Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Comparative drug susceptibility study of five clonal strains of *Trichomonas vaginalis in vitro*

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ARTICLE INFO

Article history:
Received 17 October 2010
Received in revised form 27 October 2010
Accepted 15 November 2010
Available online 20 January 2011

Keywords:
Trichomonas vaginalis
Clonal strains
Drug susceptibility
Metronidazole

ABSTRACT

Objective: To produce comparative data on a group of *Trichomonas vaginalis* clonal strains with varied drug responses using identical methods and materials. **Methods:** Five clonal strains of *Trichomonas vaginalis* were isolated from reference strain using agar plate technique. The variability of growth kinetic and susceptibility of clonal strain to metronidazole, tinidazole, satranidazole and nitazoxanide were observed in 96 well microtitre plate. **Results:** Among these clonal strains there was a good correlation between rates of growth with the relative susceptibility of the strains to drugs *in vitro*. Regarding metronidazole, tinidazole and satranidazole susceptibility, different degrees of susceptibility were determined. However, no difference in nitazoxanide susceptibility was found between the clonal strain tested and a reference strain. **Conclusions:** This is the first description of biological variability in clonal stock of *Trichomonas vaginalis*. Different degrees of drug susceptibility were determined among clonal strains tested. Further studies will be necessary to ascertain the importance of this variability in clinical infection.

1. Introduction

Trichomonas vaginalis (T. vaginalis), a flagellated protozoon with worldwide distribution, is a causative agent of human trichomoniasis. According to WHO, over 170 million people are infected with *T. vaginalis* annually worldwide[1]. The parasite generally infects the squamous epithelium of the genital tract, but sporadically it is recovered from the urethra, fallopian tubes and pelvis[2]. The incubation period is varies between 4 to 28 days in infected individuals[3]. The main clinical picture in acute infection includes vaginatis, cervicitis and urethritis in women and prostatitis and other genito-urinary syndromes in men. Other complications associated with trichomoniasis are adnexitis, pyosalpinx, and endometritis; infertility and low birth weight; and cervical erosion[4]. The prevalence and spectrum of disease in males are less well characterized; the infection appears to usually be asymptomatic but occasionally causes urethritis and prostatitis[5-7]. Since probably almost 50% of trichomonal infections are asymptomatic, the infection can spread easily. Another serious aspect of trichomoniasis is the association between *T. vaginalis* infection and an increased risk of transmission of other sexually transmitted diseases, including human immunodeficiency virus[8,9].

Despite high prevalence and associated risk of trichomoniasis, very little is known about the biological variability of the parasite. Infected individuals show a great variability in the pathological manifestations, from asymptomatic presentation to increased risk of pelvic inflammatory disease and tubal infertility[10]. The reasons for such variation and how the characteristics of every isolate impact the clinical manifestations of trichomoniasis are not well known. Experimental drug susceptibility testing is one of the parameter that can be applied to the characterization of isolates. Assays of variability have been used by various researchers in "standard" strains or clinical isolates without exact definition of their drug susceptibility differences[11,12]. However, ours is first of an attempt in clonal stocks. Establishment of long term clonal lines helps to determine the actual range of susceptibility of different parasite lines to a particular drug. These well defined sensitive and resistant lines can then be used as a reference against which field isolates can be compared. This allows

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a correlation of the *in vitro* sensitivity of the reference lines with the clinical outcome.

Metronidazole (MTZ) and a related 5-nitroimidazole drug, tinidazole (TNZ), are the treatment of choice for trichomoniasis. Satranidazole (STZ) is one of a large series of nitroimidazoles with a potent antiprotozoal activity. Nitazoxanide (NTZ) is a new thiazolide antiparasitic agent that shows excellent in vitro activity against a wide variety of protozoa and helminths[13]. Since the introduction of 5-nitroimidazoles in the 1960s, there have been reports of increases in the prevalence of metronidazole-resistant *T. vaginalis* infection[14]. So, it is of great interest to study the susceptibility these drugs to clonal strains of *T. vaginalis*.

The aim of the present study was to produce comparative data on a group of *T. vaginalis* clonal strains with varied drug responses using identical methods and materials. We also tried to correlate the growth rate of strains with the relative susceptibility to drugs *in vitro*.

2. Materials and methods

2.1. Strains of T. vaginalis

A local strain of *T. vaginalis* employed for this study was kindly provided by Professor Nancy Malla from Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India.

The clonal strains investigated in this study had been isolated using the method by Samuels[15] with certain modifications. Briefly, stock trichomonads for plating were cultured in TYI-S-33 medium. Hemocytometer counts were made with 48 h old culture of trichomonads and the organisms serially diluted with TYI-S-33 medium to the numbers desired, usually 30-50 per 0.5 mL. First base layer of 20 mL of 1.6% agar in Trichomonads modified CPLM base medium (Himedia lab, Mumbai) were poured from 20- or 25-mm tubes into sterile glass Petri dishes. Just before pouring, 0.2 mL of a common solution of 10 000 units/mL of penicillin and streptomycin (PAA lab, Austria) was added to each tube and mixed by gentle swirling. Immediately after pouring, the plates were placed in incubator at 37 ℃ and 5% CO_2 to harden for at least 30 min after the last was poured. Overlay medium with 0.8% agar, 10 mL per 20 or 25 mm tube, was melted and cooled in a water bath to 40 ℃. Warmed penicillin-streptomycin mixture (0.1 mL) and 1ml of heat inactivated adult bovine serum (PAA lab, Austria) were added, then the diluted organisms in 0.5 mL of TYI-S-33 medium at room temperature. Mixing and pouring of the overlay was done as rapidly as possible to reduce temperature shock. The plates were again set in incubator at 37 ℃ and 5% CO_2 for 3-5 days. The colony embedded in agar containing organisms was transferred to 0.5 mL of TYI-S-33 medium in a sterile 15 mL glass tube using a platinum wire, flattened in the plane of a lance-shaped loop. After release of the organisms, TYI-S-33 medium was added to a total volume of 10 mL. Five clonal strains *T. vaginalis* namely C1, C2, C3, C4, C5 were isolated. *T. vaginalis*(TV) strain from PGIMER Chandigarh was used as reference for drug susceptibility testing.

2.2. Culture medium

All strains were cultured axenically and maintained in pre-warmed TYI-S-33 medium containing 10% bovine serum in 15 mL glass tubes at 37 ℃ in an atmosphere containing 5% CO_2. Subcultures were usually done every 2-3 days.

2.3. Growth assay in 96 well microtitre plate

The numbers of trichomonads per mL in 24 h old culture of all the strains employed in study were determined using a hemocytometer chamber. The strains were then diluted using TYI-S-33 medium to give final concentration of 5×10^4 trichomonads/mL and 200 μL aliquots of each were then introduced in to microtitre plates with 96 flat-bottomed wells (CELLSTAR®, greiner bio-one). Microtitre plates were then incubated at 37 ℃ in an atmosphere containing 5% CO_2 (Heraeus instruments). Samples were taken for counting after mechanical vortexing for 5 s to 10 s, using long tipped Pasteur pipettes. The microtitre plates were tightly capped and opened only for sampling. Only motile trichomonads were counted using hemocytometer under inverted microscope (Nikon, ECLIPSE TE300). The trichomonads counts for growth study were made at 24 h interval for total of 120 h. Each experiment was performed in duplicate, with duplicate counts made for each sample. Generation time and number of generations calculated using the formula

Generation time $t_g = t/n$

$$n = \frac{\lg b - \lg a}{0.3010}$$

Where, b = No. of cells in population

a = No. of cells in initial innoculum

n = No. of generations that occured between time of inoculation to time of sampling

2.4. Drug preparation

Metronidazole (MTZ) (J.B.Pharmaceuticals, Mumbai), Tinidazole (TNZ) (Zydus Alidac, Ahmedabad), Satranidazole (STZ) (Ciba Geigy Limited, Mumbai) and Nitazoxanide (NTZ) (Ind-Swift lab, Chandigarh) were used. MTZ and TNZ were dissolved in sterile distilled water. STZ and NTZ were dissolved in DMSO. Drug solutions were freshly prepared before use by diluting with TYI-S-33 medium to appropriate concentration.

2.5. Drug susceptibility testing

Standard microtitre plates with 96 flat-bottomed wells (CELLSTAR®, greiner bio-one) were used in the test. Drugs

of various concentrations (from 0.048 to 200 μM) were added to the wells in 50 μL aliquots. A 200 μL suspension containing 1×10^4 *T. vaginalis* was then placed into each well[16]. All the drug and control studies were carried out in duplicate; a minimum of two independent experiments was conducted for each drug tested. The plates were incubated at 37 ℃ and were examined after 48 h with an inverted microscope (Nikon, ECLIPSE TE300) at 40× for motile trichomonads. The minimal inhibition concentration (MIC) is defined as the lowest drug concentration in which no motile organism was seen. Effect of DMSO on the parasite was investigated and observed that 2% (v/v) concentration had no effect on parasite motility.

3. Results

3.1. Growth curve

The growth curves of the five clonal strains and one reference strain are presented in Figure 1.

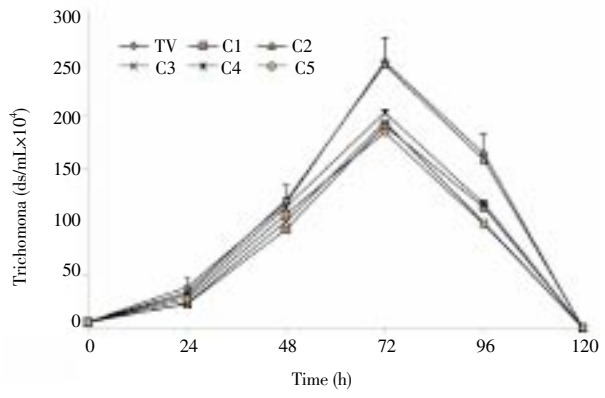

Figure 1. Growth curves of six strains of *T. vaginalis* on TYI-S-33 medium.
48 hr old culture of *T. vaginalis* that had not entered the stationary phase was used for growth study and drug susceptibility testing. Final concentration of innoculum was 5×10^4 trichomonads /mL. All the values were expressed as Mean±SEM.

The approximate doubling time of these clonal isolates was 13 to 14 h, and they reached the stationary phase of growth after about 72 h. Exponential growth occurred well beyond 48 h, resulting in maximum concentrations of organisms per mL observed. In contrast after 96 h 99% of the cultures were non-motile and presumed dead. The growth curve indicates that TV and C2 were the fastest and C1 and C5 were slowest growing strains. The growth rate of C3 and C4 falls between those of the other four. The generation time found for TV and C2 strains were 13 h 21 min; 14 h 11 min for C1 and C3; 13 h 45 minutes for C4; 14 h 27 min for C5.

3.2. Drug susceptibility testing

The *in vitro* drug susceptibility of five clonal strains tested is summarised in Table 1.

Table 1
Susceptibility of *T.vaginalis* clonal strains to MTZ, TNZ, STZ and NTZ in comparison to reference strain (TV) (μm).

Strain	MIC			
	MTZ	TNZ	STZ	NTZ
TV	6.4	0.8	3.2	12.5
C1	25.0	12.5	12.5	12.5
C2	6.4	0.8	3.2	12.5
C3	12.5	6.4	6.4	12.5
C4	12.5	3.2	6.4	12.5
C5	25.0	6.4	12.5	12.5

All clonal strains showed an inhibited growth *in vitro* after an incubation period of 48 hours with 6.4 to 25 μm of MTZ. Concentrations of TNZ ranging from 0.8 to 12.5 μm were lethal in all the strains tested. MIC values of STZ showed variation between 3.2 to 12.5 μm. Clonal strains C1 and C5 were the least sensitive to MTZ, TNZ and STZ. The response of clonal strain C2 was similar to that of reference strain. The sensitivity of clonal strain C3 and C4 were similar to all the drug tested except TNZ (C3= 6.4 μm; C4=3.2 μm). Surprisingly, no difference in NTZ sensitivity found between the clonal strains tested and a reference strain.

4. Discussion

Assays of variability in *T. vaginalis* have been used by various researchers in "standard" strains or clinical isolates without exact definition of their drug susceptibility differences[11,12]. However, ours is first of an attempt in clonal stocks.

There can be no doubt that *T. vaginalis* contains a number of strains which vary in their susceptibility to same drug. The degree of drug susceptibility can be affected by environmental conditions. A comparison of the relative drug susceptibility of various strains under different but uniform set of conditions always shows differences. One of the inherent factors which seem to affect the drug susceptibility is the rate of multiplication of the parasites in the culture media. In our study, the variability of growth kinetic of *T. vaginalis* was observed in 96 well plates. The use of multiwell plates, thus removing the tedium and unreliable reproducibility of tube assays[11]. The growth curves and generation times of the five clonal strains are compared with parent strain. It is evident that among the strains of *T. vaginalis* there is a good correlation between rates of growth with the relative susceptibility of the strains to drugs *in vitro*. On the other hand, it appears that the more drug-susceptible the strains of *T. vaginalis*, the shorter their generation time. In reference to the clonal strains of *T. vaginalis* the rates of growth in culture reflects their drug susceptibility. The effect of a new thiazolide antiparasitic agent NTZ did not differ between the slow and fast growing

strain. This suggests that the probable mode of action of NTZ is different from that of 5-nitroimidazoles. NTZ was 2 times as active as MTZ against C1 and C5 (less susceptible to MTZ) and nearly similar activity against susceptible strains. Higher the activity than MTZ seen for NTZ in relatively MTZ-refractory strains (C1 and C5) of *T. vaginalis* indicates that resistance mechanism to MTZ may be to a variable extent bypassed by NTZ. In view of NTZ unique mechanism of action, should be considered for further clinical evaluation in the treatment of MTZ resistance *T. vaginalis*. However, additional data to support this context are required.

Results of our study show that there was a striking difference in the activity of 5- nitroimidazoles against clonal strains. MIC of MTZ differed 2-4 fold between clonal strains. C1 and C5 exhibited a decreased susceptibility to two other antitrichomonad nitroimidazoles, *i.e.*, TNZ and STZ. The reported similarity in the mechanism of action of MTZ, TNZ and STZ has been confirmed in C1 and C5 showed a markedly decreased susceptibility to all three compounds. For all clonal strains, the MIC for TNZ was lower than that for MTZ, but it was evident that those clonal strains less susceptible to MTZ have decreased sensitivity to TNZ. This finding is not surprising, as both compounds are 5-nitroimidazoles.

In conclusion, this is the first description of biological variability in clonal stock of *T. vaginalis*. It is evident that among the clonal strains of *T. vaginalis* there is good correlation between rates of growth with the relative susceptibility of the strains *in vitro*. C1 and C5 were found to be less susceptible to 5- nitroimidazoles and NTZ had shown similar activity against all the strains tested. Further studies will be necessary to ascertain the importance of this variability in clinical infection.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We thank Prof. P. Rama Rao, Director, National Institute for Pharmaceutical Education and Research (NIPER), S. A. S. Nagar, for his help and continued encouragement. One of us (HC) is grateful to NIPER for providing financial assistance. The technical assistance provided by Mr. Vijay Kumar Mishra, is also acknowledged.

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