, Vaňáčová S, Kulda J, Flegr J. Concordance between genetic relatedness and phenotypic similarities of *Trichomonas vaginalis* strains. BMC Evol Biol 2001; 1:11.
56. Fraga J, Rojas L, Sariego I, Fernández-Calienes A. Characterization of specific RAPD markers of virulence in *Trichomonas vaginalis* isolates. Iran J Parasitol 2015;10(3):448-456.
57. Conrad MD. *Trichomonas vaginalis*: population structure, pathogenicity and the vaginal microbiome. Ph. D. thesis 2012, Department of Basic Medical Science, New York University, United States.
58. Cornelius DC. Population genetics, characterization of virulence factors, and epidemiology in the sexually transmitted parasite, *Trichomonas vaginalis*. Ph. D. thesis 2012, Department of Microbiology, University of Mississippi Medical Center, Jackson, United States.
59. Paulish-Miller TE, Augostini P, Schuyler JA, Smith WL, Mordechai E, Adelson ME, *et al. Trichomonas vaginalis* metronidazole resistance is associated with single nucleotide polymorphisms in the nitroreductase genes *ntr4Tv* and *ntr6Tv*. Antimicrob Agents Cemother 2014; 58(5): 2938-2943.