An in vitro metronidazole susceptibility test for trichomoniasis using the InPouch TV™ test

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Objective: An efficient anaerobic culture system, the InPouch TV™ test, was used to determine the susceptibility of *Trichomonas vaginalis* to metronidazole. Glacial acetic acid was employed as a solvent for metronidazole.

Methods: *T vaginalis* isolates were cultured from 16 symptomatic female patients. The 11 who responded to oral metronidazole, 250 mg tid for 7 days, were considered as having sensitive trichomonads; the 5 who remained infected after treatment were considered to have resistant organisms. All isolates were cultured for minimum lethal concentrations (MLC). Metronidazole was added to a series of pouches; two-fold dilution of the most concentrated was 50 μg/ml and the least was 0·4 μg/ml. The inoculum of viable trichomonads was 1 $\times$ 105/ml in each pouch. Pouches were incubated at 37°C for 48 h, examined microscopically for motile trichomonads, and then 0·5 ml was subcultured to drug free pouches. After 5 days incubation at 37°C, each subculture and culture were examined microscopically for viable trichomonads.

Results: Eleven isolates of *T vaginalis* from patients responding to metronidazole treatment had MLC between 0·4 to 3·1 μg/ml. The MLC from the 5 treatment failure patients were between 12·5 to 50 μg/ml.

Conclusions: For the 16 patients in this study, the MLC values determined with the InPouch TV™ test differentiated between infection caused by metronidazole sensitive and resistant trichomonads. The mean MLC of clinically resistant isolates was approximately eleven fold higher than the mean MLC of clinically sensitive isolates (15 μg/ml vs 1·32 μg/ml). There was a significant difference between clinically resistant and sensitive isolates ($t = 5·47$, $p < 0·0005$). This study suggests that the InPouch TV™ test could be used for distinguishing between metronidazole resistant and sensitive isolates.

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Keywords: trichomonas vaginalis; metronidazole; InPouch TV

Introduction

Metronidazole (flagyl; 1-hydroxyethyl-2-methyl-5-nitroimidazole) is the most effective drug for treating a *Trichomonas vaginalis* infection. The first evaluation of its clinical efficacy began in France in 1958.[1] Presently it is the only nitroimidazole available for treatment in the United States.[2] Prescribed treatment for trichomoniasis is either one 250 mg tablet taken orally 3 times per day for 7 consecutive days, or a single 2 g dose.[2 3] Although the drug is effective for most patients, about 8% of trichomonad vaginitis patients are refractory to treatment.[3 4] Trichomonad resistance to nitroimidazoles has been reported in various areas of the world and appears to be increasing.[2]

Some nitroimidazoles such as tinidazole, ornidazole are available in other countries. Even though alternative drugs may have longer half-lives, they are not clinically superior to metronidazole. Extended courses of topical treatments of clotrimazole, aminacrime, and sulfonamides have been evaluated in patients for whom a nitroimidazole was either contraindicated or unavailable. These treatments have been effective in less than 30% of the patients.[3]

Most treatment failures are produced by *T vaginalis* isolates with a low susceptibility to metronidazole. These isolates are resistant to the drug both in vivo and under aerobic and anaerobic conditions in vitro. Inadequate susceptibility corresponds to a high minimum lethal concentration (MLC) of drug. The MLC is defined as the minimum lethal concentration of drug in which no motile organisms are visible after 48 h incubation at 37°C.[5] These findings are confirmed by subculturing the trichomonads in a drug free medium.[2]

This current study employed the InPouch TV™ (pouch) test containing 4 ml of culture medium to evaluate metronidazole MLCs for clinical isolates of *T vaginalis*. Isolates were obtained from patients who had responded to treatment (n = 11). Isolates from patients (n = 5) who remained symptomatic after treatment with 250 mg of oral metronidazole, tid for 7 consecutive days, were considered resistant.

Materials and methods

Before beginning the drug susceptibility tests, experiments were conducted to determine the effect of glacial acetic acid (GAA) on the growth of *T vaginalis*. GAA was selected because it was recommended as the best solvent for metronidazole (personal communication, Sigma Chemical Company, St Louis, Missouri). Viability studies were performed in the pouch using 10 isolates of *T vaginalis* in their logarithmic growth phase. Each was inoculated into a pouch containing 0·1 ml of

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Table 1   Concentrations of T vaginalis in the InPouch TV test containing different concentrations (mM) of GAA (37°C)

Conc of GAA (mM)	Numbers of T vaginalis isolates									
	103	6	2	123	157	159	162	163	165	66
(1 × 10⁴ cells/ml, 24 h)										
Control	24·0	25·2	30·7	25·7	36·2	32·8	49·7	52·3	54·4	41·2
0·4	32·0	28·0	31·5	26·3	37·0	34·0	48·0	51·5	54·5	39·5
0·8	32·5	25·4	31·1	24·7	35·8	31·5	49·5	52·5	50·3	40·0
1·6	32·0	25·0	29·5	25·5	36·7	33·0	47·8	49·5	56·2	41·5
3·2	28·1	26·0	30·3	24·0	35·0	32·5	48·7	50·1	53·7	38·5
6·5	2·40	2·37	2·01	1·95	3·01	3·20	3·90	4·21	4·37	3·07
13·1	0·75	—	0·55	—	0·80	0·40	0·68	0·45	—	0·66
26·2*	—	—	—	—	—	—	—	—	—	—
(1 × 10⁵ cells/ml, 48h)										
Control	87·0	81·0	81·5	80·5	95·0	94·9	92·2	96·8	95·5	90·5
0·4	95·0	82·1	80·1	79·5	97·7	95·9	91·0	97·2	95·8	91·3
0·8	85·0	79·1	79·8	80·0	94·8	93·8	91·7	93·9	97·2	91·5
1·6	97·0	80·5	78·5	80·1	95·3	97·3	92·7	95·7	94·7	89·7
3·2	86·0	79·5	82·5	78·7	96·7	94·5	90·5	98·8	93·2	90·0
6·5	3·00	2·90	2·70	2·45	4·01	3·87	4·21	4·37	4·07	5·00
13·1	0·03	—	0·04	—	0·07	0·02	0·03	0·03	—	0·03
26·2*	—	—	—	—	—	—	—	—	—	—
(1 × 10⁶ cells/ml, 72h)										
Control	26·6	26·1	26·5	25·6	39·1	41·5	38·7	50·8	49·6	55·7
0·4	27·6	27·0	28·1	26·8	40·0	42·3	38·2	51·5	48·5	54·7
0·8	26·6	24·7	24·3	24·7	36·7	39·9	37·5	48·7	50·0	57·0
1·6	23·0	26·7	23·7	25·0	38·5	42·5	39·0	49·5	47·7	53·5
3·2	27·9	25·6	24·2	26·2	37·8	40·1	38·5	50·5	49·7	56·6
6·5	9·46	9·05	9·21	9·00	9·85	8·67	9·00	9·01	8·95	9·15
13·1	—	—	—	—	—	—	—	—	—	—
26·2*	—	—	—	—	—	—	—	—	—	—

—: No viable organisms
*: Four ml of media were mixed with 0·1 ml of GAA (17·4 M) to yield 420 mM HOAc in the most concentrated preparation. Ten less concentrated stock solutions of GAA were prepared from the 17·4 M HOAc by two fold serial dilutions into NS, that is, maximum of 1:512. Four ml of media was mixed with 0·1 ml of each of the diluted stock solution to obtain the listed concentration. Dilutions from 420 mM to 26·2 were negative for all isolates and not listed in this table.

NS (normal saline) as a control and into additional pouches with different 0·1 ml dilutions of GAA. The final concentrations of trichomonads were 1×10^4/ml, 1×10^5/ml, and 1×10^6/ml. After incubation at 37°C for 24 h, 48 h and 72 h, the organisms were counted employing a haemacytometer. The density of organisms (cells/ml) for each pouch was calculated and the results compared by the paired *t* test (table 1).

For MLC determinations both clinically resistant and sensitive motile isolates from the logarithmic growth phase (37°C, 48 h) were used as inocula (final dilution of 1×10^5/ml). Two-fold serial dilutions of metronidazole ranged from 50 µg/ml to 0·4 µg/ml. Each *T vaginalis* isolate was cultured in a pouch without metronidazole as a control, and in pouches containing different final concentrations of metronidazole. The tests were incubated at 37°C and the organisms were observed at 48 h to determine the MLC. To confirm the findings, 0·5 ml of the 3 media with the least concentrated drug which suppressed motility, were subcultured in a pouch without metronidazole and incubated at 37°C for 5 days (fig 1).

To determine the effect of metronidazole on the viability of trichomonads, one clinically sensitive isolate (SJCR 66) and a resistant isolate (KP) were each cultured in a pouch without metronidazole (containing NS as a control) and in pouches containing 12·50 µg/ml, 25·00 µg/ml, and 37·50 µg/ml of metronidazole. After exposure to metronidazole, each culture was observed microscopically at 100 ×. A haemacytometer was used to count viable organisms at 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 48 h and 72 h. The percentage of viable organisms (PVO) was determined as follows: percent viable organisms = 100% × (viable cell count/total cell count). The relationships were evaluated between the concentration of metronidazole, the PVO and exposure times.
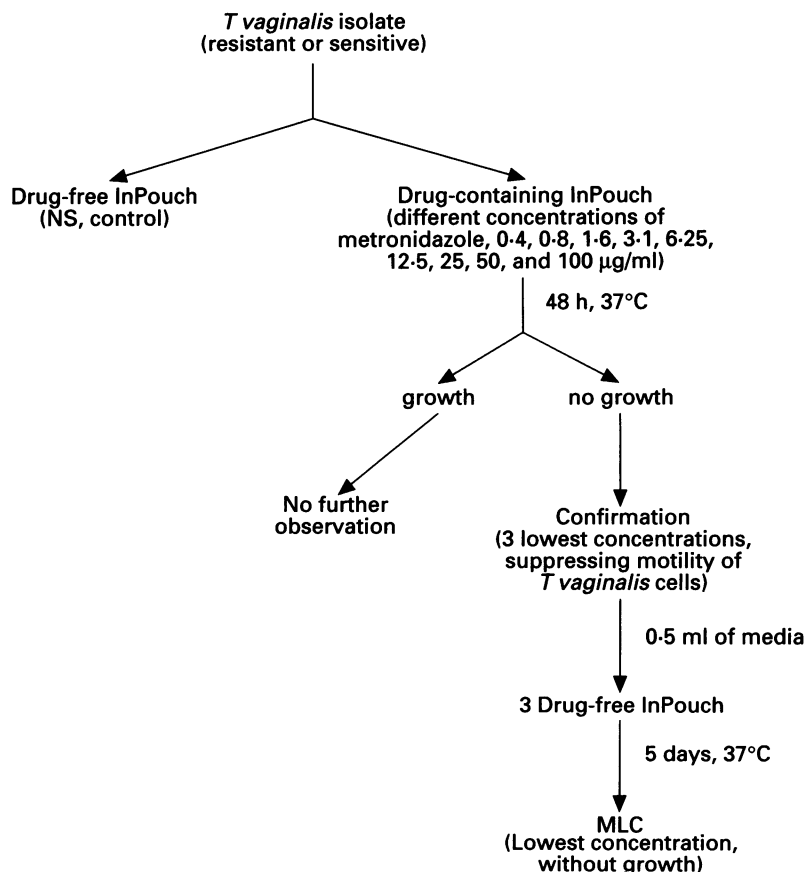

Figure 1   Flowchart of MLC determination. (The lowest concentration with no growth at the fifth day represents the MLC).

Table 2   Comparison of the influence between normal control and different concentrations of GAA on the growth of T vaginalis (37°C)

Group	Con of T vaginalis (Cells/ml)		t Value*	p Value*
	Mean	SD		
	(24 h)			
Control	3·72 × 105	1·10 × 105	—	—
0·4 mM AA	3·82 × 105	9·42 × 104	0·2183	> 0·50
0·8 mM AA	3·73 × 105	9·80 × 104	0·0215	> 0·50
1·6 mM AA	3·77 × 105	1·01 × 105	0·1059	> 0·50
3·2 mM AA	3·67 × 105	1·01 × 105	0·1059	> 0·50
6·5 mM AA	3·05 × 104	8·37 × 103	9·7891	< 0·0005
13·1 mM AA	4·29 × 103	3·04 × 103	10·5669	< 0·0005
	(48 h)			
Control	8·95 × 106	6·17 × 105	—	—
0·4 mM AA	9·05 × 106	6·86 × 105	0·3427	> 0·50
0·8 mM AA	8·87 × 106	6·63 × 105	0·2793	> 0·50
1·6 mM AA	9·02 × 106	7·16 × 105	0·2342	> 0·50
3·2 mM AA	8·90 × 106	6·74 × 105	0·1730	> 0·50
6·5 mM AA	3·66 × 105	7·96 × 104	43·6335	< 0·0005
13·1 mM AA	2·50 × 103	2·06 × 103	45·8579	< 0·0005
	(72 h)			
Control	3·80 × 107	1·09 × 107	—	—
0·4 mM AA	3·85 × 107	1·02 × 107	0·1059	>0·50
0·8 mM AA	3·70 × 107	1·13 × 107	0·2014	>0·50
1·6 mM AA	3·69 × 107	1·10 × 107	0·2246	>0·50
3·2 mM AA	3·77 × 107	1·11 × 107	0·0610	> 0·50
6·5 mM AA	9·13 × 106	3·05 × 106	8·0659	< 0·0005
13·1 mM AA	No viable organisms			

*Compared with control group.

Table 3   Comparison of MLC of metronidazole between clinically sensitive and resistant T vaginalis isolates

Group	Number	Mean (µg/ml)	SD (µg/ml)
Sensitive	11	1·32	1·14
Resistant	5	15·00	8·48
	t = 5·47	p < 0·0005	

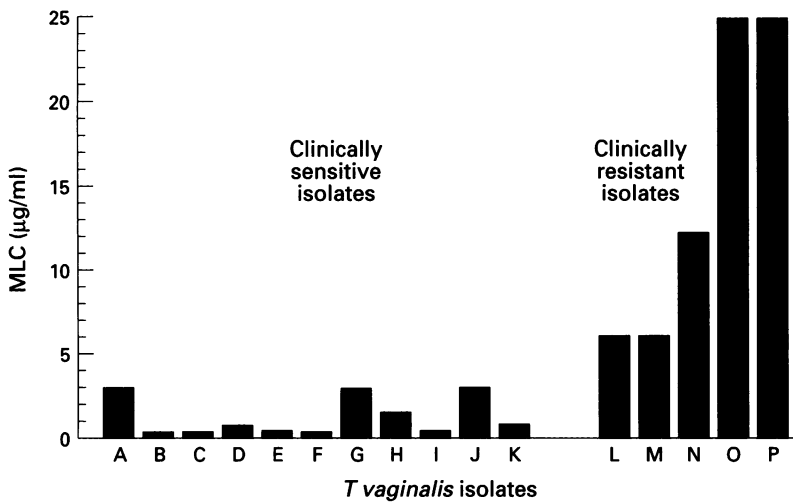
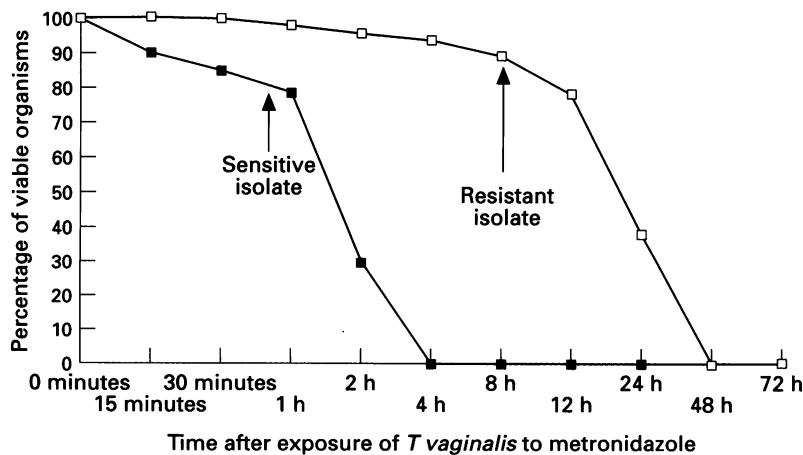Figure 2   MLCs of metronidazole for T vaginalis isolates.

Figure 3   T vaginalis viability after extended exposure to 12·5 µg/ml metronidazole.

Results

Tables 1–3 illustrate the effects of GAA on the growth of trichomonas. GAA at concentrations > 26·2 mM terminated *T vaginalis* viability at 24 h, 48 h, and 72 h (table 1). In 13·1 mM GAA no viable organisms were observed in 3 isolates (6, 123, and 165, at 24 h, 48 h, and none were viable at 72 h (table 1). GAA had no significant effect on viability at concentrations of < 3·2 mM GAA (p > 0·50, table 2). When the concentration of GAA was < 3·2 mM (that is, the pH of the medium was 8·5–8·6) the GAA did not inhibit the growth of *T vaginalis* (p > 0·50) But GAA inhibited the growth of *T vaginalis* at a concentration of > 6·5 mM (that is, the pH of the medium was < 8·0 (p < 0·0005). These results indicate that GAA did not influence the growth of *T vaginalis* in the pouch, but that pH < 8·0 inhibited growth. This suggested that the stock metronidazole should be dissolved with GAA and then diluted with NS to avoid the pH effect on growth in the pouches. In the current study the stock metronidazole solution (100 mg/ml) was prepared with GAA. After dilution with NS, the concentration of GAA in the most concentrated metronidazole (100 µg/ml) solution used in the MLC determination was 0·42 mM HOAc. This was much less than the 6·5 mM which inhibited the growth of *T vaginalis* in the pouch.

The MLCs of clinically sensitive isolates were from 0·4 µg/ml to 3·1 µg/ml and that of clinically resistant isolates were between 6·25 µg/ml and 25 µg/ml (fig 2). The mean MLC of clinically resistant isolates was about eleven fold higher than the mean MLC of clinically sensitive isolates. The difference was significant (*t* = 5·47, p < 0·0005, table 3).

There were differences in the PVO for clinically sensitive and resistant *T vaginalis* after exposure to 12·5 µg/ml, 25 µg/ml, and 37·5 µg/ml of metronidazole for as little as 15 min. At 12·5 µg/ml the PVO of the clinically resistant isolate was 100% and that of the clinically sensitive isolate was 90% (fig 3). The PVOs of the clinically resistant isolate in the pouches containing 25 µg/ml and 37·5 µg/ml were 100% and 98% respectively. But the PVOs of the clinically sensitive isolate was either 90% or 87% (figs 4, 5).

The PVO of the clinically sensitive isolate dramatically decreased after 2 h in 12·5 µg/ml of metronidazole, by 1 h both in 25 µg/ml and 37·5 µg/ml. The PVO of the clinically resistant isolate did not significantly decrease until after 8 h in 37·5 µg/ml metronidazole, or after 12 h in 12·5 µg/ml and 25 µg/ml (figs 3–5). This indicated that when the PVO of the clinical isolate did not decrease at 2 h in the pouch containing > 12·50 µg/ml of metronidazole, it could be resistant to metronidazole. Conversely, if the PVO of the clinical isolate decreased at 2 h in the pouch containing > 12·50 µg/ml of metronidazole, the isolate would be susceptible to metronidazole.

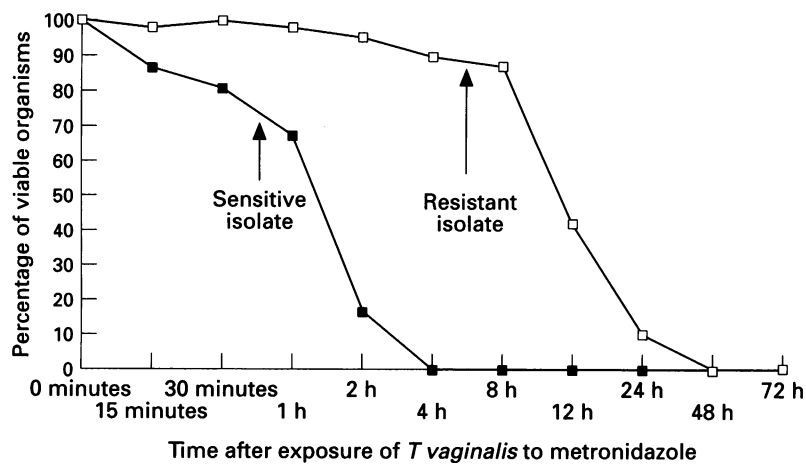## Discussion

Metronidazole treatment failures in patients

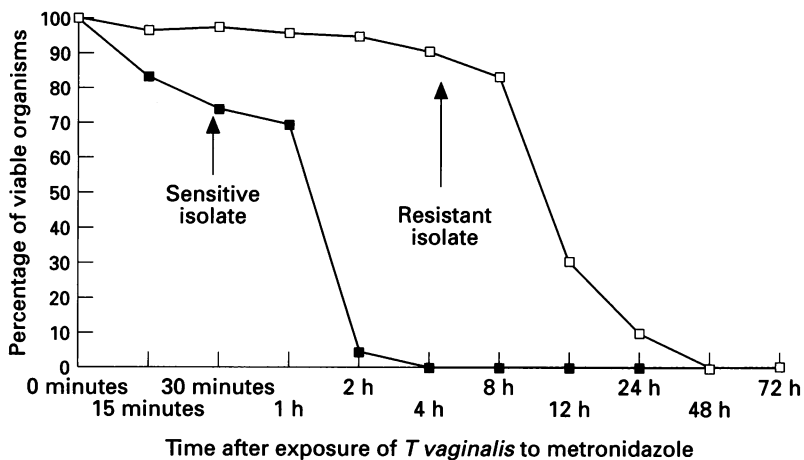Figure 4   T vaginalis viability after extended exposure to 25 µg/ml metronidazole.

Figure 5   T vaginalis viability after extended exposure to 37·5 µg/ml metronidazole.

resistant trichomonas isolated from a patient with a recurrent infection was evaluated by a method employing both stoppered tubes and multi-well plates.[9] This isolate only demonstrated resistance under aerobic conditions indicating the importance of the assay method. Another metronidazole study on resistant trichomonads used trichosel broth in tubes containing disks with various concentrations of metronidazole.[10] After incubation either aerobically or anaerobically, the results indicated that anaerobic incubation significantly decreased the MIC of the resistant isolates to metronidazole.

Lossick et al[11] reported that the anaerobic mean MLC of resistant isolates was about three-fold higher than that observed in non-resistant isolates (5·0 µg/ml vs 1·6 µg/ml). In the current study, the mean MLC of the clinically resistant isolates was about eleven fold higher than the mean of the clinically sensitive isolates (15 µg/ml vs 1·3 µg/ml). This difference was significant ($t = 5·47$, $p < 0·0005$).

In this evaluation the InPouch TV™ test provided an improved environment for distinguishing between resistant and sensitive T vaginalis. This suggests that the pouch test containing 3·1 µg/ml metronidazole differentiates between trichomonads that are either sensitive or resistant. T vaginalis remaining viable in pouches containing more than 3·1 µg/ml of metronidazole would be considered resistant.

have been attributed to non-compliance in taking the drug as prescribed, limited absorption from the gastro-intestinal tract, inadequate concentrations in the vagina, and degradation by vaginal microbial flora.[6 7] In a study of 11 patients, who were repeated treatment failures to oral metronidazole, (3 dosage levels during a 14–17 day period), high pressure liquid chromatographic analyses indicated that even at the low dosages, metronidazole and its metabolite were present in the expected concentrations both in plasma and the vagina.[8] Therefore, treatment failure of these patients was not due to any of the previously suggested causes. The current study confirmed the resistance of some isolates of T vaginalis to metronidazole.

Metronidazole resistance has been evaluated employing a variety of procedures. A

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