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Original Article

In Vitro Susceptibility of Iranian Isolates of *Trichomonas vaginalis* to Metronidazole

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Abstract

Background: Metronidazole, a 5-nitroimidazole derivative, is the main antitrichomonal agent of choice for treatment of trichomoniasis. Since 1962, some cases of treatment failure with metronidazole have been reported and recently drug resistance is now on the rise in the world. This study was aimed to determine current susceptibility of Iranian isolates of *Trichomonas vaginalis* to metronidazole.

Methods: This study was performed on 50 *T. vaginalis* isolates collected from west and central areas of Iran. After axenisation of the parasites, susceptibility testing was carried out by using serial twofold dilutions of metronidazole (400 to 0.1 µg/ml). The minimum inhibitory concentration (MIC) and the minimum lethal concentration (MLC) of the trichomonads were determined after 48 h incubation at 35.5 °C. Drug susceptibility assays of the all isolates were carried out two times in triplicate under aerobic and anaerobic conditions.

Results: Ninety-eight percent of the *T. vaginalis* isolates (49/50) were sensitive to metronidazole. Metronidazole resistance was defined as aerobic MIC ≥50 µg/ml, detected in one isolate. The means of aerobic MICs and MLCs and that of anaerobic MICs of the parasites were 2.91, 1.95 and 0.28 µg/ml, respectively.

Conclusion: This investigation showed in vitro low-level tolerance to metronidazole in a few *T. vaginalis* isolates that may be leading to the development of drug resistance.

Introduction

Trichomonas vaginalis, an anaerobic flagellated protozoan, is responsible for human urogenital trichomoniasis with notable prevalence in the worldwide. Trichomoniasis, known as a common sexually transmitted disease (STD), is often accompanied with other STDs and suspected of increased risk of infection with other microbes considerably HIV, cervical neoplasia and adverse pregnancy outcomes (1-5). The various biological behaviors of *T. vaginalis* is one of the most striking points of the parasite so that clinical features of the disease can be observed differently, ranged from severe to mild or without clinical manifestations (6).

Drug resistance and treatment failure are the two most important issues of trichomoniasis. The nitroimidazole compounds are the only drugs of choice for treatment of the infection and cleared by the Food and Drug Administration (FDA) in the United State (7). The recommended drug regimens are metronidazole or tinidazole 2 g orally in a single dose and alternative regimen is metronidazole 500 mg orally twice a day for 7 d, CDC 2010. The cure rate of metronidazole and tinidazole therapeutic regimens for trichomoniasis is 90-95% and 86-100%, respectively (7). Two to five percent of clinical trichomoniasis posses some level of drug resistance to metronidazole, the common antitrichomonal agent (7). Present of *T. vaginalis* parasite, after two recommended successive courses of metronidazole, is considered as clinical resistance reported since 1962 (8, 9). Clinical treatment failure can be due to drug inactivation by vaginal bacterial flora, defective absorption or delivery of drug to the target site, reinfection and incomplete treatment and finally, may be primarily caused by parasite-dependent activity (9).

Because of the public health importance of the issue and the existence of little information in this field, in this study we conducted drug susceptibility testing of clinical *T. vaginalis* isolates for determining drug sensitivity among Iranian isolates.

Materials and Methods

T. vaginalis isolates and culture

Parasite samples were acquired from women attending gynecology clinics in Hamadan, Toyserkan and Tehran City, during 2010 and 2011. Vaginal sampling was used to detect trichomoniasis in all participants**.** Specimens were collected from women undergoing a genital examination and parasite survey was conducted by wet mount preparation and xenic culture technique using Dorset culture medium (10). Then, *T. vaginalis* isolates cultivated in TYI-S-33 (Diamond's) medium supplemented with 10 to 15 ml of heat-inactivated adult bovine serum, antibiotic (100 IU/ml penicillin, 100 µg/ml streptomycin sulfate, 50 µg/ml gentamicine) and antifungal (1 µg/ml amphotericin B), incubated at 35.5 °C. The cell culture tubes were viewed daily under an inverted microscope and they were passaged every 48 h. Axenic culture of the parasites was achieved after several subcultures, confirmed by elimination of the antibiotics used in the culture medium and bacterial culture negative.

Determination of MIC and MLC

The minimum inhibitory concentration MIC was defined as the lowest drug concentration that the motile parasite was not observed in the culture microtiter plate well examined by inverted microscope at high magnification (×400) after 24 and 48 h incubation under both aerobic and anaerobic condition. In addition, the minimum lethal concentration (MLC) was defined as the highest dilution of medicine prevented growth of the drug-exposed parasites in fresh antibiotic-free medium.

Drug susceptibility assays

In vitro drug susceptibility testing was conducted in accordance with Meingassner meth-

od modified by the CDC (11, 12). Metronidazole powder (Sigma Chemical Co. St Louis) was dissolved in distilled water sterilized through filtration (0.22 µm pore size) and stored at 4 ˚C. Serial twofold drug dilutions, ranging from 400 to 0.1 µg/ml, were prepared using medium culture. Recommended trichomonad cells for aerobic and anaerobic susceptibility assays were 1×10^5 and 5×10^3 trophozoites per well, respectively (13). Parasite cells were added in 96-well flat-bottom microtiter test plate, containing serial dilutions of the metronidazole. The test plates incubated at 35.5 ˚C in aerobic condition and monitored microscopically after 24 and 48 h. Then, the lowest concentration of the drug in microplate wells in which no motile parasites were detected, considered as MIC. The aerobic MLC was done in this manner: following determination of aerobic MIC, 100 µl of the content of each MIC well was transferred to 5 ml of fresh medium, without metronidazole, and incubated aerobically. The tube cultures were surveyed with inverted microscope for viable cells and growth of parasites after 5 and 10 days of cultivation and then the highest dilution of the drug that had parasite growth-inhibitory effect, considered as MLC (14). For MIC anaerobic tests, the test plates were placed in anaerobic jar containing Anaerocult C pack (Merck) for generation of an anaerobic environment. All susceptibility tests of each isolate were performed three times at least twice in aerobic and anaerobic condition compared with control.

Statistical analysis

SPSS statistical software, version 16 (Chicago, IL, USA), was applied to evaluate the relationship between the level of metronidazole susceptibility and the clinical manifestations. Comparison of mean of aerobic and anaerobic MICs and MLCs of metronidazole was performed by paired sample *t*-test.

Results

Vaginal swab samples were examined by wet mount and Dorset's culture and 50 out of 950 specimens were positive for *T. vaginalis*.

Aerobic assays

After 48 h incubation, the in vitro metronidazole susceptibility of 98% of isolates showed drug sensitivity ranged from 0.2 to 25 µg/ml and the mean MICs ± standard deviation was 2.91 ± 8.39.

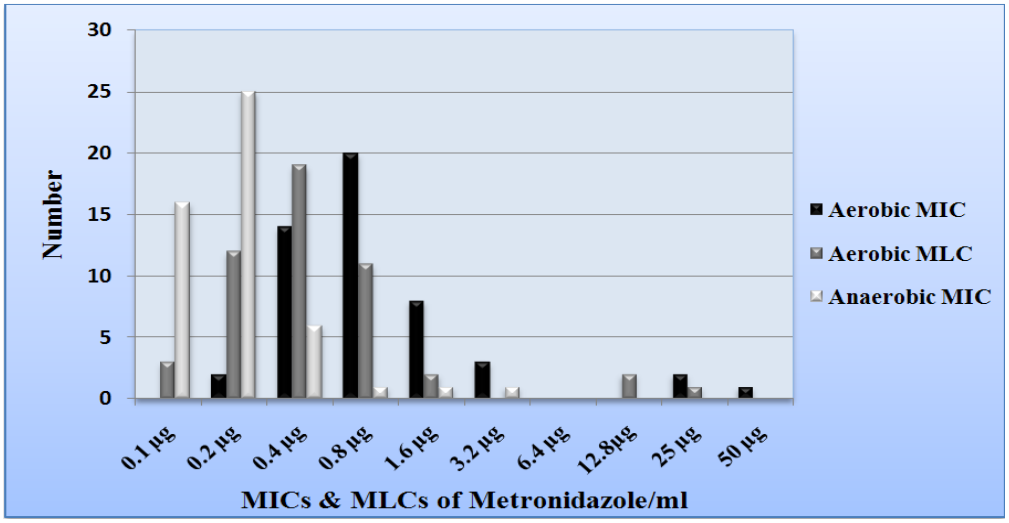

Fig .1: Comparison of metronidazole MICs and MLCs of Iranian *T. vaginalis* isolates under both aerobic and anaerobic environments

Aerobic MIC of metronidazole equal or more than 50 μg/ml considered as metronidazole resistance (12), detected in one isolate. Aerobic MLCs of metronidazole were in the range of 0.1 to 25 μg/ml with a mean of 1.45 μg/ml and a standard deviation of 4.19 μg/ml. Ninety four percent of parasite isolates (47/50) showed aerobic MLCs of metronidazole less than 1.6 μg/ml, two MLCs were 12.8 μg/ml and metronidazole resistant isolate had a MLC equal 25 μg/ml (Fig.1). Aerobic MICs after 24 h were at least one dilution higher than those after 48 h.

Anaerobic assays

Under anaerobic condition for 48 h incubation, MICs of metronidazole were achieved from 0.1 to 3.2 μg/ml with an average of 0.28 μg/ml and a standard deviation of 0.46 μg/ml. The most of isolates (47/50) had susceptibility to metronidazole less than or equal to 0.4 μg/ml. Anaerobic MIC of resistant isolate was 3.2 μg/ml.

Statistical analysis

Three *T. vaginalis* isolates had MIC ≥ 25.6 μg/ml, caused symptomatic trichomoniasis but there was no relationship between metronidazole susceptibility and clinical manifestations, at the 0.05 level (P= 0.235). Significant differences were acquired between the means of aerobic and anaerobic MICs (P=0.025) and likewise, between aerobic MICs and MLCs (P=0.009).

Discussion

Before the introduction of metronidazole in 1959, local vaginal therapy was the only way to cure trichomoniasis and it was mainly palliative in women. After development of the nitroimidazole derivative, 1-β-hydroxyethyl-2-methyl-5-nitroimidazole, a new horizon for treatment of trichomoniasis emerged and was found extremely effective in treating of severe vaginal infection. Very soon, the new curative drug encountered with reports about clinical treatment failure, in 1962. Metronidazole resistance of *T. vaginalis* was divided suggested to into two classes, aerobic and anaerobic. Ferredoxin, an iron-sulfur protein that operates as an electron carrier in biochemical reactions, is involved in aerobic resistance mechanism so that production of ferredoxin is reduced by resistance trichomonads. In anaerobic resistance, reduced activity of Pyruvate: ferredoxin oxidoreductase (PFOR) and hydrogenase, two hydrogenosomal proteins, are suspected in the phenomena (5, 6, 8).

To our knowledge, the present study is the first comprehensive effort to investigate the in vitro metronidazole susceptibility assays of clinical *T. vaginalis* isolates in Iran. In this study, drug susceptibility testing was accomplished aerobically and anaerobically on 50 *T. vaginalis* isolates from Hamadan, Tehran and Toyserkan including 35, 8 and 7 isolates, respectively. The overwhelming majority of isolate sensitive to metronidazole and 94% of them had MICs of 3.2 ≤ μg/ml. Sensitivity equal 12.8 μg/ml was detected in two isolates that can be indicated as development of drug resistance in the society. Marginal resistance or in other words, low-level tolerance (aerobic MIC=50 μg/ml), was observed in one isolate from Hamadan. A single point mutation in the rDNA gene, ITS1 C66T, may be correlated to metronidazole resistance of *T. vaginalis* (15). Three trichomonad isolates that had MICs of ≥25 μg/ml were mutant type (16).

These findings were approximately consistent with other reports of workers. According to the CDC, 2-5% of clinical status of the infected patients may be accompanied with low-level metronidazole resistance and high-level resistance rarely is found (7). Meingassner et al. reported MICs and MLCs of all 94 *T. vaginalis* isolates, utilized in the drug susceptibility in 1978, to be in the range of 0.1 to 1.6 and 0.4 to 6.4-μg metronidazole/ml, respectively (11). The MLC method was based on microscopic examination and trophozoite motility (11). A study on 91 Spanish isolates, acquired from infected patients and female sex

worker, showed 2.2% metronidazole-resistant strains with sensitivity 50 μg/ml (17). In Atlanta, drug resistance survey of *T. vaginalis* parasite was performed on adolescent female samples and exhibited two moderate level (MLC of >50 μg/ml) and three low level (MLC=50 μg/ml) metronidazole resistance isolates (18). Schwebke et al. carried out susceptibility testing of 178 *T. vaginalis* achieved from women presenting to a STD clinic in Birmingham and detected 9.6% resistance isolates including eleven very low, three low, two moderate and one high resistance with MLCs of 50, 100, 200 and >200 μg/ml, respectively (12). In 2012, five cases of refractory trichomoniasis were reported among 15 infected women attending genecology clinics in Hamadan (19).

Various threshold values for detection of metronidazole-resistant trichomonads have been offered by workers. Some researchers reported aerobically MICs of 25 and >200 μM belonged to metronidazole-sensitive and clinically resistant isolates and under anaerobic condition MICs of 3.2 and 25 μM, respectively (13, 19). Muller and colleague's demonstrated threshold MLCs of metronidazole resistance isolates based on treatment outcome of 199 *T. vaginalis*-infected women. They reported aerobic and anaerobic MLCs of >100 and >3.1 μg/ml, respectively, and they also declared that there was overlap of successful treatment among resistant and sensitive strains (20). According to Meri et al., other threshold concentration of clinical resistant *T. vaginalis* were proposed MICs of >75 and >15 μg/ml after 24 h aerobic and 48 h anaerobic cultivation, in that order (14). In addition, for clinically sensitive parasite, those were <19 and <10 μg/ml and unlike the previous study, they could not find overlap between them (14).

There is no consensus approach to performance and interpretation of drug susceptibility of *T. vaginalis*. Some workers have used the MIC and/or MLC in aerobic and/or anaerobic environment. MIC value was four times higher than MLC (9, 14). At the present study, the mean value of MICs was more than that of MLCs that this finding is similar to the previous study. It can be explained that the metronidazole-exposed parasites were irreversibly influenced by the medication at lower concentration thus; they cannot grow anymore in drug-free medium under the MLC assay condition.

In vitro drug susceptibility test of parasite is not exactly correlated with the necessary dose that is needed for treatment of trichomoniasis (20). However, it is useful for predicting and management of treatment process. Frequently, the drug resistance is not absolute and refractory trichomoniasis can be cured by high dose and long time period treatment. Treatment of infection with marginal and moderate resistance strain require 10 and 40 g metronidazole, respectively, administered orally for several days (1).

It is supposed that clinically metronidazole resistance is related to aerobic resistance phenomenon and can be created in parasites during the ordinary therapy of trichomoniasis. Thus, the aerobic susceptibility testing appears to be more discriminative and applicable than the anaerobic assay (5, 6, 8, 21).

Conclusion

Drug sensitivity testing of *T. vaginalis* with reliable methods, is required when refractory trichomoniasis is found and the other agents of treatment failure, especially poor drug compliance and reinfection are excluded. This study detected metronidazole tolerance in few Iranian *T. vaginalis* isolates thus; future study is needed to evaluate accurately correlation between in vitro drug susceptibility and in vivo treatment outcome of trichomoniasis.

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