

## Drug Susceptibility Testing of Anaerobic Protozoa

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**A simple technique for routine, reproducible global surveillance of the drug susceptibility status of the anaerobic protozoa *Trichomonas*, *Entamoeba*, and *Giardia* is described. Data collected using this technique can be readily compared among different laboratories and with previously reported data. The technique employs a commercially available sachet and bag system to generate a low-oxygen environment and log<sub>2</sub> drug dilutions in microtiter plates, which can be monitored without aerobic exposure, to assay drug-resistant laboratory lines and clinically resistant isolates. MICs (after 2 days) of 3.2 and 25 μM indicated metronidazole-sensitive and highly clinically resistant isolates of *T. vaginalis* in anaerobic assays, respectively. The aerobic MICs were 25 and >200 μM. MICs (1 day) of 12.5 to 25 μM were found for axenic lines of *E. histolytica*, and MICs for *G. duodenalis* (3 days) ranged from 6.3 μM for metronidazole-sensitive isolates to 50 μM for laboratory metronidazole-resistant lines. This technique should encourage more extensive monitoring of drug resistance in these organisms.**

One of the most important issues for future biomedical research is the development of antimicrobial resistance and the lack of coordinated multinational surveillance mechanisms (25). Overuse and abuse, inappropriate treatment regimens, and over-the-counter sales of cheap and effective drugs in many parts of the world have already rendered chloroquine, penicillin, and methicillin ineffective against *Plasmodium falciparum*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, respectively (17, 44).

Infections by the anaerobic protozoa *Trichomonas vaginalis*, *Giardia duodenalis*, and *Entamoeba histolytica* are treated with metronidazole (Flagyl) or one of the related 5-nitroimidazole family of drugs such as tinidazole (Fasigyn) (1, 21, 31, 40). Metronidazole is the only drug approved for the treatment of trichomoniasis in some countries, the only safe treatment for invasive amoebiasis, and the favored treatment for giardiasis (40). It is cheap, easy to produce, safe, and effective; as a result, it is widely prescribed for a host of ailments including the treatment of infections by anaerobic bacteria including *Helicobacter pylori* (13), for prophylaxis, and postoperatively (39, 40). It is sold over the counter in many Asian countries, where optimal courses of treatment are rarely recommended or taken (43). Albendazole (Zentel) is an alternative for the treatment of giardiasis, with 62 to 95% reported efficacy compared with 97% for metronidazole (16).

Trichomonal infection of women ranges from an asymptomatic carrier state to profound, acute, inflammatory disease (28). Infections have been linked to sterility problems, low birth weight, and preterm delivery (30). These in turn are closely associated with high infant mortality. It is now generally accepted that *T. vaginalis* infection predisposes carriers to human immunodeficiency virus (HIV) and AIDS (8, 9) and cervical cancer (41). Given the prevalence of *T. vaginalis*, and conse-

quent attributable risk to HIV transmission, screening for and treatment of *Trichomonas* could prove to be the single most cost-effective step in HIV incidence reduction (4). Giardiasis is a significant gut pathogen causing acute and chronic diarrhea and failure to thrive in children (12). In Mexico alone, approximately 10% of the population has had an episode of invasive amoebiasis and a significant number will succumb to amoebic abscesses (7).

Drug failures in trichomoniasis treatment appeared soon after metronidazole was introduced in the 1960s (40; see reference 24 for references), and there has been an alarming increase in recent years (32). Resistance has recently been confirmed among clinical isolates of *Giardia* via difficult in vivo assays (22). In a concerted effort to sustain the usefulness of this very valuable drug, uniform assays for the assessment of its susceptibility in anaerobic assays need to be established for current as well as future worldwide comparisons.

In the past, susceptibility assays have been monitored for a variety of end points and reported as 50% inhibitory concentrations (IC<sub>50</sub>) for *Giardia* ranging from 0.01 to 1 μg/ml, 50% inhibitory dose (ID<sub>50</sub>) values of 0.2 and 1.5 μg/ml for *Giardia* (see reference 39 for references), minimum lethal concentrations (MLCs) after 72 h of 50 to 100 μM for susceptible organisms (36) and 11.6 μM for the HM-1 strain of *E. histolytica* (6), and on MIC of >15 μg/ml indicating resistance in anaerobic assays after 48 h (24). The assortment of assays found in the literature prevents comparison of data among different laboratories. In some cases the method of assay is not detailed, often as a result of shortage of journal space, and the experiment is therefore irreproducible (42).

Traditionally two major choices for susceptibility assays have been available: tube assays and microtiter plate assays. Microtiter plates are problematic due to the variability of the anaerobic environment and the need to remove the plates from the anaerobic or low-oxygen environment to monitor the progress of the assay. Tube assays are too cumbersome and time-consuming, with few if any replicates possible. A variety of methods have been used to generate a low-oxygen environment,

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including nitrogen flushing of parasites in 96-well plates (2), oxygen regulation of trichomonads in 24-well plates within anaerobic jars (24), and tube assays containing an oxygen-depleting medium (15). The aim of this study is to describe a simplified new method which may be extensively used for the determination of chemosensitivity in the anaerobic protozoa. The new method uses the Anaerocult C minisystem (Merck) for *Trichomonas* and *Giardia* and the Anaerocult A system (Merck) for *Entamoeba*. The Anaerocult C mini-system generates a 9% CO<sub>2</sub>-6% O<sub>2</sub> atmosphere after 24 h (manufacturer's data sheet), while the Anaerocult A system generates 18% CO<sub>2</sub>-<0.1% O<sub>2</sub> within 150 min when used as recommended in an anaerobic jar (manufacturer's data sheet). The microtiter plates are sealed in special incubation bags provided with the Anaerocult C minisystem together with the sachet which creates the low-oxygen environment. The plates can be monitored while sealed within the bags over several days and are incubated in a nongassed 37°C incubator. Parasites are monitored for viability, and in our experience drug MICs obtained using log<sub>2</sub> drug dilutions provide clear results.

#### MATERIALS AND METHODS

For methods to be standardized worldwide, equipment must be kept to a minimum. The following assays require the use of flat-bottom, sterile, tissue culture microtiter plates, a multichannel pipettor, an incubator, and an inverted microscope. The low-oxygen environment is generated using the Anaerocult C minisystem, which comes with special incubation bags in which to seal the microtiter plates. The bags can be sealed with an Anaeroclip (Merck) or a plastic bag sealer. The Anaerocult A system was designed for an anaerobic jar, but we cut one small square from the large (four by two squares) sachet and use it in the same way as the Anaerocult C minisystem for *Entamoeba* assays.

**Parasite culture and isolates.** All parasites were grown in TYI-S-33 (10), which was supplemented with bile for *Giardia* assays (19). The parasites were subcultured three times a week, except for *Entamoeba*, which was subcultured twice a week. Parasites to be used in drug susceptibility assays were grown for 1 day following regular subculturing and were in the log phase of growth. Drug-resistant lines were grown without drug for this period.

The *T. vaginalis* isolate BRIS/92/STDL/F1623 (F1623) was obtained from a male patient with pruritus. The metronidazole-resistant line BRIS/92/STDL/F1623-M1 (F1623-M1) derived from it (5) is routinely maintained in 400 µM metronidazole. The patient was treated with one 2-g dose of tinidazole and was clear on follow-up. BRIS/98/BSHC/11147 was obtained from a female who was similarly treated and was clear on follow-up. The clinically resistant line BRIS/92/STDL/B7268 was obtained from a woman who had recurrent infection for 18 months (42).

Axenic *E. histolytica* isolates were HTH-56:MUTM (MUTM) (33) and the drug-resistant line derived from it, MUTM-M1 (29), the isolate HM1:IMSS (HM1) (ATCC 30459), and line HM1-M1, which was induced to be metronidazole resistant in the same manner and to the same level as described for MUTM-M1 (unpublished results). Both MUTM-M1 and HM1-M1 are maintained in 10 µM metronidazole.

*G. duodenalis* isolates BRIS/83/HEPU/106 (referred to as 106) and the metronidazole-resistant line BRIS/83/HEPU/106-2ID<sub>10</sub> (106-2ID<sub>10</sub>) (2, 3) were used. 106 was originally obtained from a child with chronic gastrointestinal complaints. 106-2ID<sub>10</sub> is maintained in 5 µM metronidazole (3). WB1B and the metronidazole-resistant line WB-M3, which is currently maintained in 58 µM metronidazole, are long-established cultures (34). The albendazole-resistant line WB-M3-Alb (37) is currently maintained in 2 µM albendazole. The line was one of several in which albendazole resistance was induced, and it was derived from the metronidazole-resistant line WB-M3 (34).

**Drug susceptibility assays.** Stock solutions (0.1 M) of drug (17.1 mg/ml for metronidazole; 26.5 mg/ml for albendazole [Sigma] in *N,N*-dimethylformamide (DMF; high-pressure liquid chromatography grade [Sigma]) or dimethyl sulfoxide (Sigma) were prepared and stored at -20°C. The stock solution was diluted in medium to the required concentration. A useful starting concentration was 200 µM, which yields a maximum concentration in the assay of 100 µM (17.1 µg of metronidazole per ml and 26.5 µg of albendazole per ml). In aerobic *Trichomonas* assays, a maximum concentration of at least 200 µM in the wells is

recommended. A similar dilution of DMF or dimethyl sulfoxide was prepared for control wells. A 200-µl volume of diluted drug was added to wells 1, 3, 4, 6, etc., of row A, and 200 µl of diluted DMF was added to wells 2, 5, etc., of a 96-well flat-bottom, covered tissue culture plate (Costar). A 100-µl volume of medium was added to all other wells. Double dilutions down the plate were performed, and the last 100 µl from each well of row H was discarded. A 100-µl volume of medium containing parasites of the first strain was added to wells in columns 1, 2, 3, 4, 5, and 6. Parasites of a second strain were added to wells in columns 7, 8, 9, 10, 11, and 12. Final drug concentrations in rows A to H were typically 100, 50, 25, 12.5, 6.3, 3.2, 1.6, and 0.8 µM, respectively (17, 8.5, 4.25, 2.12, 1.06, 0.52, 0.26, and 0.13 µg of metronidazole per ml). For each strain and drug concentration, there were four replicates with drug and two without. If other drugs are to be compared with metronidazole, the replicates and plate arrangement can be adjusted. However, it is important to ensure sufficient replicated wells to avoid edge effects for both control and drug-containing wells (a minimum of three wells for each concentration of drug for each strain is suggested).

For *T. vaginalis* cultures 5 × 10<sup>3</sup> trophozoites per well were required for anaerobic assays and 10<sup>5</sup> were required for aerobic assays. *G. duodenalis* assays used 4 × 10<sup>4</sup> trophozoites per well, and *E. histolytica* assays used 1 × 10<sup>4</sup> per well.

The plate was placed in the incubation bag supplied with the Anaerocult C minisachets and made anaerobic as specified by the manufacturer for *T. vaginalis* and *G. duodenalis*. For *E. histolytica* a single square of the Anaerocult A sachet (there are eight square sections in each sachet) was used. The environment was made anaerobic as specified by the manufacturer for Anaerocult C mini. The incubation bag was sealed with a plastic bag sealer or with Anaeroclips and placed in a 37°C incubator. Multiple use of Anaeroclips (more than three times) was not successful. Aerobic assay mixtures were placed directly in a nongassed incubator. Trophozoite growth was monitored on a daily basis by comparing control and drug-containing wells in the same row using an inverted microscope while the plate remained in the incubation bag. Trophozoite numbers were scored using 1+ (dead or significantly fewer [not more than 20% coverage of well surface] and significantly less active, or in the case of *Entamoeba*, >90% rounded up, than the control well), 2+ (20 to 50% coverage of the well surface and some parasite motility), 3+ (an almost confluent well, much motility), and 4+ (a confluent well). Motility can be difficult to assess in adhering cell lines of *T. vaginalis*, in which case well coverage is scored; *Giardia* was monitored for motility and confluence; and *Entamoeba* was monitored for motility and rounding-up, which is an indication of drug susceptibility. Rounding-up refers to the ball-like *Entamoeba* trophozoite with no evidence of purposeful movement and no pseudopodia. *T. vaginalis* assay results were usually very clearly defined after 48 h for both anaerobic and aerobic assays. The results for 72 h were usually the same as those for 48 h. *E. histolytica* results were usually clearly defined after 24 h. *G. duodenalis* assay results were best reported after 72 h.

MICs are defined as the lowest concentration of drug at which a 1+ score was obtained in the majority of the triplicate (or quadruplicate) wells.

#### RESULTS

***T. vaginalis*. (i) Anaerobic assays.** After 1 day small numbers of parasites were scored, but by 2 days the control wells were at least 3+. By 3 days the fast-growing drug-sensitive isolates in the control wells were overgrown while the drug-resistant lines were 4+. Trophozoites growing in 0.8 µM metronidazole were either 3+ or 4+. The MIC of metronidazole for F1623, which is regarded as a susceptible isolate, was the lowest, at 3.2 µM (most frequently obtained value, the mode) with a range of 1.6 to 6.25 µM (Table 1). The previously uncharacterized drug-susceptible isolate 11147 was intermediate between the susceptible isolate and the clinically resistant isolate, for which the minimum MIC was 25 µM. The MIC for laboratory-induced metronidazole-resistant line F1623-M1 was >100 µM (mode) (Table 1). A second worker who carried out assays with *T. vaginalis* obtained a MIC mode of 1.6 µM for F1623 (12 attempts, 83% frequency, 1.6 to 3.2 range) and a MIC mode of 50 µM for F1623-M1 (3 attempts, 66% frequency, 50 to 200 range). Differences between the data presented in Table 1 and the above data may be a result of plate scoring. Nevertheless, the MICs for F1623 were in the 1.6 to 6.3 µM range, the

TABLE 1. MIC for *T. vaginalis*, *E. histolytica*, and *G. duodenalis* in drug susceptibility assays

Isolate	MIC ( $\mu\text{M}$ ) <sup>a</sup>	No. of attempts <sup>b</sup>	Frequency <sup>c</sup> (%)	Range ( $\mu\text{M}$ ) <sup>d</sup>
<i>T. vaginalis</i>				
Anaerocult C (2 days)				
BRIS/92/STD/L/F1623	3.2	9	66	1.6–6.3
BRIS/92/STD/L/F1623-M1	>100	4	75	50–>100
BRIS/98/BSHC/11147	6.3	4	75	3.2–6.3
BRIS/92/STD/L/B7268	25	4	25	25–>100
Aerobic assay (2 days)				
BRIS/92/STD/L/F1623	25	4	75	12.5–25
BRIS/92/STD/L/F1623-M1	Dead			
BRIS/92/BHSC/11147	50	4	75	50–100
BRIS/92/STD/L/B7268	>200	4	100	>200
<i>E. histolytica</i> , Anaerocult A (1 day)				
HTH-56:MUTM	12.5	4	50	12.5–50
HTH-56:MUTM-M1	25	4	50	25–100
HM1:IMSS	25	3	100	25
HM1:IMSS-M1	25	2	50	25–100
<i>G. duodenalis</i>				
Anaerocult C (3 days)				
BRIS/83/HEPU/106	6.3	13	69	3.2–12.5
BRIS/83/HEPU/106-2ID <sub>10</sub>	25	3	100	25
WB-1B	6.3	15	80	6.3–12.5
WB-M3	50	3	100	50
WB-M3-Alb	6.3	3	100	6.3
Albendazole assay				
BRIS/83/HEPU/106	3.2 <sup>e</sup>	9	44	0.8–3.2
WB-1B	3.2 <sup>e</sup>	11	63	1.6–3.2
WB-M3-Alb	12.5 <sup>e</sup>	3	66	6.3–12.5

<sup>a</sup> Most frequently obtained MIC of metronidazole (*Giardia* only) or albendazole or lowest MIC (for BRIS/92/STD/L/B7268 and HM1:IMSS-M1).

<sup>b</sup> Number of 96-well plate assays. Each plate had at least three identical columns of test drug and at least one drug-free control column.

<sup>c</sup> Percentage of attempts for which the mode was the MIC.

<sup>d</sup> Range of MICs obtained.

<sup>e</sup> Albendazole MICs; all other values are metronidazole MICs.

drug-sensitive range, and MICs for F1623-M1 were clearly within the clinically resistant range.

(ii) **Aerobic assays.** The results of the aerobic assays confirmed the ranking of the metronidazole susceptibility of the isolates observed in the anaerobic assays. The highly metronidazole-resistant line F1623-M1 did not grow under aerobic conditions (Table 1).

*E. histolytica.* The assays were more reliable when the Anaerocult A rather than the Anaerocult C system was used and were best read after 24 h. There was no apparent difference in the mode (Table 1) of these assays between the line HM1-M1 and its parent strain. However, at least one assay of HM1-M1 indicated increased resistance (Table 1). MUTM-M1 was consistently at least twofold less susceptible to metronidazole than was the parent strain, MUTM, in the same assay (Table 1).

*G. duodenalis.* The MIC for metronidazole-susceptible lines was 6.3  $\mu\text{M}$  (mode) in these assays, and that for the resistant lines was consistently higher (Table 1). Although the concentration of metronidazole in which 106-2ID<sub>10</sub> is maintained is low, the strain can survive higher concentration of drug. The albendazole-resistant line WB-M3-Alb, which was derived from the metronidazole-resistant line WB-M3, has apparently

lost its resistance to metronidazole but is less susceptible to albendazole than its parent strain. The reportedly high in vitro susceptibility of *Giardia* isolates to albendazole in comparison with metronidazole (11) was evident (Table 1).

## DISCUSSION

One of the difficult aspects of anaerobic drug susceptibility assays is standardization of the low-oxygen environment. The Anaerocult systems allow the environment to be duplicated from experiment to experiment and from laboratory to laboratory. They allow the use of multiwell plates, thus removing the tedium and unreliable reproducibility of tube assays. These systems provide the scientific community, including diagnosticians, with a uniform method of carrying out anaerobic drug susceptibility assays of the anaerobic protozoa so that data for clinical isolates can be compared both worldwide and with future data.

More complex and sophisticated drug susceptibility assays than those described here, with more precise readouts, have been reported, but these may be difficult to reproduce in some laboratories. For example, [<sup>3</sup>H]thymidine uptake (2) has been used to estimate ID<sub>50</sub>s, but the cost and accessibility of ra-

dioactive compounds and associated equipment in some parts of the world are prohibitive. Similarly, Kang et al. (18) and Gero et al. (14) developed anti-giardial and antitrichomonal activity colorimetric assays which employ synthetic substrates of purine salvage pathway enzymes and require an enzyme-linked immunosorbent assay reader. Although Meri et al. (24) reported the use of the Anaerocult A system to create an anaerobic environment in jars, the bag system that we used will provide a more reproducible, low-cost assay. Our assays have been used successfully to assess clinical isolates in Durban, South Africa (unpublished data), and are being used in India to establish *E. histolytica* assays for clinical isolates growing xenically. Clearly, the assay using axenic lines will have to be adapted to xenic cultures, since axenic cultures of *E. histolytica* are notoriously difficult to establish.

Future considerations include assay of clinical metronidazole-resistant *Giardia* isolates such as those described by Lemée et al. (22) and identification of stable clinical metronidazole-resistant *Entamoeba* strains. These stable drug-resistant isolates, together with clinically resistant *T. vaginalis* strains, some of which are well characterized in the literature (27, 42), can be deposited or identified in the American Type Culture Collection protist bank as a source of positive control strains that can be used as standards along with laboratory-induced drug-resistant strains.

The number of trophozoites used in the assays is adjusted to provide a useful reading after a specified time. For *T. vaginalis*,  $5 \times 10^3$  trophozoites per well gave a reliable reading after 48 h; for *E. histolytica*, approximately  $1 \times 10^4$  in 24 h was successful, and for *G. duodenalis*,  $4 \times 10^4$  was reliable after 72 h. Fewer trichomonads were required because of their rapid growth in comparison with *Giardia*, while the size of the *E. histolytica* trophozoite in comparison with either *Giardia* or *Trichomonas* was the reason for its relatively low number. Two- to threefold-lower numbers of trophozoites did not alter the MIC.

It is important to report the MIC as a molar concentration to standardize the comparison of efficacy between different drugs, especially if, for example, metronidazole is being compared with another nitroimidazole with a significantly higher molecular weight.

The MICs reported for metronidazole-resistant lines in the three different species used do not necessarily correlate with the amount of metronidazole in which the so-called resistant lines grow. This emphasizes the need to rigorously report assay readouts over an established period. The MIC for *E. histolytica* in our assays suggests that the parent strains could survive in approximately 10  $\mu$ M metronidazole. This is not the case for long periods, and continued exposure to fresh supplies of drug, as indicated in our previous work (29), where the resistant line MUTM-M1 survived in 10  $\mu$ M metronidazole but the parent isolate did not, will result in death of the culture. It is possible, however, to induce levels of resistance in *E. histolytica* with parasites maintained in 40  $\mu$ M metronidazole (45).

The high level of metronidazole resistance reached by trichomonads (5, 20) reflects the ability of the parasite to down-regulate all hydrogenosomal function, thus circumventing metronidazole activation, and to use alternative metabolic pathways (5). Aerobic versus anaerobic resistance in *Trichomonas* isolates is an important consideration since most reports indicate no apparent anaerobic resistance but significant levels

of aerobic resistance (26, 27). The highly metronidazole-resistant laboratory line, F1623-M1, for which the MIC mode is  $>100 \mu$ M metronidazole, was unable to grow aerobically, as documented for other resistant trichomonads (26). An anaerobic MIC of 25  $\mu$ M after 48 h for *T. vaginalis* isolate B7268 provides a MIC for clinical resistance associated with great difficulty in patient treatment. MICs of 6.3  $\mu$ M or less appear to correlate with 5-nitroimidazole susceptibility.

Lemée et al. (22) correlated clinical resistance to a standard anti-giardial therapy with ID<sub>50</sub>s of 125, 175, and 149 mg of metronidazole per kg assayed in a mouse model. Treatment efficacy correlated with a range of ID<sub>50</sub>s from 31 to 81 mg/kg. Thus, an apparently two- to threefold increase in resistance of the parasite grown in mice is sufficient to indicate clinical resistance and treatment failures. A threefold decrease in the activity of the enzyme pyruvate:ferredoxin oxidoreductase (35) and a twofold decrease in ferredoxin activity (23) in the metronidazole-resistant laboratory line WB-M3 may therefore be sufficient for clinical resistance. Similarly, a three- to fivefold increase in MRP activity renders tumor cells resistant to chemotherapy (46). The reason for using the mouse model for the assays (22) is the difficulty in establishing *in vitro* cultures of *G. duodenalis*, with the best success rates reporting around 50% of samples finally established in culture (38).

The 50  $\mu$ M MIC for the metronidazole-resistant line WB-M3, which is maintained in 58  $\mu$ M metronidazole, reflects the differences in growth conditions in tubes versus the conditions in the microtiter plate in the Anaerocult C anaerobic environment and emphasizes the importance of using consistent and reproducible assay conditions for drug susceptibility assays. Conversely, the MIC for 106-2ID<sub>10</sub>, which is grown in 5  $\mu$ M metronidazole, is 25  $\mu$ M in the assay. We have not previously reported the decreased metronidazole resistance following long-term growth in the absence of metronidazole in the line WB-M3, as was evident in WB-M3-Alb. WB-M3 was selected from surviving trophozoites exposed to high levels of metronidazole following UV mutagenesis (34) and was one of the most successful survivors when exposed long-term to albendazole (37). WB-M3 maintains its resistance characteristics for several days without the drug but has not been tested for sensitivity after months or years in the absence of metronidazole, similarly to WB-M3-Alb. The line 106-2ID<sub>10</sub>, which was induced to be metronidazole resistant following exposure to low levels of drug, reverted to sensitivity after 22 weeks without drug the (3).

This simple assay system provides a realistic common reference assay and is the first step toward global surveillance of the development of drug resistance in the anaerobic protozoa. It can be used in any basic laboratory equipped with an incubator and sterile culture facilities and allows comparisons of the more sophisticated methods for their interpretation of aerobic and anaerobic resistance among different isolates and for their correlation with earlier techniques.

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