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ORIGINAL PAPER

The genetic diversity of metronidazole susceptibility in *Trichomonas vaginalis* clinical isolates in an Egyptian population

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Abstract Trichomoniasis is the most common curable sexually transmitted disease worldwide. Resistance to metronidazole in treating trichomoniasis is a problematic health issue. We aimed to determine the minimum lethal concentration (MLC) of metronidazole for *Trichomonas vaginalis* isolates detected in Mansoura, Egypt and studied the genotypic profile of these isolates. Vaginal swab specimens were obtained from 320 symptomatic and 100 asymptomatic females, for whom clinical examination, vaginal discharge wet mount, Giemsa stain, and culture in modified Diamond's media were performed. Metronidazole susceptibility testing by an aerobic tube assay was performed. Both sensitive and resistant isolates were examined by PCR amplification followed by restriction fragment length polymorphism (RFLP). *Trichomonas vaginalis* was identified in 49/420 (11.7%) using either culture or PCR, while wet mount and Giemsa stain detected the parasite in 8.1 and 7.6% of participants, respectively. After 48 h incubation, most isolates were sensitive to metronidazole with a minimal lethal concentration (MLC) of 1 μg/ml. Mild resistance was observed in two isolates with MLCs of 64 μg\ml and mild to moderate resistance was observed in an additional two isolates with MLCs of 128 μg/ml. The four isolates that demonstrated low to moderate metronidazole resistance displayed a unique genotype band pattern by RFLP compared to the other 45 samples that were metronidazole sensitive. Our results highlight the presence of in vitro metronidazole tolerance in a few *T. vaginalis* isolates in Mansoura, Egypt that may lead to the development of drug resistance as well as the possibility of an identifying RFLP pattern in the isolates.

Keywords *T. vaginalis* · Metronidazole · Susceptibility · MLC · PCR-RFLP

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Introduction

The protist, *Trichomonas vaginalis*, is globally the most common, non-viral, and sexually transmitted pathogen (Poole and McClelland 2013). Trichomonas infection can cause vaginitis, vulval irritation, malodorous vaginal discharge, and strawberry cervix (Byun et al. 2015). Other adverse clinical manifestations like inflammatory pelvic disease (Cherpes et al. 2006) and infertility (Stark et al. 2009) are also reported.

Presently, the 5-nitroimidazole family of drugs (especially metronidazole and tinidazole) is the only oral treatment approved for trichomoniasis. As a result, drug resistance, which has been documented and is widely reported, is of concern (Paulish-Miller et al. 2014). The majority of treatment failures are produced by *T. vaginalis* isolates with low susceptibility to metronidazole, in both in vivo and in vitro conditions (Yarlett et al. 1987).

Classification of *T. vaginalis* isolates primarily considers the sensitivity of PCR and the reliability of RFLP (Conrad et al. 2012; Bradic et al. 2017). The PCR-RFLP technique combines PCR and RFLP and can reveal minor variations in a gene where a single base substitution creates or abolishes a

recognition site for the restriction endonuclease enzyme. The technique has proven its effectiveness for strain typing of diverse organisms, like *Chlamydia trachomatis*, *Treponema pallidum*, and *Neisseria gonorrhoeae* (O'Rourke et al. 1995).

Given the growing awareness and appreciation of the serious health sequelae that are associated with *T. vaginalis* infection and the increasing rate of metronidazole resistance, *T. vaginalis* metronidazole susceptibility and its relatedness to different genotypes is still poorly understood. Thus, we chose to study phenotypic variation regarding metronidazole susceptibility of different *T. vaginalis* genotypes in Mansoura, Egypt as a critical consideration for both treatment strategies and epidemiologic studies.

Materials and methods

Ethics

This study was approved by the Institutional Review Board of the Faculty of Medicine, Mansoura University, Egypt (IRB reference no: 16.06.83). Prior to enrollment, informed consent was gained from each participant. Women with confirmed trichomoniasis were treated and followed up.

Study participants

The study was conducted in the period from December 2013 to February 2016. Vaginal swabs were collected from 320 female patients with symptoms consistent with vaginitis attending the Gynecology outpatient clinic at Mansoura University Hospital. In addition, another 100 asymptomatic women were recruited. Participants were of child bearing age (18–45 years); those who were pregnant or menstruating were excluded.

Specimen collection and processing

Participants were examined in accordance with a standard clinical protocol. Two speculum-assisted vaginal swab samples were obtained from the posterior fornix of the vagina of each participant. Specimens were assigned a study ID and, after linkage to a sociodemographic and clinical dataset, specimens were stripped off patient identifying data. The first swab was kept in a tube containing 3 ml sterile phosphate buffered saline (PBS) for wet mount microscopy, which was done within 10 min of sample collection and considered positive if motile *T. vaginalis* parasites were observed. Subsequently, PBS containing the parasite was used for Giemsa-stained smear preparations according to Radonjic et al. (2006). The second swab was transported within half an hour to the parasitology research laboratory for complete examination and incubation.

Culturing *T. vaginalis* isolates

The second swab was used to inoculate a pre-warmed culture tube containing modified Diamond's medium (Fouts and Kraus 1980) with streptomycin-penicillin at 50 μg/ml and 10% heat-inactivated horse serum. Cultures were incubated at 37 °C and assessed by daily from day 2 to day 7 by collecting a drop of culture medium aseptically and examining it microscopically at 10×, positive confirmation at 40×.

In vitro drug susceptibility testing

Positive specimens were sub-cultured in modified Diamond's medium at 48 h intervals by transferring 0.3 ml of the media to the new culture tubes till the required inoculum for drug susceptibility test was reached (Meri et al. 2000). This was done in duplicate three times a week. Trophozoites in sub-culture tubes were counted using a glass hemocytometer and Trypan blue exclusion test according to the method of Borchardt et al. (1997).

The drug susceptibility test was carried out by the aerobic tube assay method described by Kulda et al. (1982). Metronidazole (Amriya) serial dilutions at concentrations of 1, 2, 4, 8, 16, 32, 64, 128, and 256 μg/ml were prepared. The assay was run twice, using drug-free media as standard control. Hemocytometer counts were made of 48 h cultures to obtain the desired number (1×10^4 trophozoite/ml). Each culture tube was inoculated with 10^4 trophozoites, and the different concentrations of the drug were added to each isolate with a final volume of 2 ml per tube and incubated at 37 °C under aerobic conditions for 24 and 48 h. The assays were run in duplicate and repeated at least twice.

The MLC, which is defined as the lowest drug concentration at which no motile parasites were observed after 48 h incubation (Kulda et al. 1982) was determined. In vitro metronidazole resistance was defined as an aerobic MLC ≥ 50 μg/ml after 48 h incubation (Narcisi and Secor 1996).

DNA extraction, PCR amplification

After the drug susceptibility tests were performed, the remaining 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 using Thermo Scientific GeneJET Genomic DNA Purification Kit (USA) according to the manufacturer's instruction. Quantity and purity of the extracted DNA was estimated by Nanodrop (Thermo scientific NanoDrop 2000 spectrophotometer, USA), and the quality of extracted DNA was checked by 1.5% agarose gel electrophoresis.

The Internal transcribed spacer 1 (ITS1) fragment was amplified with oligonucleotide PCR primers based on *T. vaginalis* ribosomal DNA (rDNA) gene (ACCESSION AF466750); TVITS F: 5′-ACA CCG CCC GTC GCT CCT

AC-3′ and TVITS R: 5′ AAT TTG CAT TCA AAG ATT AAC-3′. These primers produce an amplified PCR product of the *T. vaginalis* ITS1. Each PCR reaction mixture contained 5 μL DNA template, 12.5 μl master mix (Tiangen Biotech, Beijing, China), and 1 μL of each of the forward and reverse primers (10 pmol) and 5.5 μL of double distilled water in a 25 μl final total volume. PCR amplification was performed according to the following procedure: initial denaturation by incubation for 5 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s and extension at 72 °C for 1 min, with a final 10 min incubation at 72 °C. The expected single PCR product (313 bp) was confirmed by electrophoresis 2% (*w*/*v*) agarose gel, stained by ethidium bromide and visualized under a UV trans-illuminator.

Restriction fragment length polymorphism (RFLP)

After amplification, the PCR products were digested with MspI restriction enzyme (Thermo Scientific FastDigest MspI #FD0544). In brief, in each PCR tube, 17 μL of sterile D.W, 2 μL (10X) of Fast digest buffer, 10 μL of amplified PCR product, and 1 μl of the restriction endonuclease (MSp1) were added. The reaction mixture was incubated at 37 °C for 15 min followed by enzyme inactivation by heating for 5 min at 65 °C in a heat block. The restriction patterns were analyzed on a 2% agarose gel, run with 50 bp DNA ladder at 100 V for 45 min and visualized using a UV Trans-illuminator.

Statistical analysis

The data were plotted and analyzed using SPSS statistical software package, version 22. Associations between categorical variables were tested by the chi-square test. A P value < 0.05 was considered statistically significant. Fisher's exact test was used when the assumptions of chi square were violated.

Results

The study involved 420 female patients, of whom 320 were symptomatic and 100 were asymptomatic, with a mean age ± standard deviation of 32 ± 5.6 years. The overall prevalence of *T. vaginalis* was 11.7% (13.8% in symptomatic patients and 5% in asymptomatic ones), primarily in the 26 to 35-year-old age group. 95.9% of participants were married, 67.3% were from rural areas, and 55.1% had a vocational level of education. Fourteen (28.6%) of the *T. vaginalis* positive cases were diabetic, which was significantly different compared to negative cases ($P = 0.046$). A burning sensation (65.9%) and pruritus vulvae (63.6%) were the most common symptoms reported by positive individuals. Of those positive for the parasite, 84.1% reported atypical vaginal discharge and 14.3% had post coital bleeding. The symptomatic characteristics of the participants are summarized in Table 1.

Culture and PCR recorded the same percentage of parasite detection among patient groups (11.7% for each), while wet mount and Giemsa stain detected fewer positives (8.1%, 7.6%, respectively). Out of 49 cases (11.7%) positive for *T. vaginalis*, 45 (91.8%) cases were sensitive to metronidazole and only 4 (8.2%) were found resistant. The degree of resistance was classified according to Kissinger et al. (2008) as follows: isolates with MLCs of 50–100, 101–199, 200–400, and > 400 mg/ml were considered to have mild, mild to moderate, moderate, and high resistance, respectively. Most susceptible cases had MLCs of 1 and 2 μg/ml of metronidazole after 24 and 48 h incubation, respectively, as shown in Table 2.

Amplification of the ITS1 fragment by PCR was applied to the 49 *T. vaginalis* positive samples. The amplified 313 bp product was digested by MspI restriction enzyme and electrophoresed on 2% agarose gel. The digested product revealed a 270 bp fragment in all 45 (91.8%) susceptible isolates compared to a 240 bp product in the 4 (8.1%) resistant isolates (Fig. 1, Table 2).

The four in vitro resistant isolates of *T. vaginalis* were from symptomatic women who had atypical discharge and previous history of taking metronidazole and on clinical examination, all demonstrated strawberry cervix. The isolate MLCs were 128, 128, 256, and 128 μg/ml and 64, 128, 128, and 64 μg/ml after 24 and 48 h, respectively.

We did not find significant differences between women infected with metronidazole-resistant compared to metronidazole-susceptible strains in terms of age, residence, marital status, educational level, symptoms, the use of contraception, parity, or having a previous diagnosis of trichomoniasis. Nevertheless, we found statistically significant difference between both groups regarding presence of typical discharge, post coital bleeding,

Table 1 Laboratory findings in clinical trichomoniasis

		Asymptomatic		Symptomatic		*P**
		No.	%	No.	%	
Wet mount	Negative	4	80	11	25	0.026
	Positive	1	20	33	75	
Giemsa stain	Negative	4	80	13	29.5	0.043
	Positive	1	20	31	70.5	
RFLP band	270 pb	5	100	40	90	1
	240 pb	0	–	4	10	
Culture	Negative	0	–	0	–	1
	Positive	5	100	44	100	
PCR	Negative	0	–	0	–	1
	Positive	5	100	44	100	

*Fisher's exact test

Table 2 Genotyping of *Trichomonas vaginalis* and MLC of metronidazole after 48 h incubation under aerobic condition

	MLC μg/ml, No. (%)								
	I						III	III	IV
FRLP	1	2	4	8	16	32	64	128	256
270	17 (34.7%)	8 (16.3%)	6 (12.2%)	7 (14.2%)	4 (8.1%)	3 (6.1%)			
240							2 (4.1%)	2 (4.1%)	

*degree of resistance according to (Kissinger et al., 2008), I (susceptible), II (Mild resistance), III (Mild to moderate resistance), IV (Moderate resistance)

lower abdominal pain, recorded partner symptoms, and history of previous treatment (Table 3). Additionally, a past history of multiple rounds of metronidazole treatment was noted in the participants infected with in vitro resistant isolates compared to the susceptible ones.

Discussion

Failure of metronidazole to cure *T. vaginalis* infections is of concern because metronidazole is currently the only oral drug approved for the treatment of trichomoniasis (Ali and Nozaki 2007). To our knowledge, this study is the first to investigate the in vitro metronidazole susceptibility assays of clinical *T. vaginalis* isolates and its relevance to *T. vaginalis* genotypes in Mansoura, Egypt.

The prevalence of trichomoniasis was 11.7% (13.8% in symptomatic and 5% in asymptomatic participants). This prevalence is in accordance with a study that showed a prevalence of *T. vaginalis* infection of 11% (Hussein et al. 2015) among symptomatic females in Benha hospital, Egypt. However, other studies demonstrated a higher prevalence of *T. vaginalis* infection ranging from 23 to 38.37% (Negm and el-Haleem 2004; Hegazy et al. 2012). The epidemiology of trichomoniasis is variable and depends on many factors like age, sexual activity, other infections, method of examination, and diagnostic technique (Grama et al. 2013).

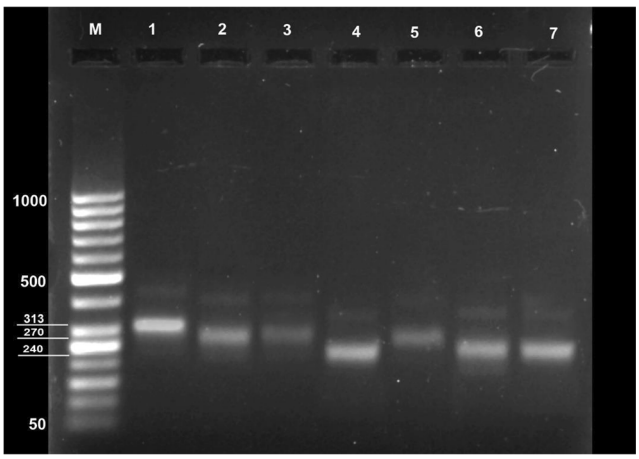

Fig. 1 Two percent agarose gel electrophoresis for PCR products and after digestion with Msp1 restriction enzyme. Fifty-base-pair DNA ladder is in lane M. Lane 1, PCR amplification product (313 bp); lane 2, 3, and 5, MspI-digested PCR product (270 bp) (wild type) and lane 4, 6, and 7, MspI-digested PCR product (240 bp) (mutant type) in resistant cases

Herein, 5% of asymptomatic cases were positive for *T. vaginalis*, a finding that reinforces the importance of conducting laboratory tests for accurate diagnosis and not only being concerned with the presence of symptoms. Among the positive cases, 28.6% (14/49) were diabetic. This information deserves attention, since diabetes is a risk factor for trichomoniasis (Younis and Elamami 2016; Kalra and Kalra 2017) and is likely due to poor glycaemic control. Hence, it is wise to follow diabetic females to ensure optimal perineal/genital and metabolic health.

Our findings showed a statistically higher frequency of discharge, post coital bleeding, lower abdominal pain, vaginal hyperemia, strawberry cervix, multiple metronidazole treatments, and increased partner symptoms in women with resistant isolates compared to those with susceptible isolates. With these findings in mind, resistance to metronidazole might be anticipated from history and examination. The prevalence of metronidazole-resistant cases (8.2%) in our locality approximates that in the study of Schwebke and Barrientes (2006) which detected 17/178 (9.6%) resistance isolates.

In our work, genotyping with PCR-RFLP using ITS1 gene revealed differences between susceptible and resistant isolates. According to Kazemi et al. (2010), the resistance may be associated with a mutation in the ITS1 gene at position 209 (C209T), in which the thymidine was replaced by cytosine. By the use of MspI enzyme, the 4 resistant isolates showed a MspI digested PCR product of 240 bp and the 45 susceptible isolates showed MspI digested PCR product of 270 bp when analyzed by agarose gel electrophoresis. Thus, our findings are in agreement with Kazemi et al. (2010).

Another study of Conrad et al. (2012) used multilocus sequence typing and microsatellite genotyping of *T. vaginalis*; the authors detected two genotypes and reported a correlation between metronidazole MLCs and *T. vaginalis* isolates using microsatellite genotyping. They found that a higher degree of metronidazole resistance was associated with the type 2 isolate. Snipes et al. (2000) used random amplified polymorphic DNA

Table 3 Characteristic manifestations of both susceptible and resistant metronidazole genotypes in positive *T. vaginalis* cases

		Susceptible symptomatic cases (40)		Resistant symptomatic cases (4)		P [a]
		No.	%	No.	%	
Itching	Absent	16	40	0	–	0.28
	Present	24	60	4	100	
Burning	Absent	15	37.5	0	–	0.28
	Present	25	62.5	4	100	
Dysuria	Absent	19	47.5	1	25	0.61
	Present	21	52.5	3	75	
Lower abdominal pain	Absent	24	60	0	–	0.03
	Present	16	40	4	100	
		Susceptible symptomatic cases (38) [b]		Resistant symptomatic cases (4)		P [a]
		No	%	No	%	
Dyspareunia	Absent	19	50	0	–	0.11
	Present	19	50	4	100	
Post coital bleeding	Absent	35	92.1	1	25	0.007
	Present	3	7.9	3	75	
		Susceptible symptomatic and asymptomatic cases (45)		Resistant symptomatic and asymptomatic cases (4)		P [a]
		No.	%	No.	%	
Previous metronidazole treatment	Absent	22	48.9	0	–	0.12
	Present	23	51.1	4	100	
Number of metronidazole courses	Single	21	46.7	0	–	0.002
	Multiple	2	4.4	4	100	
Partner symptoms [c]	Absent	40	93	1	25	0.005
	Present	3	7	3	75	

[a] Chi-square test (×2), [b] Two cases were excluded as they have no partner, [c] Only 43 cases were included as two cases have no partner

technique and found a correlation between metronidazole resistance and a point mutation in ITS1 (between 16S and 5.8S rRNA).

Conversely, other studies did not find any difference between metronidazole-susceptible and resistant *T. vaginalis* isolates using PCR (Rabiee et al. 2012) or HSP70 gene RFLP analysis by EcoRl-digestion (Hussien et al. 2005), possibly because they targeted a region outside the genes that code for the drug targets (Gandhi et al. 2014).

Conclusion

In essence, among symptomatic and asymptomatic females enrolled in our study, we found a 4.1% prevalence of both mild and mild to moderate metronidazole resistance. Resistant and susceptible isolates were differentiated by the use of PCR-RFLP of the ITS1 region. The outcomes of this study emphasize the need for periodic evaluations of *T. vaginalis* metronidazole drug susceptibility to monitor the possible emergence of resistance, empirical treatment of patients based on clinical symptoms, introduction of routine resistance testing, and pursuit of novel treatment options other than metronidazole.

Compliance with ethical standards

Ethical approval This study was approved by the Institutional Review Board of the Faculty of Medicine, Mansoura University, Egypt (IRB reference no: 16.06.83).

Statement of informed consent Prior to enrollment, informed consent was gained from each participant. Women with confirmed trichomoniasis were treated and followed up.

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