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Metronidazole resistance in *Trichomonas vaginalis* determined by molecular and conventional methods

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Abstract. *Trichomonas vaginalis* (*T. vaginalis*) is a protozoan parasite that infects the urogenital tract of both women and men worldwide. Trichomoniasis can cause serious symptoms if untreated. Metronidazole is the drug of choice for the treatment of trichomoniasis. In recent years metronidazole-resistant *T. vaginalis* has often been mentioned in clinical isolates. The aim of this study was to determine the conventional and molecular methods to determine metronidazole-resistant *T. vaginalis*, which is seen commonly and to discuss the possible reasons for this. Samples taken from patients diagnosed with *T. vaginalis from* the gynaecology and obstetrics clinic between April 2015 and June 2016 were evaluated for metronidazole-resistance using molecular and conventional methods. A total of 170 patients were examined and *T. vaginalis* was determined in 6 (3.5%) patients. Metronidazole resistance was determined in 2 (33.3%) of the 6 clinical isolates as a result of the molecular and conventional tests applied. Metronidazole resistance was determined using nitroreductase genes $ntr4_{Tv}$ and $ntr6_{Tv}$. These findings suggest that metronidazole-resistance *T. vaginalis* strains can be determined in laboratory samples of cases with trichomoniasis. This may be an important underlying factor in the unsuccessful management of recurrent cases seen in routine gynecological practice.

INTRODUCTION

T. vaginalis is an anaerobic protozoan which moves by turning its flagellated and undulating membrane around itself and causes trichomoniasis in humans. It resides in the vagina of females and in the urethra of males. Trichomoniasis is transmitted through sexual contact and the incidence is high in sexually mature females (Ozcel *et al.*, 2007). Trichomonal infection has a worldwide distribution and is reported in all racial groups and all socio-economic levels. Worldwide, there are approximately 333 million new cases per year of Sexually Transmitted Diseases (STD) and of these, 170 million cases are due to *T. vaginalis* infection (WHO, 2012).

In some individuals it may remain asymptomatic and can live in the vagina for a long time. Suppression of *Lactobacillus acidophilus* found in the vagina and the growth of some pyogenic bacteria in the vagina may result in an elevation of the vaginal pH, which is normally 4.5, and frequently and prominently rises above 5.0, which results in increase growth of *T. vaginalis* (Ozcel *et al.*, 2007). Factors for predisposition of this disease include immune suppression, HIV, cervical cancer, prostate cancer, use of contraceptives and polygamy (Lehker *et al.*, 2000).

If trichomoniasis is not treated, it can cause serious symptoms. In females, these include a feeling of burning sensation in the vulva and vagina, mild or severe itching, and

an odorous foaming vaginal discharge which can vary in colour from white to light yellow. Although there are no evident symptoms in males, occasionally a white discharge from the urethra with burning sensation on urination has been reported (Mielczarek & Blaszkowska, 2016).

The microscopic examination of vaginal discharge is the most of common method. Several studies have shown that the culture method to be more sensitive than microscopy (Tamer *et al.*, 2008). Currently, the Polymerase Chain Reaction (PCR) is the method of choice. The disadvantages of this test are that it requires a well equipped laboratory with experienced staff which may not be economical. Some researchers have reported that PCR is more sensitive than culture method as more limited number of *T. vaginalis* can be determined (Pilay *et al.*, 2004; Snipes *et al.*, 2000).

Metronidazole is the standard treatment for trichomoniasis, however metronidazole-resistant strains are cultured in a increasing number of refractory cases (Garcia *et al.*, 2017; Bradic *et al.*, 2017). Metronidazole resistant *T. vaginalis* has been determined in 4.3%–9.6% of clinical *T. vaginalis* cases in the United States (Kirkcaldy *et al.*, 2012).

The aim of this study was to determine the level of metronidazole-resistant *T. vaginalis*, using conventional and molecular methods.

MATERIALS AND METHODS

Patients

The samples used in this study were taken from clinically diagnosed patients who presented at the outpatient gynecology service of our university hospital between April 2015 and June.2016 according to standard criteria (Wolner-Hanssen *et al.*, 1989).

T. vaginalis culture

The metronidazole-resistant strain, *T. vaginalis* ATCC50143 and the metronidazole-sensitive strain, *T. vaginalis* ATCC50148, were used as positive and negative controls. Trichomonas Broth (TB, liofilchem, 610061) medium was purchased commercially and was prepared according to the manufacturer's instructions. After preparation of the TB, it was distributed into the experimental tubes and placed in the autoclave at 121°C for 15 mins, then cooled to 37°C, and 10.0% inactive horse serum (Sigma, 1234598765) was added to the medium. The samples were taken from the posterior fornix of the patients with a sterile swab and added to the TB medium and incubated for 3 days at 37°C under anaerobic conditions.

Metronidazole sensitivity test

The Minimum Lethal Dose (MLD) for the clinical isolates against metronidazole was tested in comparison with the metronidazole-sensitive strain, *T. vaginalis* ATCC50148, and the metronidazole-resistant strain, *T. vaginalis* ATCC50143. For this purpose, 96-well plates were used. The *T. vaginalis* strains produced from seeding in the TB medium at 37°C were incubated in metronidazole (Sigma, 1711544348111) concentrations of 400 µM, 200 µM, 100 µM, 50 µM, 25 µM, 12,5 µM, 0,6 µM and 0,3 µM. After 24 h, the incubated live protozoa were checked on a Thoma slide for flagellated and undulating membrane movement and were counted in a 1.0% eosin solution. A dose where no live parasites were found was determined microscopically and evaluated as MLD.

Polymerase chain reaction

All the isolates were tested with PCR. *TVK3* and *TVK7* primers were used for *T. vaginalis*. In all the strains, the presence of the nitroreductase gene ($ntr4_{Tv}$, $ntr6_{Tv}$) which was used as the metronidazole-resistant primer was tested at the molecular level and compared with the conventional method. Following optimisation of the PCR conditions, the protocol developed by Kengne *et al.* (1994), PCR procedure was applied using primer pairs formed by the gene sequence for *T. vaginalis*. Isolates which formed a band at 261 bp were evaluated as *T. vaginalis* (Table 1). As a marker of metronidazole-resistant *T. vaginalis*, the PCR procedure was applied using primer pairs formed by the gene sequence for

Table 1. The PCR procedure was applied using primer pairs

Gene	Primer sequences 5'-3'	Amplicon size (bp)
TVK3	AT TGT CGA ACA TTG GTC TTA CCCTC	261
TVK7	TCT GTG CCG TCT TCA AGT ATGC	
$ntr4_{Tv}$ F	ATGAGTGTCCTTAAGTGCATCCAA	549
$ntr4_{Tv}$ R	TTAGTCGGCATAAACTACCTTAGA	
$ntr6_{Tv}$ F	CATTGAATTTATTCGTTCAAAATT	743
$ntr6_{Tv}$ R	TTATTCAATGTATGTAACCTTTCT	

$ntr4_{Tv}$ and $ntr4_{Tv}$ as reported by Paulish-Miller *et al.* (2014) and 549 bp was accepted as positive for $ntr4_{Tv}$ and 743bp for $ntr6_{Tv}$ (Table 1).

PCR procedure

DNA extracts from the isolates were applied to a Qiagen DNA isolation kit (69506). The PCR procedure was performed as described below. As the PCR mixture, 5 x Hot Firepool Blend Master Mix (Solis Biodyne) was used. According to the kit procedure, 4 µl master mix, 1 µl primer (for each primer), and 5 µl DNA, were added to 20 µl deionised water and the PCR mixture was formed. The initial denaturisation was 15 min at 95°C then 45 cycles were formed of denaturisation at 95°C for 20 s, hybridisation at 60°C for 1 min and synthesis at 72°C for 1 min. The final cycle was set as 7 min at 72°C. PCR products were separated with electrophoresis in 1% agarose gel and stained with ethydium bromide, then maintained at 90V for 30 min and examined under a UV light.

Research ethics

Approval for this study was granted by Cumhuriyet University Ethics Committee for Clinical Investigations with the approval number of 2015-03/13.

Statistical analysis

The data obtained in the study were evaluated using SPSS v22.0 software. The 2x2 sets were evaluated with the Chi-square test and in multiple sets in dependent groups, the Chi-square (McNeman) test was used. A value of $p<0.05$ was accepted as statistically significant.

RESULTS

As a result of our study, *T. vaginalis* was determined in 6 (3.5%) of 170 samples by microscopy. All samples were cultured and evaluated after approximately 3 days at 37°C. In the evaluation, 6 (3.5%) positive samples were determined by TB. Metronidazole resistance and 2 (33.3%) of the 6 clinical isolates were determined as a result of the molecular and conventional tests applied. Metronidazole resistance was determined using nitroreductase genes $ntr4_{Tv}$ and $ntr6_{Tv}$.

At the end of the 24 h period of the metronidazole sensitivity test, the MLD of 1st and 2nd clinical isolates was determined to be 400 µM, and the MLD of the positive control was determined to be > 400 µM. In the statistical examination on the survival rates of the protozoa no significant differences were observed between the positive control and the 1st and 2nd clinical isolates ($p>0.05$). When these data were evaluated, it was determined that the 1st and 2nd clinical isolates were resistant to metronidazole. This was confirmed by comparison with the positive control strain using PCR. When the results of the PCR were examined, the presence of the nitroreductase gene was observed in the 1st and 2nd clinical isolates (Table 2, Figure 1).

After the metronidazole sensitivity test the 3rd and 4th clinical isolates were found to be sensitive. No live protozoa were observed in the negative controls or the 3rd and 4th clinical isolates after 24 h of exposure to 0.3 µM of metronidazole, which was the lowest concentration examined in this study. In

Table 2. The number of organisms of *T. vaginalis* isolates exposed to various metronidazole concentrations after 24-hour period

TV suşları	Metronidazol concentrations								
	400 µM	200 µM	100 µM	50 µM	25 µM	12.5 µM	0.6 µM	0.3 µM	0 µM
PC	9.10^3	10.10^3	8.10^3	9.10^3	10.10^3	10.10^3	11.10^3	11.10^3	12.10^3
NC	0	0	0	0	0	0	0	0	13.10^3
N1	0	5.10^3	5.10^3	6.10^3	7.10^3	6.10^3	9.10^3	11.10^3	12.10^3
N2	0	4.10^3	4.10^3	5.10^3	6.10^3	7.10^3	7.10^3	9.10^3	11.10^3
N3	0	0	0	0	0	0	0	0	12.10^3
N4	0	0	0	0	0	0	0	0	11.10^3
N5	0	0	0	0	3.10^3	5.10^3	5.10^3	5.10^3	10.10^3
N6	0	0	0	5.10^3	5.10^3	6.10^3	7.10^3	7.10^3	12.10^3

TV; *T. vaginalis*, PC; Positive Control, NC; Negative Control, N; Clinical Isolate.

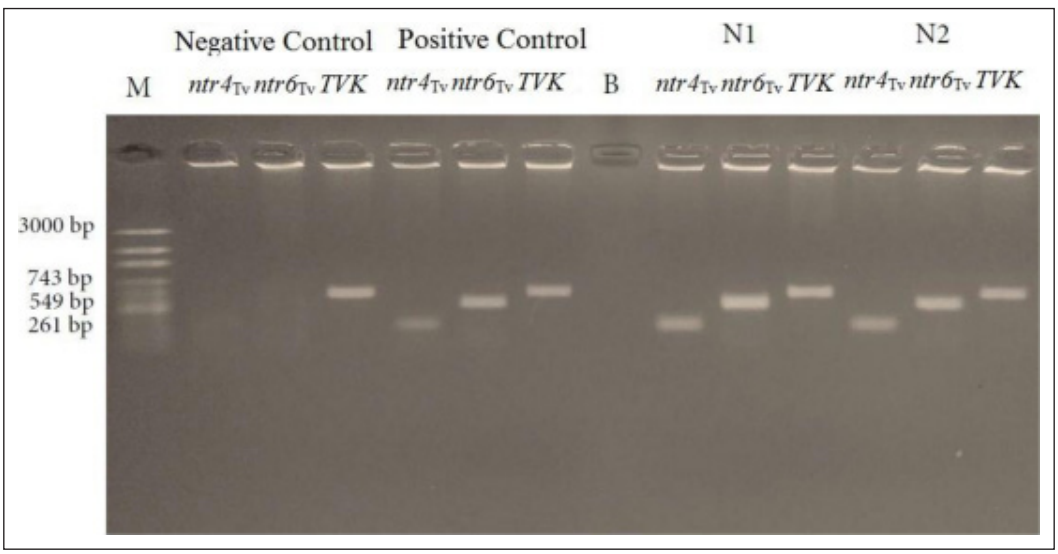

Figure 1. The Polymerase Chain Reaction result (M; Marker, Negative control (*T. vaginalis* ATTC 50148), Positive control (*T. vaginalis* ATTC 50143), N; Clinical isolates.

addition, the nitroreductase gene was not found in the negative controls or the 3rd and 4th clinical isolates when examined using PCR. In the statistical examination on the survival rates of protozoa no significant differences were observed between the negative control and the 3rd and 4th clinical isolates ($p>0.05$, Table 2, Figure 1).

The MLD of the 5th and 6th clinical isolates was determined to be 50 µM and 100 µM, respectively. Using PCR, the nitroreductase gene was also observed in the 5th and 6th clinical isolates, which showed low resistance in the conventional method (Table 2, Figure 1).

DISCUSSION

Although *T. vaginalis* is widespread throughout the world, it is more common in the under-developed and developing countries. It is found in all climates and it is one of the primary STD that lives in the

human urogenital system. The prevalence of infection varies according to the lifestyle and socio-cultural structure of the population. It has been estimated that 180–200 million people worldwide, mostly females, are infected with *T. vaginalis*. Studies in Turkey have reported a prevalance of 5.0%–10.0% of females attending private clinics, 13.0%–25.0% of women presenting at gynaecology and obstetrics clinics and 50.0%–70.0% of women in prisons or working in brothels (Daldal *et al*., 2002).

Currently, there are many studies related to the prevalence of *T. vaginalis*, which has been determined using microscopic and culture methods. In a study conducted in Sivas by Selvitopu *et al*. (2006), of the 61 vaginal samples examined by the Giemsa method and seeded in cysteine-peptone-liver-maltose (CPLM) medium, two cases (3.3%) were positive for *T vaginalis*. As seen in the two studies described above, the prevalence of *T. vaginalis* presents differently according to the region where it is being studied. These variations are related to the lifestyle and socio-cultural structure of the societies living in these regions.

Recently, metronidazole was found to be unsuccessful in some patients. According to studies in the United States, metronidazole-resistant *T. vaginalis* has been reported at rates varying between 4.3% and 9.6%, as determined by conventional methods. Kirkcaldy *et al*. (2012) evaluated 538 patients in six different cities in the United States between 2009 and 2010. All the patients were recorded as HIV-negative and 3.0% were pregnant. Using conventional methods, metronidazole resistance was determined to be low (23 cases identified, which was 4.3% of the total sample) (Schwebke & Barrientes, 2006).

In another study in Finland, 10 clinical isolates were tested positive for metronidazole resistance using conventional methods under aerobic conditions; of these, 3 (30,0%) resistant strains were identified (Meri *et al*., 2000). In a study in Iran, *T. vaginalis* was observed in 15 (2.2%) of 683 patients. Using metronidazole assays and conventional methods, five of the isolates (33.3%) were determined to be metronidazole resistant (Rabiee *et al*., 2012).

In a 1994 study in Turkey, metronidazole resistance was tested using conventional methods, but no resistant strains were identified (Belek *et al*., 1994). In another study in Turkey that was conducted in the Parasitology Laboratory of Adnan Menderes University Medical Faculty between 2009 and 2014, 40 *T. vaginalis* clinical isolates were tested for metronidazole resistance using conventional methods, and resistant isolates were determined at a rate of 7.5% (Ertabaklar *et al*., 2016). In the present study, two (33.3%) *T. vaginalis* strains were found to be resistant using conventional and molecular methods. This high rate may not be statistically significant due to the low number of patients studied. A review of the literature on this topic revealed very few studies have been carried out on metronidazole-resistant *T. vaginalis* by using molecular methods. A study by Paulish-Miller *et al*. (2014) on this subject demonstrated that the molecular identification of metronidzole-resistant *T. vaginalis* could be made with the single nucleotide polymorphisms (SNP) method. SNP analysis was applied to various *T. vaginalis* clinical isolates and it was concluded that the presence of the nitroreductase gene could be a marker of metronidazole resistance.

In the present study, the nitroreductase gene was determined to be present in the *T. vaginalis* ATCC50143 strain, which was used as the positive control, but the nitroreductase gene was not observed in the *T. vaginalis* ATCC50148 strain, which was used as the negative control. In terms of the clinical isolates, the presence of the nitroreductase ge was observed molecularly in the 1st and 2nd clinical isolates. In the 3rd and 4th clinical isolates, which were evaluated as metronidazole-sensitive, the presence of the nitroreductase gene was not determined. These findings confirm the presence of the nitroreductase gene as a sign of metronidazole resistance. When the 5th and 6th clinical isolates were examined, the presence of the nitroreductase gene was

detected by PCR test, the data obtained from the conventionally applied metronidazole sensitivity test supported the sensitivity of these two isolates. Consequently, it can be said that the molecular determination of the presence of the nitroreductase gene in *T. vaginalis* strains could be a sign of metronidazole resistance.

In conclusion, in the present study, two samples (33.3%) were found to be resistant to metronidazole. This was determined using conventional and molecular methods. The findings suggest that metronidazole-resistant *T. vaginalis* strains can be detected in clinical isolates of cases with trichomoniasis. This may be an important obstacle for the successful management of recurrent cases in routine gynecological practice.

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