

STUDIES OF METHODS OF MEASURING IN VITRO EFFECTS OF DRUGS FOR VAGINAL TRICHOMONIASIS

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INTRODUCTION

Trichomonas vaginalis, a protozoan belonging to the flagellates has a slightly higher metabolic mechanism than bacteria and is not easy to culture in various conditions¹⁻⁵. In a series of our studies of infection of the male urogenital organs with *Trichomonas vaginalis* (hereinafter referred to as TV), we have found that the new clinical isolates of TV alter in sensitivity to drug depending on the number of passages cultured, also noted were some interesting findings on the difference between the susceptibility of TV to drugs *in vitro* and the clinical effects of the drug. The results are presented below.

MATERIALS AND METHODS

In the present study 12 strains of TV freshly isolated from the urines of male patients during the period 1974 to 1975 were used. These isolates came all from patients with subclinical infection. They were discovered when the first early morning urine was collected into a test tube from each patient, and part of it was cultured with "Asami medium²" to screen it for the presence or absence of infection. All the subjects screened were male adults.

The medium was inoculated with the urine, and examined for the presence or absence of TV cells 3 days later. At the same time, it was further examined for the coexistence of bacteria, yeasts and candidas; and only the TV strains that were isolated were used for experimental purposes. These strains were preserved by culturing by pas-

sage with the Asami medium at 2 or 3 day intervals. In the experiment, each MSF (Modified Shaffer Frye) medium was seeded with about 200,000 cells of one strain; each drug was introduced at various concentrations and part of the medium was microscopically examined at 3~5 day intervals. If no cells were found on the medium, blind passage was performed until the second subculture on the Asami medium, and the medium was then microscopically examined. The reasons why this blind passage was made only until the second subculture, is mentioned in a previous paper of the author⁶.

Sensitivity tests of the strains of TV at 1 mcg/ml or even at 0.1 mcg/ml are relatively easy. However, since the present study was intended to identify significant alterations in sensitivity of TV, it was decided to check only the key points by the use of progressively doubled dilutions, namely 3-fold, 6-fold, 12-fold and 24-fold dilutions. Three drugs were used in the sensitivity tests: nitrofurantoin, metronidazole, tinidazole. Nitrofurantoin, being sparingly soluble in water, was used as a solution in as small a volume of dimethylformamide as possible. In this instance, 3 MSF media containing one drug at one concentration was used for the cells of each strain; and the control media consisted of a drug-free medium and a medium not containing the drug but containing dimethylformamide at the same concentration as the medium containing this agent at the highest concentration.

The MSF medium was used for dilution of the drugs.

Table 1. MIC of Nitrofurantoin to some T.V. strains.

Strain No.	No. of passages	Concentration mcg/ml				Clinical effect
		3	6	12	24	
678	3	+	+	+	-	No
2,011	3	+	+	-		"
1,428	3	+	+	-		"
2,463	3	+	+	+	-	"
279	3	+	+	-		"
180	3	+	-			Yes
1,083	3	+	-			"
1,452	3	+	+	-		No
514	3	+	+	-		Yes

The effects of each drug were assessed as described above. This is, if viable cells were found under a microscope or found under a microscope after culture by passage, it was defined as (+), and if only dead cells or no cells were found, it was defined as (-). For the examination, that portion of the deep part of the *in vitro* medium that appeared abundant in the number of cells was transferred onto slide glasses, and 3 slides were prepared for each sample.

The clinical effects of the drugs were assessed as described below. A urine sample was collected immediately before treatment from each of the patients from whom TV had been isolated, and cultured on the Asami medium to reconfirm the infection. It was noted from this that there can be some male cases which resolve spontaneously.

The patients were each medicated with 300 mg daily of nitrofurantoin (trade name: Furadantin C®) as microcrystals, in 3 equally divided parts, for 10 consecutive days. A urine sample was collected from each patient again 5~7 days after treatment, and cultured; and the effects of the medication were assessed. The patients were instructed to use a condom in coitus in the meantime. In the cases that could not be completely cured, the application of 500 mg $\times$ 2 $\times$ 1 day of tinidazole, that is, oral application of a single dose of this agent, was made⁷⁾. In the cases that could not still be cured, it was scheduled to apply 1.5 g $\times$ 1 $\times$ 1 day of metronidazole⁸⁾.

RESULTS

The sensitivities of the isolates to nitro-

furantoin at concentrations of 3, 6, 12 and 24 mcg/ml were studied.

The results are shown in Table 1. All the strains at the 3rd subculture were not susceptible to 3 mcg/ml; 2 susceptible to 6 mcg/ml, 6 susceptible to 12 mcg/ml, and 2 were susceptible to 24 mcg/ml: in this instance, the median was found in 12 mcg/ml. The medication with this drug was assessed effective in 3, but ineffective in 6 patients. The patients that favorably responded to the medication included those from whom 2 strains sensitive to 6 mcg/ml had been isolated. This involved strains Nos. 180 and 1083, and were limited to those from whom had been isolated strains sensitive to 12 mcg/ml, and none were included from whom had been isolated strains sensitive to 24 mcg/ml. Conversely, the patient from whom had been isolated strains Nos. 678 and 2463 with MICs of 24 mcg/ml were included in the ineffective group. They are the findings *in vitro* conformed with the clinical findings.

The MICs to the strains at the 20th~

Table 2. MIC of Nitrofurantoin to some T.V. strains.

Strain No.	No. of passages	Concentration mcg/ml			
		3	6	12	24
678	20	+	-	-	
2,011	20	+	+	-	
1,428	34	+	+	-	
2,463	20	+	+	+	-
279	19	+	+	-	
180	33	+	-		
1,083	32	+	-		
1,452	10	+	+	-	
514	32	+	-		
121	31	+	+	-	

Table 3. MIC of Nitrofurantoin to some T.V. strains.

Strain No.	No. of passages	Concentration mcg/ml				Alterations in MIC
		3	6	12	24	
678	38	+	-	-		Decreased
2,011	36	+	+	-		Unchanged
1,428	52	-	-	-		Decreased
2,463	50	+	+	+	-	Unchanged
279	35	+	+	-		"
180	51	+	-			"
1,083	50	+	-			"
1,452	50	+	+	-		"
514	49	+	-			Decreased
121	49	+	+			Unchanged

30th passages are shown in Table 2. The strains were common with the younger generations in that all of them were non-sensitive to 3 mcg/ml; however, there were 4 strains sensitive to 6 mcg/ml, and 5 sensitive to 12 mcg/ml, and it was found that two of the strains, strains Nos. 678 (at the 20th subculture) and 514 (at the 32nd subculture), became more sensitive to the drug. Nitrofurantoin was clinically found effective in the patients with infection induced with strain No. 514.

In Table 3 are shown the MICs to the strains cultured by about 40~50 passages. There appeared one strain sensitive to 3 mcg/ml (strain No. 1428), and this strain was cultured by the greatest number of passages in the present series of experiments. There were 4 strains sensitive to 6 mcg/ml, and 3 sensitive to 12 mcg/ml; thus, all the strains but strain No. 1428 showed no increased sensitivity. As a whole, there were 3 strains that showed increased sen-

Table 4. MIC of Tinidazole to some T.V. strains.

Strain No.	Concentration mcg/ml				Clinical effect on strains cultured over 20 or more generations
	3	6	8	12	
678	+	+	-		ND*
2,011	+	-	-		"
1,428	+	+	-		"
2,463	+	+	+	-	"
279	+	+	-		"
180	+	+	-		"
1,083	+	+	-		"
1,452	+	+	-		"
514	+	+	-		"
121	+	+	-		"

* Not done

sitivities with the various MICs to them while there were 7 strains whose sensitivities remained unchanged.

In Tables 4 and 5 are shown the MICs of tinidazole and metronidazole to the 20th or more subcultures of the same strains. In this instance, the sensitivities of the strains to 3, 6, 8 and 12 mcg/ml of each drug were studied.

None of the strains were sensitive to 3 mcg/ml, 2 sensitive to 6 mcg/ml, 7 sensitive to 8 mcg/ml, and 1 sensitive to 12 mcg/ml of tinidazole; and none were sensitive to 3 mcg/ml, none sensitive to 6 mcg/ml, 8 sensitive to 8 mcg/ml, and 2 sensitive to 12 mcg/ml.

When the 6 patients who failed to favorably respond to nitrofurantoin treatment were treated with tinidazole, all favorably responded to the latter. Vaginal trichomoniasis disappeared from the patients from whom had been isolated the strains with the MICs of 12 mcg/ml.

Table 5. MIC of Metronidazole to some T.V. strains.

Strain No.	Concentration mcg/ml				Clinical effect on strains cultured over 20 or more generations
	3	6	8	12	
678	+	+	-		Yes
2,011	+	-	-		"
1,428	+	+	+	-	"
2,463	+	+	-		"
279	+	+	-		"
180	+	+	+	-	ND*
1,083	+	+	-		"
1,452	+	+	-		"
514	+	+	-		"
121	+	+	-		Yes

* Not done

DISCUSSION

At present no standard method is available for the determination of the sensitivity of *Trichomonas vaginalis* to drugs and no standard culture media have been established. In our present study, the sensitivities of TV strains were determined by the use of the Asami and MSF media; however, it is known that the use of a solid medium apparently improves their sensitivities⁹⁾.

The present attempt was made as a part of a series of studies for establishing the standard method for screening newly developed drugs for anti-TV action, and chiefly aimed at (1) observing from the urologic point of view how close the *in vitro* effect is to the clinical effect, and (2) how greatly the sensitivities of TV strains vary with the culture by passage. This is often talked about, but seldom reported.

When the data is analysed from this view point, the clinical effects in male cases of TV infection tended, as shown in Tables 1~4, to conform with the *in vitro* effects. In another study, which will be published later, the sensitivities of 55 TV strains to nitrofurantoin were measured, and compared with the clinical effects of the drug, the infections induced with 21 out of 36 strains sensitive to 9 mcg/ml or less of nitrofurantoin were treated with this drug, and all cured, while the cases induced with 9 out of 19 strains sensitive to 9 mcg/ml or more of the same drug were treated likewise, but 7 failed to favorably respond to the medication. In this instance, however, the periods of preservation and the number of strains of the isolates differed from the data achieved in the present study. When the difference in sensitivity between the 3rd, approximately the 25th, and the 45th subculture was examined, there was no strain whose sensitivity was lost, but 3 strains with improved sensitivities and seven with no alterations in sensitivity. The MICs to 2 out of the 3 strains with improved sensitivities, that is, strains Nos. 678, 1428 and 514, decreased by two or more steps, which may be said to be marked alterations.

Strains Nos. 678 and 514 already showed improved sensitivities at the 20th and 32nd

passages, and the sensitivities then remained unchanged until the 38th and the 49th generation. Strain No. 1428, on the other hand, showed no change in sensitivity until the 20th generation, but showed rapidly improved sensitivity until the 52nd generation. From this data, there is no alternative but to say that the number of passages with which the improvement in sensitivity occurs greatly varies from strain to strain. There were, on the other hand, strains which showed no alternations in sensitivity even when cultured by passage to the 50th or more subculture, e.g., strains Nos. 2463, 780, 1083 and 1452.

There are reports on alterations in the sensitivities of TV strains to Trichomycin^{10,11)} and to metronidazole¹²⁾ during culture, all revealing that the sensitivities more or less varied; however, the methods used in the reports studied were not the same as the author's. Considering them together, however, it appears certain that there are some strains that show alterations in *in vitro* sensitivity.

Next, the MICs of tinidazole and those of metronidazole are compared with those of nitrofurantoin. Strains Nos. 678 and 2463 which are poorly susceptible to nitrofurantoin are not necessarily poor in sensitivity to tinidazole or metronidazole. There also appears to be no correlation between the MICs of tinidazole and those of metronidazole. The data with only ten strains are shown in the present paper, but the reader is referred to another paper of the author.

The *in vitro* effects and the clinical effects of tinidazole were then studied. The drug proved effective in treating an infection induced even with strain No. 1428 the MIC to which was 12 mcg/ml, and the drug even at such a low concentration as 1.2 mcg/ml was active on the strains: probably for this reason, the drug was assessed effective in all the patients. It should be pointed out, however, that unlike the application of nitrofurantoin, tinidazole was applied in a large single dose.

Nitrofurantoin was clinically assessed effective in 2 patients, from whom the isolates were sensitive to 6 mcg/ml, and ineffec-

tive in both of the 2 patients, the isolates from whom were sensitive to 20 mcg/ml but not sensitive to 12 mcg/ml: thus, there appears to be a splitting point which borders effectiveness and ineffectiveness between 6 and 24 mcg/ml, especially at about 12 mcg/ml. Considering the fact that the blood level of nitrofurantoin remains about 2 mcg/ml with the application of 100 mg of the drug at a time, 3 times daily¹³, the results of *in vitro* study and those of clinical study do not appear to conform well with each other; however, because the pathogen cells may be considered to exist chiefly in the seminal vesicles or the prostate gland¹⁴, it appears that the concentration of the drug in this part is influential on the results. There are few papers on the concentrations of nitrofurantoin in the seminal vesicles and the prostate gland.

From the practical point of view, this method is accompanied by the following drawbacks:

1. It is necessary to culture a TV strain at least by 3 passages from the time of its discovery from a male patient until its pure culture is obtained, that is, it takes a period of 4~10 days.

2. It takes 6~8 days to get such data as are shown in Table 1 by measuring the sensitivities of the cells to drugs.

3. Thus, it takes a total of 10~18 days, and it requires special media and techniques, and is time-consuming.

4. All the cases cannot be tested smoothly. Roughly speaking, a pure culture is impossible to achieve for about one third of the isolates because of contamination, and one quarter of the remaining ones disappear during the culture by passage. Furthermore, there are problems as to the qualities of media and contamination at passage.

5. Because it takes 10~18 days to get the data, there can be such patients whose infections have disappeared at the time when the data are available.

Therefore, the following will have to be studied in order to reflect the data in clinical treatment or to determine the

standard method:

1. Such a strain that requires less passages is desirable.

2. The media and the number of cells should be kept constant. These two factors are already known to be essential, and the following will also be necessary:

3. Study of the sensitivity of the isolates from clinically ineffective cases, and of why there arises a difference between the clinical effect of a drug and the *in vitro* sensitivity of a test strain to it.

4. Does the phenomenon, "acquisition of resistance," really occur?

5. At what concentration is a drug distributed into each of the male genitourinary organs, and which part does *Trichomonas vaginalis* infect?

For these purposes, the discovery of male patients with infection induced with TV and the preservation of the isolates from such patients are of importance.

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和文抄録

抗膣トリコモナス剤の試験管内効力測定法に関する研究

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男子尿性器由来の膣トリコモナス保存株を経代培養すると、薬剤に対する感受性が変化し、経代を重ねるほど MIC が低下する。つまり薬剤が効きやすくなることが判明した。また、*in vitro* での薬剤効果と臨床効果にも差のあることが判明した。

したがって、膣トリコモナスの薬剤感受性を測定し、それを臨床的に利用しようとするには、

1. 経代数については、なるべく代数を重ねない株がよい。

2. 培地、虫体数などは、常に一定する。ということが必要であることがわかっているが、さらに、

3. 臨床無効例より検出した虫体の感受性の検討、および試験管内感受性との間の差がなぜ起こるかにについての検討。

4. 耐性獲得という現象が本当にあるのか？

5. 男子尿性器へ薬剤が如何なる濃度で分布するか、また膣トリコモナスが、どこに感染するか？等についての追及が必要となろう。

それには、資料として、男子 TV 感染者の発見と、その株の保存が大切である。

本論文の要旨は第22回日本化学療法学会総会において発表した。

訂正: Table 4 で Strain No. 1428 は ++++, 2463 は +++ です。次の欄は

Yes, Yes, Yes, Yes, Yes, ND*, ND*, ND*, ND*, Yes です。

Table 5 で No. 1428 は ++, 2463 は ++++ です。次の欄はすべて

ND* です。