In Vitro Susceptibilities of 25 *Giardia lamblia* Isolates of Human Origin to Six Commonly Used Antiprotozoal Agents

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The role drug resistance plays in the occurrence of chronic and recurrent giardiasis has not been established. Extensive data on the susceptibility to antimicrobial agents of living *Giardia* spp. trophozoites from human origin are lacking. We have determined with a macrodilution method in semisolid medium the in vitro susceptibility of 25 *Giardia lamblia* isolates, all obtained by routine cultivation of the duodenal fluid of children to six commonly used antiprotozoal drugs. The results showed tinidazole to be the most active drug (all isolates have MICs of ≤0.5 μg/ml). Metronidazole was equally active on all but one isolate, for which an MIC between 0.5 and 1 μg/ml was found. Furazolidone was the most active nonimidazole compound tested. More than 50% of the isolates were very susceptible to paromomycin, pyrimethamine, and chloroquine. Two of the strains presented an MIC for paromomycin higher than 10 μg/ml, and six strains needed more than 50 μg of pyrimethamine per ml to be inhibited. Decreased susceptibility of several of the isolates to different agents appears to be linked.

Although susceptibility testing is not the principal problem in *Giardia* spp. research, it is not known whether drug resistance or drug ineffectiveness plays a role in chronic and recurrent giardiasis. This is mainly due to the fact that no easy technique was available for in vitro cultivation of the microorganism.

Earlier described studies on the susceptibility of *Giardia* spp. to antimicrobial agents were technically very instructive (2, 4, 6, 11), but interpretation of the data was difficult: the number of strains tested was low (2, 4, 6, 11), and the isolates were mostly not of human origin (4, 6, 11). Moreover, the organisms were often maintained for a long time in axenic cultures before testing. This may have caused changes in important factors like generation time.

Recently, we developed a technique enabling the routine cultivation of *Giardia lamblia* trophozoites from human duodenal aspirates (5). By determining systematically the in vitro susceptibility of these isolates with a macrodilution method in semisolid medium (4), we can evaluate the activities of different antiprotozoal drugs on a large population of clinical *Giardia* spp. isolates of human origin. Since the test can be performed on fresh cultures and carried out in a few days, the results could have clinical implications.

This paper presents the results of the determination of the MICs of six commonly used antiprotozoal drugs on 25 clinical isolates of *G. lamblia*.

MATERIALS AND METHODS

**Microorganisms.** During a 10-month period, 25 *G. lamblia* strains were isolated by routine culture from duodenal fluid of children attending our pediatric gastroenterology department. A selective medium, based on a modification of TPS-1 medium (3), was used to grow the isolates axenically with a technique extensively described earlier (5). The cultures were maintained in the same medium without antibiotics (3, 5) and dispensed in 16-ml screw-capped glass tubes. Once the cultures were fully grown, the tubes were placed in ice water for 5 min to detach the microorganisms from the glass, centrifuged, and suspended in 2 ml of phosphate-buffered saline. A trophozoite count was performed in a Fuchs-Rosenthal chamber, and the suspension was adjusted to a final concentration of 3 × 10⁶ trophozoites per ml.

**Antimicrobial agents.** The following antiprotozoal drugs were tested: tinidazole (Fasigyn; Roerig), metronidazole (Flagyl; Rhone-Pouilenc), chloroquine (Nivaquine; Rhone-Pouilenc), pyrimethamine (Daraprim; Wellcome Research Laboratories), furazolidone (Furoxone; Norwich), and paromomycin (Hematin; Parke, Davis & Co.). Fresh stock solutions of 2,000 μg/ml were prepared by dissolving pure substance of each test drug in 3 ml of distilled water (for chloroquine and paromomycin) or dimethyl formamide (for tinidazole, metronidazole, pyrimethamine, and furazolidone).

**Susceptibility test.** A macrodilution method in semisolid medium was used to determine the activity of the different drugs (4). Glass tubes were filled with 6 ml of modified TPS-1 medium (5) without antibiotics. The medium was prepared no later than 5 days before the test to avoid degradation and oxidation and was stored at 4°C. Just before the experiment, various amounts of stock solution of each antiprotozoal drug were added to obtain final test concentrations of 0.5, 1.0, 5.0, and 10 μg of active substance of tinidazole, metronidazole, and furazolidone per ml and 1.0, 10.0, 50.0, and 100.0 μg of paromomycin, pyrimethamine, and chloroquine per ml in the test tubes. At least one control tube in which the active drug was substituted by phosphate-buffered saline was included for each drug and each strain.

Subsequently, an agarose III solution (Sigma Chemical Co.) in phosphate-buffered saline was added to the test tubes brought at 40 to 42°C to give a final concentration of 0.16% agar. An inoculum of 50,000 trophozoites per ml was obtained by transferring under laminar flow 0.1 ml of the
suspension containing $3 \times 10^6$ organisms per ml to each test tube. The medium was homogenized by inverting the tubes twice and then solidified by chilling for 3 min in ice water. Finally, all test tubes together with the control tubes were incubated at 37°C and inspected macroscopically every day. As soon as growth was detected, the number of colonies was estimated daily up to 7 days. The presence and growth of *G. lamblia* trophozoites were always confirmed with an inverted microscope at a magnification of $63 \times$. The MIC of a drug was defined as the lowest concentration for which no visible growth was detected before or until day 7 of the experiment. The same procedure was repeated afterward for each strain and each drug to confirm the reproducibility of the test.

## RESULTS

The interpretation of growth or no growth in the test tubes was clear cut. Massive growth occurred in control tubes without antibiotics from day 3 on and was detectable as "flock formation," each flock corresponding to a microcolony of living *G. lamblia* trophozoites, as confirmed by microscopic examination. All noninhibited organisms, exposed to drug concentrations lower than the MIC, grew massively in the test tubes no later than 2 days after the control became positive.

Table 1 displays the frequency distribution of the MICs of the 25 *G. lamblia* isolates for metronidazole, tinidazole, and furazolidone. Growth of the trophozoites was completely inhibited by the lowest concentration of tinidazole (MIC, $\geq 0.5 \mu g/ml$). In a further experiment, all isolates were resistant to a concentration of 0.1 $\mu g/ml$. The results for metronidazole were comparable, with the exception of one isolate, which needed 1 $\mu g/ml$ for growth inhibition. Although most isolates were susceptible to 0.5 $\mu g$ of furazolidone per ml, 2 of 25 were at least 10 times less susceptible to the drug.

Table 2 shows the frequency distribution of the MICs for paromomycin, pyrimethamine, and chloroquine. Higher concentrations were needed to inhibit some of the isolates: between 1.0 and 10 $\mu g$ of paromomycin, pyrimethamine, and chloroquine per ml was needed to inhibit 5, 2, and 12 isolates, respectively. One strain presented an MIC between 10 and 50 $\mu g/ml$ for paromomycin, and 1 and 6 strains showed an MIC higher than 50 $\mu g/ml$ for paromomycin and pyrimethamine, respectively.

Certain isolates appeared simultaneously less susceptible (MIC of at least 10-fold higher than most of the other strains tested) to several of the drugs tested. The two strains with decreased susceptibility to furazolidone were simultaneously less susceptible to paromomycin and pyrimethamine. For one strain, decreased susceptibility to paromomycin implied also an MIC higher than 50 $\mu g/ml$ for pyrimethamine. Moreover, it is striking that the isolate presenting an MIC of 1 $\mu g/ml$ for metronidazole showed also a decreased susceptibility to paromomycin and pyrimethamine.

## DISCUSSION

Chronic and recurrent giardiasis raises many therapeutic problems for the clinician. Few data are available on the in vitro susceptibility of *G. lamblia* to different antiprotozoal agents from earlier published papers (2, 4, 6, 11). The combination of a technique for routine cultivation of *Giardia* spp. trophozoites from human clinical samples, previously described (5), and the present method for susceptibility testing enables one to gather information on a large scale on the activity of the different drugs, commonly used in the treatment of giardiasis. The test is very accurate and easy to perform. Cumbersome setups to determine the viability of the *Giardia* spp. organisms, like the incorporation of $[^3]$Hthymidine in microorganisms (2), dye exclusion, or counts of moving trophozoites in the medium (4, 6, 12), are not needed. In contrast to the techniques with liquid medium, the presence of living organisms after exposure to antibiotics in this test is not due to overgrowth of a single trophozoite, since each colony is formed originally by one organism. The choice of which criterion to use to evaluate the organisms’ susceptibility, as described in other papers (e.g., the 50% inhibitory concentration [4, 11], 50% inhibitory dose [2], minimal lethal concentration [4], or MIC [present work]) is less important, since all of the described techniques, including the present one, are meant to compare the in vitro activity of certain drugs to each other. Therefore clinical conclusions should be drawn very carefully out of these in vitro results. No consistent data are available on important factors like drug levels at the site of infection or bioinactivation of the drug by duodenal fluid. Also, lower concentrations than the in vitro MIC may be sufficient to eliminate the parasite in vivo, due to synergy of the drug with host defense mechanisms.

Tinidazole and metronidazole were the most active compounds in this series. The MIC of tinidazole was lower than 0.5 $\mu g/ml$ for all tested strains and was comparable to the results found by Boreham et al. (2) and Jokipi et al. (6). In the future however, lower concentrations of metronidazole and tinidazole should be tested with more strains to evaluate differences in activity between both nitro-imidazoles.

Interesting is the fact that during the investigation of a clinical case of treatment failure of giardiasis with metronidazole, Smith et al. (11) determined an MIC of 0.8 $\mu g/ml$ for the infecting strain. This value is comparable to the MIC of 1.0 $\mu g/ml$ that we found in the least susceptible isolate, for which clinical data are unfortunately lacking.

The MIC values found for furazolidone, paromomycin, pyrimethamine, and chloroquine were far less uniform. Furazolidone is widely used in Belgium because it has fewer side effects than the imidazoles. Whether the higher failure rate in the treatment of giardiasis with this drug (1, 8, 9, 13) is related to the higher MIC values found in this study for

### TABLE 1. Frequency distribution of MIC values ($n = 25$)

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<tr>
<th>MIC range (µg/ml)</th>
<th>Frequency distribution with antibiotic:</th>
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<tbody>
<tr>
<td></td>
<td>Tinidazole</td>
</tr>
<tr>
<td>0.1&lt;--&lt;0.5</td>
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</tr>
<tr>
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<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>5.0&lt;--&lt;10</td>
<td>0</td>
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</table>

### TABLE 2. Frequency distribution of MIC values ($n = 25$)

<table>
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<tr>
<th>MIC range (µg/ml)</th>
<th>Frequency distribution with antibiotic:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Paromomycin</td>
</tr>
<tr>
<td>&lt;1.0</td>
<td>18</td>
</tr>
<tr>
<td>1.0&lt;--&lt;10.0</td>
<td>5</td>
</tr>
<tr>
<td>10.0&lt;--&lt;50.0</td>
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<td>50.0&lt;--&lt;100.0</td>
<td>1</td>
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some isolates is unclear and can only be demonstrated by systematically determining the susceptibility in the future.

The highest MICs were found for paromomycin, another nonabsorbable antidiarrheic compound, and for pyrimethamine, with values well over 50 μg/ml.

Combined decreased susceptibility to both paromomycin and pyrimethamine is suggested, but remains to be confirmed. The same phenomenon is noticed for paromomycin and furazolidone and for pyrimethamine and furazolidone. It is noteworthy that for the isolate with decreased susceptibility to metronidazole, all other non-imidazole drugs, except furazolidone, were less active. To our knowledge, the possibility is noteworthy that for the isolate with decreased susceptibility, the phenomenon can only be demonstrated by systematically determining the MIC values for paromomycin.

The same phenomenon is noticed for paromomycin and furazolidone and for pyrimethamine and furazolidone. It is noteworthy that for the isolate with decreased susceptibility to metronidazole, all other non-imidazole drugs, except furazolidone, were less active. To our knowledge, the feature of linked in vitro decreased susceptibility to different drugs has not been described for intestinal protozoa and is hard to explain theoretically since the drugs have different mechanisms of action. If confirmed however, it opens a new dimension in the approach of giardiasis treatment.

Chloroquine was included to investigate a specific problem. Chloroquine is widely recommended in antimalarial prophylaxis for travelers at a daily dose of 100 mg. Since many travelers nevertheless return from tropical countries infected with Giardia spp., different hypotheses arise: one might speculate that the prophylactic dose of 100 mg a day does not give protection against all Giardia spp. isolates, especially since both Lerman et al. (7) and Schneider et al. (10) describe an excellent in vivo activity of chloroquine in giardiasis at a daily dose of 600 mg a day. It might be interesting to see whether Giardia spp. strains isolated from patients who took this drug as antimalarial prophylaxis present higher MIC values for chloroquine.

Although many other factors than in vitro activity play a role in the effectiveness of a treatment, we feel that the systematic determination of the susceptibility of routinely isolated G. lamblia strains will enable the collection of essential information on the mechanisms involved in the success or failure of giardiasis treatment with both classical and experimental drugs.

LITERATURE CITED