IN VITRO BIOCHEMICAL VARIABILITY OF SUSCEPTIBLE AND NON SUSCEPTIBLE ISOLATES OF TRICHOMONAS VAGINALIS TO METRONIDAZOLE

By

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ABSTRACT

Background: Although metronidazole is the drug of choice for treatment of trichomoniasis, resistant *Trichomonas vaginalis* isolates have been identified. **Aim of the study:** So, the aim of our study is to investigate the in vitro susceptibility of *T. vaginalis* against metronidazole in our locality.

Methods: Vaginal swabs were collected from 420 cases attending the outpatients' clinics of the gynecology department, Mansoura university hospital. The patients were subjected to wet mount smear examination and Giemsa staining

followed by culture on modified Diamond's media. In vitro susceptibility testing was done for 49 *trichomonas vaginalis* positive cases to determine minimal lethal concentration (MLC) of metronidazole in each case.

Results: Most susceptible isolates (34.7%) were dead at MLC 1 µg/ml followed by MLC 2 µg/ml at which 8 (16.3%) isolates were killed, followed by 6 (12.2%) isolates at 4 µg/ml, 7 isolates (14.2%) at 8 µg/ml, 4 isolates (8.1%) at 16 µg/ml, and 3 (6.1%) isolates at 32 µg/ml while in resistant isolates 2 were killed at concentration 64 µg/ml and 2 iso-

lates were sensitive at concentration 128 µg/ml.

Conclusion: In our locality, most isolates were susceptible to low dose of metronidazole (lower than or equal 32 µg/ml) with few resistant isolates that could be explained by development of drug resistance in future.

Keywords: Trichomonas vaginalis, Metronidazole, Resistance, Susceptibility, in vitro biochemical testing.

INTRODUCTION

Trichomoniasis, the clinical disease refers to the *T. vaginalis* pathogen, has become the most prevalent non-viral sexually transmitted infection (STI) in the United States [1]. The worldwide incidence of trichomoniasis was estimated to be 276.4 million new cases per year in 2008 [2]. In Egypt, the reported rate of prevalence ranges from 5% to 79.16% [3].

In women, trichomoniasis has a wide spectrum of clinical presentations ranging from asymptomatic to acute, or subacute vaginitis with malodorous vaginal discharge [4]. Other

symptoms include dysuria, dyspareunia, or vulvovaginal soreness. Common signs include vulvovaginal erythema, frothy yellowish-gray vaginal discharge, increase pH of the vagina (>6), and rarely a strawberry cervix [5]. Infection may be complicated with preterm labor and increased perinatal morbidity [6].

Diagnosis of *T. vaginalis* is established by the traditional wet mount test, in which "corkscrew" motility is observed [7]. Culture has long been the gold standard for diagnosing *T. vaginalis* infection, with a sensitivity range from 85-95% [8]. Other used methods for diagnosis include enzyme-linked immunosorbent assay, staining methods, latex agglutination, and nucleic acid amplification tests. [9]

Metronidazole is the drug of choice for the treatment of trichomoniasis. A single oral dose of 2-g achieves a cure rate of 90-95% [10]. Low-level resistance of *T. vaginalis* to metronidazole has been reported in a range of 2-5% [11].

Metronidazole resistance is defined clinically as failure to cure infection after two successive courses

of treatment. Once other causes of treatment failure have been ruled out including medication noncompliance and reinfection, the possibility of a resistant vaginal trichomoniasis must be considered [12]. Although various therapeutic regimens have been developed and recorded, there is no consensus on therapy for metronidazole resistant vaginal trichomoniasis [13].

The aim of this work is to detect the magnitude of *T. vaginalis* resistance to metronidazole in our locality and its implications on patients' outcome.

SUBJECTS & METHODS

2.1. Study design

The study was carried out in Medical Parasitology Department Faculty of Medicine, Mansoura University and Gynecology outpatient clinic of Mansoura University Hospital, during the period from December 2013 to February 2016.

2.2. Patients' selection

A total of 320 symptomatic female patients were included in this study. Another 100 patients were included in the study screening for trichomonas vaginalis in non-symptomatic patient.

Criteria for symptomatic patients:

Women in their childbearing age (18:45 years) who complained of vaginal discharge of any type, itching, burning sensation or both, and with any other gynecological manifestations suggestive of trichomoniasis such as dyspareunia and or dysuria.

Criteria for asymptomatic patients: Patients matching the same age group were included.

Exclusion criteria: Pregnant women, during menstruation, vaginal douching for at least 2–3 days before the day of examination.

3.2. Data and sample collection

An informed written consent was obtained from each patient after explanation of the procedure and all experiments have been examined and approved by Mansoura University ethics committee.

History taking included age, parity, presenting symptoms such as vaginal discharge, bad odor, itching, dysuria, dyspareunia, burning sensation and post coital bleeding, also obstetric history as regard delivery of low birth weight infant and preterm delivery

Clinical examination involved per vaginal (PV) non lubricated dry sterile speculum for each case to obtain two vaginal swab.

4.2. Methods

Two Vaginal specimens were collected using sterile cotton swab from the posterior fornix during speculum examination to be examined as following : one swab for wet mount microscopic examination according to Mahmoud et al [14]. Swab was mixed in 1:2 ml phosphate buffer saline solution and then examined microscopically under dark ground illumination for evidence of motile trichomonas vaginalis within 0.5 – 3 hours after sampling time (Figure 1).

The other swab used for culture of *T. vaginalis* on Modified Diamond medium [15]. The medium was supplemented with 10% heat inactivated bovine serum. Microorganisms were eliminated by the aid of sodium penicillin G, streptomycin sulphate, amphotericin. All strains were maintained in 10 ml screw cap glass tube at 37°C. The detection of *T. vaginalis* trophozoites was considered if object of approximately 7 to 10 µm in diameter was observed showing the characteristic jerky motility (Figure 2).

Subculture was usually done every 48h by adding 0.3 ml from the suspension to fresh tube containing culture media [16].

5.2. In vitro resistance tests

We used metronidazole vial (Amerizole) (Amriya for pharmaceutical industries) each 100 ml contain 500mg metronidazole. Drug susceptibility test was carried out by aerobic tube assay method as described by Kulda et al [17]. Metronidazole was used in our study in a serial dilution at concentration of 1,2,4,8,16,32,64,128,256 µg/ml. The assay was run twice with drug free media as standard control. Haemocytometer count was made with 48h old culture of trichomonads to obtain the desired number (1×10^4 trophozoite /ml).

Each one of 9 culture tubes were 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 48 hours. The control tubes for each isolate were incubated simultaneously under the same conditions, the drug susceptibility of the different isolates was recorded

by determining the minimal lethal concentration (MLC), which is defined as the lowest drug concentration at which no motile organisms were observed [17, 18]. The viability of the parasite was assessed by re-culture of the inoculate previously exposed to the drug in drug-free media [17]. Metronidazole resistance case was defined as aerobic MLC $\geq$ 50 μ g/ml [18, 19].

6.2. Statistical analysis

Data were analyzed on a personal computer running IBM-SPSS for windows "Statistical-Package for Social-Scientists" Release-22. Statistical tests were two-tailed. A p-value < 0.05 was interpreted as statistically significant. For description of qualitative variables, the frequency distribution was utilized with tabulation including the number of cases and percentage. For description of measurable parameters, the mean, and standard-deviation were used to show central tendency and dispersion. Relations between categorical variables were tested by the Chi-Square Test (X^2). Fishers' exact-test was utilized when the prerequisites of Chi-square test were violated.

RESULTS

From 320 Symptomatic patients, 44 (13.8%) patients were positive for infection with *T. vaginalis* and from 100 asymptomatic patients 5 (5%) patient was positive for infection as shown in (Table 1.). There is high percent in the presence of *T. vaginalis* infection in symptomatic cases vs. asymptomatic cases (13.8% vs. 5%; $P=0.02$), and the difference was statistically significant, the overall prevalence of *T. vaginalis* among the studied cases was (11.7%).

The mean age of studied cases as shown in Table 2 was 32 ± 5.6 years, *T. vaginalis* positive cases were more prevalent among age group 26-35 years (53.1%). The difference between presence and absence of *T. vaginalis* among different age groups was of no statistically significant difference ($p=0.08$).

As shown in (Table 3), (From 44 symptomatic (40 susceptible and 4 resistant) not include 5 cases which is asymptomatic) percent of typical type of discharge is higher in resistant than susceptible cases. The difference reveal high statistically significant difference (100% vs. 7.5%; $P=0.001$).

According to (Table 4), (From 44 symptomatic patients from which there was 1 patient widow and one patient divorced (40 susceptible and 4 resistant) not include 5 cases which is asymptomatic) post coital bleeding and pain were significantly higher in resistant cases.

Table 5 from total 49 positive symptomatic and asymptomatic cases, there was a high percent in presence of preterm delivery in resistant than susceptible cases (50% vs. 2.2%) and the difference was statistically significant ($p=0.015$). Also, there was a high percent in presence of low birth weight infant in resistant than susceptible cases (50 vs. 4.4%) and the difference was statistically significant ($p=0.03$).

In the present study, MLC of different concentration of metronidazole under aerobic condition after 48 h incubation period as shown in Figure 3 was as following, most susceptible isolates 17/49 (34.7%) were sus-

ceptible at MLC 1 $\mu\text{g/ml}$ followed by MLC 2 $\mu\text{g/ml}$ at which 8/49 (16.3%) isolates were died, followed by 6/49 (12.2%) isolates at 4 $\mu\text{g/ml}$, followed by 7/49 isolates (14.2%) at 8 $\mu\text{g/ml}$, followed by 4/49 isolates (8.1%) at 16 $\mu\text{g/ml}$, followed by 3/49 (6.1%) isolates at 32 $\mu\text{g/ml}$ while in resistant isolates 2 were died at concentration 64 $\mu\text{g/ml}$ and 2 isolates were died at concentration 128 $\mu\text{g/ml}$ and no isolates were died at concentration 256 $\mu\text{g/ml}$.

The prevalence of low-level metronidazole resistance was (4.1%) and the prevalence of mild to moderate resistance was (4.1%) according to Kissinger et al [19], degree of resistance was detected as follow, mild resistance occurs with MLCs of 50:100 $\mu\text{g/mL}$, mild-to moderate resistance occur with MLCs of 101:199 $\mu\text{g/mL}$, moderate resistance occurs with MLCs of 200:400 $\mu\text{g/mL}$ and high resistance occur with MLCs of 400 $\mu\text{g/mL}$ of isolates.

Table 1 Detection rate of *Trichomonas vaginalis* among studied cases

Group	Total No	Positive	%	P*
Symptomatic Cases	320	44	13.8%	0.02
Asymptomatic Cases	100	5	5%	
All cases included in the study	420	49	11.7%	

*Chi Square test (χ^2)Table 2 *Trichomonas vaginalis* according to age groups of studied cases :

Age group	Trichomonas +ve N (%)	Trichomonas -ve N (%)	Total number	P*
18-25	9 (18.3%)	107 (28.8%)	116	0.08
26-35	26 (53.1)	143 (38.5%)	169	
36-45	14 (28.6)	121 (32.6%)	135	

*Chi Square test (χ^2)Table 3 Comparison of susceptible and resistant isolate of *Trichomonas vaginalis* symptomatic cases as regard presence of discharge and bad odor:

		Susceptible (40)		Resistant (4)		P**
		No	%	No	%	
Discharge	Typical	3	7.5%	4	100.0%	<0.001
	Atypical	37	92.5%	0	0.0%	
Bad odor	Absent	18	45.0%	0	0.0%	0.13
	Present	22	55.0%	4	100.0%	

**Fisher's Exact test

Table 4 : comparison of susceptible and resistant isolates of *Trichomonas vaginalis* symptomatic cases as regard other symptoms:

		Susceptible (40)		Resistant (4)		p**
		No	%	No	%	
Itching	Absent	16	40.0%	0	0.0%	0.28
	Present	24	60.0%	4	100.0%	
Burning	Absent	15	37.5%	0	0.0%	0.28
	Present	25	62.5%	4	100.0%	
Dyspareunia	Absent	19	50.0%	0	0.0%	0.11
	Present	19	50.0%	4	100.0%	
Post coital bleeding	Absent	35	92.1%	1	25.0%	0.007
	Present	3	7.9%	3	75.0%	
Dysuria	Absent	19	47.5%	1	25.0%	0.61
	Present	21	52.5%	3	75.0%	
Pain	Absent	24	60.0%	0	0.0%	0.03
	Present	16	40.0%	4	100.0%	

**Fisher's Exact test

Table 5 : comparison of susceptible and resistant of *Trichomonas vaginalis* positive cases as regard obstetric history:

		Susceptible cases (45)		Resistant cases (4)		p**
		No	%	No	%	
Parity	Nulliparous	14	31.1%	0	0.0%	0.31
	Has Children	31	68.9%	4	100.0%	
Preterm	Absent	44	97.8%	2	50.0%	0.015
	Present	1	2.2%	2	50.0%	
Abortion	Absent	43	95.6%	3	75.0%	0.22
	Present	2	4.4%	1	25.0%	
Low Birth Weight	Absent	43	95.6%	2	50.0%	0.03
	Present	2	4.4%	2	50.0%	

**Fisher's Exact test

Figure. 1: Shows *T. vaginalis* trophozoite in wet mount preparation

Figure. 2: *T. vaginalis* observed in Modified Diamond's culture

*Figure 3 Minimal lethal concentrations of metronidazole on *T. vaginalis* isolates.*

DISCUSSION

Trichomonas vaginalis is a parasite of the urogenital tract of human, with a worldwide occurrence and significant implications for global public health. It has been implicated with preterm labor, pelvic inflammatory disease, cervical intraepithelial neoplasia [20].

The overall prevalence of *T. vaginalis* among women included in this study was 11.7% (49/420), among symptomatic woman was 13.8% (44/320) and among asymptomatic woman was 5% (5/100). This corroborates findings by a previous study from Benha the prevalence of *T. vaginalis* in this study was 11%, [21]. However, higher rates of infection were recorded in Cairo and Mansoura with prevalence of 23% and 38.37%, respectively [22, 23]. On the other side, studies from Africa, Iraq and Cairo demonstrated a lower prevalence of *T. vaginalis* infection ranging from 4.1%- 5.4% [14, 24, 25]

The variations in the prevalence of infection related to many factors including age, sexual activity, number of sexual partners, other sexually transmitted diseases, menstrual cycle phases, examination techniques,

specimen collection, sample size, inclusion and exclusion criteria and laboratory technique. Also, may be due to socio-demographic characters of the communities that change from a country to another and from a society to another [26].

In this study the Infection was considerably more common in symptomatic than in asymptomatic women with statistically significant difference (13.8% vs. 5%; $P=0.02$), and this finding matched with other study [27]

The mean age of studied cases was 32 ± 5.6 years, *T. vaginalis* positive cases were more prevalent among age group 26-35 years (53.1%), followed by age group 36-45 years (28.6%), followed by age group 18-25 years (18.3%).

In the present study, percent of typical type of discharge is higher in resistant than susceptible cases and the difference was of high statistical significance (100% vs. 7.5%; $P=>0.001$), also there was a high percent in the presence of post coital bleeding in resistant versus susceptible cases and the difference between was statistically significant (7.9% vs. 75%; $P=0.007$), also there

was a high percent in the presence of lower abdominal pain in resistant versus susceptible cases and the difference between was statistically significant (40% vs. 100%; $P=0.03$), also there was a high percent in presence of low birth weight infant in resistant than susceptible cases (50 vs. 4.4%) and the difference between was statistically significant ($p=0.03$)

To our Knowledge this is the first study to compare the susceptible and resistant isolates of *T. vaginalis* from the previous point of view and from this result it will be a valuable finding used to suspect resistant *T. vaginalis* isolates in patient with profuse frothy yellow to gray malodorous discharge [28], post coital bleeding, lower abdominal pain, history of preterm delivery and low birth weight infant especially if all present in combination.

According to Narcisi and Secor [18], in an aerobic 48 hours assay in the present study *T. vaginalis* isolates with MLC of 32 $\mu\text{g/ml}$ and less were considered susceptible to metronidazole, while those with MLC of 64 $\mu\text{g/ml}$ and greater were considered resistant.

From the previous finding the prevalence of resistance in our study was 8.2%, this finding correlate with other study in US where 9.6% only were resistant to metronidazole among total Trichomoniasis cases [11]. Variation in susceptibility was also reported in other studies [29].

The prevalence of low-level metronidazole resistance was (4.1%) and the prevalence of mild to moderate resistance was (4.1%), this was in agreement with other study as regard low level resistance [30].

Conclusion

- *T. vaginalis* resistance to metronidazole is an announcing problem in the world, the incidence rate of metronidazole resistance is 8.2% in our locality.
- The presence of typical discharge, pain and post-coital bleeding point are significantly associated with resistance to metronidazole.
- *T. vaginalis* resistance to metronidazole is associated with significantly higher obstetric complications including preterm labor and low birth weight.
- It is recommended to do a pharmacokinetic study of metronidazole.

zole to detect its level in the vaginal mucosa; a concentration higher than 64ug/ml at 48 hours is needed to overcome resistance.

- Searching for another drug in *T. vaginalis* resistance should be considered in this prospect.

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