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

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*Trichomonas vaginalis* is a globally common sexually transmitted human parasite. Many strains of *T. vaginalis* from around the world have been described to be resistant to the current drug of choice, metronidazole. However, only a few cases of metronidazole resistance have been reported from Europe. The resistant strains cause prolonged infections which are difficult to treat. *T. vaginalis* infection also increases the risk for human immunodeficiency virus transmission. We present a practical method for determining the resistance of *T. vaginalis* to 5-nitroimidazoles. The suggested method was developed by determining the MICs and minimal lethal concentrations (MLCs) of metronidazole and ornidazole for *T. vaginalis* under various aerobic and anaerobic conditions. Using this assay we have found the first three metronidazole-resistant strains from Finland, although the origin of at least one of the strains seems to be Russia. Analysis of the patient-derived and previously characterized isolates showed that metronidazole-resistant strains were also resistant to ornidazole, and MLCs for all strains tested correlated well with the MICs. The suggested MICs of metronidazole for differentiation of sensitive and resistant isolates are >75 μg/ml in an aerobic 24-h assay and >15 μg/ml in an anaerobic 48-h assay.

*Trichomonas vaginalis* is a flagellated protozoan and is the most common parasite that causes sexually transmitted disease (24). The World Health Organization has estimated that there are annually approximately 170 million infections worldwide (43). The main clinical manifestations of *T. vaginalis* infections are vaginitis, urethritis, and prostatitis. In addition, infection with trichomonads can increase the risk of pelvic inflammatory disease and tubal infertility (2). Since probably almost 50% of infections with HIV-1 (20, 36). Only under 20 metronidazole-resistant strains have been described from Europe (3, 7, 12, 16, 19, 26, 40, 41). In addition, some preliminary reports have been published, for example, from Russia (18) and Africa (13). To overcome the clinical problems of metronidazole resistance, higher doses of metronidazole have usually been used to treat the patients (11, 22). Also, despite the cross-resistance with other 5-nitroimidazoles, tinidazole (10) and ornidazole (19) have been used to treat some patients infected with metronidazole-resistant strains of *T. vaginalis*. Furazolidone, a nitrofuran, has also shown trichomonicidal activity in vitro (30). Paromomycin has been used as a topical treatment for some patients with allergy to metronidazole or infections caused by metronidazole-resistant strains of *T. vaginalis* (3, 31).

Resistance to nitroimidazoles has been studied by growing *T. vaginalis* strains in the presence of different drug concentrations under aerobic and anaerobic conditions in vitro. Resistance has been suggested to be dependent on one or more of the following: a reduced PFOR enzyme activity (19), an altered conformation of the hydrogenosome (39), a ferrodoxin with an exceptional redox potential (44), or a reduced amount of intracellular ferrodoxin (27). In the resistant strains the intracellular amounts of ferrodoxin are decreased by over 50% and the rate of ferrodoxin gene transcription is reduced by as much as 40 to 65% compared to those in sensitive isolates (33). Many of the suggested mechanisms lead to fermentation of pyruvate in the cytosol instead of the hydrogenosome, which means that the action of the 5-nitroimidazole drug is inhibited. The aerobic resistance to nitroimidazoles is thought to be enhanced by free O2 in the cytosol, as it is a potent electron acceptor from nitro radicals and thus could inhibit the action of the drug (6). The suggested mechanisms that lead to an excess of intracellular oxygen include decreased amounts of oxidases and a decreased affinity of terminal oxidases to oxygen (39).

In this report we suggest a practical in vitro method for determination of the metronidazole susceptibility of *T. vaginalis* in advanced clinical microbiology laboratories and report on...
three clinically metronidazole-resistant and in vitro metronida-
zole-resistant strains of T. vaginalis from Finland. The suscept-
pibility to metronidazole was tested under various oxygen con-
centrations. The susceptibilities of the characterized strains of
T. vaginalis to metronidazole and ornidazole were compared to
those of a set of patient-derived strains.

MATERIALS AND METHODS

Reagents. Metronidazole and ornidazole were purchased from Sigma Chem-
ical Co. (St. Louis, Mo.) and were dissolved in 0.15 M phosphate buffer (pH 6.4) for
the susceptibility assays just before use. Dimethyl sulfoxide (DMSO) was pur-
bought from Merck & Co. (Darmstadt, Germany).

T. vaginalis strains and cultivation. T. vaginalis strains were cultured in 7-mL
Wassermann glass tubes containing 5 mL of Trypticase-yeast extract-maltose
(TYM) medium (4) complemented with 10% heat-inactivated horse serum and
a combination of 100 IU of streptomycin (Sigma) per mL and penicillin (Orion
Diagnostica, Espoo, Finland).

The characterized metronidazole-resistant (28) and -sensitive (29) strains of
T. vaginalis were obtained from the American Type Culture Collection (ATCC;
ATCC 50143 and ATCC 50148, respectively) and were treated as described
below for the patient-derived isolates.

Patient-derived strains of T. vaginalis were obtained from female Finnish
patients. The vaginal samples were originally plated into VagiCult tubes (Orion
Diagnostica), which have been developed for the diagnostic cultivation of T. vagi-
nalis and yeast. To continue the culture an aliquot of 0.5 mL was taken from each
positive culture and was transferred to TYM medium. Aliquots of the culture
suspensions containing live T. vaginalis trophozoites (0.2 to 1.0 mL) were trans-
ferral to the bottoms of prewarmed new culture tubes three times a week. After
1 to 2 weeks aliquots of each strain were frozen (–70°C with DMSO) by a previ-
ously described method (42). For each assay new vials were thawed. After
thawing the strains were cultured for approximately 7 days to collect enough
trophozoites. The possible presence of bacteria or yeasts was examined by cul-
vating samples of all T. vaginalis cultures in a thiglycolate medium (37°C for
7 days) and on plates with blood or chocolate agar (CO2 atmosphere) or fastid-
ious to metronidazole (anaerobic atmosphere). Samples from thiglycolate medi-
um-containing tubes with any cloudy patches were cultured on the plates. All
cultivations were performed by standard bacteriological methods.

Species identification by PCR. The species of all the strains was analyzed by
PCR. In the PCR assay a highly conserved repeated DNA stretch specific for
T. vaginalis was amplified with primers TVK3 and TVK4, which have been de-
dscribed elsewhere (17).

5-Nitroimidazole susceptibility assays. To examine the susceptibilities of the
T. vaginalis strains to metronidazole and ornidazole under aerobic and anaerobic
conditions, the trophozoites were cultured on 24-well tissue culture plates
(Greiner, Gloucestershire, United Kingdom) in airight jars with adjustable ven-
tilators on the lid. Three plates with duplicate wells for each drug concentra-
tion were used in each susceptibility assay. All assays were run twice. Fresh TYM
medium (200 mL) was mixed with 200 mL of preplated 5-nitromidazole (0.15
M phosphate buffer [pH 6.4]) in each well, and subsequently, 100 mL of medium
containing live T. vaginalis trophozoites was added to each well. The con-
centration of motile trophozoites was adjusted to approximately 10^7/mL in
each well. The drug dilution buffer (phosphate buffer [pH 6.4]) was used as a posi-
tive control on each plate.

Anaerobic circumstances were created with the chemical oxygen consumption
plate Anaerocult A (Merck & Co.) and were monitored with an anaerobic indicator
(Anaerob, Hampshire, England). All incubations were performed at 37°C.

The aerobic conditions were created by two different methods. The first assay
used the same jars used for the anaerobic assay, but the ventilators were left open
for access to room air. In the second assay the susceptibility of T. vaginalis to
5-nitroimidazoles was tested in the presence of different concentrations of oxy-
gen in the culture atmosphere. The amounts of oxygen, nitrogen, and carbon
dioxide were adjusted by using bottled gas (AGA Corp., Helsinki, Finland) and
separate flow meters (Brooks Instrument B.V., Veenendaal, The Netherlands).
The purities of the gases used were 98, 99, and 97% for N2, O2, and CO2, respec-
tively. The N2 and CO2 were free of O2. To prepare an anaerobic envi-
noment, 95% N2 and 5% CO2 were used. To prepare various aerobic environ-
ments, different amounts of O2 (0.5, 5, 10, and 30%) were used to partially
replace the N2. The gas mixture was allowed to flow through the inlet and outlet
of the anaerobic jar for 30 min at a rate of 1,600 mL/h after the incubation and
after the check at 24 h. Anaerobic circumstances were monitored with an anaerobic
indicator (Anaerob).

Determination of MICs and MLCs. The viabilities of the trophozoites in the
culture plate wells were assessed by examining visually the motilities of the cells
with an inverted microscope at a ×400 magnification after 24 and 48 h of
incubation. The MIC was the lowest concentration of the drug in the well in
which no motile cells were detected. To check whether the evaluation of viability
(MIC) reflected the effectiveness of the drug at actually killing the cells, the
minimal lethal concentrations (MLCs) were also tested after cultivation of the
trhopozoites in the presence of different oxygen concentrations. The contents of
each of the wells were inoculated into the bottoms of tubes containing 5 mL of
fresh TYM medium, and the tubes were examined for the presence of motile
T. vaginalis trophozoites after 5 and 10 days of cultivation.

Clinical data. The first (strain 1) and second (strain 2) clinically metronida-
zole-resistant isolates of T. vaginalis were obtained from female patients living
in the Helsinki district in Finland. The third strain (3) was from a female
patient who is from the eastern part of Finland and whose husband had often
visited Russia. All patients suffered from recurrent trichomoniasis and were
prescribed with several courses (6, 3, and 10 courses for patients 1, 2, and 3,
respectively) of metronidazole with a standard or an increased dose. All three
patients were hospitalized and were treated with metronidazole intravenously
(patient 1 with 1 g a day for 3 days, patient 2 with 500 mg four times a day for
7 days, and patient 3 with 750 mg three times a day for 5 days). After the treatment
patients were symptomless for a follow-up period of 3 months, and the cultures
of their samples were also negative for T. vaginalis.

RESULTS

Anaerobic MICs of metronidazole. The MICs of two 5-ni-
troimidazoles (metronidazole and ornidazole) for the different
T. vaginalis strains were obtained under both aerobic and ana-
erobic conditions. The MICs of metronidazole were also
determined under various oxygen concentrations. The MLCs
were determined by cultivating the trophozoites in fresh nitro-
imidazole-free medium after incubation with metronidazole.

In the anaerobic assay the MICs of metronidazole were
determined by cultivating the known resistant isolate (strain
R), clinically resistant isolates (strains 1 to 3), the known sen-
titive isolate (strain S), and clinically sensitive isolates (strains
4 to 10) of T. vaginalis in the presence of different concentra-
tions of metronidazole. Parasite motility was evaluated micro-
scopically after 24 and 48 h. At the 24-h time point the MIC of
metronidazole was 145 μg/mL for the known resistant strain
(strain R), 23 μg/mL for clinically resistant strain 1, over 250
μg/mL for clinically resistant strains 2 and 3, 20 μg/mL for the
known sensitive strain (strain S), and from 8.5 to 12 μg/mL for
clinically sensitive strains 4 to 10. At the 48-h time point the
MICs were 39 μg/mL (strain R), 16 μg/mL (strain 1), 24 μg/mL
(strain 2), 36 μg/mL (strain 3), and from 1.0 to 9.8 μg/mL
(strains 4 to 10) (Fig. 1A).

Aerobic MICs. In the first aerobic assay the sensitivity of
T. vaginalis to metronidazole was tested under ordinary room
air conditions at 37°C. The MICs of metronidazole at the 24-h
time point were 135 μg/mL for the known resistant strain
(strain R), 77 μg/mL for clinically resistant strain 1, over 250
μg/mL for strains 2 and 3, 18 μg/mL for the known sensitive
strain (strain S), and from 3.5 to 10 μg/mL for strains 4 to 10.
After 48 h of cultivation the MICs were 8.4 μg/mL (strain R),
7.8 μg/mL (strain 1), 48 μg/mL (strain 2), 140 μg/mL (strain 3),
1.0 μg/mL (strain S), and from 1.0 to 2.0 μg/mL (strains 4 to 10)
(Fig. 1B).

As the results of anaerobic and aerobic resistance for some
strains, especially clinically resistant isolate 1, differed, a sec-
ond aerobic sensitivity assay was performed to examine the
effect of oxygen on drug tolerance. The T. vaginalis tropho-
zotes were cultivated in the presence of different amounts of
metronidazole under various concentrations of oxygen (0, 5,
10, and 20%) in the atmosphere. It was found that the MIC
declined with increasing oxygen concentrations (Fig. 2). The
MOICs of metronidazole for clinically resis-
tant strain 1 were approximately 20 times higher than that for
sensitive strains 6 and 7.

MICs of ornidazole. The MICs of ornidazole were deter-
mined for the known resistant strain (strain R), clinically
resistant strain 1, the known sensitive strain (strain S), and
clinically sensitive strains 4 to 7 in both the aerobic and the
anaerobic assays. In the anaerobic assay the MICs of ornidazole

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after 24 h were 133 μg/ml for strain R, 37 μg/ml for strain 1, 2.0 μg/ml for strain S, and from 4.0 to 8.0 μg/ml for strains 4 to 7. After 48 h of cultivation the MICs of ornidazole were 8.4 μg/ml (strain R), 17 μg/ml (strain 1), 2.0 μg/ml (strain S), and from 4.0 to 8.0 μg/ml (strains 4 to 7) (Fig. 3A). In the aerobic assay the MICs were 200 μg/ml (strain R), 78 μg/ml (strain 1), 2.0 μg/ml (strain S), and from 2.0 to 4.0 μg/ml (strains 4 to 7) after 24 h of cultivation and 10 μg/ml (strain R), 18 μg/ml (strain 1), 1.0 μg/ml (strain S), and from 1.0 to 2.0 μg/ml (strains 4 to 7) after 48 h of cultivation (Fig. 3B).

MLCs of metronidazole. The MLCs were measured from the second aerobic sensitivity assay. While the MIC was determined by microscopic evaluation on the basis of cell motility at different drug concentrations, the MLC was assayed to reveal the actual lethal concentration on the basis of the growth after 5 and 10 days in fresh medium after exposure to different concentrations of the drug. When no oxygen was present the MLC for strains 1 and R was 6.0 μg/ml and the MLC for the clinically sensitive strains (strains S, 6, and 7) was 0.75 μg/ml (Table 1). The MLCs varied from 6 to 100% of the corresponding MICs. When the concentration of oxygen was ≥10% the sensitive strains died even in the absence of the drug.

**DISCUSSION**

In the present study we have tested different in vitro conditions for determination of the 5-nitroimidazole resistance of T. vaginalis and suggest a relatively simple and easily interpretable method for laboratory testing of strains suspected of being metronidazole resistant. We describe the analysis of the first three metronidazole-resistant T. vaginalis strains, all of which were finally eradicated by intravenous metronidazole therapy.

Testing of drug susceptibility in vitro is necessary to ensure that the long-lasting T. vaginalis infection is really caused by a nonsensitive strain and that the patient is not experiencing a recurrent infection. We tested the susceptibilities of different resistant and sensitive strains of T. vaginalis under various conditions and at various time points. As a conclusion, we suggest that a combination of an aerobic 24-h cultivation and an anaerobic 48-h cultivation of patient-derived strains in the presence of different concentrations of metronidazole can be used to determine whether their susceptibilities to metronidazole are decreased. One of the patient-derived resistant strains (strain 1) described in this report was concluded to be a resistant isolate only from the aerobic assay. Anaerobic 24-h MICs were only slightly elevated for clinically resistant strain 1 and
TABLE 1. Effect of oxygen on MLCs of metronidazole for five strains of *T. vaginalis* after 48 h of incubation in the presence of different oxygen concentrations in the atmosphere

<table>
<thead>
<tr>
<th>% O₂</th>
<th>Strain R</th>
<th>Strain S</th>
<th>Strain 1a</th>
<th>Strain 2b</th>
<th>Strain 3b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MLC (µg/ml)</td>
<td>MLC/MIC (%)</td>
<td>MLC (µg/ml)</td>
<td>MLC/MIC (%)</td>
<td>MLC (µg/ml)</td>
</tr>
<tr>
<td>0</td>
<td>6.0</td>
<td>100</td>
<td>0.75</td>
<td>100</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>33</td>
<td>1.5</td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>24.0</td>
<td>16</td>
<td>1.5</td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td>10</td>
<td>48.0</td>
<td>6</td>
<td>1.5</td>
<td>100</td>
<td>48</td>
</tr>
<tr>
<td>20</td>
<td>48.0</td>
<td>6</td>
<td>C−</td>
<td>C−</td>
<td>48</td>
</tr>
<tr>
<td>30</td>
<td>48.0</td>
<td>6</td>
<td>C−</td>
<td>C−</td>
<td>24</td>
</tr>
</tbody>
</table>

a Treatment-resistant patient-derived strain of *T. vaginalis*.
b Treatment-sensitive patient-derived strains of *T. vaginalis*.
c C−, control cells incubated without metronidazole.

differed from those for the drug-susceptible control (strain S) only by 3 µg/ml.

Aerobic resistance has also been induced in vitro, and these strains were also drug sensitive under anaerobic conditions (38). Thus, both assays are needed in the laboratory analysis (5). A suitable anaerobic susceptibility testing protocol for diagnostics (InPouchTV test) is commercially available (1), but it should be complemented by an aerobic assay.

Interpretation of the MICs or MLCs for *T. vaginalis* has not been based on any widely accepted scheme. Some investigators have used a method in which the MIC is the lowest concentration of drug at which cell growth takes place and the MLC is the lowest concentration with motile cells. Both definitions are based on visual observation after a 48-h cultivation (23, 25, 28, 29, 38). In some studies the MLC is the original lowest drug concentration at which growth of the parasites in a drug-free medium no longer takes place (1, 12, 19). According to Narcici and Secor (30), the MIC of metronidazole was approximately fourfold higher than the concentration (MLC) that killed the isolates, but no data were presented. It seems that in the MLC assay the trophozoites had suffered irreversible damage but still retained motility. Narcici and Secor (30) used a 48-h cultivation for the determination of possible growth in drug-free medium. However, for some isolates it may take longer to recover from the effect of the drug because we found viable trophozoites after 5 days of cultivation. According to the present study the MIC for *T. vaginalis* is a suitable indicator of the actual MLC and can be used for diagnostic assessments.

Different threshold concentrations of the 5-nitroimidazole drugs for resistance have been proposed by many investigators. Müller et al. (28) have reported MLCs for 199 *T. vaginalis* strains and the treatment outcomes for the corresponding patients. According to their interpretation, the MLCs for a treatment-resistant strain were >100 µg/ml in an aerobic assay and >3.1 µg/ml in an anaerobic assay. They also found that treatment success overlapped between susceptible and resistant isolates. We found that following an aerobic 24-h cultivation and an anaerobic 48-h cultivation the MICs for clinically resistant isolates were >75 and >15 µg/ml, respectively. For clinically sensitive strains MICs were considerably lower in both assays (<19 and <10 µg/ml, respectively), and these values did not overlap with the values for the resistant strains. Many different methods are used in laboratories to test for drug susceptibility, and it is not possible to define accurate threshold values for resistant and sensitive strains. There is also uncertainty whether the MIC or MLC can be used as such to determine the suitable drug dosage for the patient (23, 34). We suggest that a patient-derived strain is judged to be resistant after comparing it to a known, highly resistant strain by an easily interpretable method.

In this study we found three patient samples with *T. vaginalis* strains that were clearly resistant to metronidazole in vitro. The finding correlated well with the clinical data for resistance to treatment. In earlier reports patients who carry resistant strains of *T. vaginalis* have usually been treated with longer courses of standard doses of metronidazole or with a considerably higher dosage (23). All patients described in this paper were initially treated with several courses of metronidazole per os. Finally, trichomonads were eradicated by using metronidazole intravenously. Some reports have suggested that other 5-nitroimidazoles, like tinidazole, ornidazole (19), or furazolidone (30), are curative for metronidazole-resistant isolates, and some reports have shown the opposite (7). However, oral metronidazole as a single dose of 1.5 g is still the drug of choice for the treatment of trichomoniasis (37), and the susceptibility assay is indicated only when the standard metronidazole dosage is repeatedly incapable of eradicating the infection from the patient and the partner(s). When the MIC for a patient-derived strain is high by a reliable assay, we suggest that the patient be treated with as high a dosage of the drug as possible to treat the infection and to prevent it from spreading.

The origins of our first two treatment-resistant *T. vaginalis* strains (strains 1 and 2) are not exactly known. The third strain (strain 3) came from a patient in the eastern part of Finland and is likely to have originated from a partner who had visited Russia several times. Tourism between Western Europe and Russia has increased a lot, and part of it is sex tourism in and out of Russia. No statistical data from Russia on how common *T. vaginalis* infections are and the current status of drug resistance in Russia are available. In Russia self-treatment with the usual doses of metronidazole is possible; thus, the time lag between a new diagnosis and effective treatment is increased.

In Europe it is very important to start diagnosing possible resistance in vitro after, for example, 2 months of “recurrent” infections, to report the resistant strains, and to trace the sexual contacts, if possible. Recent epidemiological and in vitro evidence suggests that *T. vaginalis* infection may enhance the risk of HIV-1 transmission (36). If this connection is confirmed, *T. vaginalis* susceptibility testing becomes even more important in countries where infections are both frequent and inadequately controlled, for example, in Russia and tropical Africa. Patients infected with a resistant strain of *T. vaginalis* should be tested for HIV infection.

In summary, using metronidazole sensitivity testing under various oxygen concentrations, we found the first three metronidazole-resistant strains of *T. vaginalis* from Finland. It appears that the metronidazole resistance of *T. vaginalis* is an emerging threat in Europe. Thus, testing for metronidazole susceptibility in vitro and introduction of rapid and intense medication should be implemented more often. The in vitro
diagnostic assays that we suggest can be relatively simply adopted for use in advanced clinical microbiology laboratories.

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