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# In Vitro Testing of *Trichomonas vaginalis* Drug Susceptibility: Evaluation of Minimal Lethal Concentrations for Metronidazole and Tinidazole That Correspond With Treatment Failure

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**Background:** The only drugs approved by the US Food and Drug Administration for oral treatment of trichomoniasis belong to the 5-nitroimidazole group. Most individuals infected with *Trichomonas vaginalis* can be cured with a standard treatment of metronidazole or tinidazole, but it is estimated that more than 159,000 people fail treatment each year. Although a minimal lethal concentration (MLC) corresponding to treatment failure has been reported for metronidazole, the MLC for tinidazole associated with treatment failure has not been determined. We conducted a study using *T. vaginalis* isolates from women with reported treatment success or failure to determine these values.

**Methods:** We measured MLCs of 47 isolates obtained from women who had failed metronidazole treatment, 33 isolates from women who had failed tinidazole treatment, and 48 isolates from women successfully cured with metronidazole. The cutoff was calculated as the 95th percentile of MLCs of susceptible isolates for each drug.

**Results:** Our data confirmed that the MLC previously associated with metronidazole treatment failure is ≥50 μg/mL and identified the MLC associated with tinidazole treatment failure as ≥6.3 μg/mL. For metronidazole, the agreement between laboratory result and treatment outcome was 93.7%; for tinidazole, this agreement was 88.9%.

**Conclusions:** The *T. vaginalis* susceptibility assay is useful for determining whether 5-nitroimidazole treatment failure in persons with trichomoniasis can be attributed to drug resistance. These results are useful for establishing interpretive guidance of test results, and MLC levels can help guide appropriate patient treatment.

*Trichomonas vaginalis* is a flagellated, facultative anaerobic protozoan that is the causative agent of trichomoniasis, the most common nonviral sexually transmitted disease. An estimated 2.6 million infections occur annually in the United States, and the World Health Organization (WHO) estimates more than 156 million infections worldwide.[1,2] Although many *T. vaginalis* infections in women are asymptomatic, trichomoniasis can range from mild irritation to copious, malodorous discharge, and inflammation of the cervix with punctate lesions.[3] In pregnancy, trichomoniasis has been associated with preterm labor and low birth weight.[4] Men are rarely symptomatic but can experience urethritis, prostatitis, balanoposthitis, epididymitis, and infertility.[5] In the United States, trichomoniasis disproportionately affects women compared with men. There is also a disproportionate burden associated with Black men and women compared with other races, persons who have less education, and individuals living below the poverty level.[6] *T. vaginalis* infections also increase the transmission risk of HIV.[7]

Effective oral therapy is limited to a single class of drugs, the 5-nitroimidazoles. In the United States, metronidazole, tinidazole, and, recently, secnidazole are Food and Drug Administration approved for treatment of *T. vaginalis* infections.[8] Treatment failures with metronidazole were reported shortly after its introduction, and in vitro resistance has been reported in 4% to 10% of all infections.[9,10] Combined with the overall prevalence of trichomoniasis, we estimated that there are more than 159,000 people in the United States who require some sort of alternative therapy each year.[11] Intravaginal treatment alternatives may be used, but cure rates can be variable.[12]

The Centers for Disease Control and Prevention (CDC) offers antimicrobial susceptibility testing of *T. vaginalis* isolated from women who fail standard 5-nitroimidazole therapy (cdc.gov/laboratory/specimen-submission). A swab collected from patients who have failed treatment is inoculated into commercial transport/growth media (InPouch TV; Biomed Diagnostics, White City, OR) and submitted to the CDC. Parasites are cultured and tested over a range of concentrations of metronidazole and tinidazole to determine the minimal lethal concentration (MLC) for each drug. Results are then used in conjunction with the infected person's treatment history to make a treatment recommendation, which is usually successful to effect cure.[12]

An in vitro assay MLC corresponding to metronidazole treatment resistance was established in 1988,[13] before tinidazole was approved for treatment of trichomoniasis. The objective of this study was to measure MLCs from *T. vaginalis* isolates obtained from women who either cured or failed 5-nitroimidazole treatment to determine the MLCs associated with metronidazole and tinidazole treatment failure.

## METHODS

### Isolates and Reagents

*T. vaginalis* isolates from patients who had treatment failure with metronidazole or tinidazole were collected through CDC's Trichomoniasis Drug Susceptibility testing service. All viable isolates submitted to the CDC between July of 2014 and October of

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2015 from infected, nonpregnant, treatment compliant women who had failed to clear their infections after 1 or more treatments with metronidazole or tinidazole were defined as persistent infections. Use of these isolates for method validation was approved under CDC institutional review board protocol no. 6756. Susceptible isolates were collected in a separate study at Emory University (institutional review board no. 48502)[14] from infected women who were treated with a single 2-g dose of metronidazole and shown to have microbiologic cure (negative by both culture and polymerase chain reaction [PCR]) 3 months later. Because metronidazole is the usual first-line treatment for trichomoniasis, similar isolates susceptible to tinidazole were not available. Investigators from the CDC were considered nonengaged in human subjects research with these study participants. We evaluated 47 isolates from women with metronidazole treatment failure, 33 isolates from women with tinidazole treatment failure, and 48 isolates with susceptibility to metronidazole. Isolates were maintained by serial passage in Diamond's[15] trypticase, yeast extract, and maltose media by transferring actively dividing cells every 48 hours into fresh media and incubating at 37°C. Diamond's trypticase, yeast extract, and maltose media was made in-house by CDC's Division of Scientific Resources.[15] Metronidazole and tinidazole for use in the susceptibility assays were obtained from Sigma Chemical Corporation (St. Louis, MO). Dimethyl sulfoxide, which was used to solubilize the drugs, was also obtained from Sigma.

## Drug Resistance Assay

Testing of isolate susceptibility to metronidazole and tinidazole was performed under aerobic conditions using a modification of the assay originally developed by Meingassner and Thurner[16] and Narcisi and Secor[17] as previously described. Briefly, *T. vaginalis* isolates were incubated with serial 2-fold dilutions of the test drugs from a concentration of 0.2 to 400 μg/mL in a 96-well, U-bottomed microtiter culture plate; $10^4$ parasites were added per well. Assays are conducted under aerobic conditions because isolates demonstrate a wider dynamic range than under anaerobic conditions, thus providing a more distinct delineation between susceptible and resistant isolates.[17] Each drug concentration was tested in triplicate, and the assay was repeated twice for each isolate. Reference drug-resistant and drug-susceptible isolates were included in each assay, as were media controls containing equivalent concentrations of dimethyl sulfoxide. Plates were incubated for 48 hours at 37°C and examined using an inverted microscope. Results are expressed as the MLC, or the lowest concentration at which no parasite motility can be observed.

## Determination of MLCs Associated With Treatment Success or Failure and Assay Performance

The median and 95th percentile MLCs of susceptible isolates were calculated for both metronidazole and tinidazole using GraphPad Prism 7.03 (GraphPad Software, San Diego, CA). In addition, the MLC distributions of isolates from successfully treated individuals and those failing treatment with metronidazole or tinidazole were graphed to visualize the distributions of each group of isolates. Using the 95th percentile MLC of susceptible isolates as the cutoff for potential in vitro resistance as used for determining cutoffs with some molds and yeast,[18] we compared the agreement of the in vitro metronidazole and tinidazole MLC for each isolate with the corresponding treatment outcome. We assessed reproducibility by testing 5 different isolates against both metronidazole and tinidazole 6 times each (2 different people 3 times each) and comparing the resulting MLCs relative to the cutoffs determined for the 2 drugs.

# RESULTS

## Metronidazole MLC

Metronidazole MLC data from 47 *T. vaginalis* isolates that had been obtained from women after metronidazole treatment failure were compared with metronidazole MLC data from 48 women who had been treated for trichomoniasis and were subsequently negative by culture and PCR testing. The median metronidazole MLC of the isolates from successfully treated individuals was 6.3 μg/mL, and the 95$^{th}$ percentile was 38.8 μg/mL, which rounds up to 50 μg/mL in the assay configuration. There were no isolates recovered from women successfully treated with standard therapy that had an MLC >50 μg/mL (Fig. 1), consistent with the value

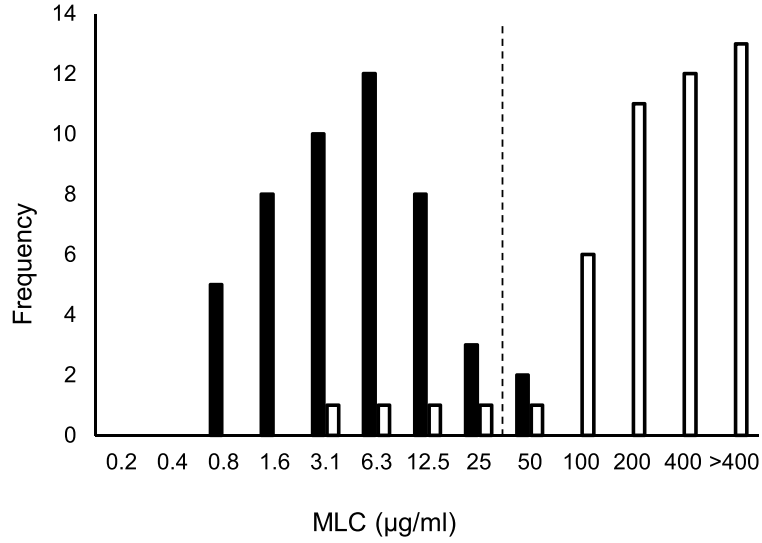


**Figure 1.** Metronidazole MLC distributions of isolates obtained from women with *T. vaginalis* infections who were successfully (black bars) or unsuccessfully (white bars) treated with metronidazole. The dashed line represents the cutoff based on the 95th percentile metronidazole MLCs of isolates from women who were successfully treated.

**TABLE 1.** Categorization of Isolates From Women Successfully or Unsuccessfully Treated With Metronidazole According to in Vitro MLC

| Metronidazole MLC, μg/mL | Metronidazole Treatment Failure | Metronidazole Treatment Success |
|---|---|---|
| <50 | 4 | 46 |
| ≥50 | 43 | 2 |

observed in 1985.[13] Applying the 50-μg/mL MLC cutoff to the data collected on the isolates from women successfully or unsuccessfully treated with metronidazole (Table 1), 91.5% (43 of 47) of isolates from women who failed metronidazole treatment had MLCs ≥50 μg/mL, and 95.8% (46 of 48) of the isolates from women who were successfully cured with metronidazole treatment had MLCs <50 μg/mL. Overall agreement between laboratory result and clinical outcome was 93.7% (89 of 95).

## Tinidazole MLC

Tinidazole MLC data from 33 *T. vaginalis* isolates that had been obtained from women who had tinidazole treatment failure were compared with tinidazole MLC data from the 48 women who had been successfully treated with metronidazole and were subsequently negative by culture and PCR testing. The median of tinidazole MLCs of the isolates from women successfully treated with metronidazole was 1.6 μg/mL, and the 95$^{th}$ percentile was 6.3 μg/mL, with no isolate from successfully treated women having a tinidazole MLC >6.3 μg/mL (Fig. 2). Of the isolates from women who had failed tinidazole treatment, 84.8% (28 of 33) had an MLC ≥6.3 μg/mL (Table 2). By contrast, 92% (44 of 48) of the women who were cured with metronidazole had tinidazole MLCs <6.3 μg/mL. Agreement between laboratory result and treatment outcome was 88.9% (72 of 81).

## Reproducibility

Five isolates that had been submitted to the CDC for susceptibility testing were randomly selected for reproducibility testing. One of the isolates had an MLC less than the potential MLC cutoff for both drugs, and 4 of which had MLCs higher than each drug's potential cutoff. The MLCs were determined for both metronidazole and tinidazole a total of 6 times each by 2 different testers (3 times per tester). For both drugs, the results were 100% reproducible based on the categorical assessments (less than or greater than 50 μg/mL for metronidazole and 6.3 μg/mL for tinidazole). Isolate MLC values remained within 1 doubling dilution across all assays.

# DISCUSSION

The *T. vaginalis* susceptibility assay provides qualitative levels of parasite sensitivity to 5-nitroimidazole drugs that correspond to the treatment outcomes of *T. vaginalis* infections to both metronidazole and tinidazole. This is the only laboratory test for quantifying drug susceptibility levels for *T. vaginalis*. A PCR test is commercially available that evaluates the presence of genetic mutations that are associated with resistance,[19] but it is unable to indicate a level of resistance in the same way the culture-based assay can and would miss resistance associated with other mechanisms. Isolates from women who have repeatedly failed metronidazole or tinidazole treatment usually have higher in vitro MLC values in the *T. vaginalis* drug susceptibility test than do isolates from women who respond to treatment. However, pharmacokinetic and treatment compliance factors may be involved such that not every isolate from women who fail standard treatment has high MLC values. For example, unexpectedly high rates of trichomoniasis treatment failure have been observed in pregnant women.[20] In vitro testing of isolates from women who have failed standard treatment is useful to determine whether the parasites causing the infection are truly resistant, and provides guidance for treatment regimens that can assist in the choice of subsequent treatment regimens. Usually, this manifests as higher doses of nitroimidazole drugs for a longer time because *T. vaginalis* resistance to nitroimidazoles is relative and not absolute. Testing and use of the CDC alternative therapy recommendations result in successful cure of >80% of persons who failed treatment before testing.[12]

Historically, based on the work of Müller et al.,[13] metronidazole treatment failure was associated with an in vitro aerobic metronidazole MLC ≥50 μg/mL. No previous work has been performed to establish an aerobic in vitro MLC for tinidazole that correlates with treatment outcomes. In determining an MLC associated

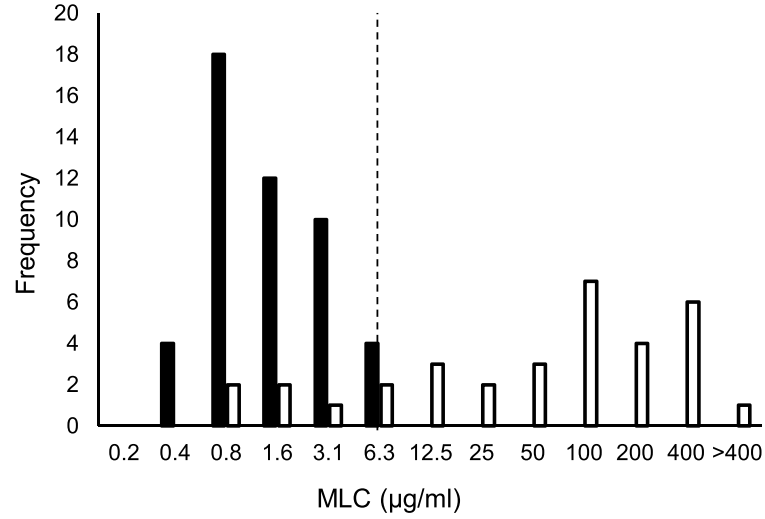


**Figure 2.** Tinidazole MLC distributions of isolates obtained from women with *T. vaginalis* infections who were successfully treated with metronidazole (black bars) or unsuccessfully treated with tinidazole (white bars). The dashed line represents the cutoff based on the 95th percentile tinidazole MLCs of isolates from women who were successfully treated with metronidazole.

**TABLE 2.** Categorization of Isolates From Women Successfully Treated With Metronidazole or Unsuccessfully Treated With Tinidazole According to in Vitro MLC

| Tinidazole MLC, μg/mL | Tinidazole Treatment Failure | Metronidazole Treatment Success |
|---|---|---|
| <6.3 | 5 | 44 |
| ≥6.3 | 28 | 4 |

with treatment failure for tinidazole, we used data collected from our metronidazole-sensitivity assay service activity along with the results of a research study performed in collaboration with Emory University that provided *T. vaginalis* isolates from confirmed positive women who tested negative for infection 3 months after having received a standard metronidazole treatment. Because metronidazole is generally used as the first-line treatment and is available in a generic form commonly covered under most insurance plans and Medicaid, we did not have access to isolates from women who were successfully treated with tinidazole. However, the tinidazole values from the isolates from women successfully treated with metronidazole should still provide appropriate data, as neither we nor others have previously observed an isolate with a higher MLC for tinidazole than metronidazole.[21]

Confirmation of the MLC associated with metronidazole treatment failure as ≥50 μg/mL and establishing the MLC associated with clinical tinidazole resistance as ≥6.3 μg/mL improves the ability to correctly identify persistent infections due to drug resistance and helps to identify alternative treatment regimens that are more likely to affect cure. Unfortunately, there is inadequate laboratory capacity to serve the large number of women who may have 5-nitroimidazole–resistant infections.[11] The CDC conducts susceptibility testing under Clinical Laboratory Improvement Amendments regulations to support patient treatment. In recent years, there has been an increase in requests for drug susceptibility testing for *T. vaginalis.* We believe that this is likely attributable to the recent availability of nucleic acid amplification tests for *T. vaginalis,* which are much more sensitive than wet mount or culture, resulting in recognition of more persistent infections. Greater availability of *T. vaginalis* drug susceptibility testing is needed, and CDC can provide technical assistance to laboratories interested in establishing the assay. This test is also useful for evaluating new drugs for treating trichomoniasis,[22,23] which is important because isolates resistant to metronidazole can demonstrate cross-resistance to tinidazole and the exact mechanism(s) of resistance remains poorly understood.[24] Identifying new drugs that can be administered orally and are effective against *T. vaginalis* infections (e.g., secnidazole and non-nitroimidazole drugs) is an important step toward an appropriate public health response to this common sexually transmitted disease.

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