Studies on strain sensitivity of *Trichomonas vaginalis* to metronidazole

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SUMMARY Ninety-four strains of *Trichomonas vaginalis* isolated from unselected patients in Vienna during the period from November 1974 to November 1975 were subjected to sensitivity tests against metronidazole *in vitro* and *in vivo*. Nearly 90% of the isolates examined showed *in vitro* growth inhibition at concentrations of 0·4 to 1·6 µg metronidazole/ml and only about 10% at concentrations of less than 0·4 µg/ml. All isolates showed some inhibition of growth after incubation for 48 hours with 3·2 µg metronidazole/ml. Tests on treatment carried out on experimentally infected mice revealed that all strains of *T. vaginalis* examined were sensitive to metronidazole (MDE: $<3 \times 35$ mg/kg peroral).

Introduction

Reports on treatment failures with metronidazole (Robinson, 1962; de Carneri *et al.*, 1963; Arnold, 1966; de Carneri, 1966; Diddle, 1967; Aure and Gjønnaess, 1969; Kurnatowska, 1969; Giannone, 1972) have been the subject of much discussion and a possible drug resistance of *T. vaginalis* has been questioned (Jennison *et al.*, 1961; Kane *et al.*, 1961; Squires and McFadzean, 1962; Nicol *et al.*, 1966b; McFadzean *et al.*, 1969; Keighley, 1971; Korner and Jensen, 1976). These observations vary primarily because of divergent findings on the susceptibility of isolates from so-called 'therapeutical non-starters' to metronidazole as demonstrated by Jennison *et al.* (1961), Robinson (1962), de Carneri *et al.* (1963), Nicol *et al.* (1966b), Kurnatowska (1969), and McFadzean *et al.* (1969).

However, experimental studies have revealed that trichomonad strains whose resistance to metronidazole was proved by tests on treatment in laboratory animals, hardly differed from sensitive reference strains when they were tested for *in vitro* sensitivity (de Carneri *et al.*, 1969; Benazet and Guillaume, 1971). Thus, the value of such sensitivity tests appears to be in doubt (de Carneri *et al.*, 1969). A possible explanation for these observations has recently been found in our investigations (Meingassner and Lindmark, 1977): it was shown that a strain of *Tritrichomonas foetus* (KV$_1$/M100), the causative agent of urogenital trichomoniasis in cattle, developed resistance to metronidazole (Meingassner and Mieth, 1976) in mice after several

syringe passages under increasing drug concentrations. However, as with strains of *T. vaginalis* showing *in vivo* resistance to metronidazole, this strain exhibited the same sensitivity to metronidazole *in vitro* as the sensitive parent strain when examined under anaerobic test conditions. Under aerobic test conditions the KV$_1$/M100 strain also proved to be highly resistant to metronidazole.

In response to these findings we have tested unselected isolates of *T. vaginalis* for their sensitivity to metronidazole in laboratory animals as well as under aerobic test conditions *in vitro* in order to ascertain the range of variation of the sensitivity of *T. vaginalis*.

Material and methods

ISOLATION AND CULTURE OF *T. VAGINALIS*
Vaginal swabs were collected from 100 untreated patients with symptomatic trichomoniasis. The swabs were rinsed in a trichomonas medium (Oxoid, CM 161) containing antibiotics and the medium was then incubated for three days. In order to eliminate the concomitant microbial flora subpassages were made with 1000 iu of penicillin, 1000 µg of streptomycin, and 10 iu of nystatin per ml medium. Eventually 94 isolates were cultivated under axenic conditions. Determination of the sensitivity was made on these cultures within 12

passages. All strains subjected to the sensitivity test had been isolated in Vienna during the period from November 1974 to November 1975.

EVALUATION OF THE *IN VITRO* SENSITIVITY

The sensitivity of each isolate to metronidazole was estimated by determining the minimal inhibitory concentration (MIC) and the minimal lethal concentration (MLC) in a serial dilution test.

BROTH DILUTION TEST PROCEDURE

The cups of sterile cell-culture plates (Linbro Chemical Co. Inc., New Haven, Conn./USA, model 96 CV-TC) were filled with 1·4 ml of the diluted overnight cultures of the isolates (final concentration about 50 000 trichomonads/ml CM 161-medium) and with 0·1 ml of metronidazole solution. The plates were then sealed with a pressure-sensitive tape (Scotch) and incubated for 48 hours at 37°C. The evaluation of each isolate was made in one test with two replications. The concentration (using a twofold dilution schedule) ranged between 0·05 and 100 μg of metronidazole/ml.

In order to determine the MIC and MLC the plates were examined in an inverted microscope with a 40× magnification. The growth was estimated subjectively in accordance with the system + to +++, whereby the lowest concentration with + growth was regarded as the MIC. (Single motile trichomonads per cup were read as + and the culture density corresponding to the growth in untreated controls as +++). The MLC was the lowest concentration in which no motile trichomonads could be detected. The final evaluation was the result of the majority of the individual findings. A sensitive strain of *T. vaginalis* (A, isolated in 1971) was used as a control in every test.

EVALUATION OF THE *IN VIVO* SENSITIVITY

The chemotherapeutic sensitivity of the isolates was determined on ectopically-infected mice which were checked for the presence or absence of lesions and parasites six days postinfection after three peroral treatments.

IN VIVO TESTING PROCEDURE

Female NMRI-mice weighing 10–12 g were treated orally with metronidazole 2, 18, and 24 hours after a double infection with $1·2 \times 10^6$ trichomonads intraperitoneally and 4×10^5 trichomonads subcutaneously. For the isolates nos 1–70 dosage schemes of 3×15, 25, and 35 mg of metronidazole/kg body weight were applied using six mice per concentration, while only one dose of 3×25 mg/kg on 12 mice per concentration was used for isolates

nos 71–94, as it had been found that all these dosage schemes were statistically equally reliable.

The animals were killed on the sixth day after infection. The criteria of antitrichomonal efficacy of metronidazole were based on the absence of macroscopically visible lesions in the subcutis (abscesses) and on the absence of motile trichomonads in 48-hour cultures of the abdominal cavity. To compare the dose responses the score system shown in Table 1 was used:

Table 1 *Score system*

Score	Culture density/abdomen	Size of abscesses/subcutis	
		Description	Approximate size (mm)
0	0	Normal	
1	(+)	Pinhead	2–3
2	+	Lentil	4
3	++	Pea	5–6
4	+++	Bean	>6

As a control, infected, untreated groups were used to determine the pathogenicity of the isolates for mice, as well as treated groups infected with a reference strain (E, isolated in 1971) to compare the sensitivity.

Smears from abscesses were examined microscopically for motile trichomonads.

STATISTICAL TREATMENT

The total frequency distribution of the MIC and MLC of the 94 isolates was compared with that of the control strain A, estimated by means of the χ^2 test. This test was also applied for assessing the metronidazole efficacy *in vivo*, compared with the reference strain E.

Results

The *in vitro* metronidazole sensitivity of all isolates tested is summarised in Figs 1 and 2. Growth inhibition was achieved in nearly 90% with concentrations ranging from 0·4 to 1·6 μg metronidazole/ml medium, and in about 10% with concentrations of less than 0·4 μg/ml. All isolates showed an inhibited growth *in vitro* after an incubation period of 48 hours with 3·2 μg of metronidazole/ml (Fig. 1). The values of the MLC showed a similar variation in metronidazole sensitivity. Concentrations ranging from 1·6 to 3·2 μg were lethal in 85% of the isolates whereas lower concentrations were trichomonacidal in only 15%. In a single isolate—although its MIC did not differ from those of the remaining isolates— the lethal concentration was found to be >3·2 μg (Fig. 2). No marked differences in metronidazole

sensitivity could be found between the isolates tested and the reference strain after 14 separate tests.

The findings of the sensitivity tests *in vivo* are summarised in Tables 2 and 3. The results of treatment in ectopically infected mice showed that all strains had the same degree of sensitivity to metronidazole. The total healing rate ascertained with doses of 25 mg metronidazole/kg body weight was 81·7% by the use of the pathological findings and 78·4% according to the results of reisolation. Not more than 33·3% of mice per treatment group remained infected after treatment with 3×25 mg

metronidazole/kg. The result for the groups treated with dosages of 3×35 mg/kg body weight was 99% (Table 2). The infected untreated controls showed that all isolates were pathogenic for mice.

The sensitivity of the isolates obtained from the ectopic infection model *in vivo* did not differ significantly from that of the reference strain E.

A comparative analysis of the efficacy of metronidazole at the two sites of infection is presented in Table 3.

In 77·6% of mice treated with 3×25 mg metronidazole/kg body weight both infection sites were cured. In 17·7% the subcutaneous infection was
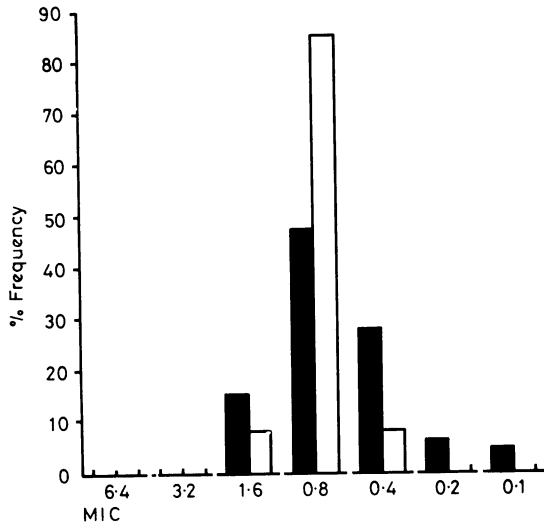

Fig. 1   In vitro *activity of metronidazole against* 94 *isolates and a reference strain of* T. vaginalis: *minimal inhibitory concentration (MIC) in* μg/ml.
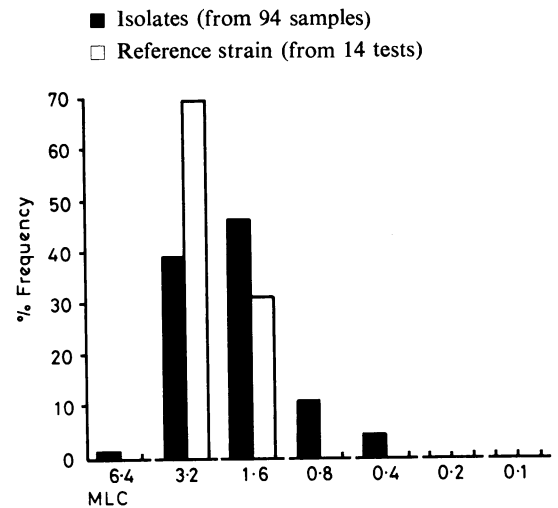

Fig. 2   In vitro *activity of metronidazole against* 94 *isolates and a reference strain of* T. vaginalis: *minimal lethal concentrations (MLC) in* μg/ml.

Table 2   *Efficacy of metronidazole against* 94 *isolates and a reference strain of* T. vaginalis *in mice: incidence of the individual findings scored with* 0–4 *after peroral treatment*

Samples of T. vaginalis	Dosage (mk/kg body weight)	Score (% incidence)					No. of animals
		0	1	2	3	4	
Isolates	3 × 15	5·0	69·2	23·5	2·3	0·0*	302‡
		1·7	3·6	38·1	54·6	2·0†	
	3 × 25	81·7	17·8	0·3	0·2	0·0*	870
		78·4	7·1	13·3	1·3	0·0†	
	3 × 35	99·1	0·9	0·0	0·0	0·0*	325‡
		99·4	0·3	0·3	0·0	0·0†	
Infected controls	Untreated	0·0	10·5	52·0	37·5	0·0*	564
		0·0	0·0	0·0	51·6	48·4†	
Reference strain E	3 × 25	88·6	11·4	0·0	0·0	0·0*	
		81·5	3·8	14·7	0·0	0·0†	184‡

* Lesions in the subcutis
† Density of trichomonads in cultures of the abdominal cavity
‡ Several groups comprised fewer than 6 or 12 animals

Table 3 *Comparison of the efficacy of metronidazole in mice according to the mode of infection: incidence of different findings after peroral treatment of subcutaneous or intraperitoneal infections*

Samples of T. vaginalis	Dosage (mg/kg body weight)	Differences in the scoring system (% frequency)			No. of animals
		Intraperitoneal < subcutaneous*	Intraperitoneal = subcutaneous†	Intraperitoneal > subcutaneous‡	
Isolates	3 × 15	89	9·3	1·7	302§
	3 × 25	17·7	77·6	4·7	870
	3 × 35	0·6	98·5	0·9	325§
Reference strain E	3 × 25	18·5	80·4	1·1	184§

*Clearance better in subcutaneous infections than in intraperitoneal infections
†Clearance identical in subcutaneous and intraperitoneal infections
‡Clearance better in intreaperitoneal infections than in subcutaneous infections
§Several groups comprised fewer than 6 or 12 animals

cleared and the peritoneal was not, whereas the reverse was true in only 4·7%.

Discussion

In vitro sensitivity tests carried out on 94 isolates of *T. vaginalis* revealed that all specimens were sensitive to metronidazole to almost the same degree. This was also shown very clearly by the results from the experiments in mice. The MLC value of only one strain was 6·4 µg/ml, although its growth sensitivity and susceptibility to metronidazole *in vivo* did not differ from the remaining isolates. The reason we employed the double-infection method was that while subcutaneous infections with strains of *T. vaginalis* of unknown virulence can more easily be established than intraperitoneal infections, intraperitoneal infections do permit a more reliable evaluation of treatment. However, in our assay all intraperitoneal infections proved to be successful. Distinct therapeutic differences owing to the mode of infection were found in the dosage group of 3 × 15 mg/kg which, we believe, might be explained by the different modes of assessment used. The results of treatment in mice infected subcutaneously were assessed macroscopically (which may be considered a rather rough evaluation), while intraperitoneally infected mice were evaluated by reisolating trichomonads from the abdominal cavity which proved to be far more reliable.

Various workers have pointed out (Nicol *et al.*, 1966a; McFadzean *et al.*, 1969) that it is possible that metronidazole may be inactivated by the vaginal bacterial flora, so we used only decontaminated cultures for our studies. Those samples which could not be cultivated axenically with penicillin, streptomycin, and nystatin after 12 subpassages were therefore rejected.

Considering the fact that we conducted our studies on unselected patients suffering from vaginal discharge and it may therefore be assumed

that they comprised primary cases and reinfections as well as exacerbations or uncured cases (although no amnanestic details were known), we feel that the results were remarkably uniform. In neither test system did the isolates significantly differ one from another or from the reference strains regarding their sensitivity. Resistant and 'relatively' resistant strains were not found with our assay system, nor did the strains show a gradual decrease of susceptibility compared with the reference strains as described by Giannone (1972).

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