Use of a Time-Kill Technique for Susceptibility Testing of 
*Trichomonas vaginalis*

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The emergence of metronidazole-resistant *Trichomonas vaginalis* and questions about the safety of metronidazole are significant concerns in treatment of trichomoniasis. At 24 h, a microtiter assay was used to test antimicrobial susceptibility of 16 recent isolates; the MICs of metronidazole ranged from <0.06 to 25 µg/ml. Observable motility as an endpoint correlated imperfectly with survival as measured in pour plates. Quantitative pour plate cultures of six *T. vaginalis* isolates after timed exposures to antimicrobial drugs demonstrated exquisite sensitivity to metronidazole with minimal trichomonadal concentrations of 0.025 to 0.100 µg/ml. Killing of some *T. vaginalis* isolates by clotrimazole and rosoxacin occurred only at concentrations of 100 µg/ml. Resistance to both rosoxacin and clotrimazole correlated with increasing resistance to metronidazole (*P* < 0.01).

*Trichomonas vaginalis* is a protozoan pathogen of worldwide importance best known for its ability to produce vaginitis (8). At least 2.5 million U.S. women develop symptomatic trichomonal vaginitis per year (2). Trichomoni- asis is a sexually transmitted disease which is also associated with nongonococcal urethritis, prostatitis, and other conditions in male partners of infected women (11).

For the last 20 years metronidazole has been the drug of choice for treatment of urogenital trichomoniasis (6). Widespread use of metronidazole is cause for concern since the drug is mutagenic for bacteria (6, 15) and carcinogenic for laboratory rodents (25, 26). Therefore, use of metronidazole should be avoided in pregnancy, particularly during the first trimester (23). Another problem is that metronidazole-resistant *T. vaginalis* strains have recently been isolated from patients with refractory trichomoniasis (18, 19, 21). There is an unresolved need for better methods to evaluate potentially toxic drugs for their antitrichomonal activity and to monitor development of drug resistance in *T. vaginalis*. For these reasons, we developed reproducible methods for determining the antimicrobial sensitivity of *T. vaginalis*. These methods were used to determine the metronidazole sensitivity of recent *T. vaginalis* isolates and the activity of two potentially useful antitrichomonal agents, clotrimazole and rosoxacin.

**MATERIALS AND METHODS**

**Organisms, culture medium, and conditions.** *T. vaginalis* strains were isolated from women attending the Albermarle County Venereal Disease Clinic, Charlottesville, Va., and the King County Venereal Disease Clinic, Seattle, Wash., with the use of Feinberg-Whittington liquid medium containing penicillin (1 × 106 U/liter), gentamicin (100 mg/liter), and amphotericin B (2.5 mg/liter) (12). Cultures were maintained under anaerobic conditions in brewer jars containing hydrogen and 4 to 7% carbon dioxide (Gas-Pak; BBL Microbiology Systems, Cockeysville, Md.) at 37°C. Routine subcultures were done every 3 to 4 days. Trichomonad isolates were passed at least three times in culture to assure that they were free of contaminating microorganisms. All clinical isolates were tested for antibiotic sensitivity within 4 weeks of initial isolation. Midlogarithmic-phase cultures of *T. vaginalis* were examined microscopically before susceptibility testing. Characteristic morphology and ≥95% motility were prerequisites for additional evaluation.

Rosoxacin (Win 35213) was kindly supplied by the Sterling-Winthrop Research Institute (Rensselaer, N.Y.). Clotrimazole (BAY b 5097) was kindly provided by Schering-Plough (Kenilworth, N.J.). Metronidazole was obtained from Searle Laboratories (Chicago, Ill.).

**Microtiter system.** Trichomonads were washed in Hanks balanced salt solution and then adjusted to a concentration of 2.5 × 106 ± 0.5 × 106/ml of antibiotic-free Feinberg-Whittington liquid medium. Disposable polystyrene microtiter plates (Dispore-Tray, no. 96-002-05; Linbro Scientific, Camden, Conn.) were inoculated with 0.10 ml of *T. vaginalis* culture per well. An additional 0.10 ml of antibiotic-free medium was then added to each well.

Stock solutions of metronidazole (20 and 500 µg/ml), clotrimazole (500 µg/ml), and rosoxacin (500 µg/ml) were prepared in Feinberg-Whittington liquid medium containing no other antimicrobial agents. The first two rows of a microtiter plate transfer plate (no. 1-220-43; Dynatech Laboratories, Alexandria, Va.) were inoculated with 0.05 ml of the appropriate stock solutions. Routinely the lower concentration of metronidazole stock solution was used. However, for more resistant organisms the higher metronidazole concentration was also employed. An additional 0.05 ml of medium was added to the second row of the transfer plate. Serial dilutions were carried out from the second through seventh wells with a Rotatiter with an 0.05-ml Microdiluter (no. M-303-01 and no. M-222-23, respectively; Dynatech). The eighth row was inoculated with 0.05 ml of growth medium containing no antimicrobial drugs as a control. The transfer plate containing 0.05 ml of antimicrobial solution per well was then inserted into the microtiter plate, containing 0.20 ml of culture medium with 2.5 × 106 *T. vaginalis* organisms per well (final concentration, 104 *T. vaginalis* organisms per ml). The transfer plate was agitated gently, then removed. Thus, serial twofold dilutions of each antimicrobial agent were obtained ranging from 100 to 1.56 µg/ml for rosoxacin.

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or clotrimazole and routinely from 4 to 0.06 \( \mu g/ml \) for metronidazole. Isolates demonstrating relatively higher resistance were also tested with the more concentrated metronidazole stock solution, resulting in final metronidazole concentrations from 100 to 1.56 \( \mu g/ml \).

Duplicate plates were incubated either in anaerobic brewer jars or in room air at 37°C for 6, 12, 24, or 48 h. The plates were then examined with a phase-contrast inverted microscope (Nikon; distributed by Ehrenreich Photo-Optical Industries, Garden City, N.Y.). The lowest concentration of an antimicrobial agent that completely inhibited all trichomonad motility was recorded for each isolate at each time period. Reproducibility of the assay was evaluated by testing half the isolates two or three times in blinded fashion.

The viability of trichomonads remaining in microtiter wells after incubation was evaluated by inoculating the contents of microtiter wells at the MIC ± 1 dilution onto 60- by 15-mm Falcon polystyrene petri plates (Becton Dickinson Labware, Oxnard, Calif.). Nine milliliters of Diamond medium containing 0.5% purified agar (Oxoid; distributed by K. C. Biologic, Inc., Lexena, Kans.) was added; after hardening, the plates were incubated anaerobically in brewer jars at 37°C for 5 days (12). Colonies were then counted with a dissecting microscope. The minimal trichomonicidal concentration was defined as the lowest concentration of a drug that killed all trichomonads after 24 h exposure.

**Time-kill system.** Protozoa in 48-hour-old cultures were harvested by centrifugation (250 × g for 10 min), washed twice in Hanks balanced salt solution, and then counted with a hemocytometer. The concentration of trichomonads was adjusted to 2.5 × 10⁶ ± 0.5 × 10⁶ organisms per ml of Feinberg-Whittington medium containing no antibiotics. Cultures were then maintained at 37°C in either an anaerobic workbench (no77A; Germfree Laboratories, Inc., Miami, Fla.) or in room air, depending on the requirements of the experiment. Feinberg-Whittington medium (5 ml) containing the antibiotic to be tested was inoculated with 0.1 ml of T. vaginalis culture (final concentration, 4 × 10⁶ to 6 × 10⁶ T. vaginalis organisms per ml). After 0, 4, 8, and 24 h of incubation at 37°C, the tube cultures were vortexed for 10 to 20 s. A 0.5-ml sample was inoculated onto each of three 60- by 15-mm petri plates, and Diamond medium containing 0.5% purified agar was added. Hardened pour plates were incubated anaerobically in brewer jars at 37°C for 5 days. T. vaginalis colonies were then counted with a dissecting microscope.

Six different T. vaginalis isolates were evaluated for susceptibility to metronidazole, and four isolates were evaluated for susceptibility to the other drugs by using this quantitative time kill system. Susceptibility to metronidazole (0, 0.025, 0.05, 0.1, 0.25, 0.50, 1.0, and 5.0 \( \mu g/ml \)) was evaluated under both aerobic and anaerobic conditions. Susceptibility to both rosoxacin (0, 2.5, 5.0, 10, and 100 \( \mu g/ml \)) and clotrimazole (0, 25, 50, and 100 \( \mu g/ml \)) was evaluated only under aerobic conditions.

**Statistical methods.** The effect of antimicrobial agents on T. vaginalis survival appeared to follow first-order kinetics. For each drug concentration, the percent survival was plotted semilogarithmically against time (Fig. 1a). The slope of the linear regression line for these points measures the rate of T. vaginalis killing by the agent. The slope of individual regression lines was then plotted against antibiotic concentration (Fig. 1b) (12, 24). This analysis facilitated comparisons of T. vaginalis susceptibility to different drugs. The significance of the apparent increase in rosoxacin or clotrimazole resistance accompanying increased resistance to metronidazole was examined by using Kendall’s coefficient of concordance (5).

**RESULTS**

**Microtiter MIC determination.** Sixteen T. vaginalis isolates were evaluated for antibiotic sensitivity under either aerobic or anaerobic conditions for various intervals. Maximal antibiotic effects occurred after 24 h. Three isolates demonstrated ≥4 serial twofold dilutions higher resistance to metronidazole under aerobic than under anaerobic conditions. Because of the potential clinical significance of these findings, 24 h of incubation under aerobic conditions was selected as the time for determination of MICs. Reproducibility of the microtiter assay was evaluated by repeating the MIC determinations for eight isolates within 2 weeks of the initial evaluation with a third determination within 3 weeks for six isolates. The MIC for all antimicrobial drugs re-
mained within a twofold dilution of the initial value for all isolates.

The range of metronidazole MICs was from <0.06 to 25 \( \mu \text{g/ml} \), whereas the MIC for 90% of the isolates was 6.25 \( \mu \text{g/ml} \) (Table 1). Rosoxacin MICs varied from 1.57 to 100 \( \mu \text{g/ml} \), and the range of clotrimazole MICs ranged from 3.13 to >100 \( \mu \text{g/ml} \). The MIC for 50% of isolates was 50 \( \mu \text{g/ml} \), whereas the MIC for 90% of the isolates was >100 \( \mu \text{g/ml} \) for both drugs. Resistance to both rosoxacin and clotrimazole increased along with increasing resistance to metronidazole (\( W = 0.84, \chi^2 = 37.63, P < 0.005 \)).

Pour plates were made from \( T. \text{vaginalis} \) cultures containing antibiotics in microtiter wells after 24 h of incubation under aerobic conditions at 37°C. Colonies of \( T. \text{vaginalis} \) grew from 24 of 30 microtiter wells that contained motile trichomonads. \( T. \text{vaginalis} \) colonies also grew from 2 of 15 wells that contained no motile trichomonads on microscopic examination. Therefore, trichomonad motility by direct examination of microtiter cultures, at the MIC \( \pm 1 \) dilution, had a sensitivity of 92% and a specificity of 68% for predicting the presence of CFU in pour plates. There was no significant correlation between the degree of antibiotic sensitivity of individual isolates as determined in microtiter plates and the posterior probability of CFU in pour plates.

**Time-kill studies.** All six isolates used for these studies were killed rapidly by metronidazole at concentrations of \( \leq 1.56 \mu \text{g/ml} \). Additional studies determined that the minimal trichomonacidal concentrations of metronidazole were 0.025 (three strains), 0.050 (one strain), and 0.100 \( \mu \text{g/ml} \) (two strains). Because of these strain differences, linear regression lines were calculated for metronidazole concentrations of \( \geq 0.25 \mu \text{g/ml} \) for comparison to the other drugs. The survival of a representative \( T. \text{vaginalis} \) isolate in growth media containing different concentrations of metronidazole is presented in Fig. 1a. The slope of the least mean squares linear regression line for each concentration of metronidazole represents the rate of killing of \( T. \text{vaginalis} \) (Fig. 1b).

The survival of six \( T. \text{vaginalis} \) isolates in growth media containing metronidazole under aerobic and anaerobic conditions is summarized in Fig. 2. Killing of trichomonads by metronidazole was rapid and occurred at similar rates under both aerobic and anaerobic conditions. Clotrimazole and rosoxacin were markedly less effective than metronidazole. Killing of trichomonads by either rosoxacin or clotrimazole only occurred at concentrations of 100 \( \mu \text{g/ml} \) for three of the four isolates tested. The fourth isolate was not killed by either drug at 100 \( \mu \text{g/ml} \).

**DISCUSSION**

Metronidazole was the first antitrichomonad drug effective in over 90% of infected patients (6, 7). Treatment failures have historically been attributed to reinfection from untreated partners, poor patient compliance, variations in pharmacokinetics, or inactivation of metronidazole by other microorganisms (4, 10, 17). Recently, metronidazole-resistant \( T. \text{vaginalis} \) strains have been isolated from patients who failed conventional therapy (21, 29).

Metronidazole underwent clinical evaluation before current requirements for extensive preclinical evaluation. Subsequent studies demonstrated that metronidazole was mutagenic in bacteria and induced tumors in rodents (6, 14, 25, 26). Carcinogenic urinary metabolites have been detected during therapy in patients (9, 14). For these reasons, metronidazole must be regarded as a potential carcinogen and teratogen in humans. Thus, there was a clear need for in vitro systems to determine the metronidazole susceptibility of \( T. \text{vaginalis} \) isolates and to evaluate other antitrichomonad drugs.

Direct microscopic evaluation of motility in the microtiter assay required only 24 h for susceptibility testing of logarithmic-phase \( T. \text{vaginalis} \) cultures. In agreement with previous studies, aerobic incubation was necessary to demonstrate the highest levels of resistance to metronidazole (20, 21). The MICs of metronidazole ranged from <0.06 to 25 \( \mu \text{g/ml} \), roughly approximating values previously reported for unselected \( T. \text{vaginalis} \) isolates by a variety of qualitative methods (10, 17, 22, 28). However, determination of trichomonad viability by direct observation of parasite motility correlated imperfectly with production of colonies in pour plates. Time-kill studies enumerating CFU after measured exposures may demonstrate subtle differences in the kinetics of \( T. \text{vaginalis} \) killing by antitrichomonal agents such as zinc salts (12) and proved useful for evaluating susceptibility of \( T. \text{vaginalis} \).
vaginalis to antimicrobial agents. The six isolates evaluated all demonstrated exquisite susceptibility to metronidazole under both aerobic and anaerobic conditions of incubation. Each time-kill study required 15 pour plates per isolate and 5 days of incubation for colony growth. Therefore, microtiter studies may prove to be more useful for rapid susceptibility testing of T. vaginalis isolates, whereas time-kill studies might be used for in-depth evaluation of selected isolates.

Biological characteristics of trichomonads may be changed by cultivation in vitro. Prolonged passage of trichomonads may result in reduced virulence in animal models (8). Other investigators selected T. vaginalis strains that were resistant to zinc salts from initially susceptible populations (12). In the studies reported herein T. vaginalis isolates were tested for susceptibility to antimicrobial drugs within 4 weeks of isolation. Repeated determinations of susceptibility to antimicrobial drugs during a subsequent 3-week period for six isolates did not reveal any alterations in susceptibility. Further studies of antimicrobial agent susceptibility of trichomonads immediately after isolation with repeated evaluation of the same isolates after prolonged passage in culture would be of interest.

Previous studies suggested that the antimicrobial and mutagenic effects of metronidazole and related drugs depended on the activation of the 5-nitro group (1, 15). Because increased resistance of trichomonads to metronidazole was associated with increased resistance to other 5-nitroimidazoles (19), we chose to compare the antitrichomonal effects of drugs from other classes with metronidazole. Clotrimazole is an imidazole compound without a 5-nitro group. In older clinical studies, clotrimazole (100-mg intravaginal tablet daily for 6 days), reportedly cured 48 to 89% of women with trichomoniasis (13, 16, 27). Rosoxacin is a synthetic quinoline derivative with activity against many gram-negative pathogens, including β-lactamase producing gonococci (3). Both clotrimazole and rosoxacin demonstrated antitrichomonal activity against some strains in the microtiter system. High drug concentrations were necessary to achieve killing of recent T. vaginalis isolates; the MIC for each drug was >100 μg/ml for 90% of the isolates. Quantitative time-kill studies confirmed the observations that clotrimazole- and rosoxacin killed T. vaginalis only at high concentrations. These findings are in accord with recent clinical observations that short-course clotrimazole therapy cured only 13 to 27% of women with trichomonal vaginitis (W. M. McCormack, M. R. Spence, M. F. Rein, and M. R. Danzig, 13th International Congress of Chemotherapy, Vienna, Austria, August 28 to September 2, abstr. no. 74, 1983). Because clotrimazole is administered topically and has few adverse effects, it may be possible to improve clinical results by increasing the local drug concentration or the duration of treatment. Rosoxacin achieves peak serum concentrations of approximately 10 μg/ml after oral administration and is therefore unlikely to be useful for treatment of trichomoniasis. The trend toward increasing levels of resistance to both clotrimazole and rosoxacin associated with increasing resistance to metronidazole (P < 0.005) suggests that neither drug will prove highly effective in treatment of patients with metronidazole-resistant T. vaginalis infections.

LITERATURE CITED


