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Axenic Culture of *Trypanosoma congolense*: Application to the Detection of Sensitivity Levels of Bloodstream Trypomastigotes to Diminazene Aceturate, Homidium Chloride, Isometamidium Chloride and Quinapyramine Sulphate

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ABSTRACT An axenic culture system for *Trypanosoma congolense* bloodstream trypomastigotes was used for in vitro detection of their sensitivity to diminazene aceturate (DA), homidium chloride (HC), isometamidium chloride (IC) and quinapyramine sulphate (QS). Bloodstream trypomastigotes of 4 stocks (IL2079, IL2466, IL3266 and IL3338), 4 clones (IL1180, IL2642, IL3000 and IL3035) and 16 clones obtained in vitro from IL2079, IL2466, IL3000, IL3266 and IL3338, were propagated in vitro using HMI-93 medium (Hirumi and Hirumi 1991). Among these populations IL1180, IL2466, IL2642 are known to be sensitive to DA and/or IC as tested in mice and/or cattle, while IL3035 and IL3338 are highly resistant to DA, IC and/or HC when examined in cattle. Each well of a 24-well culture plate received 100μl of distilled water containing various amounts of the drugs. Test plates were then freeze-dried and stored at room temperature. Levels of the resistance for each drug were then expressed in 10 steps from 10 to 1, denoting the following concentrations: DA at 600, 500, 400, 300, 200, 100, 80, 60, 40 and 20 ng/ml; HC, IC and QS at 10-fold serial dilutions from 10μg to 10 fg/ml. Five hundred μl aliquots of trypanosome suspension in the medium, containing $4x10^5$ trypanosomes/ml, were then placed in each well and maintained at 34°C in a CO_2 incubator for 5 days without medium change. Effects of the drugs were examined by phase-contrast microscopy every 24h. Growth inhibition of trypanosomes could be detected by day 3 and affected trypanosomes died during the next 24-48h. In contrast, trypanosomes which were resistant to the given concentrations continued to grow reaching the maximum population density by day 3-5, and died during the next 24h due to overgrowth. The pH indicator, phenol red, in medium in wells which contained affected populations indicated pH 8.0-8.5, while that in wells in which trypanosomes reached the maximum population density indicated pH6.5 by day 3-5. Colorimetry of the media on day 5 was thus also used to distinguish the drug sensitivity of trypanosome populations. Resistance levels of the 4 stocks and the 4 clones against DA, HC, IC and QS were IL1180: 4,7,6 & 6, IL2079: 5,6,6 & 6, IL2466: 4,6,6 & 8, IL2642: 2,4,3 &5, IL3000: 5,7,7 & 6, IL3035: 8,7,6 & 8, IL3266: 4,6,5 & 5 and IL3338: 8,7,7 & 6, respectively. The levels of resistance of the in vitro cloned populations were similar to those of their parental populations. The information obtained in this system using 3 plates per drug per trypanosome population for 5 days provided an equivalent amount of information about the drug sensitivity of a trypanosome population as an in vivo test which uses 36 mice and lasts 2 months.

INTRODUCTION

African trypanosomiases are known as sleeping sickness in man and nagana in livestock. These diseases are caused by protozoan parasites that are mainly transmitted by tsetse-fly vectors (*Glossina* ssp.). Major species involved are *Trypanosoma brucei rhodesiense* and *T. b. gambiense* in man, and *T. b. brucei*, *T. congolense*, *T. simiae* and *T. vivax* in livestock. At present, chemotherapy and chemoprophylaxis are still the main methods for controlling the diseases (Kaminsky 1990). However, the extensive use of a limited number of commercially available trypanocides during the last several decades, particularly for the control of bovine trypanosomiasis, has resulted in the appearance of drug-resistant parasites in many parts of Africa (Leach and Roberts 1981). Improved surveillance for drug resistance encountered in the field is essential for more efficient control of the diseases (Kaminsky 1990) since, in some situations, the resistance has become so prevalent that the drugs concerned have been withdrawn from general use (Leach and Roberts 1981). The standard

method which is still widely in use for assessment of drug resistance is testing of the susceptibility of trypanosomes to trypanocidal drugs in experimental animals (Kaminsky 1990). These tests often require a number of animals and are time-consuming, in general taking 30-60 days. Thus, simple but reliable alternative methods for detecting the sensitivity of trypanosomes to the drugs in vitro are highly desirable. The establishment of in vitro methods for cultivating bloodstream trypomastigotes of *T. b. brucei* (Hirumi et al. 1977), *T. congolense* (Hirumi and Hirumi 1984) and *T. vivax* (Brun and Moloo 1982, Hirumi et al. 1983) in the presence of mammalian feeder layer-cells has led to the development of systems for determining drug sensitivity (Borowy et al. 1985a, 1985b, 1985c, 1988, Zweygarth and Kaminsky 1990). However, the requirement for feeder layer-cells in these culture systems has hampered their use in studying trypanocidal activities in vitro (Kaminsky 1990). This problem has recently been overcome by the development of feeder layer-free culture systems for bloodstream trypomastigotes of *T. brucei* (Baltz et al. 1985, Duszenko et al. 1985, Hirumi and Hirumi 1989), *T. congolense* (Hirumi and Hirumi 1991), *T. simiae* (Zweygarth et al. 1992) and *T. vivax* (Hirumi et al. 1991a, Zweygarth et al. 1991). Following the introduction of these axenic culture systems, considerable progress in the in vitro assessment of trypanocidal activities of various compounds in bloodstream trypomastigotes of *T. brucei* (Bouteille et al. 1988, Brun and Kunz 1989, Brun et al. 1989, Kaminsky and Zweygarth 1989a, 1989b, 1991, Kaminsky et al. 1989, Zinsstag et al. 1991, Zweygarth and Kaminsky 1991, Zweygarth et al. 1990) and, to a limited extent, in *T. vivax* (Zweygarth et al. 1991b) has been made. In *T. congolense*, on the other hand, in vitro trypanocidal activities have only been examined to date by using the insect-vector stage (procyclic forms) (Brun and Rab 1991, Elrayah and Kaminsky 1991, Ross and Taylor 1990) and the incorporation of [^{3}H]-hypoxanthine by bloodstream trypomastigotes in short-term culture systems in the absence (Brun and Rab 1991) or the presence (Ross and Taylor 1990) of feeder layer-cells. This paper describes an application of the recently established axenic culture system for *T. congolense* bloodstream trypomastigotes (Hirumi and Hirumi 1991) to the in vitro detection of sensitivity levels to 4 trypanocidal drugs which are commonly in use.

MATERIALS AND METHODS

Trypanocidal drugs. Trypanocidal drugs tested were diminazene aceturate (DA) (Jensch 1958) (Berenil^R, Hoechst AG, Germany), homidium chloride (Watkins and Woolfe 1952) (HC) (Novidium^R, RMB Animal Health Ltd., England), isometamidium chloride (Berg 1960) (IC) (Samorin^R, RMB Animal Health Ltd, England) and quinapyramine sulphate (Davey 1950) (QS) (Trypacide Sulphate^R, May & Baker, England).

Trypanosomes. Histories of 4 stocks, 4 clones, and 16 clones obtained in vitro from the stocks and one of the clones during the course of the present study, are listed in Table 1. Among these populations, IL1180, IL2466 and IL2642 were known to be sensitive to DA and/or IC as tested in mice and/or cattle (Peregrine et al. 1988, 1991, Pinder and Authié 1984, Sones et al. 1988, Sutherland et al. 1991), while IL3035 and IL3338 are highly resistant to DA, IC and/or HC when examined in cattle (Codjia et al. in press, Sutherland et al. 1992).

Test plates. Prior to the drug sensitivity tests, a comparative test for the effect of each drug at various concentrations on the growth of trypanosomes was made in groups of test plates (24-Well Tissue Culture Cluster, Costar, MA, USA) which were prepared in 2 different ways. In group 1, serial dilutions of each drug at 5-times test concentrations were prepared in culture medium and placed in wells (100 μl per well) which subsequently received trypanosome suspensions in culture medium (400 μl per well). In group 2, serial dilutions of the drugs were prepared in distilled water at the same concentrations as in group 1, placed in wells (100 μl per well) and freeze dried (BETA I vacuum freeze dryer, Heraeus-Christ GmbH, Osterode, Germany) under sterile conditions. Trypanosome suspensions in culture medium were then placed in wells (500 μl per well). No detectable differences were observed between the 2 groups regarding the effects of drugs on the growth of trypanosomes at the same concentrations. Thus, all test plates used in this study were prepared by the freeze-dry method which considerably simplified the preparatory procedure of the test. The freeze-dried test plates were wrapped with aluminium foil and stored at room temperature until use. Test plates could be kept at least 6 months under these conditions without any detectable

Table 1. History of *Trypanosoma congolense* stocks and clones.

Designation	Original designation	Isolation			Reference
		Host	Place	Year	
IL1180(C*)	Serengeti/71/STIB212	Lion	Tanzania	1971	Geigy & Kauffman 1973
IL2079(S**)	Serengeti/71/STIB249	Lion	Tanzania	1971	Geigy & Kauffman 1973
IL2079.1(C)	(Cloned in vitro from IL2079)***				
IL2079.2(C)	(Cloned in vitro from IL2079)***				
IL2079.3(C)	(Cloned in vitro from IL2079)***				
IL2466(S)	Farakoba/78/CRTA/19	Cow	Burkina Faso	1978	Pinder & Authié 1984
IL2466.1(C)	(Cloned in vitro from IL2466)***				
IL2466.2(C)	(Cloned in vitro from IL2466)***				
IL2466.3(C)	(Cloned in vitro from IL2466)***				
IL2466.4(C)	(Cloned in vitro from IL2466)***				
IL2642(C)	EATRO209	Cow	Uganda	1962	Morrison et al. 1978
IL3000(C)	Trans Mara I Strain	Cow	Tanzania/Kenya	1966	Wellde et al. 1974
IL3000.I(C)	(Re-cloned in vitro from IL3000)***				
IL3000.II(C)	(Re-cloned in vitro from IL3000)***				
IL3000.III(C)	(Re-cloned in vitro from IL3000)***				
IL3035(C)	M1068	Cow	Kenya	1985	Sutherland et al. 1992
IL3266(S)	Zaria/67/LUMP/69	Cow	Nigeria	1969	Luckins et al. 1986
IL3266.1(C)	(Cloned in vitro from IL3266)***				
IL3266.2(C)	(Cloned in vitro from IL3266)***				
IL3266.3(C)	(Cloned in vitro from IL3266)***				
IL3266.4(C)	(Cloned in vitro from IL3266)***				
IL3338(S)	Ghibe/89/ILRAD/1392	Cow	Ethiopia	1989	Codjia et al. in press
IL3338.1(C)	(Cloned in vitro from IL3338)***				
IL3338.2(C)	(Cloned in vitro from IL3338)***				

* Clone, ** Stock, *** Cloned in vitro in HMI-93 medium following the method by Hirumi et al. (1980), except in the absence of feeder-layer cells.

deterioration of the trypanocides' efficacy. Levels of the resistance of each trypanosome population were expressed in 10 steps from 10 to 1 at the following final concentrations: DA at 600, 500, 400, 300, 200, 100, 80, 60, 40 and 20 ng/ml; HC, IC and QS at 10-fold serial dilutions from 10 μg to 10 fg/ml.

Propagation of bloodstream trypomastigotes. Bloodstream trypomastigotes of each population were propagated at 34^{o}C in 25cm^{2} T-type (T-25) culture flasks (Costar) following the method by Hirumi and Hirumi (1991) using HMI-93 medium which was modified by supplementing Iscove's modified Dulbecco's modified Eagle's MEM (Iscove's DMEM, Flow Laboratories, Irvine, Scotland) with bloodstream form-supporting (BSF-S) factors: 0.05mM bathocuproine sulphonate (Sigma, St. Louis, MO, USA), 1.5mM L-cysteine (Sigma), 0.5mM hypoxanthine (Calbiochemi, La Jolla, CA, USA), 0.12mM 2-mercaptoethanol (BDH chemicals, Poole, England), 1mM sodium pyruvate (Sigma) and 0.16mM thymidine (Sigma); 5% (v/v) Serum Plus^{TM} (Hazleton Biologics, Lenexa, KS, USA) and 20% (v/v) young goat serum (prepared at ILRAD).

Drug sensitivity tests. Five hundred microlitres of trypanosome suspension in medium, containing 4x10^{5} trypanosomes/ml, were placed in each well and maintained at 34^{o}C in a CO_2 (5%) incubator (Heraeus B5060 EC/CO_2, Hanau, Germany) for 5 days without medium change. Effects of the drugs were examined by phase-contrast microscopy (Nikon DIAPHOT-TMD, Nippon Kogaku, Tokyo) every 24 h and colorimetric reactions of culture medium assessed by eye.

RESULTS

Seeding density. Prior to the drug-sensitivity tests, general growth patterns of bloodstream trypomastigotes of *T. congolense* IL2642 and IL3338 were examined in 24-well culture plates in the absence of drug for 5 days without medium change in order to establish the optimum seeding density for the tests. The test wells were seeded with 10 different amounts of trypanosomes ranging from 10^6 to $1.5x10^4$ trypanosomes in 500 μl HMI-93 medium. Wells which received $2x10^5$ trypanosomes of either IL2642 or IL3338 supported the growth pattern which appeared to be most suitable for the drug tests. In these wells, trypanosomes attached to the bottom of the well within 3h, continued to grow, reaching the maximum population density on day 3, and died during the next 24h due to overgrowth. In the wells which received higher amounts, many trypanosomes detached from the bottom of the well, forming a number of clumps. Thereafter, they rapidly reached the maximum density and died before day 3. This seeding density appeared to be too high to test drug sensitivity in vitro. In contrast, in wells which received less than $2x10^5$ trypanosomes, trypanosome populations reached the maximum density on day 5 or later, indicating that the seeding density

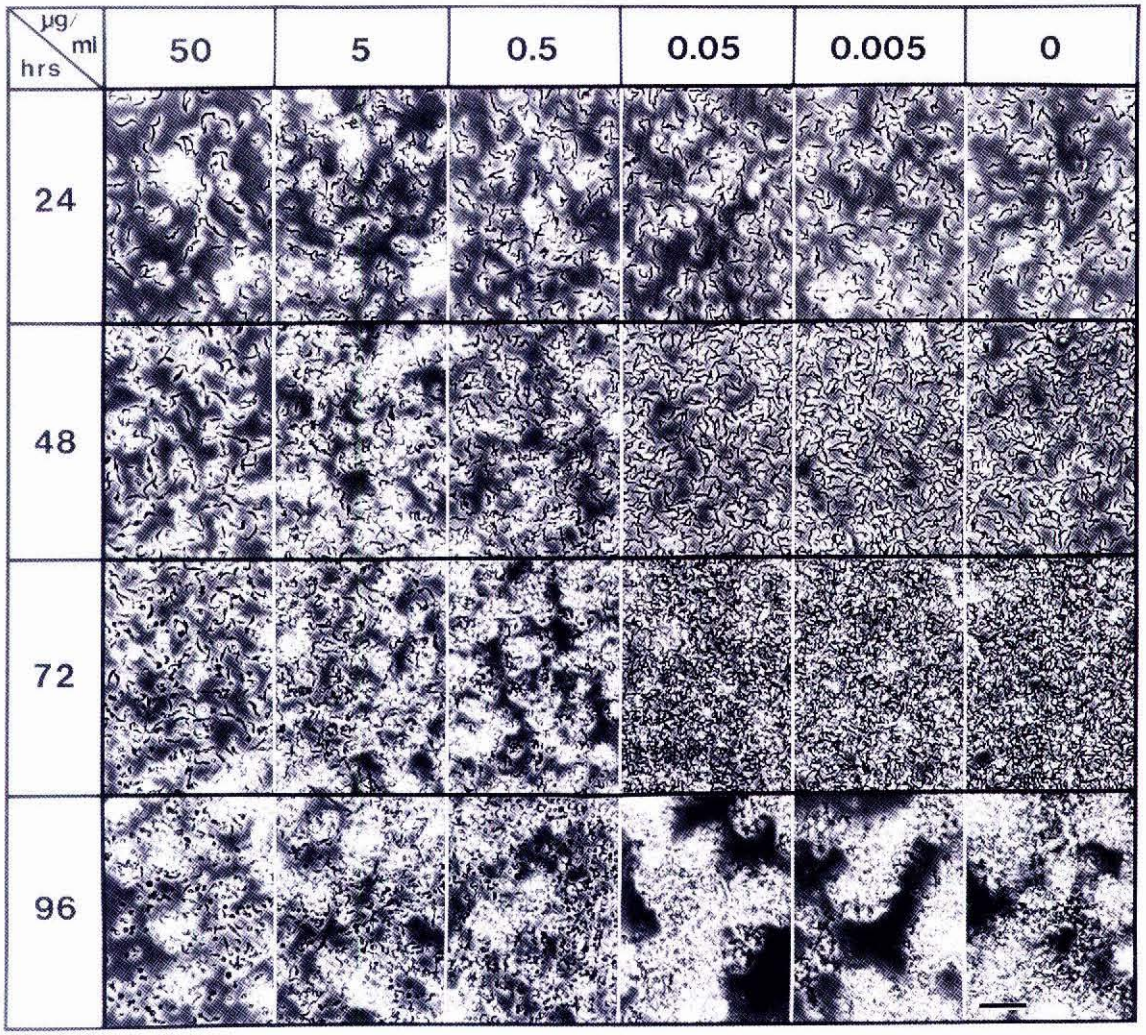

Fig.1. Basic growth patterns of bloodstream trypomastigotes of *Trypanosoma congolense* stock IL3338 in the drug sensitivity test wells (24-well culture plate), which contained diminazene aceturate in 10-fold serial dilutions from 50 to 0.005μg/ml. Each well received $2x10^5$ trypanosomes in 500μl HMI-93 medium and were incubated at 34°C in a CO_2 incubator for 96h without medium change. Phase-contrast micrographs. Bar indicates 50μm.

would be too low for the test. Thus, the seeding density of $2x10^5$ trypanosomes in 500 μl HMI-93 medium per well was used in all the remaining experiments.

Basic growth patterns. The basic growth patterns of bloodstream trypomastigotes of *T. congolense* in the drug-sensitivity test wells were examined in the presence of various amounts of the drugs in HMI-93 medium for up to 5 days without medium change. Figure 1 shows the result of one such test with stock IL3338 and DA in 10-fold dilutions from 50 to 0.005 μg/ml. Growth inhibition of the trypanosomes was clearly observed at the concentrations of 50, 5 and 0.5 μg/ml by day 3, and the affected trypanosomes died during the next 24h. In contrast, trypanosomes in the wells containing 0.05 and 0.005 μg/ml continued to grow up to day 3, increasing in numbers in a similar manner to the control wells which contained no drug. The maximum population density was attained by day 3. During the next 24h the population died due to overgrowth. The results indicated that drug-affected trypanosome populations were clearly distinguishable from unaffected populations by means of phase-contrast microscopy by day 5 under the conditions tested.

Growth response of resistant and sensitive populations. The maximum resistance levels of stock IL2642 (highly sensitive to DA in mice [Peregrine et al. 1991]) and stock IL3338 (highly resistant to DA in cattle [Codjia et al. in press]), were examined using 2-fold serial dilutions of DA, from 640 to 40 ng/ml, and with a control containing no drug. The result on day 3 demonstrated that the maximum concentration to which IL2642 trypanosomes were resistant was 40 ng/ml, whereas the maximum concentration to which IL 3338 trypanosomes were resistant was 320 ng/ml (Fig.2). The growth of both IL2642 and IL3338 populations, maintained in wells containing higher concentrations, was severely inhibited by day 3, and all the affected trypanosomes died during the next 48h. In contrast, unaffected trypanosomes of both IL2642 and IL3338 continued to grow, reaching the maximum population density by day 3 or 4, and thereafter died during the following 24h due to overgrowth. These results not only confirmed the previous observation on the basic growth patterns but also revealed that different levels of drug resistance between the sensitive and the resistant populations could be readily detected in vitro by means of phase-contrast microscopy. The results also suggested that a simple method for quantifying the drug sensitivity of *T. congolense* populations could be developed if an optimum range of test concentrations was established for each drug.

Optimum ranges of drug concentrations. The maximum resistance concentrations of DA, HC, IC and QS to all the trypanosome population of the 4 stocks and the 4 parental clones were,

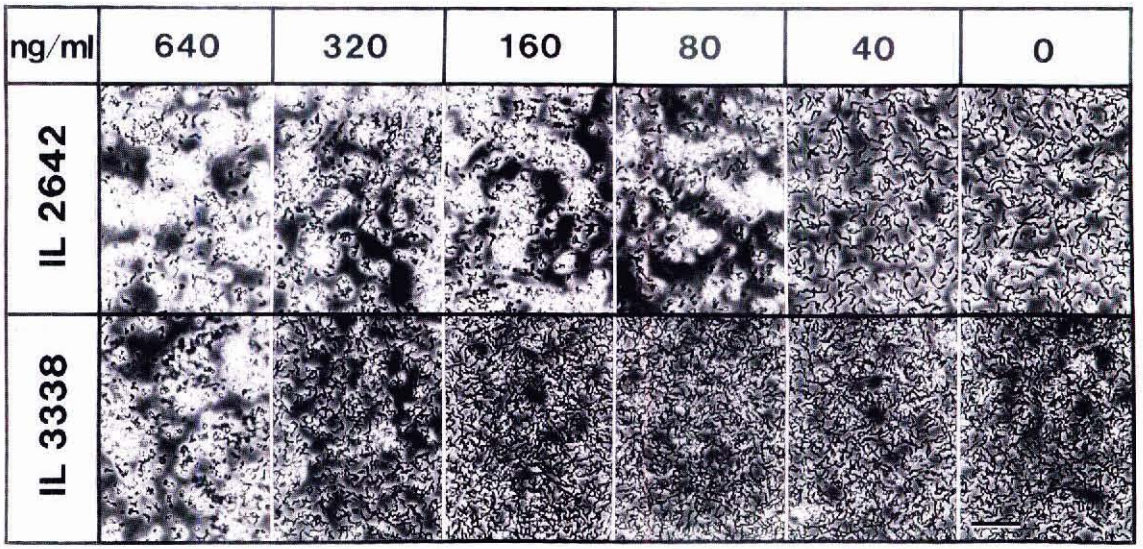

Fig. 2. Growth responses of bloodstream trypomastigotes of *Trypanosoma congolense* stock IL2642, known to be highly sensitive to diminazene aceturate (DA), and stock IL3338, known to be highly resistant to DA, to 2-fold serial dilutions of DA from 640 to 40 ng/m', and 0 (control), in HMI-93 medium on day 3 under the same test conditions mentioned in Fig.1. Phase-contrast micrographs. Bar indicates 50μm.

therefore, examined by testing each population with 10-fold, 5-fold and 2-fold serial dilutions of each drug, ranging from 1 mg/ml to 1 fg/ml, with 5-20 steps. The results are summarized in Table 2. The difference of the maximum resistance concentrations of DA between the most sensitive population (IL2642: 4x10 ng/ml) and the most resistant populations (IL3035, IL3338: $4x10^2$ ng/ml) was 10-fold, whereas those of HC, IC and QS were 10^3-, 10^4- and 10^3-fold, respectively. In consideration of the possible existence of trypanosome populations that may exhibit lower and/or higher levels of sensitivity than those detected in the present study, the following ranges of test concentrations for the 4 drugs were chosen for the standardized drug-test system: DA at 600, 500, 400, 300, 200, 100, 80, 60, 40 and 20 ng/ml; HC, IC and QS at 10-fold serial dilutions from 10^4 to 10^{-6} ng/ml; and a well with no drug. Figure 3 shows the growth patterns of trypanosomes of clone IL3000 on day 3 in a standardized isometamidium chloride sensitivity test plate. The growth patterns of affected trypanosomes at the resistance levels 10, 9 and 8, and those of unaffected populations at the levels from 6 to 0 were, in general, identical to the basic growth patterns observed earlier. However, at the level 7, many trypanosomes were affected and died during the initial 3 days, whereas some of them were unaffected and continued to grow, reaching the maximum population density 2 days later (by day 5) than in the unaffected population. This type of delayed growth was occasionally seen in test plates for all 4 drugs, depending on the resistance level of certain trypanosome populations. The recovery of the trypanosome populations appeared to be similar to relapse infections in host animals. Trypanosome populations that recovered in this

Table 2. The maximum resistance concentrations of diminazene aceturate (DA), homidium chloride (HC), isometamidium chloride (IC) and quinapyramine sulphate (QS) for the most sensitive and the most resistant stocks and/or clones among the 8 *Trypanosoma congolense* populations tested.

Drug	Maximum resistance concentration (ng/ml)		Difference
	Most sensitive population	Most resistant population	
DA	4 x 10 (IL2642)	4×10^2 (IL3035, IL3338)	10 -fold
HC	10^{-2} (IL2642)	10 (IL1180, IL3000, IL3338)	10^3-fold
IC	10^{-3} (IL2642)	10 (IL3000, IL3338)	10^4-fold
QS	10^{-1} (IL2642, IL3266)	10^2 (IL2466, IL3035)	10^3-fold

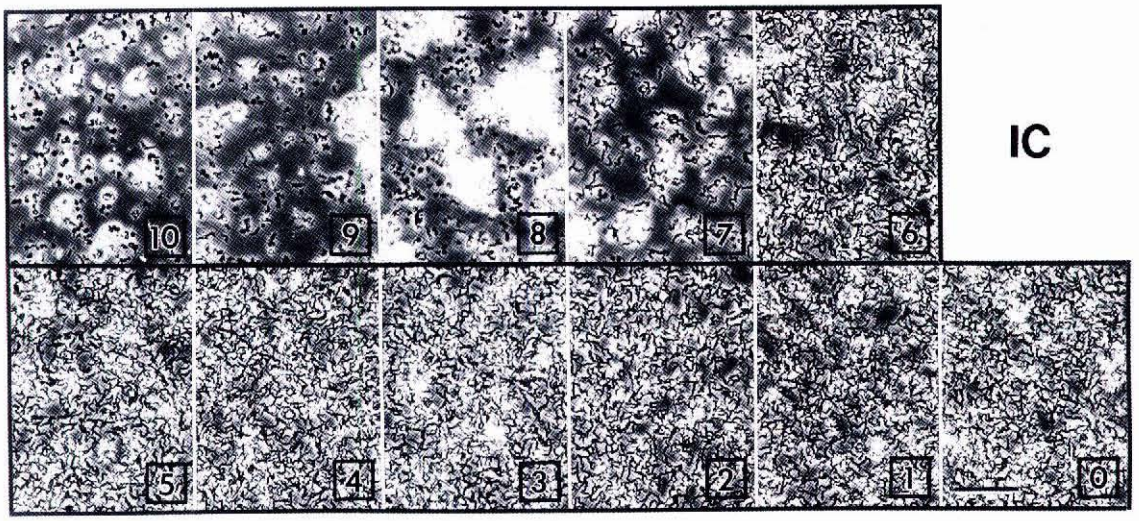

Fig. 3. Growth of bloodstream trypomastigotes of *Trypanosoma congolense* clone IL3000 in an "isometamidium chloride (IC) test plate" on day 3. The test wells contained 10-fold serial dilutions of IC from 10μg to 10 fg/ml which were expressed in 10 drug resistance levels from 10 to 1, respectively, and 0 (control without drug). Other test conditions were the same as those mentioned in Fig.1. Phase-contrast micrographs. Bar indicates 50μm.

manner were, therefore, considered to be resistant to the concentration tested.

The consistency of test results was also compared in 24-well and 96-well (96-Well, Tissue Culture Seroclusters with Flat Bottom Wells, Costar) plates. The results showed that the maximum resistance levels detected in the wells of 96-well plates often varied in ± 1 level from test to test, whereas the results in the wells of 24-well plates were consistent. Thus, the 24-well plates were used in the standardized test system.

Colorimetric reaction of culture media. During the course of this study it became evident that the pH indicator, phenol red, in the culture medium in the wells in which the growth of trypanosomes was inhibited by given concentrations of drugs indicated pH 8.0-8.5 (pinkish color), while those in the wells in which trypanosomes were unaffected and died due to overgrowth indicated pH 6.5 or lower (yellowish color) by day 3-5 (Fig.4). The indicator in the wells in which the trypanosomes were partially affected by day 3 (Fig.3, resistance level 7), but recovered later, indicated pH 7.8-8.0 (amber color) (not shown). These colorimetric reactions were readily detected with the naked eye 1h (or longer) after removal from a CO_2 incubator, and were stable under normal room conditions for several days. The colorimetry of the culture media on day 5 was thus also used to distinguish the drug sensitivity of trypanosome populations in addition to the phase-contrast microscopy in the standardized test system.

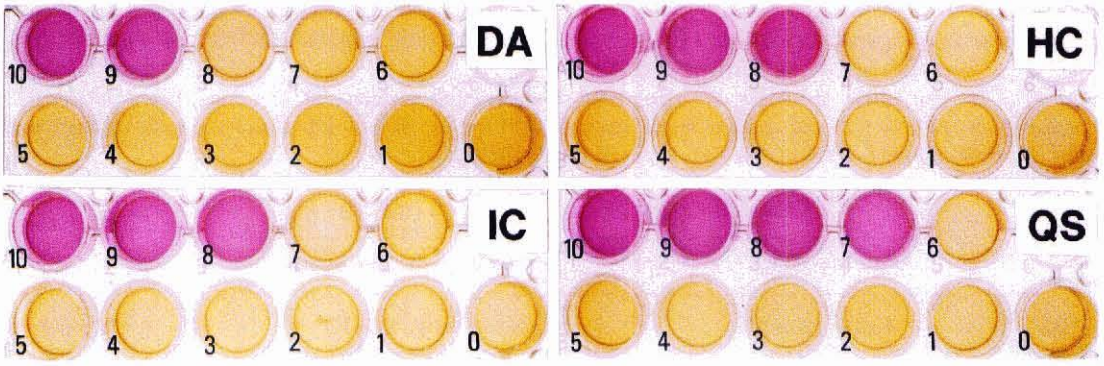

Fig.4. Colorimetric responses of culture media to the growth of bloodstream trypomastigotes of *Trypanosoma congolense* clone IL3338.1 in the presence of various concentrations of diminazene aceturate (DA), homidium chloride (HC), isometamidium chloride (IC) and quinapyramine sulphate (QS) on day 5 under the same test conditions mentioned in Fig.1. "Drug resistance levels" were expressed in 10 steps, from 10 to 1, at the following final concentrations: DA at 600, 500, 400, 300, 200, 100, 80, 60, 40 and 20 ng/ml; HC, IC and QS at 10-fold serial dilutions from 10μ to 10 fg/ml, respectively, and 0 (control without drug). In culture media which indicated pH8.0 or higher (pink), the growth of trypanosomes was inhibited, while in those which indicated pH6.5 or lower (yellow), unaffected trypanosomes continued to grow for 3-4 days, and died by day 5 due to overgrowth.

Characterization of drug sensitivities. All the 4 trypanosome populations, including 16 clones obtained in vitro, were characterized for their sensitivity to all the 4 drugs. The results are summarized in Fig.5. The results revealed that the maximum resistance levels of the population IL2642, known to be very sensitive to DA and IC in mice and/or cattle (Peregrine et al. 1988, 1991), to DA, HC, IC and QS were generally low (2, 4, 3 & 5, respectively), whereas those of IL3035 and IL3338, known to be very resistant to DA, HC and/or IC in cattle (Codjia et al. in press, Sutherland et al. 1992), were high (IL3035: 8, 7, 6 & 8, IL3338: 8, 7, 7 & 6, respectively). The levels of the population IL1180, known to be sensitive to DA and IC in mice (Peregrine et al. 1991) and to IC in cattle (Sones et al. 1988), were intermediate overall (4, 7, 6 & 6, respectively) between the highly sensitive and the highly resistant populations. However, no specific correlations between the resistance levels to the 4 drugs in each trypanosome population were observed; the levels of resistance to DA and IC were 8 and 8 in IL3035, but 8 and 6 in IL3338, respectively. In another "sensitive" population (IL2466), known to be sensitive to IC in cattle (Pinder and Authié 1984), the maximum resistance level to IC was low (4), while that to QS was high (8). These results suggest that independent mechanisms may underlie the different sensitivities observed with different *T. congolense* bloodstream trypomastigotes to the different drugs.

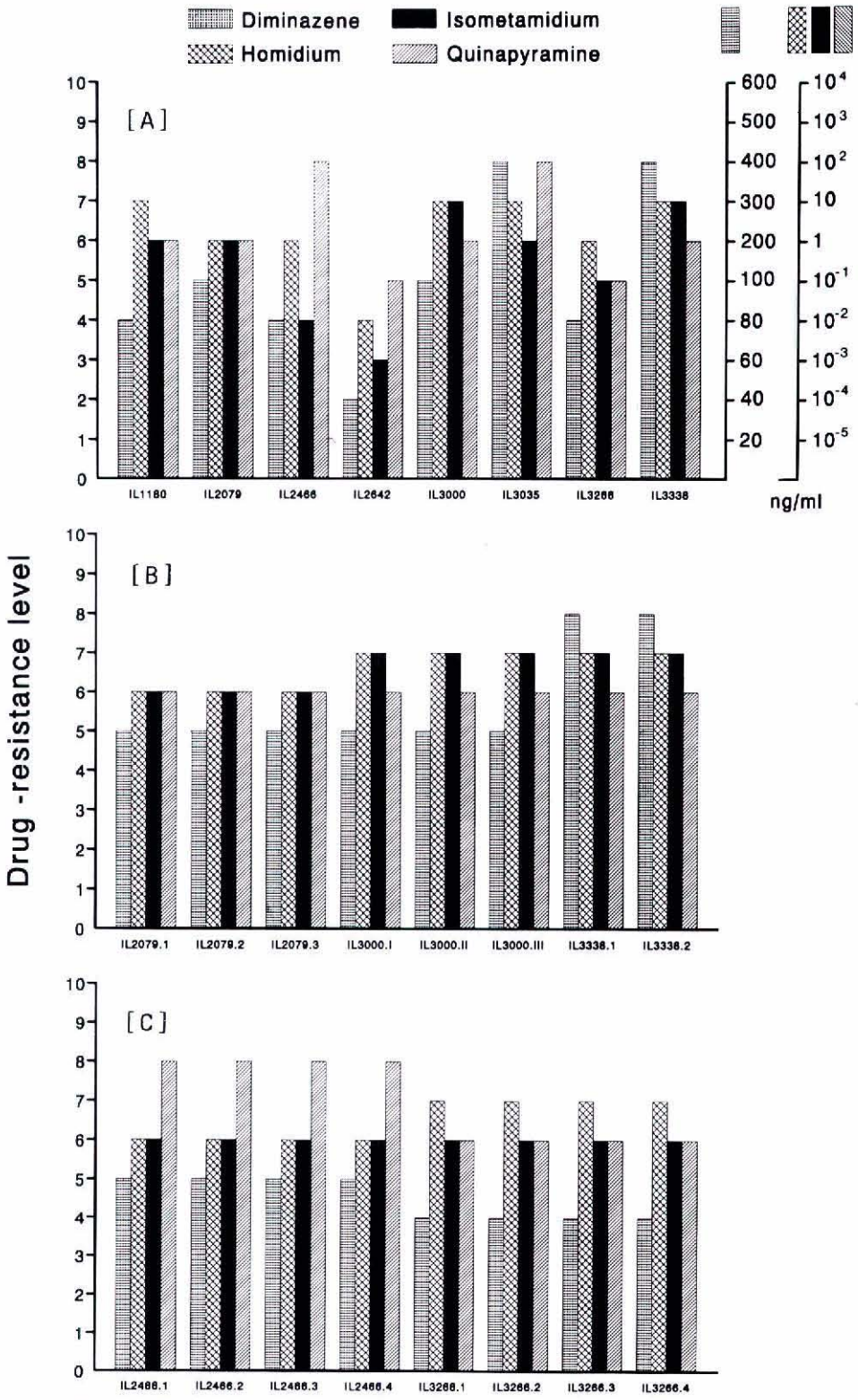

Fig.5. Drug resistance levels of the original 4 stocks and 4 clones [A] and 16 clones [B&C] obtained in vitro from the stocks and clones in [A] against 4 drugs shown on the top in [A]. Ten levels indicated in the left hand column were tested at the concentrations indicated on the right hand columns. Example: the maximum resistance levels of IL1180 against the 4 drugs were 4(80ng/ml), 7(10 ng/ml), 6(1ng/ml) and 6(1ng/ml), respectively.

The 16 clones which were cloned, or re-cloned, in vitro from the 5 parental populations (IL2079, IL2466, IL3000, IL3266 and IL3338) expressed the same levels of resistance to the 4 drugs as the parental populations (Fig. 5 B&C). Furthermore clones IL2079.1, IL3000.I and IL3338.1 expressed the same levels of resistance detected in their parental populations, after being maintained in vitro over 6 months. The re-cloned IL3000.I population, after being passaged 4 times in mice, also showed the same levels of resistance seen in the original IL3000.I population. Bloodstream trypomastigote populations of IL3000.II and IL3000.III also expressed the same levels of resistance as their parental population (IL3000) after having undergone complete cyclical transformation in culture following the method by Hirumi and Hirumi (1991). The sensitivities of IL3338 against DA and IC were also re-examined in mice after maintaining the population in culture for 1 month. The 50% curative doses (CD_{50}) and their 95% confidence interval (95% C.I.) (Peregrine et al. 1991) of IC and DA were 8.4mg kg^{-1} body weight (95% C.I.: 5.9-12.0 mg kg^{-1} body weight) and 60.0 mg kg^{-1} body weight (no 95% C.I. was obtained since the CD_{50} value was the maximum tolerated dose of the drug), respectively. The curative doses obtained were similar to those observed with the original IL3338 population in mice before in vitro cultivation. These results suggest that the sensitivity of the *T. congolense* populations to the 4 drugs are stable, under the experimental conditions examined here.

DISCUSSION

In the present study, direct correlations between the maximum resistance concentrations of DA, HC, IC and QS and the maximum resistance doses in host animals have not been established, although the overall levels of sensitivity observed in vitro were, in general, in agreement with the ranges reported in vivo (Codjia et al. in press, Peregrine et al. 1988, 1991, Pinder and Authié 1984, Sones et al. 1988, Sutherland et al. 1991). It therefore appears that the in vitro test system reported here is a potentially useful tool for the screening of trypanosome populations isolated from naturally infected animals in the field. The results obtained in this study also indicate that the system would be a useful laboratory tool for (1) the screening of new drugs, (2) the selection of experimentally induced drug-resistant populations for molecular biological studies, and (3) the study of growth promoting, as well as inhibiting, factors.

All the populations of bloodstream trypomastigotes of *T. congolense* used in this study had undergone complete cyclic transformation in vitro (Hirumi and Hirumi 1991) prior to testing and were maintained in vitro for various periods ranging from 1 to 10 months. No attempt to test *T. congolense* isolates which had not undergone in vitro transformation (such as stocks isolated directly from naturally infected animals) has yet been made. Further investigation regarding the applicability of the in vitro detection system to such *T. congolense* populations is needed.

In a preliminary study, the in vitro detection described here has been applied to several *T. b. brucei* and *T. vivax* populations, known to be either sensitive or resistant to trypanocidal drugs, using the axenic culture systems for bloodstream trypomastigotes of *T. brucei* (Hirumi and Hirumi 1989) and *T. vivax* (Hirumi et al. 1991). The results obtained so far indicate that the system would also be applicable to these populations. The yellowish color of colorimetric reaction in *T. vivax* populations was similar to that observed in *T. congolense* populations, although the reaction in *T. b. brucei* was somewhat less distinct (amber).

Most problems that have hampered the application of in vitro systems, available earlier, to the assessment of drug sensitivity of trypanosome populations (Kaminsky 1990) have been overcome by using the simplified axenic culture system for *T. congolense* bloodstream trypomastigotes. In this culture system, the trypomastigote populations can be maintained without medium change until they reach the maximum population density ($>5x10^6$ trypanosomes/ml). A few trypanosomes will also continue to grow without medium change even in the presence of large numbers of dead trypanosomes for at least 10 days. Thus, cultures initiated with a single trypomastigote, either in T-25 flasks or in wells in 24-well plates, can also be maintained without medium change for up to 10 days. This ability of the system to support the growth of the trypomastigotes without medium change for 10 days is particularly advantageous in detecting the maximum resistance concentration of trypanocidal drugs, since it selects for a small number of unaffected (resistant) trypanosomes

from the affected majority which are sensitive to a given concentration of test drug. The maintenance of the axenic culture system is also simple, although certain skills are still required to initiate the culture from the original isolates of *T. congolense* and/or laboratory stocks which have not been adapted to in vitro culture (Hirumi and Hirumi 1991).

The colorimetric reaction of the culture media, similar to that described here, has been reported earlier for testing trypanocidal activity in *T. b. brucei* bloodstream trypomastigotes using 96-well plates and an ELISA reader (Zinsstag et al. 1991). In the in vitro assay described in the present study, the effects of trypanocidal drugs should be examined by means of phase-contrast microscopy every 24h during the initial 3 days and, in general, the final detection of the end points could be made by colorimetry of the culture media by naked eye on day 5. Only the wells in which the medium shows the intermediate color (amber) should be re-examined by phase-contrast microscopy. If a small number of trypanosomes were still alive and in "good shape", attaching to the bottom of wells, such wells should be further examined by phase-contrast microscopy during the following 2-5 days in order to ensure the continuous recovery or subsequent death of the populations. The final detection in these wells may be made by day 10 (at the latest).

Another advantage of the in vitro assay described here is the requirement for only relatively simple laboratory equipment, such as a CO_2 incubator, a phase-contrast microscope and a vacuum freeze dryer. There is, thus, no requirement for expensive equipment, such as a spectrophotomer, enzyme-linked immunosorbent assay (ELISA) reader, liquid scintillation counter and a Coulter cell counter, used in earlier work (Brun and Kunz 1989, Brun and Rab 1991, Kaminsky and Zweygarth 1989b, Kaminsky et al. 1989, Ross and Taylor 1990, Zinsstag et al. 1991).

It has been pointed out that in vitro assay systems to characterize the drug sensitivity of African trypanosomes would be excellent alternatives to the in vivo tests which require large numbers of experimental animals and are time-consuming (Kaminsky 1990). The information obtained in the present in vitro system using 3 test plates (triplicate) per drug per trypanosome population and lasting 5 days, provided an equivalent amount of information about the drug sensitivity of a trypanosome population as in vivo tests which use at least 36 mice and last 2 months.

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