STUDIES ON *IN VITRO* ASSESSMENT OF "DRUG RESISTANCE" IN *TRYPANOSOMA VIVAX*

By

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DEDICATION

This work is dedicated to my late beloved father, Salum Masoud Maulaga Kitosi, who passed away nineteen years ago.

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ABSTRACT:

Six stocks of *Trypanosoma vivax* were investigated for their drug sensitivity to isometamidium chloride and diminazene aceturate. It was evaluated whether *in vitro* tests had the potential to distinguish between drug-sensitive and drug-resistant stocks of *T. vivax*. Four *in vitro* tests were employed in drug sensitivity assessment: (1) Long Term Viability Assay (20 days) with insect forms at 27°C, (2) ^{3}H-Hypoxanthine Incorporation Test (48 hrs) with bloodstream forms at 34°C, (3) Drug Incubation Infectivity Test (DIIT) with bloodstream forms at 37°C and (4) Drug Incubation Survival Test with bloodstream forms at 34°C. All tests were able to show an antitrypanosomal effect of both, isometamidium chloride and diminazene aceturate on *T. vivax in vitro*. The antitrypanosomal effects were detected by rounding and death of epimastigotes, inhibition of ^{3}H-Hypoxanthine incorporation by bloodstream forms, loss of infectivity to mice and reduction in the number of survivors.

The Long Term Viability Assay and the Drug Incubation Survival Test showed potential to distinguish between isometamidium chloride-sensitive and isometamidium chloride-resistant stocks or clones of *T. vivax*. Results on drug sensitivity of *T. vivax* stocks obtained with these tests correlated with results in cattle or mice. A correlation between *in vitro* and *in vivo* results was not observed regarding diminazene aceturate.

When cattle were infected with *in vitro* produced metacyclics, resistance of bloodstream forms of *T. vivax* stock CP 2171 was retained to treatment with 0.5 and 1.0 mg/kg isometamidium chloride after continuous *in vitro* propagation of epimastigotes for 30 months. The reduced sensitivity of epimastigotes of CP 2171 to isometamidium *in vitro* was similar in cultures initiated from these cattle compared with results obtained in cultures initiated from cattle infected with the original stabilate.

1. INTRODUCTION

The African continent has more than 170 million cattle and the human population is about 514,317 million. However, while the annual growth rate for cattle is 0.41%, that of human increases at the rate of 3.05% per annum (FAO, 1985). Hence, cattle production is of vital importance for the rapidly expanding human population on the African continent to provide the needed animal protein in forms of meat and milk.

Among the major constraints that hinder improvements in the cattle production are livestock diseases. One of the major diseases affecting cattle is trypanosomiasis (ILRAD, 1990).

The causative agents of bovine trypanosomiasis are *Trypanosoma brucei brucei*, *T. vivax* and *T. congolense*. It is estimated that 10 million square kilometres of land in Africa are infested by tsetse flies (*Glossina* species), the invertebrate vector of these parasites. This area covers 38 African countries with a total of 147 million cattle, 30 percent of which are exposed to trypanosomiasis (ILRAD, 1987). As consequences of tsetse infestation, most of land cannot be used for livestock production, thus, concentrating many animals on few suitable areas leading to overstocking problems and soil erosion in other areas. It has been estimated that as a result of the extensive distribution of this disease on the continent, Africa is producing 70-times less animal protein per hectare than Europe (Allsopp *et al*., 1985). The loss in meat production due to trypanosomiasis is US$5 billion per year excluding milk, traction power, manure, biogas and hides (ILRAD, 1987).

Chemotherapy is still considered to be the most promising approach for improvements in the control of trypanosomiasis (Doyle *et al*., 1984).

Nearly 30 million doses of trypanocidal drugs are used annually to treat 120 million cattle (ILRAD, 1987). As promising as it sounds, chemotherapy is associated with major difficulties because success depends on only three trypanocidal drugs. Among these drugs, only isometamidium chloride and quinapyramine show chemoprophylactic activity against trypanosome infection. The extensive use of the limited number of drugs has unavoidably led to the development of drug resistance in pathogenic trypanosomes (Leach and Roberts 1981; Mbwambo et al., 1988; Pinder and Authie 1984; Rottcher and Schillinger, 1985).

Drug resistance has been reported for most of the trypanocidal drugs in current use. Röttcher and Schillinger (1985) have reported on the multiple drug resistance in T. vivax in the Tana River district of Kenya. The stocks reported were resistant to diminazene aceturate, quinapyramine, homidium bromide and isometamidium chloride. Seven isolates of T. vivax isolated from Kenya and Somalia were found to be resistant to isometamidium chloride (Schönefeld et al., 1987). Hence, in order to effect proper prophylactic and therapeutic regimes, improvement of chemotherapy requires surveillance of drug sensitivity.

Various tests are now available for determination of drug sensitivity of trypanosomes to standard trypanocides. The standard mouse test is suitable for T. b. brucei and some stocks of T. congolense but not for T. vivax or T. simiae. The standard mouse test is done by infecting mice with a particular stock of trypanosomes, treating them using specified doses of trypanocidal drugs and monitoring the infection for a period of 30-60 days. The problem with T. vivax and T. simiae and to a lesser extent T. congolense, is that many strains of these species are not infective to rodents (Anosa, 1983; Kalu et al., 1986).

In vitro assays for assessment of drug sensitivity have been used successfully for *T. brucei* spp. (Borowy *et al*., 1985; Hirumi *et al*., 1977a, b; Kaminsky & Zweygarth, 1989b; Kaminsky *et al*., 1989b). However, employing *in vitro* assays on *T. vivax* is difficult, because parasites are very difficult to adapt to *in vitro* conditions.

In this work, *in vitro* tests were investigated to determine their ability to detect drug resistance in various stocks of *T. vivax* from East and West Africa. The findings were compared with *in vivo* findings to determine if there was any correlation between *in vitro* and *in vivo* assessment of drug resistance in trypanosomes.

The principal objectives of this project were:

(1) To study the effects of isometamidium chloride and diminazene aceturate on *T. vivax* bloodstream and insect forms in *in vitro* systems,

(2) to evaluate whether the *in vitro* tests can be used to distinguish between drug-sensitive and resistant stocks of *T. vivax*,

(3) to establish whether there is any correlation between *in vivo* and *in vitro* methods of assessing drug resistance in *T. vivax*.

In particular, the following *in vitro* assays were investigated:

(a) Long term Viability Assay using insect forms (Kaminsky *et al*., 1989a).

(b) ^{3}H-hypoxanthine Incorporation Test using bloodstream forms (Brun & Kunz, 1989).

(c) Drug Incubation Infectivity Test - DIIT (Kaminsky *et al*., 1990).

(d) Drug Incubation Survival Test.

The *in vivo* sensitivity of some of the *T. vivax* stocks tested in *in vitro* was investigated by conducting drug sensitivity tests in cattle or mice.

2. LITERATURE REVIEW

2.1. TRYPANOSOMIASIS

2.1.1. Morphological characteristics and pathogenicity of trypanosomes

Trypanosomes are flagellated protozoa which belong to the family trypanosomatidae. The family consists of several genera and many species, all being parasitic in habit. Most of the species which parasitize vertebrates require a vector for transmission. Trypanosomes live in the blood of their mammalian hosts but may also invade other body fluids such as cerebrospinal fluid. For morphological characteristics (See Table 2.1.).

Mammalian trypanosomes fall into two sections depending on the location of the metacyclic trypanosomes in the insect vector. In the Stercoraria group (sterco = faeces), the metacyclics occupy a posterior position in the gut and are passed out in the insect's faeces, e.g., *T. cruzi*; infection is therefore contaminative. In the Salivaria group, the metacyclics are in the insect's proboscis and infection is therefore inoculative, e.g., pathogenic trypanosomes, *T. b. brucei*, *T. congolense* and *T. vivax*.

2.1.2. Salivarian trypanosomes

These trypanosomes are found in tropical Africa within the latitude 14°N and 30°S where they are associated with their vector, *Glossina*, the tsetse fly. Some species, however, have become independent of the tsetse fly and have spread to other continents where they are transmitted mainly by biting flies, eg., *T. evansi* and *T. vivax*.

Salivarian trypanosomes are not host specific and occur as parasites in many species of wild ungulates, but they are frequently pathogenic to domestic animals (Table 2.3.).

Salivarian trypanosomes can be separated into three groups or subgenera (Table 2.1.) according to their morphology in the blood and their pattern of development in the tsetse fly. These are:

(1) Trypanosomes of the Brucei-group or subgenus Trypanozoon.

(2) The Congolense group or subgenus Nannomonas.

(3) The Vivax-group or subgenus Duttonella.

Table 2.1.

Morphological characteristics of trypanosomes (FAO, 1986)

Subgenus	Group	Species	Site of development	Free flagellum	Kinetoplast	Undulating membrane	Size (uM) and motility in wet film and dark ground microscopy examination
Duttonella	vivax	T. vivax T. uniforme	Proboscis only	Present	large terminal	Not prominent	20-26, large, extremely active, traverse the whole field very quickly, pausing occassionally.
Nannomonas	congolense	T. congolense T. simiae	midgut and proboscis	Absent	medium subterminal or marginal	not prominent	9-18, small, sluggish active, adheres to red blood cells by anterior end.
Trypanozoon	brucei	T. brucei * T. rhodesiense * T. gambiense ** T. evansi ***T. equiperdum	midgut and salivary gland None None	Present in all but stumpy forms	small, subterminal centre	Prominent	12-35, large, rapid movement in confined areas.

Key
- * Causal agents of human sleeping sickness
- ** No cyclical transmission, carried by biting flies
- *** Transmitted during sexual contact
- + Polymorphic; slender, intermediate and stumpy forms

Table 2.2. Susceptibility of domestic animals to the various pathogenic trypanosomes (FAO, 1986).

Domestic Animals	Trypanosome species					
	T.congolense	*T.simiae*	*T.vivax*	*T.brucei*	*T.evansi*	*T.equiperdum*
Cattle	+	+	+	+	+	–
Sheep	+	+	+	+	+	–
Goats	+	+	+	+	+	–
Pigs	+	+	–	–	–	–
Horses	+	+	+	+	+	+
Dogs	+	–	–	+	+	–
Camels	+	+	–	+	+	+

Key: + susceptible

– not affected

Table 2.3. The occurence of pathogenic African trypanosomes of veterinary importance (FAO, 1986).

Trypanosome species	Domestic animals affected	Reservoir hosts	Experimental hosts
T. congolense	Cattle Horses Dogs Sheep Goats Pigs	Wild game Species	Rats Mice Rabbits Guinea-pigs
T. simiae	Pigs	Warthog Bushpigs	Rabbits Monkeys
T. vivax	Cattle Sheep Goats Horses	Wild game Species	None (Mice for West African stocks)
T. brucei brucei	Horses Dogs Sheep Goats Cattle Pigs	Wild game species	Rats Mice Guinea pigs Rabbits
T. b. evansi	Camels Cattle Water Buffalo Horses Dogs		Rats Mice Guinea-pigs Pigs Rabbits
T. b. equiperdum	Horses	None	None

2.1.3. Cyclical development of *T. vivax* in tsetse fly; biochemical and morphological changes occuring in trypanosomes during transformation from bloodstream to insect forms.

The classical theory of cyclical development of *T. vivax* in *Glossina* is that development in tsetse is entirely confined to the proboscis i.e. labrum and hypopharynx (Bruce *et al*., 1910; Lloyd and Johnson, 1924). Thus, it has been assumed that when tsetse feed on a host infected with *T. vivax*, some blood trypomastigotes attach to the wall of the hypopharynx. They subsequently transform to epimastigotes which multiply at the foci of attachment (Vickerman, 1973) to form rossettes of epimastigotes. Invasion of the hypopharynx by some of these forms leads to further transformation to the infective coated metatrypanosomes (Lloyd and Johnson, 1924; Gardiner *et al*., 1986). However, *T. vivax* parasites have been observed in the pharynx (cibarium) of *G. palpalis* which had mature forms of the parasites in the proboscis (Bruce *et al*., 1911), and a similar observation has recently been made in *G. tachnoides* although no *T. vivax* were found in the cibaria of infected *G. m. morsitans* (Jefferies *et al*., 1987). The role of cibarial *T. vivax* in the cyclical development of the parasite in tsetse has never been investigated. In a series of experiments conducted by Moloo and Gray (1989) it was revealed that cyclical development initially occurs in the cibarial/oesophagial region from where parasites migrate to the food canal of the proboscis and development is completed to infective metatrypanosomes in the hypopharynx.

Trypanosoma brucei brucei bloodstream trypomastigotes differ vastly from the insect forms in morphology and ultrastructure (Steiger, 1973), and biochemistry (Bowman, 1974). The inactive mitochondrion of

bloodstream trypanosomes, in the form of a single cristae canal, lacks a functional citric acid cycle and shows no spectral evidence for cytochromes (Fulton and Spooner, 1959; Ryley, 1956). Energy is produced via glycolysis with nicotinamide-adenine dinucleatide phosphate (NADH) reoxidized by L-α-glycerophosphate oxidase (Grant and Sargent, 1960). Vector midgut forms (established procyclic trypomastigotes) possess a fully functional mitochondrion complete with Krebs cycle intermediates and cytochrome electron transport system (Hill, 1976) as well as L-α-glycerophosphate oxidase. The transformation from bloodstream to procyclic trypomastigotes may be accomplished in vitro as revealed by experiments conducted with T. b. brucei (Brun et al., 1981; Cunningham, 1977) and T. congolense (Steiger et al., 1977).

In a natural transformation situation, the trypanosomes transfer from a glucose-rich environment (bloodstream) to a proline rich environment (tsetse fly). Nutritional studies conducted by Bienen et al. (1981) indicated that proline was one of the requirements for T. b. brucei growth after transformation has occured. Proline stimulates cell growth after transformation.

2.1.4. Transmission and Economic effects of the disease caused by T. vivax.

Geographically, the most extensive pathogenic trypanosome (with the exception of T. evansi) is T. vivax. In Africa, T. vivax is predominantly transmitted following cyclical development by tsetse, Glossina species, although mechanical transmission of T. vivax by biting flies other than tsetse does also occur in Africa (Mulligan, 1970; Roeder et al., 1984). T. vivax can be transmitted by at least nine species of

tsetse (Hoare, 1972) in which the development of metacyclic trypanosomes can take 5-13 days depending on temperature (Lloyd and Johnson, 1924) and is entirely confined to the proboscis and cibarial/oesophagial region of the tsetse (Bruce et al., 1910; Lloyd & Johnson; 1924; Moloo & Gray 1989). Since T. vivax generally produces high infection rates in tsetse compared to T. congolense or T. brucei (Lloyd and Johnson, 1924; Buxton, 1955), it would appear that this organism is better adapted to development in tsetse than other salivarian trypanosomes. T. vivax is a major pathogenic trypanosome of cattle (Table 2.3.) and is the dominant species in West Africa (Godfrey et al., 1965). The infections are accompanied by mortality, weight loss, reduced milk yields, stilbirths and abortions (Anosa, 1983). Sheep, camels, goats and horses can also suffer following T. vivax infection. It is commonly held that stocks of T. vivax from East and West Africa differ in their pathogenicity (Fairbairn, 1953) and with the exception of a hemorrhagic syndrome in East Africa which accompanies some T. vivax infections (Fairbairn; 1953; Wellde et al., 1983), East African stocks tend to produce mild infections in livestock in good condition. By contrast, in West Africa the disease is more often acute with untreated cases ending fataly. Whether the severe hemorrhagic syndrome is caused by particular stocks of trypanosomes or by accompanying infectious agents eg. viruses or rickettsiae, has not been critically determined. However, severe non-hemorrhagic T. vivax stocks have been noted to depress platelet numbers in infected hosts (Davis, 1982). Some degree of interference with the efficiency of blood clotting may therefore be a normal corollary of T. vivax infection, causing disseminated intravascular coagulation-like complications in severe cases (Wellde et al., 1983).

There is no doubt that trypanosomiasis has a severe detrimental effect on the economic performance of the African cattle industry (Finelle, 1974), as determined using models (Habtermariam, 1983) or field data (Wilson *et al*., 1986).

2.1.5. Diagnosis

The disease can be diagnosed by the following methods:

- Clinical methods
- Parasitological methods
- Serological techniques
- Molecular biology techniques (DNA probing technique)

2.1.5.1. Clinical methods

Clinical methods involve the examination of the history given by the owner of the animals, examination of the environment in which the animals live and examination of the clinical signs of the animal. The problem with clinical signs is that there are no specific signs for trypanosomiasis (pathognomomic signs) and hence differential diagnosis is required to rule out other diseases, and again the diseases may run a subclinical course.

2.1.5.2. Parasitological methods

The incidence, prevalence, species and sequence of appearance of different species of trypanosomes can be evaluated by examination of the blood by a variety of parasitological techniques which include:

(1) Dark ground/phase contrast buffy coat method - DG method.

(2) Hematocrit centrifugation technique (Woo, 1971) - HCT or WOO method.

(3) Blood films (thin, thick, wet)

(4) Inoculation of laboratory animals, particularly mice.

(5) Lanham column (DE 52 cellulose) - (Lanham & Godfrey, 1970).

Parasitological diagnostic methods in animal trypanosomiasis have been reviewed by Molyneux (1975) and comparative studies have been made by several authors (Murray et al., 1977; Leeflang et al., 1978, Paris et al., 1982; Kalu et al., 1986). Paris et al. (1982) found the buffy coat dark ground technique more effective than other current methods in bovine trypanosomiasis to diagnose T. congolense and T. vivax, while mouse inoculation followed by hematocrit centrifuge technique (HCT) were best for T. brucei. Also in cattle, the buffy coat dark ground method shows several advantages over standard techniques (Murray et al., 1977). Leeflang et al. (1978) suggested a combination of the HCT or thick film with thin smear for epidemiological studies on T. vivax under field conditions. In goats with subclinical infection, Kalu et al. (1986) compared eight methods and HCT gave the best diagnosis for T. vivax and T. brucei, while the buffy coat technique was superior to all other methods for T. congolense.

2.1.5.3. Serological methods

At present the serological assays in use such as complement fixation test, formol gel test, mercuric chloride test, indirect haemaglutination test, indirect immunofluorescent antibody test (IFAT) and enzyme linked immunosorbent assay (ELISA), are for the detection of anti-trypanosome antibodies. The problem with these assays is that they do not indicate active infections but show that the animal had been or was exposed to parasites.

Recently, a new method has been developed by Nantulya and co-workers. This is the use of enzyme immunoassays which are species specific and are able to detect circulating antigens of *T. congolense*, *T. vivax*, and *T. brucei* spp. (Nantulya, 1988; Nantulya and Lindqvist, 1989; Nantulya *et al*., 1989). These assays are based on monoclonal antibodies that identify trypanosome species specific non-variable somatic antigens (Nantulya *et al*., 1987).

2.1.5.4. DNA Probing

This technique uses a set of highly repetitive DNA sequence probes in the detection, and identification, of trypanosome species infecting a natural population of tsetse flies or animals in an area. The probes are specific, sensitive and identify the respective trypanosome species present in an individual infected tsetse fly or animal host (Majiwa and Otieno, 1990).

2.2. CONTROL OF TRYPANOSOMIASIS

There are 3 main approaches to the control of animal trypanosomiases.

1. Combating the vector by control or eradication of tsetse fly populations.
2. Combating at host levels by using chemotherapy and/or chemoprophylaxis.
3. Combating by using and breeding for trypanotolerant (genetic resistant) cattle.

2.2.1. Control of tsetse

The oldest and traditional method of control has been selective bush clearing. A good number of trees are cleared and the area is used for other purposes such as settlement and/or cultivation of crops. With population explosions in Africa and with an increased demand for farm lands, this is surely going to keep off some species of tsetse from rivers, plains and forests.

Modern methods of control incorporate the use of insecticides. Both residual and non-residual insecticides are utilized for this purpose. However, due to the toxic nature of some of the residual insecticides such as endosulphan (toxic to fish), use is made of non-residual insecticides such as the synthetic pyrethroids e.g. deltamethrin, cypermethrin and pemethrin. The application of these insecticides ranges from the use of mechanical hand sprayers to motorised knapsack handsprayers. With advances in the production of ultralow volume aerosols (ULV), aircrafts and helicopters were employed in the control compaigns for both savanna and riverine groups of tsetse (MacLennan, 1967; Davis, 1971; MacLennan & Cook, 1972, Lee et al., 1978).

Use has also been made of traps and screens which were formerly used for population survey studies. With improvements in terms of both simplicity and greater catching efficiency and with the incorporation of chemical attractants, and insecticides such as deltamethrin, traps and screens can now be used to augment other control methods such as aerial spraying (Hassanali et al., 1986; Jordan, 1985; Owaga, 1986; Vale, 1980; Vale, 1983). These targets in combination with aerial spraying of ULV endosulphan are currently being used for large scale eradication of tsetse in Zimbabwe, Zambia, Malawi and Mozambique (Jordan, 1985).

Pour-on acaricides such as deltamethrin are currently being tested to see whether they can simultaneously be used for control of tsetse, especially in areas where cattle serve as the main food source (Wilson, 1988) in confirmed areas such as the cattle ranches.

The capture, sterilize and release method has also been advocated as a control method. Compounds such as Bisazir have been incorporated in traps and screens to sterilize and kill the flies. But all these methods are predominantly of scientific interest since chemosterisation does not prevent transmission of pathogens and their wide spread use would eventually lead to environmental pollution (House, 1982; Hall & Langley, 1987; Langley et al., 1982; Langley & Carlson 1986).

Sterile insect release is a practical method for eradication or control of tsetse populations (Simpon, 1958; Dame and Schmidt, 1970). This method has been employed with some success in some areas of Africa such as Tanzania (Williamson et al., 1983) and Nigeria (Takken et al., 1986). Like other methods this method is used as a support to others such as aerial spraying.

Minor methods of lesser application include the removal by killing of game animals which serve as sources of blood meal for tsetse, the catching or gathering of flies or pupae and multiplication of tsetse enemies (biological control).

2.2.2. Chemotherapy and chemoprophylaxis

The treatment of trypanosomiases may be either curative, using a drug which gives little or no residual action e.g. diminazene aceturate, homidium chloride, homidium bromide, quinapyramine sulphate and isometamidium chloride, or curative and preventive as in the case of

the quinapyramine sulphate. The difference between cure and prevention depends upon the drug being used and in some cases upon the dosage rate at which it has been administered. Some drugs e.g. isometamidium chloride, are stored in the tissues and by a slow release into the circulation they maintain a concentration of the active compound sufficient to control the trypanosomes (FAO, 1986).

Curative drugs are used when the incidence is low or sporadic; when only a few cases appear in a herd during the course of a year or where the threat occurs mainly during one season, usually corresponding with the rains. Prophylaxis or prevention is required where the livestock are under a more constant threat and where the disease occurs and relapses at a high level throughout the year.

2.2.2.1. Curative drugs

These drugs are used with the intention of curing individual infected animals and not with the idea of protecting a whole herd or group for a long period. These compounds are:

1. Homidium compounds (Novidium®, Ethidium®)
2. Quinapyramine sulphate (Trypacide sulphate®)
3. Diminazene aceturate (Berenil®, Ganaseg®, Veriben®)
4. Isometamidium chloride (Samorin®, Trypamidium®)
5. Suramin (Naganol®).

Homidium

Homidium is available in two forms:

(i) Homidium bromide (Ethidium ®),

(ii) Homidium chloride (Novidium ®).

Both homidium chloride and homidium bromide are crimson powders dispensed as tablets which, when dissolved, produce a deep red solution. Homidium chloride is more soluble than the bromide and the latter requires warm water to ensure that it is completely dissolved (Table 2.4.). These two drugs are used at the dosage of 1 mg/kg body weight.

Quinapyramine sulphate

Quinapyramine sulphate is dispensed as a white powder which dissolves in water producing a clear solution.

Dosage: It is used as a 10% solution at a dosage rate of 5 mg/kg by subcutenous injection. This is equivalent to a practical dosage rate of 1.0 mls/20 kgs.

Diminazene aceturate (Berenil [R], Ganaseg [R])

This is a yellow powder which dissolves in water to produce a clear yellow solution.

Dosage: It is used as a 7% solution at a dose rate of 3.5 mg/kg body weight by subcutenous or intramuscular injection. This corresponds to 1 ml/20 kg bodyweight of the prepared solution.

Isometamidium chloride (Samorin [R], Trypamidium [R])

This is a dark red powder which readily dissolves in cold water to produce a brownish red solution. It is a remarkable trypanocide in that it may be used at dose rates ranging from 0.25-1.0 mg/kg. It is curative at the lower dosage rate and both curative and preventive at the higher levels. The preventive action and the use for that purpose will be described in the next section.

Suramin (Naganol[R])

Suramin is a white powder which is hygroscopic and therefore must be kept in a tight stoppered bottle. It is soluble in cold water.

Dosage: Suramin is used as a 10% solution at a dosage rate of 10 mg/kg usually by intravenous route. Some workers administer suramin by intramuscular injection in camels.

2.2.2.2. Prophylactic drugs

These are used when the trypanosome risk is so high that the health of the livestock cannot be maintained by the use of curative compounds alone. Prophylactic regimes prevent those other uninfected animals from contracting trypanosomiasis for a period of time.

At the moment, two recognised prophylactic drugs are in use to control animal trypanosomiasis:

1. Quinapyramine prosalt (Trypacide Prosalt[R])
2. Isometamidium chloride (Samorin[R], Trypamidium[R])

Quinapyramine prosalt (Trypacide Prosalt[R]).

This is a white powder which when mixed with water gives an opaque creamy suspension. It consists of a mixture of the soluble dimethylsulphate (a curative drug) and the insoluble chloride in the proportion of 3:2 (quinapyramine sulphate three parts, quinapyramine chloride two parts).

The soluble sulphate exerts immediate curative effects and the insoluble chloride persist at the site of injection being slowly absorbed, thus maintaining the plasma concentration of the active compound within the effective range.

Dosage: The drug is given at the rate of 7.40 mg/kg body weight using a 16.7% suspension. For practical purposes 25g of the powder are suspended in 150 mls water (or 250g in 1.50 litres of water) and the suspension is given at the rate of 2 mls/45 kg body weight or 2.25 mls/50 kg body weight.

Quinapyramine chloride is not commercially available, but which can be obtained by special order, was used in the prevention of *T. simiae* infection in pigs (FAO, 1986). It is one of the very few drugs which is effective against this type of infection.

Isometamidium (Samorin^R, Trypamidium^R)

It may be used at different dosage rates ranging from 0.25-0.5 mg/kg as a curative and at 0.5-1.0 mg/kg as a prophylactic.

2.3. Trypanotolerance (genetic resistance)

It has been shown that wild bovidae are able to withstand infection by trypanosomes. This is due to their innate ability to control parasitemia (Murray *et al.*, 1982). This is partly due to the efficiency with which their phagocytic cells are able to clear the infection by phagocytosis (Rurangirwa *et al.*, 1986). This ability is also possessed by some of the cattle breeds from West and Central Africa which include breeds such as N'dama and Muturu (Murray *et al.*, 1982). There is also evidence for the presence of similar breeds in East Africa such as the Orma Boran (Njogu *et al.*, 1985). These animals when infected are able to control parasitemia and remain productive.

Efforts are being made to study the genetic markers for the trypanotolerance trait and to use this as a tool in the breeding of

tolerant animals by crossing with local (sensitive) breeds of cattle. This is part of the ILCA/ILRAD project on cross-breeding and improvement of performance by indigenous herds of cattle.

2.4. THE PROBLEM OF TRYPANOCIDAL DRUG RESISTANCE

The constant exposure of trypanosomes to therapeutic agents has led to wide dissemination of resistant strains (Schillinger, 1985). Resistance has become established throughout the tsetse belt and has been observed in all species of pathogenic trypanosomes (Leach and Roberts, 1981; Mbwambo *et al*., 1988; Pinder and Authie, 1984; Röttcher and Schillinger 1985; Schönefeld *et al*., 1987).

Detailed information on the distribution of drug resistance is not available. The situation has worsened in the last few years with respect to both single or multiple drug resistance. Drug resistance is likely to persist for a long time even when the drugs are no longer used (Schillinger, 1985). Furthermore, there is a considerable degree of cross resistance between the commonly available drugs (Williamson, 1960). However, there are several important factors which may reduce the veterinary impact of this problem in the field. Firstly, cross-resistance between the two most commonly used drugs, isometamidium chloride and diminazene aceturate is very rarely observed in the field. Secondly, resistance to Berenil, although reported, occurs relatively infrequently. Thirdly, there is some evidence from the field that drug resistant strains may often be of comparatively low pathogenicity (Goble *et al*., 1959; Stephen, 1962; Sones *et al*., 1989) but this requires confirmation.

It is also important to define the various forms of drug-resistance which have been described. While drug resistance may be relatively straight forward in the sense of a very limited or ineffectual response of parasites to treatment, there are other situations in which the term "drug resistance" requires qualification. For example, resistance may be observed at only relatively low levels of drug dosages. It is also possible that relapse may occur after drug therapy, which is directly related to the interval of time between infection and treatment. Such a phemomenon has been reported in mice by Jennings et al., (1977a,b) who showed that there was a correlation between duration of infection with Trypanosoma brucei and efficacy of chemotherapy. The mice treated soon (3 days) after infection were permanently cured. However, if treatment was delayed until 14-21 days after infection, none of the available trypanocidal drugs produced a permanent cure, even if used at dose rates far in excess of those recommended. Treatment 14-21 days after infection was followed by a period without parasitemia, but eventually (upto 7 months later) trypanosomes reappeared in the blood. During aparasitemic period the only organ in the body of the treated mouse capable of transferring infection to an uninfected recipient was the brain (Jennings et al., 1979). Current evidence supports the view that this was because insufficient amounts of drug passed the blood brain barriers to eliminate infection.

Whether or not this form of relapsing infection following chemotherapy occurs in cattle has yet to be determined. However, it is of interest that there are reports from Nigeria (Gray and Roberts 1971a; MacLennan 1971; MacLennan & Naïsa 1970) and Kenya (Whitelaw et al., 1985, 1988) describing infections of T. simiae, T. vivax, T. congolense and T.

brucei which generally relapsed 10-40 days after chemotherapy. Whitelaw et al. (1988) described such relapses in goats in which the central nervous system and aqueous humour of the eyes were sources of relapses after chemotherapy in goats infected with T. vivax.

2.4.1. Stability of drug resistance

In 1934 Van Zwieten found that Suramin resistance remained for one year or longer in T. evansi infected horses. In Kenya, it has been observed that quinapyramine resistance in T. evansi in camels exists even though the drug has been unavailable for years (Schillinger and Gordon, 1984). In cyclical transmission by tsetse flies, Gray and Roberts (1971a, b) showed that the stability of drug resistant T. vivax and T. congolense strains in cattle was not changed after 29 months and 12 months respectively. Kaminsky and Zweygarth (1989a) found that for a T.b. brucei stock resistant to diminazene aceturate, isometamidium chloride, quinapyramine sulfate and Mel B "(Melarsoprol)", there was little, if any change of drug sensitity after in vitro propagation as bloodstream forms for 120, 177 and 272 days respectively and after in vitro transformation of bloodstream forms into procyclic, epimastigote and finally metacyclic forms.

2.4.2 Drug resistance and other reasons for drug failure.

The success of chemotherapy can be limited by factors other than induced drug resistance. Factors causing failure of trypanosomiasis chemotherapy are:

-depressed immune responses of the host

-extravascular trypanosomes inaccessible to treatment

-drug tolerance (natural resistance)

-drug resistance (induced resistance) (Schillinger, 1985).

2.4.2.1 Depressed Immune Status of the Host.

It is known that an antibody response by the host to the surface antigen of trypanosomes is necessary for rapid elimination of parasites after chemotherapy. This is particularly true if a drug is trypanostatic instead of trypanocidal. Recently this was proved with an experimental drug, as the treatment effect of the ornithine decarboxylase inhibitor Eflornithine (DL-alphadifluoromethylornithine, DMFO, Merrell Dow Company) difluoromethylornithine (DFMO, Merrell Dow Company) was greatly reduced in immuno-suppresed mice (De Gee *et al*., 1983). Since a depressed immune response resulting from other parasitic infections or due to malnutrition is a frequent occurence in tropical livestock (Griffins *et al*., 1980, 1981; Holmes *et al*., 1974; Mackenzie *et al*., 1975), the response to treatment should be considered in relation to the immune status of the host.

2.4.2.2. Extravascular trypanosomes resisting treatment.

Trypanosomes, in particular members of the subgenus Trypanozoon like *T. brucei brucei* and *T. brucei evansi*, and subgenus Nannomonas like *T. congolense* and *T. simiae* can invade body compartments other than the vascular system. With the exception of melarsoprol (Arsobal[R], Specia), an arsenical used for human sleeping sickness, the available trypanocides are not able to cross the blood-brain barrier. Therefore, in some extravascular areas, the trypanocidal level is insufficient or non-existent. Those areas can represent the site of escape from

chemotherapy (Gray *et al.*, 1982; Jennings *et al.*, 1977a, b; Poltera *et al.*, 1981; Whitelaw *et al.*, 1985, 1988) and form one reason for relapses. If the chemotherapy is not trypanocidal for all parasites, multiplication of the surviving trypanosomes is blocked by drugs retained in the host until their concentrations fall below the trypanostatic level when relapse may begin.

2.4.2.3. Drug tolerance (Natural resistance).

One of the mechanisms which reduces effective chemotherapy is the ability of parasites to tolerate certain trypanocides without previous contact with the drug: this is called drug tolerance or natural resistance. Leach and Roberts (1981) gave the following definition for this phenomenon: "Natural resistance or cross resistance of a trypanosoma strain or species to a drug is that variation in drug sensitivity that is not dependent on previous exposure to the drug concerned". Thus, two of the drugs used for treatment of African trypanosomiasis in cattle: diminazene aceturate (Berenil^{R}) and isometamidium chloride (Samorin^{R}), produced insuffient or only moderate effect against some strains of *T. evansi* in camels (Leach, 1961; Schillinger and Gordon, 1984). In East Africa, homidium treatment tends to cure *T. vivax* infections more effectively than *T. congolense* while diminazene produces the opposite effect (Williamson 1960; Killick-Kendrick and Godfrey 1963; Jones-Davies and Folkers 1966a, b; Jones-Davies 1967 a,b).

2.4.2.4. Drug resistance (induced resistance).

Drug resistance in trypanosomes can be defined as "the ability of a trypanosome strain, formely exposed to a drug, to survive, despite

the administration and absorption of a drug given in doses equal to or higher than those usually recommended" (Schillinger, 1985). Drug resistance is not an absolute state but is correlated to a particular dosage. Between complete sensitivity and complete resistance is a gradation in the response of trypanosomes to trypanocides, ranging from a loss of effect demonstrable only by occassional reappearance of parasites in the blood, to a level of resistance at which the drug apparently has no effect. Unlike the chemotherapy of malaria, there is no established grading system for resistance in the chemotherapy of trypanosomiasis. The level of drug resistance which becomes particularly important in the field is resistance to the standard curative dose (Hawking 1963b). This is especially important when we consider the narrow therapeutic index of the available trypanocides, where a small increase in plasma concentration produces toxicity and a small decrease results in loss of effect.

Cross resistance differs from multiple drug resistance. The former results when trypanosome populations not previously exposed to a drug exhibit resistance to a different but chemically related drug (Schillinger, 1985). For example, isometamidium shows a resistance pattern similar to all other members of the phenanthridine family (homidium and prothidium) (Schillinger, 1985). However, there is no cross resistance between phenanthridines and the diamidine, diminazene aceturate. These findings led Whiteside (1960, 1962) to advise the use of a "Sanative pair" such as homidium and diminazene and since neither of the two drugs produces cross resistance to the other, they can be used alternately. If there is resistance to one drug, by using the second drug the resistant trypanosomes can be eliminated. However, since more

and more multiple drug resistance is developing, the use of a "sanative pair" principle is losing its importance.

2.5. DETECTION OF DRUG RESISTANCE IN TRYPANOSOMES.

In the field, drug resistance can be detected by monitoring the treatments of animals over a period of time. By keeping proper records of treatments, animals can be monitored for any signs of relapses which may serve to indicate the appearance of drug resistance.

Another method to obtain dose related information on drug resistance is the mouse sensitivity test. Mice are infected with trypanosomes, treated 24 hours later and monitored for any appearance of trypanosomes in the blood for a period of 30-60 days. This test can be carried out with *T.b. brucei*, *T. congolense*, *T. evansi* and with West African species of *T. vivax* (rodent infective). *Trypanosoma vivax*, which does not generally grow in mice can also be tested by inoculation into cattle or goats under strict fly-free conditions followed by treatment and follow-up for a period of 3 months.

Drug resistance can also be analysed *in vitro*. Various assays for screening laboratory stocks have been introduced (Borowy *et al.*, 1985; Kaminsky & Zweygarth, 1989b; Kaminsky *et al.*, 1989b). Further studies are required to ascertain the correlation between the results of *in vivo* and *in vitro* tests. Although the *in vitro* tests are still in their early development they may become as important as sensitivity tests are in bacteriology (Rottcher, 1983).

2.6. THE USE OF IN VITRO CULTURES IN THE ASSESSMENT OF DRUG RESISTANCE IN T. VIVAX.

Trypanosoma vivax is pathogenic to livestock (Table 2.2.) and affects productivity of hosts. Studies on isolates of this parasite have been limited since they normally do not infect laboratory rodents (Anosa 1983; Kalu *et al*., 1986). However, *in vitro* culture systems offer good alternatives. To maintain trypanosomes in the *in vitro* culture system would facilitate detailed investigations on their drug sensitivity patterns and on their mechanisms of drug resistance.

However, the problems of *in vitro* techniques for *T. vivax* are the difficulties of propagating this parasite *in vitro*. So far only one mouse infective stock of *T. vivax* has been grown continuously as blood stream forms (Brun & Moloo, 1982). Trager (1959) successfully propagated insect forms of *T. vivax* in a trypanosome-tsetse tissue culture system. More recently a number of stocks were propagated as insect-form cultures (Hirumi *et al*., 1987). However, the method described by Hirumi *et al*. (1987) of propagation of insect forms involves a somewhat complicated culture system including a combination of feeder layer cells, scratching of plastic and the addition of Matrix Gel Green A Beads. Trials are still going on at ILRAD to develop a method which would allow the transformation of *T. vivax* bloodstream forms into insect forms and to propagate these at 27°C in a liquid medium only. Previous efforts succeeded in transforming bloodstream forms to insect forms and these were utilized in the long-term drug-incubation viability assay using *T. vivax* insect form cultures (Kaminsky *et al*., 1989a).

Table 2.4. Use of curative drugs (Manual of Tropical Veterinary Parasitology, 1989)

Drug	Aqueous solution	Injection (route)	Dose	Volume of injected solution	Parasites controlled	Animal Species
Suramin Sodium	10% cold	IM or IV	7-10 mg/kg	10% solution	*T. evansi*	Camelidae, Equidae canidae
Homidium bromide	1-2.5% hot water	SC (dewlap)	1 mg/kg	10mls/100kgs	*T. vivax* *T. congolense*	Cattle, small ruminants
Quinapyramine (Methylsulphate)	10% cold water	SC	5mg/kg	5ml/100kg	*T. vivax* *T. congolense*	Cattle, small ruminants
					T. brucei *T. evansi*	Equidae, canidae, camelidae
Diminazene	7% cold water	SC or IM	3.5mg/kg	5ml/100kg	*T. vivax* *T. congolense* *T. brucei*	cattle, equidae
					T. evansi	Camelidae
Isometamidium chloride	1% cold water	IM	0.25 mg/kg 0.5-1mg/kg	2.5mls/100kg 5-10ml/100kg	*T. vivax* *T. congolense*	cattle, equidae
					T. brucei	cattle, equidae, canidae

SC = Subcutenous
IM = Intramuscular
IV = Intravenous

Table 2.5. Use of prophylactic drugs (Manual of Tropical Vet. Parasitology, 1989).

Drug	Aqueous	Injection	Dose	Volume of injected soln.	Parasites controlled	Duration of protection	Animal species
Isometamidium chloride	1-2 % cold water	deep IM	0.5-1mg/kg	5-10mls/100kg	T. vivax T. congolense	2-4 months	Cattle, small ruminants, equidae, canidae
		IM or IV	0.5-0.75mg/kg	5-7.5ml/100kg	T. brucei	2 months	
Pyrithidium bromide	2% boiling water	deep IM	2mg/kg	10mls/100kg	T. vivax T. congolense	2-4 months	Cattle, small ruminants, canidae.
Quinapyramine prosalt	3.5g/15mls cold water	SC	7.4mg/kg	5ml/100kg	T. brucei T. evansi	2 months	Equidae, canidae
Homidium bromide	2.5% hot water	SC (dewlap)	1mg/kg	4ml/100kg	T. vivax T. congolense	1 month	Beef cattle
Suramin Sodium Quinapyramine complex	5% cold water	SC	40 mg/kg Quinapyramine	4ml/5kg	T. simiae	3 months (piglets) 6 months (adults)	Pigs

IM = intramuscular
SC = subcutenous
IV = intravenous

3. MATERIALS AND METHODS

3.1. INITIATION AND PROPAGATION OF FEEDER LAYER CELLS

The following feeder layer cells have been used:

- Bovine thymus cells (BT),
- Bovine aorta endothelial cells (BEC),
- Bovine bone marrow cells (BM),
- Mouse embryo fibroblast-like cells (MEC)
- Insect cells, (*Toxorhynchitis amboinensis*) (abbreviated as TOXO.)

BT, BEC, BM, TOXO cells previously established were frozen as stabilates at the start of the project. MEC cell cultures were initiated as described below.

3.1.1. Initiation of Mouse embryo fibroblast-like cells (MEC)

Materials:

- 2 sharp (point) forceps
- 2 blunt forceps (tissue forceps)
- two small sized scissors
- 2 curved tissue forceps
- 2 petri dishes
- Balanced salt solution (BSS) without calcium and magnesium (GIBCO, Paisley, UK).
- Concentrated trypsin /Ethylenediaminetetraacetic acid (EDTA) solution (Flow Laboratories, Scotland, U.K.).
- Medium for primary growth which consisted of 78% Minimum Essential Medium (MEM), 20% fetal bovine serum, 1%

antibiotics (final concentration 200 IU Penicillin and 150 ug/ml of Streptomycin) and 1% L-glutamine per 500 mls of the culture medium.

- Medium for maintenance of cells after initiation: MEM with 10% bovine fetal serum, 1% glutamine and 1% antibiotics (final concentration 200 IU penicillin and 150 ug/ml of streptomycin per 500 mls of culture medium).
- MEM with 1% antibiotics but without serum (for washing the tissues)
- 14 day old pregnant mouse.
- A haemocytometer (Superior[R], Heinz Henenz, West Germany).

3.1.2. Procedures:

a) A pregnant mouse (2 weeks old pregnancy) was anaesthetized and dissected along the ventral midline to expose the gravid uterus.

b) The uterus was incised to expose the fetuses which were gently removed using tissue forceps and placed into "Roswell Park Memorial Institute (RPM1)" - 1640 medium.

c) The fetuses were minced (choped) into smaller pieces which were washed several times in 10% balanced salt solution (BSS).

d) The fetal pieces were then transferred to another flask, and 1:3 EDTA/Trypsin solution added to the fetal mixture.

e) The tissue was incubated while stirring for 30 minutes at $37^{o}C$ and the frothing materials which appeared were discarded.

f) 5 mls of EDTA/trypsin solution was added and trypsinization was continued. The supernate was collected.

g) The supernate was then filtered through muslin gauze.

h) The filtrate was spun at 1000 r.p.m. for 5 minutes.

i) The pellet was collected and diluted in 5mls (stock solution) and 1 ml of it mixed at the ratio of 1:10 with trypan blue and the living cells counted using the haemocytometer (Superior^R, West Germany).

j) The cells were then seeded into tissue culture flasks at the rate of 1.5×10^5 living cells per ml, each flask containing 5 mls.

k) The cells were incubated at 37^oC until they attached at the bottom of the flasks.

l) After 1 hour the initiation medium was removed and replaced with maintenance medium (5 mls of fresh maintenance medium was put into each flask).

m) The cells were passaged after 7 days as described below.

3.1.3. Passaging of cells

The existing maintenance medium in the flask was removed and the attached cells were washed three times with changes of 1.5 mls of BSS. Subsequently, the process was repeated, this time washing three times with changes of 1.5 mls of the concentrated EDTA/trypsin solution.

Fresh 1.5 ml aliquots of EDTA/trypsin solution were put into each flask and the cells trypsinized by gently washing them away from the flask. This was done with the aid of a pasteur pipette.

The suspension of cells was then withdrawn and seeded into 4 tissue culture flasks each containing 4.5 ml of primary medium. The cells were then incubated for 1 hour at 37°C.

After cells had attached the primary medium was removed and replaced by maintenance medium and the cells incubated at 37°C, ready for use after 7 days.

3.2. PREPARATION OF MEDIA AND *IN VITRO* PROPAGATION OF *T. VIVAX* INSECT FORMS

3.2.1. Chemicals and Reagents

- Iscove's medium (Iscove's modification of Dulbecco's Medium) from ILRAD stores with pH 7 and osmolality 303 obtained as powder from Flow Laboratories, (Irvine, U.K.) and put in solution with double distilled water.
- Minimum Essential Medium (MEM) with 25 mM HEPES with Earle's salts but without L-glutamine (GIBCO, Paisley, U.K.).
- RPMI-1640 medium (GIBCO, Paisely, U.K.).
- Fetal bovine sera (sterile) with lot numbers 058075 (Flow Laboratories, U.K.) and 1111794 (Hyclone, U.S.A.)
- L-glutamine, (GIBCO, Paisley, U.K.
- Adenosine, (Hoechst, Germany)
- Sodium pyruvate as a 100 mM solution (Sigma Chemical Co., Dorset, U.K.).
- L-proline (Merck, Darmstadt, Germany.)
- MEM amino acids (GIBCO, Paisely, UK)
- MEM Essential vitamins (GIBCO, Paisley, U.K.)
- MEM non essential amino acids (GIBCO- Paisley, U.K.)
- 2-mercaptoethanol (Fluka Chemie, Switzerland), (as a 20mM solution).
- Bovine plasma prepared from cattle blood.
- Heparin (Heparin Novo, Denmark).

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3.2.2. Equipment:

- A laminar flow hood
- A bunsen burner for sterilizing tissue culture flasks and bottles
- Tissue culture flasks 25 cm^2 (50 mls) COSTAR[R], Massachusets, USA, FALCON[R], Becton Dickinson, and Company, Switzerland).
- Measuring cylinders (250 ml, 500 ml).

Pipettes (Stripettes[R])

- 5 mls pipettes (COSTAR[R], Massachusets, USA).
- 10 mls pipettes (COSTAR[R], Massachusets, USA).

Filters for sterilization of media

- 0.45 um filters (Nalgene[R])
- 0.22 um single unit filters (Minisart[R]/NML), Sartorius GmbH, Gottingen, West Germany).
- 115 ml Nalgene[R] filters with 0.22 um pore size.
- 500 ml Nalgene[R] filters with 0.22 um pore size (Nalgene[R] Brand Products, New York, USA).

3.3. Procedures for the preparation of various media

3.3.1. Sterilization Procedures

Most of *in vitro* experiments performed demanded complete sterility. Thus, all work procedures were performed in a Laminar flow hood (Edgegard[R], The Baker Company, Sanford, Maine). Before any activity was done, the hood was sterilized by scrubbing its floor and lateral walls with 70% ethanol.

This ensured sterility during subsequent steps in the preparation and conduction of various tests.

Glass-ware was sterilized by autoclave and Costar^{R} Nalgene^{R} and Falcon^{R} products provided were sterilized by gamma radiation. Liquids were sterilized by filtration using 0.22 or 0.45 um filter units. Water was sterilized by triple distillation.

3.3.2. Preservation and storage of chemicals and reagents.

With the exception of 2-mercaptoethanol, all other chemicals and reagents were preserved by keeping them at $4^{o}C$. Mercaptoethanol was left at room temperature but in a dark place. Sera and plasma were dispensed into 50 mls ($25cm^{2}$) culture (COSTAR^{R}) flasks and stored at $4^{o}C$ for 10 days. If not in use, they were dispensed into 100 ml glass bottles and frozen at $-20^{o}C$. Prior to use, they were thawed.

3.3.3. Preparation of the media

Various media were prepared by adding additives as shown in the table below.

Table 3.1. DIFFERENT MEDIA EMPLOYED FOR VARIOUS IN VITRO TESTS

Additives (Percentage by volume)		Final Concentration	Normal	T1	T2	MT2	DIIT	Incorporation tests
Medium	(73-75%)		Iscoves	Iscoves	Iscoves	Iscoves	Iscoves	Iscoves
Bovine fetal serum	(20%)		+	+	+	+	+	+
Sodium pyruvate	(2%)	2 mM	+	+	+	+	+	+
L-glutamine	(1%)	2 mM	+	+	+	+	+	+
Hypoxanthine	(1%)	0.1 - 1 mM	+	+	+	+	+	-
Adenosine	(1%)	0.075 mM	+	+	+	+	+	+
L-proline	(1%)	4.4 - 44 mM	-	-	+	+	-	-
2-mercaptoethanol	(1%)	0.2 mM	-	-	-	-	+	+
Penistrepto	(1%)	*200 IU penicillin	-	-	-	-	+	+
mycin antibiotic		*150 ug streptomycin						

* per 500 mls of the medium

3.2.4. TRYPANOSOME STOCKS AND CLONES USED IN THIS STUDY

IL 2337.

Trypanosoma vivax stock IL 2337 was isolated in 1978 from a naturally infected cow in Galana, Malindi (Kenya). It was given the stabilate number KETRI 2392. After serial passages in 3 cattle it was reisolated and given the ILRAD code number IL 1480. IL 1480 was further passaged in cattle to the present IL 2337. Its drug sensitivity was not known.

IL 2242.

Trypanosoma vivax stock IL 2242 was isolated in 1978 from a naturally infected cow at Likoni, Mombasa, Kenya. It was given the code number KETRI 2375. After 18 serial passages in cattle it was reisolated and given the code number KETRI 2388 which was stabilated as IL 2242. Its drug sensitivity was not known.

IL 2133.

Trypanosoma vivax stock IL 2133 was isolated from a naturally infected cow at Kilifi, Kenya. Since then the stock has been serially passaged in cattle, and tsetse, before a final passage in goats. Its drug sensitivity was not known.

IL 3185.

Trypanosoma vivax stock IL 3185 was isolated in 1970 from a naturally infected tsetse fly. After passage in cattle it

was given the code number EATRO 1721. Further passage in cattle resulted in stocks J 154 and IL V.3. The latter was further passaged in 13 cattle, in tsetse (G. m. centralis) and in goats to be re-isolated as stock IL 2946. This was further passaged in 8 goats, cloned in goats and passaged further in 10 goats to get the present clone IL 3185. Its drug sensitivity was not known.

IL 1392.

Trypanosoma vivax stock IL 1392 was isolated in 1973 from a naturally infected cow in Nigeria, (Leeflang et al., 1976). After 4 passages in mice it was given a code number ZARIA Y 486. Further passages in mice, goats, cattle and tsetse resulted in stocks IL V.9, IL 10-EV, IL V-17, IL 7-E2, IL 7-E6, IL 20E-11, IL 417, IL 560 and eventually IL 1392. This stock is rodent infective and was cured in cattle with 0.25 mg/kg isometamidium chloride (Kaminsky et al., 1990).

CP 2171.

Trypanosoma vivax stock CP 2171 was isolated in 1986 from a naturally infected Friesian cow in Bamburi, Mombasa, Kenya. In cattle this stock has been found to be resistant to 0.5, 1 and 2 mg/kg isometamidium chloride and sensitive to both 3.5 and 7 mg/kg diminazene aceturate (Schönefeld et al., 1987). IL 3060 which is a derivative of CP 2171 was obtained after serial passages in cattle and tsetse.

CP 2331.

Trypanosoma vivax stock CP 2331 was isolated in 1986 from a naturally infected Zebu calf in the Kipini area, Tana River District, Kenya. This stock has been found to be resistant to isometamidium chloride at 2 mg/kg, quinapyramine sulphate at 5 mg/kg and sensitive to diminazene aceturate at both 3.5 and 7.0 mg/kg (Schönefeld *et al.*, 1987).

IL 2005.

Trypanosoma vivax clone IL 2005 was isolated from a naturally infected cow in Teso, Uganda. It was serially passaged in 6 goats, and/or 6 cattle and given the code number EATRO 2058. It was further passaged in 9 sheep and given the code numbers EATRO 2244 and IL V4. After passage in 19 goats, the code number was given as IL V21. The stock IL V21 was cloned in a goat to get a clone of trypanosomes which after serial passages in 10 goats, was reisolated and given the code number IL 2005. Its drug sensitivity was not known.

3.3. IN VIVO EXPERIMENTS.

3.5.1. Infection of Cattle and Mice.

Cattle were infected by one of the two ways:

- needle infection
- tsetse infection

i) Needle infection (transmission).

This was done by injecting stabilates of different stocks of *T. vivax* through the intravenous route. Frozen stabilates were taken, thawed and trypanosomes assessed for viability before transmission into cattle. The ages of cattle used in these experiments ranged from 6 - 12 months. The route of injection was through the jugular vein.

ii) Tsetse transmission.

The animals were prepared by shaving the appropriate areas which in this case, were the left flanks of the animals i.e. midway between the spinal cord and ventral side of the abdomen and 6 inches from the last rib. Infection was done by attaching cages containing tsetse for at least 10-15 minutes. During this process of feeding, tsetse transmitted the appropriate stock of *T. vivax* parasites into the animal concerned.

3.4. ISOLATION OF TRYPANOSOMES FROM BLOOD.

This was performed by differential centrifugation similar to the method of Staak (1975) but with minor modifications.

Blood was mixed with plain medium containing 1% of Penicillin and streptomycin antibiotics at the ratio of 1:2 for blood from cattle and 1:4 for blood from mice. The mixture of blood and medium was then dispensed into 50 ml Falcon plastic tubes (cattle blood) or 5 ml Wheaton bottles (mice blood). A minifuge[R] centrifuge (Heraeus Christ, Bremen, Germany) was used for isolation of trypanosomes from smaller volumes of blood i.e. those not exceeding 120 mls of blood, while a Sorvall[R] RT 6000 centrifuge, (Du Pont, Newtown, Connecticut) was used for bigger volumes of blood i.e. those exceeding 120 ml of blood. Blood was spun twice at 1200 rpm for 20 minutes (cattle blood) or 10 minutes (mice blood). At each spin, supernatants were taken and preserved while the sediments after first spin were reconstituted by filling the tubes with fresh plain medium. The supernatant from both spins were pooled into 50 ml plastic tubes (cattle blood) or 10 ml sterilin tubes (mice blood) and spun at 3000 rpm for 20 minutes (50 ml tubes) or for 10 minutes (sterilin tubes). The supernatants obtained after spinning were discarded and the pellet of trypanosomes resuspended with a medium of a particular purpose up to a desired volume depending on the experiment. Trypanosomes were counted by the hemocytometer (Superior[R], West Germany) and diluted accordingly, depending on the objectives of the experiment.

3.5. LONG TERM VIABILITY ASSAY

Suspensions of isolated bloodstream form trypanosomes were dispensed into 25 cm^2 tissue culture flasks, (COSTAR[R]).

The minimum number of trypanosomes per flask was 2.5×10^6 trypanosomes per ml and each flask was dispensed with 5 ml of the trypanosome suspension.

For some of the experiments which required the use of feeder layer cells, the trypanosomes were dispensed into 25 cm^2 tissue culture flasks (Falcon^R, Becton Dickinson & Co., Switzerland) which contained the feeder layer cells. The concentration and the amount of trypanosomes were the same as in those flasks without feeder layer cells.

The flasks were then placed in a 27^oC incubator and checked daily for the transformation of trypanosomes from bloodstream trypomastigotes to epimastigotes.

Successful cultures were further sub-cultured by scratching or scraping the trypanosomes with a disposable cell scraper (COSTAR^R, Mass. USA) and seeding them into new tissue culture flasks with or without feeder layer cells. Sub-cultures were prepared when it was found that the trypanosome colonies had merged to form a continuous carpet on the bottom of the tissue flasks.

Final cultures for setting up drug viability assay were prepared by scraping the trypanosomes from the bottom of the flasks without feeder layer cells and seeding them into tissue culture flasks without feeder layer cells. The medium used for drug trials was normal Iscove's (Table 3.1.).

The trypanosomes were exposed to trypanocides at the following concentrations:

Berenil[R]	Samorin
(Diminazene aceturate)	(Isometamidium chloride)
10 ug/ml	0.01 ug/ml
1 ug/ml	0.001 ug/ml
0.1 ug/ml	0.0001 ug/ml
0.01 ug/ml	0 ug/ml (controls)
0 ug/ml (controls)	

Viability assessment was made by observing changes in:

- the motility of trypanosomes
- consumption of the media by observing changes in the pH, (colour change of phenol red in the medium).
- rounding or death of trypanosomes,
- production of metacyclic forms

The medium was changed 3 times a week. This was done by removing all the media and replacing it with the same amount of fresh medium containing drug.

Scratches were made on the bottom of the flask when the carpet was formed by merging of growing colonies to allow further growth of trypanosome colonies. This was done with the aid of a pasteur pipette or a disposable cell scraper as mentioned earlier.

3.6. TESTING FOR THE PRODUCTION OF METACYCLICS

These tests were conducted on day 6, 8, 10, 12, 13, 15 and 17 from the day of setting up cultures for long term viability assays.

Trypanosomes were harvested by pooling the supernatants of culture flasks together. Supernatants from the flasks with the same drug concentration were pooled together. Since the supernatant contained a mixed population of epimastigotes and metacyclics, 10% bovine plasma from a clean (uninfected) animal was added according to the method of Hirumi et al. (1985). Before use, the plasma was filtered through 0.45 um filter (NALGENE^R). The culture suspension after incubating in plasma for 1-2 hrs was filtered through a 5.0 um filter allowing only metacyclics to pass through. Before filtration the filters were wetted with mouse PSG with a pH of 7 and the PSG that passed was drained off. The same buffer was used in rinsing the filters at the end of each experiment. The filtrate was spun at 2800 r.p.m. for 10 minutes, the supernatant was discarded and the pellet mixed with normal Iscove's medium to make up 1 ml. The presence of metacyclics was checked by placing a drop of the mixture on a microscope glass slide and observing under inverted microscopes at x 100 magnification. The metacyclics were then counted with the aid of a hemocytometer (Superior^R, West Germany).

3.6.1. Infectivity tests in cattle and mice.

This test was done by infecting cattle with a specific number of metacyclics pooled from flasks containing the same concentration of drug in question.

Infection was done by injecting cattle via the intravenous route (jugular veins). Mice were infected via intraperitoneal route.

The animals infected were then monitored for any signs of infection for a period of 30 days for mice and 4 months for cattle. This was done by taking blood 3 times a week and checking for parasites by using the following methods:

- wet blood smears
- dark ground/phase contrast buffy coat method (DG method)
- hematocrit centrifugation technique (HCT or Woo method).

3.7. DETERMINATION OF THE MINIMUM NUMBER OF TRYPANOSOMES (INSECT FORMS) REQUIRED FOR SUBPASSAGING OF IN VITRO CULTURES.

A stock suspension containing 10^6 trypanosomes per 4.5 ml of insect forms of _T. vivax_ was taken and serially diluted (10 folds) until a dilution 10^1 was reached.

All dilutions starting from 10^6 dilution to dilution 10^1 were dispensed into 25 cm^2 (50mls) tissue culture flasks (COSTAR) and incubated at 27°C. The setting included 2 further dilutions which did not show any trypanosomes when observed at x 400 magnification.

3.8. DRUG INCUBATION INFECTIVITY TESTS (DIIT)

Mice were infected with various stocks of *T. vivax* through intraperitoneal route and checked for infection using wet blood films. When infection was confirmed and when the parasitemia was 10 trypanosomes per field at x400 magnification or more the trypanosomes were separated from blood by differential centrifugation as already explained (Section 3.4.). The trypanosomes were counted and diluted in culture medium to get a concentration of 2.5×10^5 trypanosomes per ml. The trypanosomes were then mixed with the test drug so as to get the required concentrations of 0, 0.01 and 0.001 ug/ml of the drugs. Then, 1 ml of the trypanosomes-drug suspensions were dispensed into each well of a 24 well plate. Each drug concentration was tested in 5 replicates. The plate was then incubated at 37 ^{o}C for 24 hours. At the end of 24 hours, 0.8 ml of the suspension of each well was injected into different mice through intraperitoneal route. Observation for infection was done 3 times per week by taking blood from the tips of tails of the inoculated mice and observing it microscopically at x 400 magnification using normal light microscopy.

3.8.1. The effect of blood plasma/cells on DIIT

An experiment was done to determine the effects of proteins and red blood cells/plasma on the efficiency of DIIT.

The experiment consisted of 3 sets:-

1. DIIT using trypanosomes which have been isolated from blood by differential centrifugation.

2. DIIT using a definite number of isolated trypanosomes mixed with a definite number of washed red blood cells.

3. DIIT using diluted whole blood.

3.8.2. DIIT using trypanosomes after differential centrifugation

Trypanosomes were isolated from blood by differential centrifugation as explained under section 3.4. The suspension was diluted in culture medium to get a concentration of 2.5 x 10^5 trypanosomes per ml and these were dispensed into wells of a 24-well plate. Drug was added to give final concentrations of 0, 0.01 and 0.001 ug/ml of isometamidium chloride. The plate was then incubated at 37°C for 24 hours. After 24 hours the contents of each well was injected into a mouse through intraperitoneal route. Mice were observed 3 times a week for trypanosome infection for the period of 30 days by taking tail blood and examining it under the microscope for evidence of parasitemia.

3.8.3. DIIT using a definite number of isolated trypanosomes mixed with a definite number of washed erythrocytes.

Trypanosomes were obtained after differential centrifugation of infected blood. They were counted in a hemocytometer (Superior[R], West Germany) and diluted accordingly using modified Iscove's medium to get a concentration of $2.5x10^5$ trypanosomes per ml. Blood was taken from a healthy uninfected mouse and washed twice by differential centrifugation using plain Iscove's medium (Section 3.4.) The pelleted erythrocytes were made into a suspension and 400ul of the red blood cells suspension was added to 20 ml of a trypanosome mixture to give a final concentration of 2.5×10^5 trypanosomes and 2.5×10^5 red blood cells per ml. The mixture was then dispensed into 3 rows of a 24-well plate. The first row was left without drug so as to act as a control, while each well of the second row received 10ul of isometamidium chloride from a dilution containing 0.1ug/ml of isometamidium chloride to give a final concentration of 0.001 ug drug/ml. Each well of the third well was dispensed with 10ul of isometamidium chloride from a dilution containing 1ug/ml to give a final drug concentration of 0.01ug/ml of drug. The plate was incubated at 37°C for 24 hours. After 24 hours, 0.8 ml of the contents from each well was injected into mice through intraperitoneal route and the mice were checked 3 times a week for a period of 30 days for evidence of infection by observing wet blood films from tail blood under an inverted microscope at x400 magnification.

3.8.4. DIIT using diluted whole blood.

Mice were injected with a particular stock of *T. vivax*. When the parasitemia was 10 trypanosomes per field (x400 magnification), blood was taken by heart puncture and diluted with modified Iscove's medium to give a final suspension containing 2.8 x 10^5 trypanosomes/ml. The suspension was mixed with isometamidium chloride to achieve 0.001 and 0.01 ug/ml of the drug. The diluted blood was transferred into wells of 24-well plate (1 ml/well) and setting was done as above. After 24 hours of incubation the contents of each well were injected intraperitoneally into mice The mice were checked 3 times per week for a total period of 30 days for evidence of infection. Diagnosis was done by checking wet blood films from tail blood under the microscope as described above.

3.9.1. IN VITRO TESTING FOR INCORPORATION OF RADIO LABELED HYPOXANTHINE ([3H] HYPOXANTHINE) BY SENSITIVE AND RESISTANT STOCKS OF T. VIVAX.

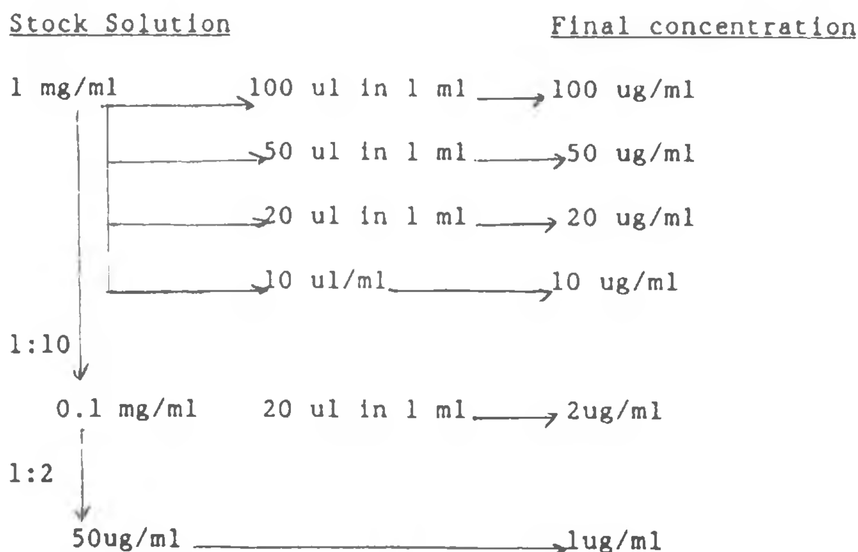
3.9.1. Preparation of the Drugs for Incorporation Tests

The following drug concentrations were employed in the test:

Isometamidium chloride	Diminazene aceturate
50 ug/ml	50 ug/ml
25 ug/ml	25 ug/ml
10ug/ml	10ug/ml
5ug/ml	5ug/nl
1ug/ml	1ug/ml
0.01ug/ml	0.01ug/ml
0.001ug/ml	0.001ug/ml

The concentrations were obtained from serial dilutions of the stock solutions as follows:

Table 3.10.1.: Dilution for incorporation tests: Isometamidium chloride



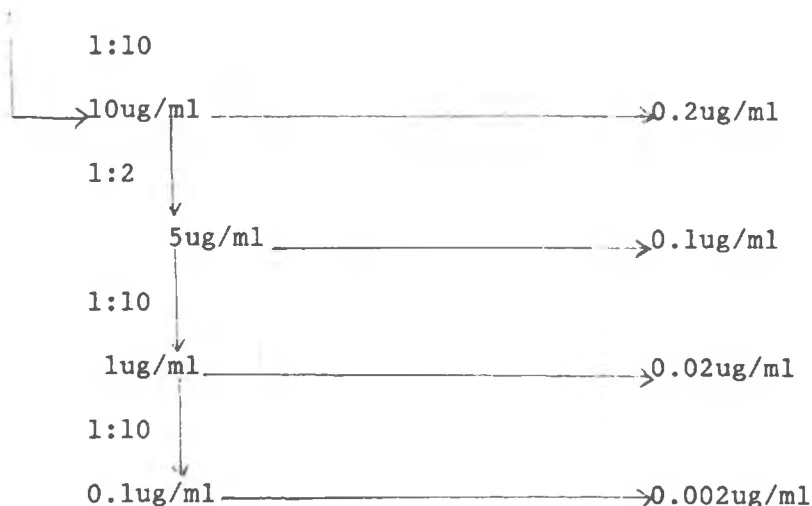


Table 3.10.2.: Dilution for incorporation tests: Diminazene aceturate

Stock Solution	Final Concentration
22.4mg/ml powder of Berenil[R]	
1:10 → by adding 100 ul in 1 ml	100 ug/ml
→ 50 ul in 1 ml	50 ug/ml
→ 20 ul in 1 ml	20 ug/ml
→ 10 ul in 1 ml	10 ug/ml
0.1mg/ml by adding 20 ul in 1 ml	2ug/ml
1:2 ↓ 50ug/ml →	1ug/ml
1:10 ↓ 10 ug/ml →	0.2ug/ml
1:2 ↓ 5ug/ml →	0.1ug/ml
1:10 ↓ 1 ug/ml →	0.02ug/ml
1:10 ↓ 0.1ug/ml →	0.002ug/ml

Final solutions for use in the incorporation tests were prepared by addition of the drug dilutions to each lml of the culture medium (as indicated in Tables 3.10.1. and 3.10.2. above).

3.9.2. Setting the Experiment for Incorporation Tests

Tests were performed in 96-well microtiter plates. The first two wells in each row were dispensed with medium without drug as controls and the other wells were dispensed with culture medium containing drug with final concentrations of 50, 25, 10, 1, 0.01, 0.001ug/ml of the drug. This was done by dispensing 50 ul of medium containing drug into each well.

A trypanosome suspension containing a defined number of trypanosomes/ml was prepared in culture medium and 50 ul was added to each well of the experiment plate. Each well was dispensed with approximately 1 to 8 x 10^5 bloodstream forms. The plate was incubated at 37^oC for 16 hours.

After 16 hours, 15ul of labelled precursor in a serum-free medium was added to each well and the plate incubated for a further 7 hours. After incubation the trypanosomes were harvested with a Skatron cell harvester on glass fibre filters (Flow Laboratories, Scotland, UK). The samples were washed with deionized water, dried, placed in 10 ml Aquasol (Biotechnology System, Massachusets, USA) and counted for 1 minute in a MR 300 liquid scintilation counter (Kontron Instruments, Becton Instruments Inc., Irvine, USA). Counts per minute (cpm) were expressed for each drug concentration as a

percentage of the controls. Each experiment was repeated twice and the results were recorded. The average background cpm were substracted from the mean cpm of each test.

3.11. DRUG INCUBATION SURVIVAL TEST ON DRUG SENSITIVE AND RESISTANT STOCKS OF T. VIVAX.

The experiment was set like the one for radiolabelled [3H] hypoxanthine, but this time without radiolabelled hypoxanthine. Instead, the normal Iscove's medium was employed and cultures were incubated for 24 and 48 hours at 34°C.

At the end of each time period, 10 ul of culture from each drug concentration were taken and the living trypanosomes were counted using a hemocytometer (Superior^{R}, West Germany). The criterion used for identifying the living trypanosomes was motility, hence motile trypanosomes were counted.

4. RESULTS

4.1. INITIATION OF IN VITRO CULTURES OF T. VIVAX INSECT FORMS FOR LONG TERM VIABILITY ASSAY

Apart from stimulating transformation of bloodstream forms to insect forms of stock IL 3060, normal medium (Table 3.1.) failed to stimulate transformation of stocks IL 2337, IL 2242 and clone 3185 (Table 4.1.). With stock IL 3060, a derivative of stock CP 2171, epimastigotes appeared after 5 days and died 10 days later. These experiments were performed at least in duplicate and were repeated several times and produced similar results.

By changing the molarity of hypoxanthine from 0.1 mM (normal medium) to 1 mM, the medium T_1 favoured the transformation of stock IL 3060 and IL 2337. Epimastigotes appeared after 5 days and died 7-9 days later. Repeated tests produced the same results.

Introducing a new additive to the normal medium, L-proline, at the rate of 4mM, the medium obtained (T_2) was able to stimulate transformation of all the three stocks of _T. vivax_ IL 3060, IL 2337 and clone IL 3185. Bloodstream forms transformed to epimastigotes after 5-7 days but the epimastigotes died after 10 days. Finally the medium was optimized by reducing the concentration of L-proline from 4.0 mM to 0.4 mM.

This medium (MT_2) was able to stimulate transformation of bloodstream forms of stocks IL 2242 (Figure 4.1.1.), IL 3060, IL 2337, IL 1392 and clone IL 3185 to epimastigotes. Epimastigotes of IL 2242 (Figure 4.1.2.), IL 3060, clone IL 3185 and IL 1392 were able to remain viable in culture for more than 35 days, time

sufficiently long enough for setting of long term viability assays. Stocks IL 1392, IL 3060, which is a derivative of stock CP 2171 (Figure 4.1.3.) and clone IL 3185 were still in culture after 116 days as epimastigotes (to the time of writing of these results).

Table 4.1. The effect of using different media on transformation of bloodstream forms to epimastigotes of different stocks of *T. vivax*.

Type of media	IL 3060/ CP 2171	IL 2242	IL 2337	IL 3185	IL 1392
Normal	+	-	-	-	-
TI	+	-	+	-	-
T2	+	+	+	+	-
MT2	+*	+*	+	+*	+*

\+ transformation from bloodstream forms to insect forms.

\- no transformation to insect forms.

* cultures remained viable as epimastigotes for more than 30 days and were used for drug sensitivity assays.

4.2. MAINTENANCE OF IN VITRO CULTURES OF T. VIVAX INSECT FORMS: MINIMUM NUMBER FOR SUB-PASSAGE

A stock suspension of epimastigotes was serially diluted from 10^6 to 10^1 trypanosomes per flask. Each dilution was done in duplicate and each flask was dispensed with 4.5 mls of trypanosome suspension and the flasks were incubated at 27°C. Daily checks were made to see the way the insect forms multiplied by growth from single trypanosomes to the formation of colonies (which sometimes merged to form a carpet on the bottom of the flask); depending on their number and density. The viability of such trypanosomes from lowest to highest dilutions was observed and noted as shown in Table 4.2.

Table 4.2.

MAINTENANCE OF IN VITRO CULTURES OF T. VIVAX INSECT FORMS OF CP 2331: MINIMUM NUMBER FOR SUBPASSAGE

Inoculum size: total number of trypanosomes in 4.5 mls of culture medium	Time period after setting up the experiment	Number of trypanosomes per field (x100 magnification)	Number of colonies per field	Merging of colonies to form a carpet
10^6	After 24 hours	>100	80	+
10^5		140	15	-
10^4		40	2	-
10^3		3	0	-
10^2		0	0	-
10^1		0	0	-
10^6	After 7 days	>100	100	+
10^5		>100	97	+
10^4		30	9	-
10^3		8	1	-
10^2		0	0	-
10^1		0	0	-
10^6	After 14 days	>100	>100	+
10^5		>100	>100	+
10^4		54	7	-
10^3		20	1	-
10^2		15	1	-
10^1		0	0	-
10^6	After 21 days	>100	>100	+
10^5		>100	>100	+
10^4		90	9	-
10^3		30	2	-
10^2		20	2	-
10^1		0	0	-
10^6	After 28 days	>100	>100	+
10^5		>100	70	+
10^4		>100	23	+
10^3		30	2	-
10^2		30	2	-
10^1		15	2	-
10^6	After 35 days	>100	>100	+
10^5		>100	>100	+
10^4		>100	>100	+
10^3		>100	>100	-
10^2		>100	1 bigger colony	-
10^1		>100	10 smaller colonies	-

+ formation of carpet by merging of colonies.
- no colony was seen.

Experiments with established insect forms of stock CP 2331 (Kipini) indicate that starting with a low number of inoculum such as 10 trypanosomes, it is possible to get an established culture after 35 days. Single trypanosomes from such an inoculum were not detectable until after 21 days. By increasing the size of the inoculum from 10 to 100, insect form trypanosomes were detectable after 14 days.

4.3. IN VIVO EXPERIMENTS IN CATTLE AND MICE

4.3.1. Drug susceptibility of various stocks of T. vivax in cattle and mice

Cattle and mice were infected with different stocks of T. vivax via needle or tsetse transmission. Diagnosis was done to determine positive infections (2.1.5.2.). Animals showing infection were treated with either isometamidium chloride or diminazene aceturate. The treated animals were kept under close supervision for a period of 4 months for cattle and 30 days for mice. These animals were examined after every 2 days for any relapse of infection. The trypanosome stocks that showed relapse infection to a particular dosage of the drug would suggest some degree of resistance. Table 4.3.1. indicates the sensitivity of various stocks to isometamidium chloride and diminazene aceturate.

Table 4.3.1. Drug Susceptibility of T. vivax stocks in cattle and mice

Species of animal used for the test	Animal identification number	T. vivax stock number	Mode of infection	Prepatent period (days)	Treatment (days after infection)	Isometamidium (chloride mg/kg)			Diminazene aceturate (mg/kg)			Remarks
						0.25	0.5	1	3.5	7.0	10.0	
Cattle (Bovine)	F 308	IL 3060	Needle	7	9	–	–	–	S	–	–	
	G 88	IL 2337	Needle	7	Died before treatment							
	G 150	IL 2337	Tsetse	11	14	–	S	–	–	–	–	
	G 156	IL 2337	Tsetse	11	X	X	X	X	X	X	X	
	G 106	IL 2242	Needle	7	10	–	–	–	R	–	–	
	G 222	IL 2242	Tsetse	9	X	X	X	X	X	X	X	
	G 232	IL 2242	Tsetse	9	11	–	S	–	–	–	–	
	G 107	IL 3185	Needle	6	8	–	–	–	S	–	–	
	G 163	IL 3185	Tsetse	10	12	–	S	–	–	–	–	
	G 217	IL 3185	Tsetse	10	X	X	X	X	X	X	X	
	G 72	IL 2133	Needle	14	16	–	–	–	R	–	–	
	–	CP 2171	–	–	–	–	R	R	S	S	–	Observed by Schonefeld et al., 1985
	–	CP 2331	–	–	–	–	R	R	S	S	–	Observed by Schonefeld et al., 1985
Mice	–	IL 1392	Needle	5-7	24 hrs	S	S	S	R	R	R	Observed by Kaminsky et al., 1990.
	–	IL 2005	Needle	3-5	24 hrs	S	S	S	R	R	–	

X = Indicate control animals; R = Resistant to; S = Sensitive to
– = Not done

4.3.2. Infection of cattle with metacyclics

A yearling animal (bovine) was infected by needle infection with 9×10^6 metacyclics of *T. vivax* stock CP 2171 (Bamburi) which had been in culture for a period of 2 1/2 years. When infection was established, trypanosomes (bloodstream forms) were isolated by differential centrifugation. Six calves were infected by needle infection each with 6.5×10^5 trypanosomes. These animals were divided into three groups each comprising of two animals. The first group was left without treatment (controls) while the second group animals were treated with 0.5 mg/kg isometamidium chloride and the third group with 1 mg/kg of the same drug. These animals were kept under observation for a period of three months and results are as shown in Table 4.3.2. below.

Table 4.3.2. Drug susceptibility in cattle infected with metacyclics of *T. vivax* stock CP 2171

Isometamidium chloride dosage	Animal identification number	Response to treatment	Days between treatment and relapse
0	BH 11	control	
	BH 13	control	
0.5 mg/kg	BH 7	-	23
	BH 9	-	26
1 mg/kg	BH 8	+	
	BH 12	-	51

Key: (+) = cured
(-) = not cured

Results indicate that of all the 4 treated animals BH 7, BH 9 and BH 12 treated with 0.5 and 1 mg/kg of isometamidium chloride were resistant to the drug at that dose.

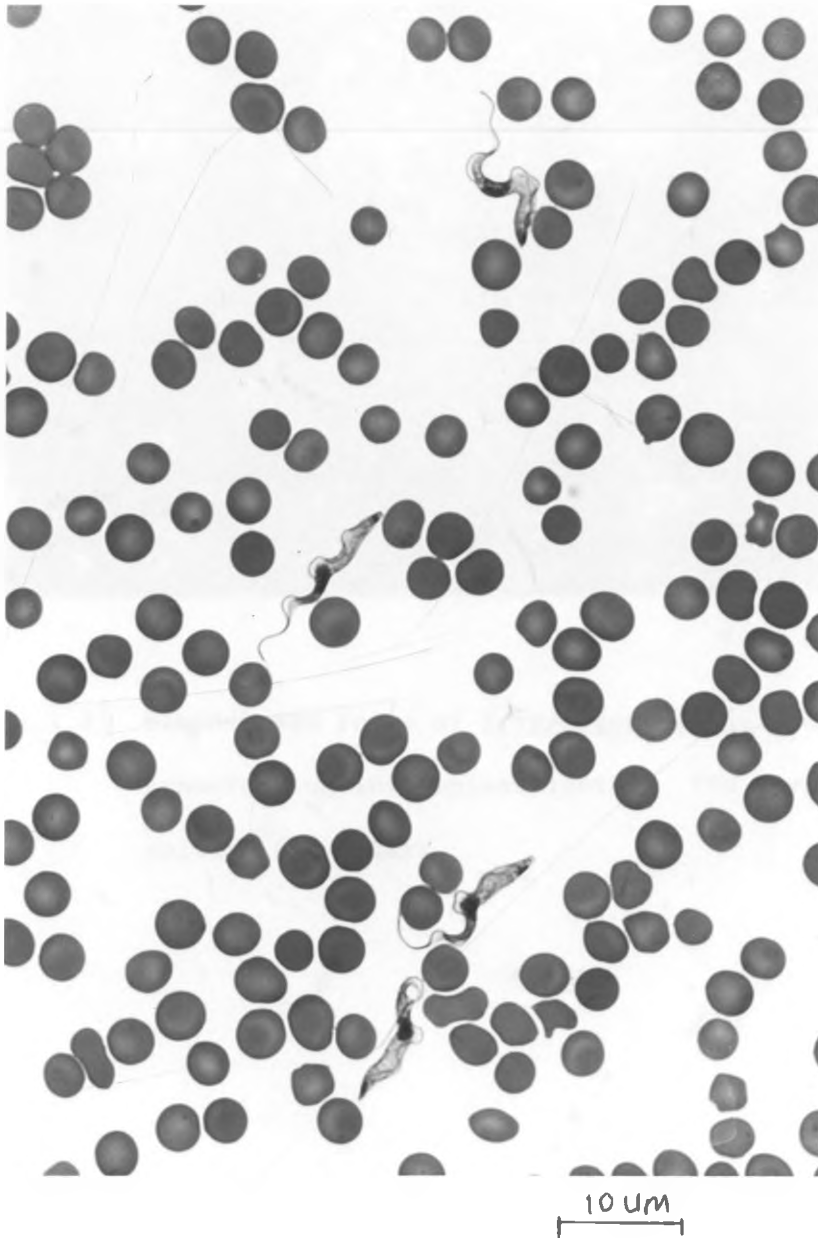


Figure 4.1.1. Bloodstream forms of Trypanosoma vivax stock IL 2242.

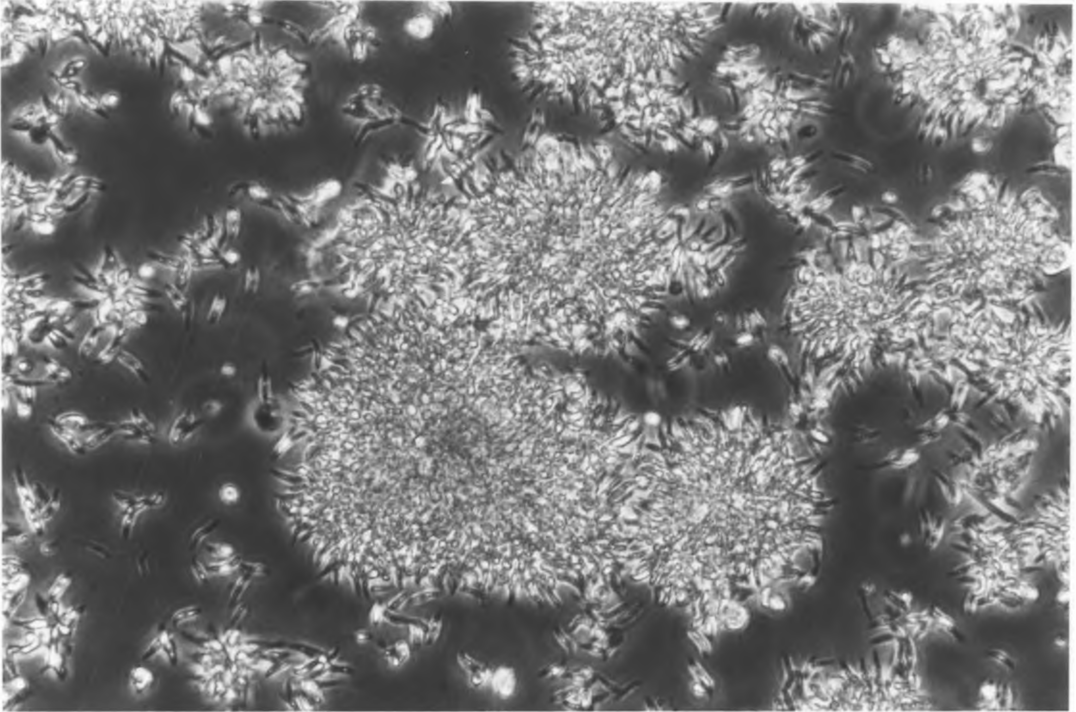


Figure 4.1.2. Bloodstream forms of *Trypanosoma vivax* stock IL 2242 transforming into epimastigotes. The trypanosomes were in culture for 4 days.

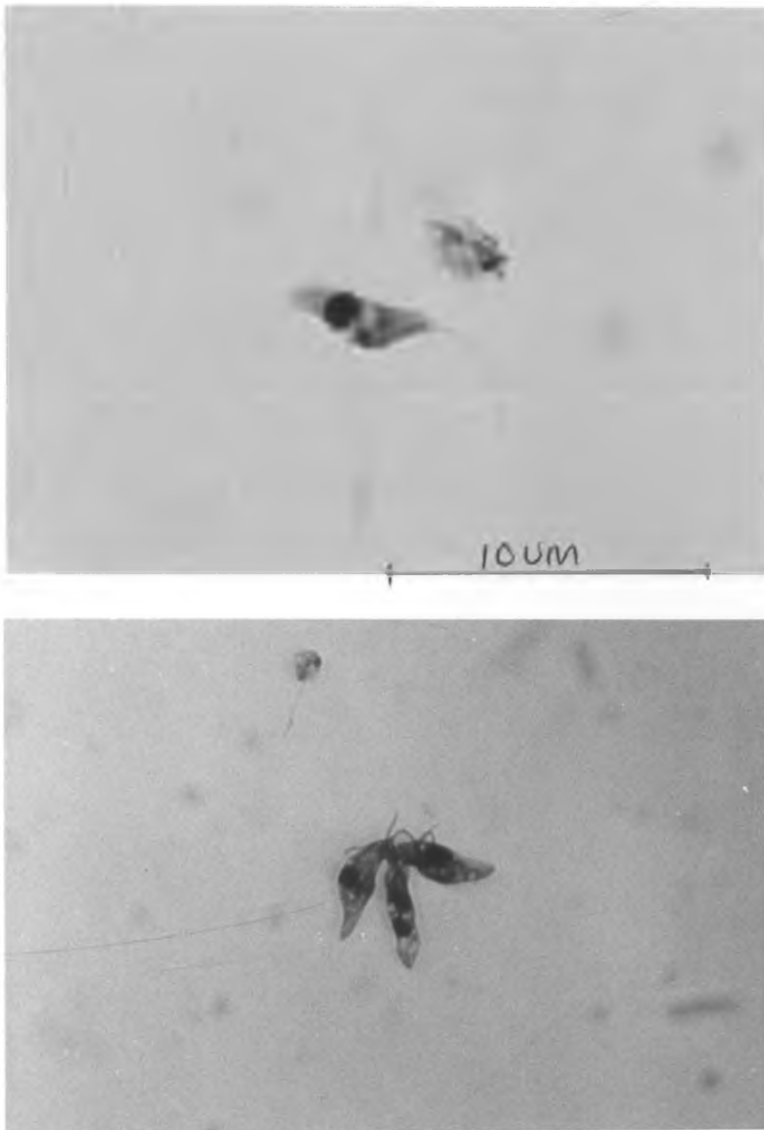


Figure 4.1.3. Epimastigotes of Trypanosoma vivax stock CP 2171. The trypanosomes had been propagated in culture for 38 days after isolation from a calf.

Figure 4.3.2. Metacyclics of Trypanosoma vivax stock CP 2171 used in infecting cattle during in vivo drug sensitivity tests. The cultures had been maintained for 2 1/2 years.

4.4. LONG TERM VIABILITY ASSAY.

4.4.1. Long term viability assay with insect forms of *T. vivax* exposed to isometamidium chloride

In this test, 4 stocks of *T. vivax* insect forms were tested. Two of the four stocks ie stock IL 1392 and clone IL 3185 were found to be sensitive to isometamidium chloride (0.25 and 0.5 mg/kg) as indicated by *in vivo* tests conducted in mice and cattle (Table 4.3.1.). Similarly, stocks CP 2331 and CP 2171 were found to be resistant to isometamidium chloride (0.5 mg/kg) as indicated by *in vivo* tests. By the time these *in vitro* tests were terminated, stock IL 1392 had been in culture for 44 days, clone IL 3185 had been in culture for 48 (1st isolation) and 51 (2nd isolation) days, stock CP 2171 for 570, 760 and 780 days for the first isolation and 38 days for second isolation, and CP 2331 for 480, 600 and 660 days respectively. The experiments were repeated twice for clone IL 3185, and thrice for stock CP 2171 and stock CP 2331. All 4 stocks were exposed to different concentrations of the drug ranging from 0.0001 to 0.01 ug/ml of the test drug in the medium. Each test was done in duplicates and the assessment of viability is shown in Table 4.4.1.

Table 4.4.1.

Viability of *T. vivax* insect forms in the presence of isometamidium chloride at 27°C.

T. vivax Stock Number	*In vitro* propagation (days) up to the termination of the test	Isometamidium chloride ug/ml	Days in culture with drug							
			6	8	10	12	13	15	17	20
IL 1392 (S)	44 days	0	+	+	+	+	+	+	+	+
		0.001	+	+	(+)	–	–	–	–	–
		0.01	+	(+)	–	–	–	–	–	–
IL 3185 (1st isolation) (S)	48 days	0	+	+	+	+	+	+	+	+
		0.0001	+	+	+	+	+	(+)	–	–
		0.001	+	+	+	(+)	–	–	–	–
IL 3185 (2nd isolation) (S)	51 days	0	+	+	+	+	+	+	+	+
		0.001	+	+	+	(+)	(+)	–	–	–
		0.01	+	+	(+)	–	–	–	–	–
CP 2171 (R)	570 days, 760 days, 780 days.	0	+	+	+	+	+	+	+	+
		0.0001	+	+	+	+	+	+	+	+
		0.001	+	+	+	+	+	+	+	+
		0.01	+	+	+	+	+	+	±	±
CP 2171 (2nd isolation) (R)	38 days	0	+	+	+	+	+	+	+	+
		0.001	+	+	+	+	+	+	+	+
		0.01	+	+	+	+	+	(+)	–	–
CP 2331 (R)	480 days, 600 days, 660 days.	0	+	+	+	+	+	+	+	+
		0.0001	+	+	+	+	+	+	+	+
		0.001	+	+	+	+	+	+	+	+
		0.01	+	+	+	+	+	+	+	(+)

Key: S = sensitive in cattle/mice, R = Resistant in cattle. Each experiment was done in duplicate and each was repeated at least twice.

\+ = denotes viable trypanosomes.

(+) = denotes dying trypanosomes as indicated by rounding in shapes and poor consumption of media.

– = denotes dead trypanosomes.

± = trypanosomes are dying or dead depending on a test.

Whereas clone IL 3185 and stock IL 1392 died after 12 and 17 days respectively, when incubated in medium containing 0.001 ug/ml of isometamidium chloride, stocks CP 2171 and CP 2331 were not affected. When the insect forms of stock IL 1392 and clone IL 3185 were exposed to the medium containing 0.01 ug/ml of isometamidium chloride they died after 10 and 13 days respectively, while those of CP 2171 and CP 2331 died after 17 and 20 days respectively.

4.4.2. Long term viability assay with insect forms of T. vivax exposed to diminazene aceturate.

In this test 4 stocks of *T. vivax* insect forms were tested: Stock CP 2331, CP 2171 and clone IL 3185 were found to be sensitive to diminazene aceturate (3.5 mg/kg) as indicated by the *in vivo* experiments conducted in cattle (Table 4.3.1.), Stock IL 1392 was less sensitive to the drug as indicated by the *in vivo* experiments performed in mice (Table 4.3.1.). By the time these *in vitro* tests were terminated, stock IL 1392 had been in culture for 60 days, clone IL 3185 for 51 days, stock CP 2171 for 720 and 780 days and stock CP 2331 for 660 days respectively. The experiment was not repeated twice for stocks IL 1392, CP 2331 but repeated twice for clone IL 3185 stock CP 2171. The insect forms were exposed to the medium containing 0, 0.01, 0.1, 1 and 10 ug/ml of diminazene aceturate for a period of 20 days. The test was done in duplicate and the results of the viability of the trypanosomes is shown in table 4.4.2.

Table 4.4.2.

Viability of *T. vivax* insect forms in the presence of diminazene aceturate at 27°C.

T. vivax Stock Number	*In vitro* propagation (days) up to the termination of the test	diminazene aceturate ug/ml	Days in culture with drug							
			4	6	8	10	12	13	17	20
CP 2331 (S)	660 days	0	+	+	+	+	+	+	+	+
		0.01	+	+	+	+	+	+	+	+
		0.1	+	+	+	+	+	+	+	+
		1	+	+	+	(+)	−	−	−	−
		10	+	+	+	±	−	−	−	−
CP 2171 (S)	720 days, 780 days	0	+	+	+	+	+	+	+	+
		0.01	+	+	+	+	+	+	+	+
		0.1	+	+	+	+	+	+	+	(+)
		1	+	+	+	+	(±)	−	−	−
		10	+	+	(+)	−	−	−	−	−
IL 3185 (S)	51 days	0	+	+	+	+	+	+	+	+
		0.01	+	+	+	+	+	+	+	+
		0.1	+	+	+	+	+	+	(+)	−
		1.0	+	(+)	−	−	−	−	−	−
		10	(+)	−	−	−	−	−	−	−
IL 1392 (R in mice)	60 days	0	+	+	+	+	+	+	+	+
		0.01	+	+	+	+	+	+	+	+
		0.1	+	+	+	+	+	+	(+)	−
		1	+	(+)	−	−	−	−	−	−
		10	(+)	−	−	−	−	−	−	−

Key: S = sensitive in cattle/mice.

R = Resistant in cattle.

The experiment was done in duplicates and each marker represents one experiment done in duplicate.

\+ = living trypanosomes.

(+) = abnormal growth or abnormal shape of trypanosomes.

− = dead trypanosomes.

The results (Table 4.4.2.) show that all the 4 stocks of *T. vivax* insect forms were sensitive to diminazene aceturate when incubated in the medium containing 10 ug/ml of the drug. Stock IL 1392 and clone IL 3185 showed marked sensitivity as both cultures died within 6 days, while stock CP 2171 and CP 2331 died after 8 and 10 days, respectively. When exposed to the medium containing 1 ug/ml, both stock IL 1392 and clone IL 3185 died after 8 days while, stock CP 2171 and CP 2331 died after 10 and 12 days. Both cultures of stocks CP 2171 and CP 2331 were not affected when these were exposed to the medium containing 0.1 ug/ml of diminazene aceturate. When incubated in the medium containing 0.1 ug/ml of the drug, stocks IL 1392 and clone IL 3185 died after 17 and 15 days, respectively.

4.5. DRUG INCUBATION INFECTIVITY TEST

4.5.1. The effect of blood plasma/cells on DIIT

Bloodstream forms of *T. vivax* stock IL 1392 were exposed to isometamidium chloride under varying conditions of trypanosome purification to determine whether isolation of trypanosome was necessary for the DIIT at 37°C. Trypanosomes were exposed to medium containing 0, 0.001 and 0.01 ug/ml of the drug and the results of infectivity to mice and the prepatent period are as shown in Table 4.5.1.

Table 4.5.1. DIIT: The effect of blood plasma/cells on DIIT with isometamidium chloride on IL 1392 at 37°C

Drug Conc. ug/ml		Isolated tryps 2.5×10^5 tryps/ml	Separated trypanosomes + washed red blood cells (RBC): 2.5×10^5 tryps/ml 2.5×10^5 RBC/ml	Trypanosomes in blood diluted with culture medium: 2.8×10^5 tryps/ml
0	Infectivity	5/5	5/5	5/5
	Prepatent period-pp (days)	7	7	9
0.001	Infectivity	0/5	0/5	0/5
0.01	Infectivity	0/5	0/5	0/5

Infectivity is expressed as the number of mice with infection over the total number of mice in the group.

Infectivity to mice was inhibited by incubating bloodstream forms of stock IL 1392 in medium containing 0.001 and 0.01 ug/ml of isometamidium chloride.

4.5.2. DIIT with stock IL 1392 and clones IL 2005 and IL 3185 incubated with isometamidium chloride at 37°C.

In this test 3 stocks of *T. vivax* bloodstream forms were tested by exposing them to culture medium containing different concentrations of isometamidium chloride ranging from 0 to 0.01 ug/ml of the drug and incubated at 37°C for 24 hours.

The prepatent period and their ability to infect mice was examined and the results are shown in Table 4.5.2.

Table 4.5.2. DIIT: Blood stream forms of stock IL 1392 and clones 2005 and 3185 incubated at 37°C for 24 hours in isometamidium chloride

Drug Conc. ug/ml		Trypanosome Stocks/clones		
		IL 2005	IL 3185	IL 1392
0	Infectivity	4/5	4/5	5/5
	prepatent period-pp(days)	13	6	7
0.001	Infectivity	0/5	0/5	0/5
0.01	Infectivity	0/5	0/5	0/5

Infectivity to mice of bloodstream forms of stocks IL 1392 and clone IL 2005 and IL 3185 was abolished when these were incubated in the medium containing 0.001 and 0.01 ug/ml of the drug.

4.5.3. DIIT: Bloodstream forms of stock IL 1392 and clone IL 2005 incubated at 37°C with diminazene aceturate.

T. vivax stock IL 1392 and clone IL 2005 bloodstream forms were incubated at 37°C in medium containing diminazene aceturate at 0, 0.001, 0.01, 0.1, and 1 ug/ml of the drug at 37°C for 24 hours

The prepatent period and their ability to infect mice was examined and the results are shown in table 4.5.3.

Table 4.5.3. Showing the infectivity and prepatent period (pp) of IL 1392 and Clone IL 2005 following their incubation with diminazene aceturate at 37°C.

Drug concen-tration ug/ml		Trypanosome stock/clone	
		IL 2005	IL 1392
0	Infectivity	5/5	5/5
	pp (days)	4	7
0.001	Infectivity	5/5	5/5
	pp	4	10
0.01	Infectivity	5/5	-
	pp	10	-
0.1	Infectivity	0/5	-
	pp	-	-
1	Infectivity	0/5	-
	pp	-	-

\- = Not done
pp = Prepatent period (days)

Infectivity to mice was not abolished when bloodstream forms of stock IL 1392 and clone IL 2005 were incubated in the medium containing 0.001 and 0.01 ug/ml of diminazene aceturate. They maintained their infectivity to mice. At 0.1 and 1 ug/ml of the drug concentration, the infectivity of both stock IL 1392 and clone IL 2005 was abolished.

4.6. INCORPORATION OF ^{3}H-HYPOXANTHINE BY T. VIVAX BLOODSTREAM FORMS UNDER DRUG PRESSURE

4.6.1. The effect of temperature on the 3H-Hypoxanthine incorporation by T. vivax bloodstream forms of clone IL 3185.

The ^{3}H-Hypoxanthine incorporation by *T. vivax* bloodstream forms of stock IL 3185 were set as explained in materials and methods (section 3.9.2.). ^{3}H-Hypoxanthine incorporation into trypanosomes were examined at 27, 34 and 37°C in medium containing 0 to 50 ug/ml of isometamidium chloride. Counts per minute (cpm) and the percentage of incorporation of ^{3}H-Hypoxanthine compared to controls are shown in Tables 4.6.1.1., 4.6.1.2., 4.6.1.3. and Figure 4.6.1.

Table 4.6.1.1. Incorporation of ^{3}H-Hypoxanthine by IL 3185 at 27°C.

Number of trypanosomes at start = 8x10^{5}/ml

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
11	0	28080.1 ± 4796	100±17
9	10	28690.7 ± 10256	102±37
12	0.001	59567 ± 12346	212±44

Table 4.6.1.2. Incorporation of ^{3}H-Hypoxanthine by IL 3185 at 34°C.

Number of trypanosomes at start = 8x10^{5}/ml

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
11	0	20763.4 ± 12895	100±62
12	10	19888.2 ± 5723	96±28
11	0.001	16438.9 ± 2897	79±14

Table 4.6.1.3. Incorporation of ^{3}H-Hypoxanthine by IL 3185 at 37°C.

Number of trypanosomes at start = 8x10^{5}/ml

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
10	0	3133.2 ± 463	100±15
11	10	1761.4 ± 325	56±10
11	0.001	5268.7 ± 948	168±30

Maximum incorporation of ^{3}H-Hypoxanthine occurred when the plates were incubated at 27°C (28080 cpm in control cultures). This was followed by 34°C (20763 cpm in control culture) and the incorporation was minimum when plates were incubated at 37°C, (3133 cpm in control cultures).

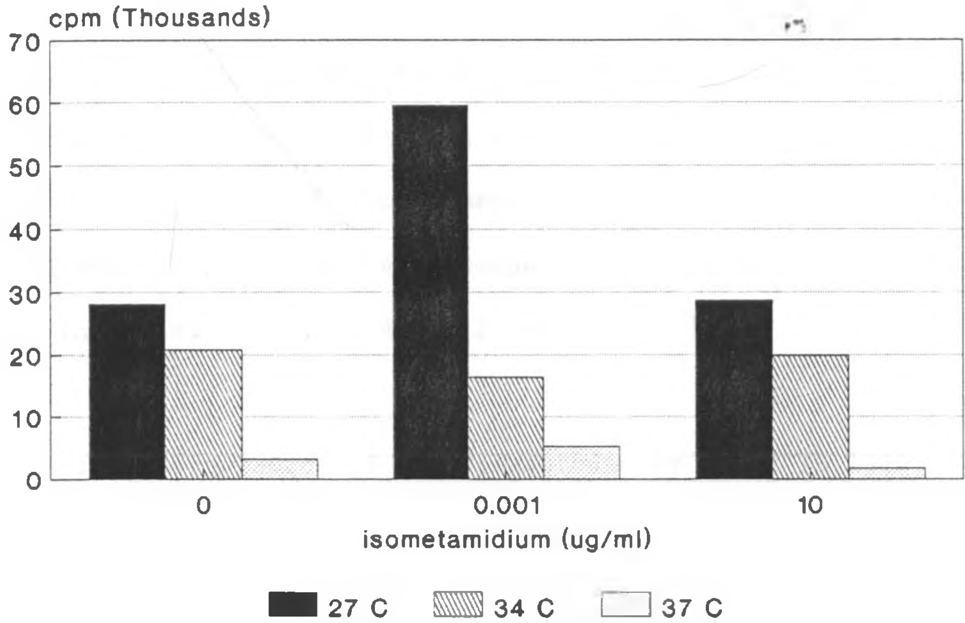


Figure 4.6.1. The effect of temperature on ^{3}H-Hypoxanthine uptake by <u>T. vivax</u> bloodstream forms of stock IL 3185 incubated at 27, 34 and 37°C in culture medium containing isometamidium chloride.

4.6.2. Incorporation of ^{3}H-Hypoxanthine by stock IL 1392 and clones IL 2005 and IL 3185 at 37°C in the presence of isometamidium chloride.

The blood stream forms of stock IL 1392 and clones IL 2005 and IL 3185 were incubated at 37°C in culture medium containing different concentrations ranging from 0 to 50 ug/ml of isometamidium chloride. Assessment of incorporation as indicated by cpm and the percentage of incorporation over the controls are shown in Tables 4.6.2.1., 4.6.2.2. and 4.6.2.3.

Table 4.6.2.1.: Incorporation of ^{3}H-Hypoxanthine by stock IL 1392 bloodstream forms at 37°C in the presence of isometamidium chloride.

Number of trypanosomes at start = 2.5×10^{6}/ml

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
2	0	2695 ± 81	100±3
5	10	237 ± 99	9±4
5	5	528 ± 184	19±7
6	1	697 ± 180	26±7
6	0.1	1456 ± 431	54±16
6	0.01	2861 ± 570	106±21
5	0.001	3677 ± 449	136±17

Table 4.6.2.2.: Incorporation of ^{3}H-Hypoxanthine by IL 3185 at 37°C.

Number of trypanosomes at the start = 2.5x10^{6} tryps/ml

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
8	0	10733 ± 2477	100±23
8	20	2256 ± 1462	21±14
8	10	4702 ± 1104	41±10
8	5	5732 ± 1606	53±15
8	1	6103 ± 1563	57±15
7	0.1	8622 ± 2434	80±23

Table 4.6.2.3.1.: Incorporation of ^{3}H-Hypoxanthine by IL 2005 at 37°C.

Number of trypanosomes at start = 2.5x10^{6} trypanosomes/ml

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
6	0	1227 ± 256	100±21
6	10	370 ± 183	30±15
5	5	635 ± 199	52±16
6	1	709 ± 172	58±14
6	0.1	751 ± 134	61±11
4	0.01	1673 ± 299	136±24
5	0.001	1784 ± 354	145±12

The uptake of ^{3}H-Hypoxanthine by bloodstream forms of stocks IL 1392, clones IL 2005 and IL 3185 at 37°C continued to fall with the increasing concentration of the drug in the medium. At very low concentrations of 0.01 and 0.001 ug/ml of the drug, the uptake of ^{3}H-Hypoxanthine exceeded that of the controls.

4.6.3. Incorporation of ^{3}H-Hypoxanthine by stock IL 1392 and clone 3185 exposed to isometamidium chloride at 27°C.

This test was performed as indicated in the materials and methods (3.9.2.). Bloodstream forms of stock IL 1392 and clone IL 3185 were exposed to different concentrations of isometamidium chloride ranging from 0 to 50 ug/ml of drug. Incubation temperature was 27°C. Assessment was made on the incorporation as determined by counts per minute (cpm) and the percentage of incorporation compared to controls was obtained as indicated in Tables 4.6.3.1., 4.6.3.2. and 4.6.3.3.

Table 4.6.3.1. Incorporation of ^{3}H-Hypoxanthine by stock IL 1392 at 27°C in the presence of isometamidium chloride.

Number of trypanosomes at start = 2.5×10^{6} trypanosomes/ml

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
13	0	2576 ± 1195	100±46
6	50	178 ± 235	7±9
5	25	1302 ± 1193	51±46
6	10	1636 ± 643	64±25
6	5	1854 ± 496	72±19
5	1	2051 ± 1022	77±40
8	0.1	2148 ± 557	83±22
8	0.01	2405 ± 1305	93±51
8	0.001	2239 ± 1058	87±41
8	0.0001	2288 ± 1053	89±41

Table 4.6.3.2. Incorporation of ^{3}H-Hypoxanthine by IL 3185 at 27°C.

Number of trypanosomes at start = $2x10^{6}$ trypanosomes/ml

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
8	0	54095 ± 14447	100±27
8	20	26907 ± 9113	50±17
8	10	34546 ± 10611	64±20
8	5	46479 ± 12714	86±24
8	1	59130 ± 11852	109±22
8	0.1	53819 ± 14944	99±28

Table 4.6.3.3. Incorporation of ^{3}H-Hypoxanthine by IL 3185 at 27°C.

Number of trypanosomes at start = $2x10^{6}$ trypanosomes/ml

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
4	0	31086 ± 6331	100±20
6	0.01	44339 ± 13523	141±44
6	0.001	50442 ± 10135	160±33
6	0.0001	47279 ± 14167	150±46

At very low dilutions of the drug, i.e. 0.01, the difference in the incorporation of ^{3}H-Hypoxanthine was not significant. At 10 ug/ml there was a fall in the amount of ^{3}H-Hypoxanthine incorporated by bloodstream forms of both stocks IL 1392 and clone IL 3185.

4.6.4. Incorporation of ^{3}H-Hypoxanthine by stock IL 1392 exposed to isometamidium chloride at 34°C.

The test was set as described in the section for materials and methods (3.9.2.). Bloodstream forms of stock IL 1392 were

exposed for 24 hours in culture medium containing different concentrations of isometamidium chloride ranging from 0 to 50 ug/ml of the drug. Incubation temperature was 34°C. Assessment was done on incorporation as indicated by counts per minute (cpm) and the percentage of incorporation compared to controls are shown in the Table 4.6.4.

Table 4.6.4. Incorporation of ^{3}H-Hypoxanthine by stock IL 1392 exposed to isometamidium chloride at 34°C.

Number of trypanosomes at start = $2.5x10^6$

Number of samples	Isometamidium chloride ug/ml	Mean cpm	Percentage Incorporation
6	0	4653 ± 2328	100±50
3	50	221 ± 493	5±11
4	25	1086 ± 112	23±2
3	10	3256 ± 1649	70±35
2	5	3669 ± 1742	79±37
4	0.1	4212 ± 1231	91±26
4	0.01	4342 ± 2590	93±56
2	0.001	5048 ± 2889	108±62

The amount of incorporation of ^{3}H-Hypoxanthine continued to decrease from a drug concentration of 0.001 ug/ml to 10 ug/ml. At concentration of 10 ug/ml and above there was a significant reduction in the incorporation of ^{3}H-Hypoxanthine.

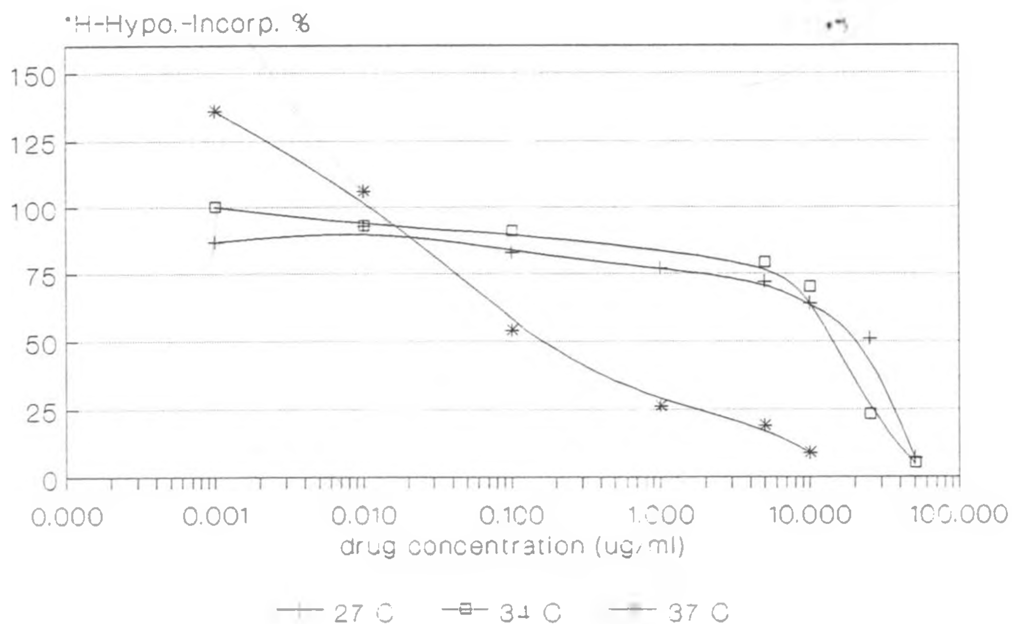


Figure 4.6.2. The effect of temperature on uptake of ^{3}H-Hypoxanthine by T. vivax stock IL 1392 incubated at 27, 34 and 37°C in culture medium containing different concentrations of isometamidium chloride.

4.7. DRUG INCUBATION SURVIVAL TEST

4.7.1 Drug incubation survival test for stock IL 1392, CP 2171, IL 2242, and clone IL 2005 exposed to isometamidium chloride at 34°C.

The tests were set as explained in the section for materials and methods (Section 3.10.). Bloodstream forms of stock IL 1392 and clone IL 2005 were exposed to different concentrations of isometamidium chloride ranging from 0 to 50 ug/ml of the drug. Counting of trypanosomes was performed after 24 and 48 hours of incubation to determine the percentage of survival compared to controls. Results obtained are as shown in Table 4.7.1. and Figures 4.7.1 and 4.7.2.

...le 4.7.1. DRUG INCUBATION SURVIVAL TEST AT 34ºC WITH STOCKS IL 1392, CP 2171, IL 2242 AND CLONES IL 2005 and IL 3185.

...etamidium ...oride ...g/ml)	0	50	25	10	5	1	0.1	0.01	0.001
...er of trypanosomes at the start ($\times 10^4$)/ml									
2242	150								
2242	106								
2005	119								
1392	113								
3185	140								
2171	175								
2171	185								
...er of trypanosomes ($\times 10^4$)/ml after 24 hours									
2005	60±11	0	0	0	0	0	4±2	15±4	33±6
1392	82±8	0	0	15±2	21±2	31±4	52±3	89±11	80±22
3185	180±41	0	0	28±2	89±13	193±11	180±14	188±5	142±8
2242	173±20	2±1	5±2	19±7	53±3	154±7	180±11	186±11	182±21
2242	146±10	43±9	44±2	39±10	85±8	145±5	155±21	150±14	149±15
2171	272±33	227±30	193±27	208+24	252±32	213±18	258±41	242±16	-
2171	125±5	22±2	31±5	82±9	79±15	90±7	113±12	122±8	-
...er of trypanosomes ($\times 10^4$)/ml after 48 hours									
2005	61±6	0	0	0	0	0	0	4±4	43±7
1392	47±7	0	0	0	0	0	4±3	22±5	45±10
3185	22±3	0	0	0	0	1±1	10±3	20±3	19±7
2242	83±9	0	0	0	0	23±4	96±13	99±13	84±14
2242	28±5	0	0	0	2±2	15±4	21±8	24±5	27±5
2171	215±6	0	3±3	43±8	198±10	253±14	226±15	209±24	-
2171	48±10	0	0	0	36±12	66±8	54±8	60±11	57±8

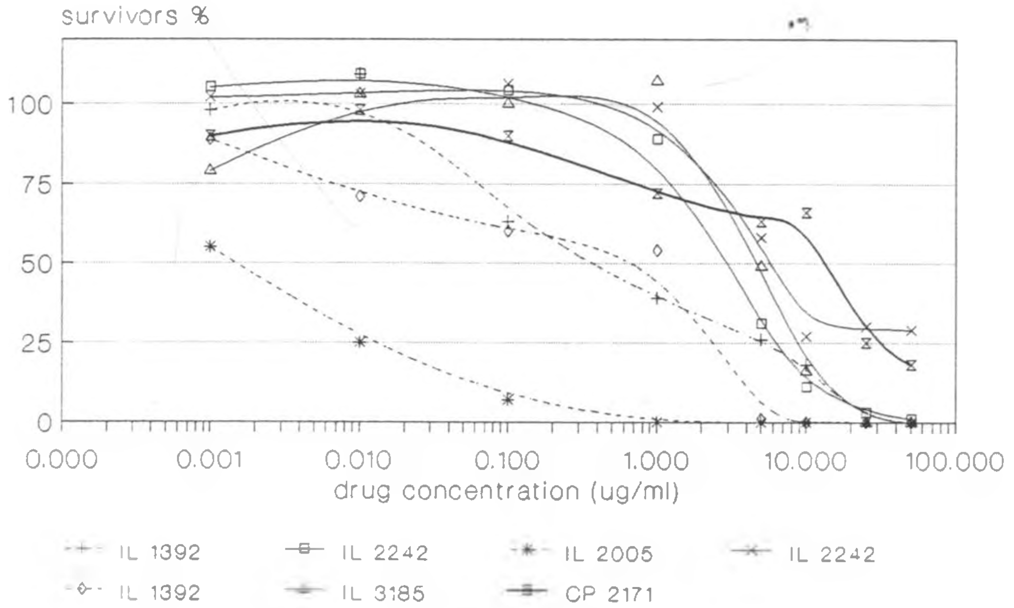


Figure 4.7.1. Drug Incubation Survival Test: Isometamidium Chloride on *T. vivax* bloodstream forms at 34°C (24h)

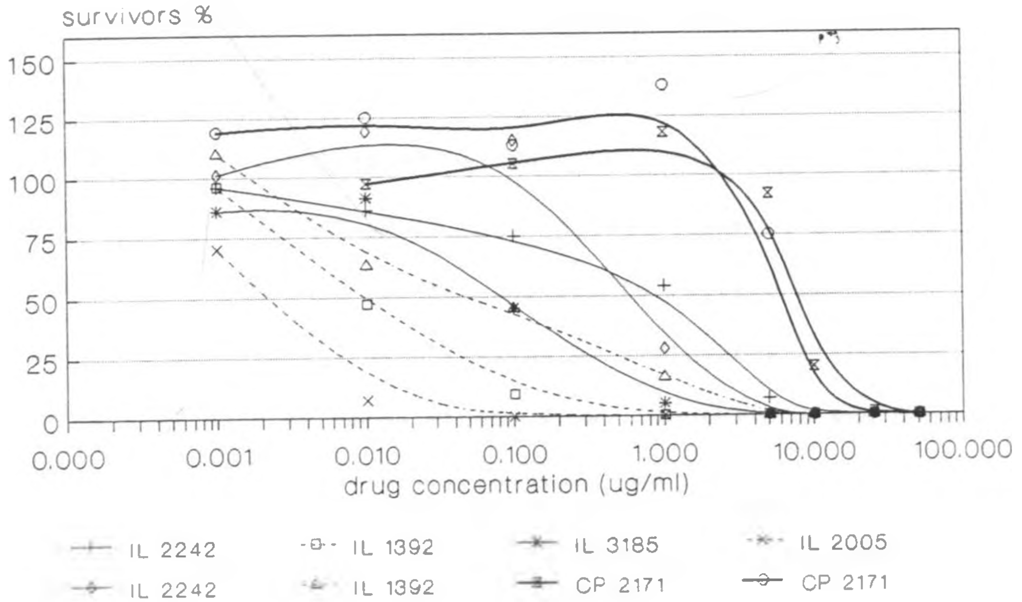


Figure 4.7.2. Drug Incubation Survival Test: Isometamidium Chloride on T. vivax bloodstream forms at 34°C (48h)

4.7.2. Drug incubation survival test with stock IL 1392. IL 2242. CP 2171 and clones IL 2005 and IL 3185 exposed to diminazene aceturate at 34°C.

The test was set as explained in the section for materials and methods (Section 3.10.). Bloodstream forms of stock IL 1392, CP 2171 and clone IL 2005 were exposed to culture medium containing different concentrations of diminazene aceturate ranging from 0 to 50 ug/ml of the drug. Incubation was done at 34°C. Counting of viable trypanosomes was done after 24 and 48 hours of incubation to determine the number and percentage of survivors compared to controls. The results are shown in the Table 4.7.2. and Figures 4.7.3. and 4.7.4.

The results indicate that all five stocks were affected when incubated in the medium containing different concentrations of the drug. Stocks IL 1392 and Clones IL 3185 and IL 2005 died within 24 hours when incubated in the medium containing 25 and 50 ug/ml of the drug but stocks IL 2242 and CP 2171 were not affected. Fourty-eight hours later, stocks IL 1392 and clones IL 3185 and IL 2005 died in all drug concentrations except those below 1 ug/ml of the drug. However, Stocks CP 2171 and IL 2242 were still viable at 25 ug/ml of the drug (Table 4.7.2.).

le 4.7.2.: DRUG INCUBATION SURVIVAL TEST AT 34°C WITH STOCKS IL 1392, CP 2171, IL 2242, AND CLONES IL 2005 AND IL 3185.

inazene aturate g/ml)	0	50	25	10	5	1	0.1	0.01	0.00
ber of trypanosomes (x10^4)/ml at the start									
2171	106								
2171	175								
1392	80								
1392	184								
2005	175								
2005	100								
3185	140								
2242	156								
2242	106								
ber of trypanosomes (x10^4)/ml after 24 hours									
2005	193±14	0	0	6±1	5±2	13±2	153±5	189±19	183±8
2005	166±17	0	0	0	2±1	6±4	105±12	207±27	220±7
3185	196±19	0	0	25±4	76±13	178±14	162±16	187±20	186±29
1392	77±10	0	0	34±6	35±4	46±6	61±5	62±11	61±11
1392	224±28	0	0	27±7	46±20	106±10	196+20	205±20	204±49
2242	163±26	10±3	48±7	82±8	83±22	128±6	163±19	178±11	170±27
2242	147±20	56±3	42±14	134±24	133±14	149±15	144±13	147±14	153±11
2171	59±7	68±8	72±11	67±12	74±6	82±15	79±15	106±28	-
2171	144±11	218±32	271±12	259±43	265±30	238±24	160±13	178±16	-
ber of trypanosomes (x10^4)/ml after 48 hours									
2005	155±41	0	0	0	0	0	33±4	155±22	157±17
2005	138±14	0	0	0	0	0	63±5	118±6	139±7
3185	17±7	0	0	0	0	0	13±1	16±4	17±6
1392	20±5	0	0	0	0	0	13±3	12±4	13±5
1392	62±11	0	0	0	0	0	32±7	62±8	62±6
2242	103±15	0	0	9±2	10±4	34±7	80±9	83±16	87±6
2242	19±5	0	1±1	4±3	7±2	14±1	18±7	21±6	18±4
2171	44±6	0	24±5	49±12	51±3	49±6	50±7	67±10	-
2171	58±12	69±5	206±22	180±20	183±20	241±19	74±4	67±12	-

The percentage of survivors in both sets of experiments increased with the dilution of the drug in the medium. At very low dilutions of the drug, eg 0.001 ug/ml the number of surviving trypanosomes approached those in the controls but the number continued to decrease with increase in the concentration of the drug in the medium.

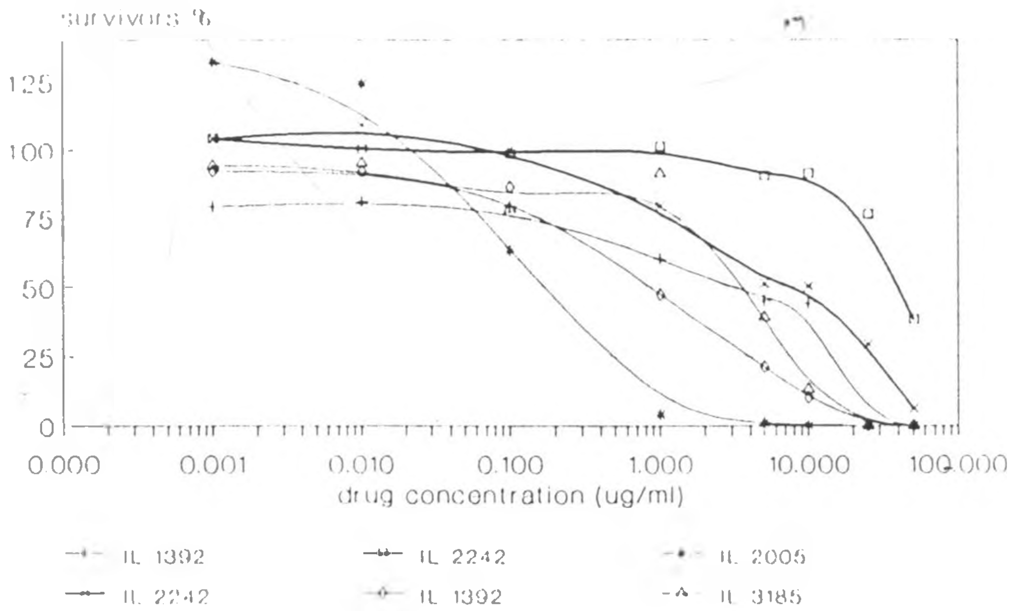


Figure 4.7.3. Drug Incubation Survival Test: Diminazene aceturate on *T. vivax* bloodstream forms at 34°C (24 h)

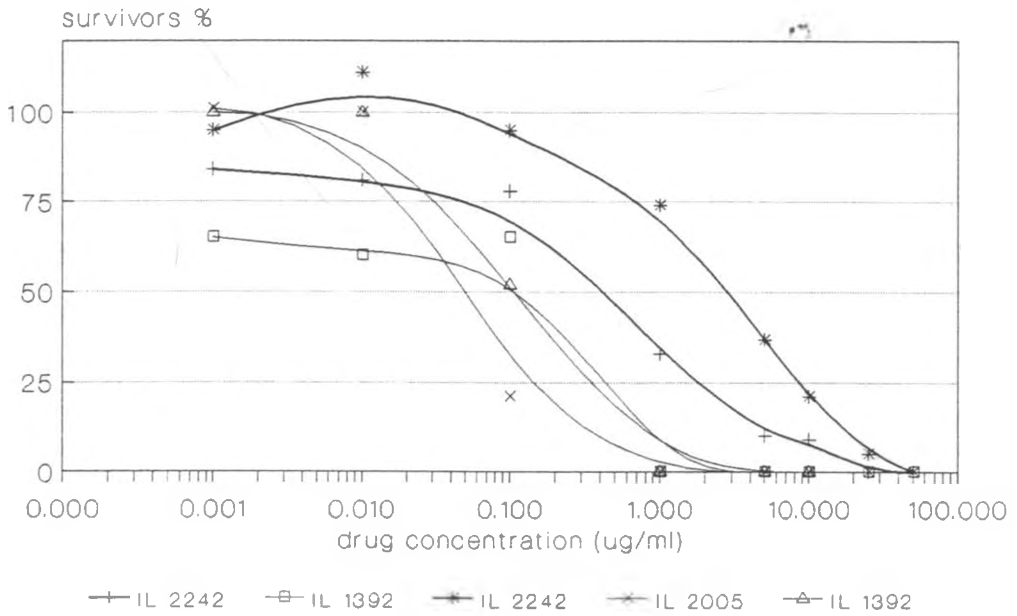


Figure 4.7.4. Drug Incubation Survival Test: Diminazene aceturate on *T. vivax* bloodstream forms at 34°C (48h)

5. DISCUSSION

5.1. Long Term Viability Assay

From the results of the long term viability assay with stocks IL 1392, CP 2171, CP 2331 and clone IL 3185 it can be stated that both diminazene aceturate and isometamidium chloride showed antitrypanosomal activity on insect forms of T. vivax. In the presence of the drugs, trypanosomes became rounded and eventually cultures died. This assay was able to distinguish between isometamidium chloride-sensitive and resistant stocks. Stock IL 1392 and clone IL 3185 died earlier (day 10 and 12) than stocks CP 2171 and CP 2331 (day 17 and 20) in 0.01 ug/ml. Stocks CP 2171 and CP 2331 were resistant to both 0.5 and 1.0 mg/kg of isometamidium chloride in cattle (Schönefeld et al., 1987). Both stock IL 1392 and clone IL 3185 were sensitive to 0.25 and 0.5 mg/kg of isometamidium chloride in mice and cattle.

The results confirmed earlier observations by Kaminsky et al. (1989a) on the effect of isometamidium chloride on T. vivax stocks IL 1392, CP 2171 and CP 2331. Incubation with culture medium containing 0.001 ug/ml of isometamidium chloride showed antitrypanosomal activity on insect forms of stock IL 1392 but had no antitrypanosomal activity on stocks CP 2171 and CP 2331. At 0.01 ug/ml of the drug both cultures of stocks CP 2171 and CP 2331 died on day 17 and 20 while those of stock IL 1392 died 7 days earlier (Kaminsky et al., 1989a). The results of the experiments with the long term viability assay indicated that there is a positive consistent correlation between in vitro and in vivo methods of assessing resistance to isometamidium chloride

using insect forms. These results were further confirmed by experiments with tsetse flies. When tsetse infected with isometamidium chloride-sensitive T. vivax IL 1392 were fed on a Boran steer previously treated with 1.0 mg/kg of the drug, trypanosome infection rates were greatly reduced (Kaminsky et al., 1989a). In contrast, infection rates in Glossina morsitans centralis infected with drug resistant T. vivax CP 2171 were unaffected when fed on the same drug-treated animal (Kaminsky et al., 1989a).

A suppressive action of isometamidium chloride on the cyclical development of T. vivax has also been demonstrated previously when tsetse were fed on blood containing isometamidium chloride (Hawking 1963a; Agu 1984, 1985; Moloo and Kamunya, 1987). The long term viability assay was not, however, able to distinguish between diminazene aceturate-sensitive and resistant stocks of T. vivax insect forms. Stock IL 1392 died earlier than stocks CP 2171, CP 2331 and clone IL 3185. Stocks CP 2171, CP 2331 and clone IL 3185 were sensitive to both 3.5 and 7.0 mg/kg of diminazene aceturate in cattle (Schönefeld et al., 1987). Stock IL 1392 was found to be less sensitive to 3.5 and 7.0 mg/kg of diminazene aceturate in mice. Hence there was no correlation between in vitro and in vivo methods in the assessment of sensitivity to diminazene aceturate.

Unlike procyclics of T. congolense and T. b. brucei (Elrayah and Kaminsky, 1989; Kaminsky et al. 1989b), epimastigotes of T. vivax can therefore be used to differentiate sensitivity of trypanosomes to isometamidium chloride. One major

setback is the long duration necessary to adapt trypanosomes to *in vitro* conditions, from the isolation of bloodstream forms from the infected animal to the setting of the test using insect forms. It takes 5-7 days for trypanosomes to transform from bloodstream forms to insect forms, an additional 7 days elapse before the culture can be passaged and the experiment set and 20 days for the long term viability assay. This makes a minimum period of 34 days before the results of a test are known. For this reason the assay is best suited as a tool for epidemiological study rather than short term curative purposes. More research is, therefore, required on the media and conditions which support transformation which would shorten the adaptation time to *in vitro* conditions by the trypanosomes.

5.2. Drug Incubation Infectivity Test - DIIT.

Using isometamidium chloride, there was a complete loss of infectivity to mice when bloodstream forms of stock IL 1392 and clones IL 3185 and IL 2005 were incubated in the culture medium containing 0.001 and 0.01 ug/ml of isometamidium chloride. Compared to other stocks of *T. b. brucei* and *T. b. evansi*, the tested *T. vivax* stock IL 1392 and clones IL 2005 and IL 3185 behaved like sensitive stocks of *T. b. brucei*, stock ILTat 1.4 and IL 2064 and *T. b. evansi* CP 1354 (Kaminsky *et al.*, 1990).

The presented study did not show, however, whether the test is able to distinguish between isometamidium chloride-sensitive and resistant stocks of *T. vivax*, since all the stocks of *T. vivax* used in this test were sensitive to isometamidium chloride

at the concentrations used . The possibility of testing isometamidium chloride resistance for comparison is limited by the poor infectivity of T. vivax stocks to mice (Anosa 1983, Kalu, et al., 1986).

Using diminazene aceturate, there was loss of infectivity to mice when bloodstream forms of stock IL 1392 and clone IL 2005 were incubated in the culture medium containing 0.1 and 1.0 ug/ml of diminazene aceturate. The infectivity was, however, not lost when trypanosomes were incubated in the culture medium containing 0.01 and 0.001 ug/ml of the drug. These results were similar to those obtained with diminazene aceturate sensitive stocks of T. b. brucei (Kaminsky et al., 1990). Like DIIT with isometamidium chloride, this study was not however able to distinguish between diminazene aceturate-sensitive and resistant stocks of T. vivax since both stock IL 1392 and clone IL 2005 were less sensitive and there was no diminazene aceturate-sensitive or resistant stock for comparison.

5.3. Incorporation of ^{3}H-Hypoxanthine by T. vivax bloodstream forms under drug presssure.

According to the results (Tables 4.6.1.1., 4.6.1.2. and 4.6.1.3.), highest incorporation of ^{3}H-Hypoxanthine occured when T. vivax bloodstream forms were incubated at 27°C, followed by incubation at 34°C and incorporation was lowest when cultures were incubated at 37°C (Figure 4.6.1.).

When dose (drug concentration) was plotted against response (uptake of ^{3}H-Hypoxanthine by trypanosomes) both incubations at

$27^{o}C$ and $34^{o}C$ produced sigmoidal shaped dose-response curves compared to incubation at $37^{o}C$ which produced linear shaped dose-response curves (Figure 4.6.2.). Incubation at $27^{o}C$ was, however, accompanied by transformation of bloodstream forms to insect forms (clone IL 3185) and the temperature did not support viability of some stocks of T. vivax (clone IL 2005). For this reason, it was decided to use $34^{o}C$ instead of $27^{o}C$ and $37^{o}C$ for experiments.

As the "shadow test" (Drug-Incubation-Survival-Test) would later reveal (Section 5.4.), the amount of ^{3}H-hypoxanthine uptake by bloodstream forms varied according to the survival rate of trypanosomes in culture medium which contained different concentrations of trypanocides. With higher concentrations of trypanocides (25 and 50 ug/ml) nearly all trypanosomes were killed (with the exception of resistant trypanosomes) while at very low drug concentrations (0.01 and 0.001 ug/ml) trypanosomes managed to survive and continued with their normal physiological functions eg. reproduction (increased in their numbers).

Similar results were obtained with T. b. brucei (Brun and Kunz, 1989) and T. congolense (Ross and Taylor, 1989). In both cases it was found that the response of trypanosomes to drug varied according to the concentration of isometamidium chloride in the culture medium.

Results presented here indicate that the test can be used to assess isometamidium chloride sensitivity of T. vivax bloodstream forms. The test is rapid. However, the disadvantage is the availability of sophisticated equipment such as a cell

harvester, radiolabelled chemicals and a liquid scintillation counter. These setbacks limit applicability of the test for broader use.

The ^{3}H-Hypoxanthine incorporation test was conducted according to the optimal test procedures found by Brun and Kunz (1989) for T. b. brucei and T. b. rhodesiense bloodstream forms incubated at 37°C. However, the differences in the amounts of ^{3}H-Hypoxanthine incorporation at different temperatures (Figure 4.6.1.) call for the standardization of the test to determine the optimal temperature and preincubation time for T. vivax bloodstream forms as compared to T. brucei isolates used by Brun and Kunz (1989).

5.4. Drug Incubation Survival Test

This test was set by incubating bloodstream forms at 34°C. This temperature was chosen because it did not support the transformation of bloodstream forms to insect forms and did not favour viability of some stocks of T. vivax. When the effects of the drugs on the survival of trypanosomes were analysed it was found that incubation at 34°C produced sigmoidal shaped curves contrary to incubation at 37°C which produced linear shaped curves.

From results on tables 4.7.1. and 4.7.2., comparison of controls after 24 hours of incubation showed that there was a marked drop in the percentage of survivors, from 100% at the start of the experiment to 36% for stock IL 1392, 25% for clone IL 2005 and 34% for Stock CP 2171. After 48 hours of incubation

the percentage of survivors were; 20% for stock IL 1392; 26% for clone IL 2005 and 13% for stock CP 2171. The percentage of survivors for clone IL 2005 did not change after 24 and 48 hours of incubation (25 to 26%) compared to stock IL 1392 (36 to 20%) and stock CP 2171 (34 to 13%).

Results with both isometamidium chloride and diminazene aceturate indicate that both drugs showed antitrypanosomal activity on T. vivax bloodstream forms. The survival of trypanosomes was higher in cultures which were incubated in the medium containing drugs in concentrations which were lower than 1 ug/ml. At high concentrations (25 and 50 ug/ml) the percentage of survivors was higher in cultures of resistant stocks. Using isometamidium chloride, counting after 24 hours indicated that stock CP 2171 was able to survive at both 25 and 50 ug/ml of drug concentrations which completely eliminated cultures of IL 1392 and IL 2005. After 48 hours of incubation, trypanosomes of stock CP 2171 could still survive at 5 ug/ml, a drug concentration which could not sustain any survival of stock IL 1392 and clone IL 2005. Stock CP 2171 proved to be more resistant compared to the other 2 stocks (Figures 4.7.1. and 4.7.2.).

Stock CP 2171 is resistant to isometamidium chloride at 0.5 and 1.0 mg/kg in cattle (Schönefeld et al., 1987) while stock IL 1392 and clone IL 2005 are sensitive to 0.25 and 0.5 mg/kg of isometamidium chloride in mice. Hence the results indicated that the test has the potential to distinguish between isometamidium chloride-sensitive and resistant stocks and this correlated well with in vivo experiments.

When using diminazene aceturate results showed that the drug exerted more effects on the viability of _T. vivax_ bloodstream forms of the stocks tested. The effects were markedly so after 24 hours of incubation. Both 25 and 50 ug/ml concentrations were trypanocidal for stocks IL 1392, IL 3185 and clone IL 2005, while stocks CP 2171 and IL 2242 were not affected (Figure 4.7.3. and 4.7.4.). Stocks CP 2171 and IL 2242 continued to survive in the medium containing 25 and 50 ug/ml. After 48 hours of incubation stock CP 2171 was still viable in the culture medium containing 50 ug/ml, which did not support the survival of other stocks.

Comparing with _in vivo_ results (Table 4.3.1.) stock CP 2171 was found to be sensitive to both 3.5 and 7.0 mg/kg of diminazene aceturate in cattle (Schönefeld _et al._, 1987), while stock IL 2242 was less sensitive to 3.5 mg/kg of diminazene aceturate and Stocks IL 1392 and clone IL 2005 were less sensitive to 3.5 and 7.0 mg/kg of diminazene aceturate in mice.

Comparing _in vivo_ with _in vitro_ results, it was observed that there was no correlation between the two sets of findings. Stock CP 2171 would have been expected to have low chances of surviving at 25 and 50 ug/ml concentrations compared to the (resistant) stock IL 1392, IL 2242 and clone IL 2005. This indicated that the test was not able to distinguish between diminazene aceturate- sensitive from resistant stocks.

This test has a better potential of being a useful test regarding isometamidium chloride sensitivity. The test is simple, rapid and does not demand elaborate/sophisticated

equipments other than a 96-well microtiter plate, and a hemocytometer. The only disadvantage being that the test is more laborious and time consuming. Before the test is put for field use more data is required on its validity. This involves testing a good number of trypanosomes from stocks with known *in vivo* drug sensitivities, which would then act as standards.

5.5. Stability of drug resistance after prolonged *in vitro* cultivation of *T. vivax* stock CP 2171 (Bamburi)

Results with *T. vivax* stock CP 2171 showed that after continuous propagation of this stock for a period of 30 months, the stock was still resistant to 0.5 and 1 mg/kg of isometamidium chloride. These observations confirm the findings of Gray and Roberts (1971a; 1971b) who demonstrated that transmission of trypanosomes through tsetse flies did not change their drug sensitivity patterns in cattle and Schönefeld *et al*., (1987) who showed that a prophylactic dose of isometamidium chloride did not prevent infection of cattle when challenged with metacyclic trypanosomes of tsetse infected with a resistant *Trypanosoma vivax*. Brown *et al*. (1987) found that adaptation of *Trypanosoma congolense* stocks to *in vitro* culture did not change their sensitivity to isometamidium chloride, although the period of cultivation was not mentioned.

These findings are also in agreement with that of Kaminsky and Zweygarth, 1989a) who observed that *in vitro* propagation of *T. b. brucei* did not change the drug sensitivity patterns of the trypanosome populations for at least 275 days.

However, under some conditions, changes in drug sensitivity of *T. congolense* have been observed (Nyeko *et al*., 1985; Peregrine *et al*., 1990.

5.6. CONCLUSIONS

5.6.1. *In vitro* Assessment of Isometamidium Chloride Resistance in *T. vivax*.

Looking at the four *in vitro* tests employed in this study, only three *in vitro* tests proved to be of potential use in assessing isometamidium chloride resistance in *T. vivax*. These tests are the long term viability assay at 27°C using insect forms of trypanosomes (LTVA), ^{3}H-hypoxanthine incorporation test at 34°C and the drug-incubation survival test at 34°C. The drug-incubation infectivity test (DIIT) at 37°C was shown to be of limited use compared to the other 3 *in vitro* tests because many stocks of *T. vivax* are not rodent infective, so the application of DIIT is limited to very few rodent-infective stocks, mainly from West Africa. Of the 3 *in vitro* tests mentioned before, the drug incubation survival test using bloodstream forms at 34°C proved to be the simplest and most rapid. Although not rapid, but simple and reliable was the long term viability assay using epimastigotes at 27°C. Both tests were able to demonstrate the antitrypanosomal effect of isometamidium chloride and distinguished between isometamidium chloride sensitive (IL 1392, IL 2005 and IL 3187) and-resistant stocks (CP 2171 and CP 2331). They showed a consistent positive correlation between tests in cattle and mice and *in vitro* tests in assessing isometamidium chloride resistance in *T. vivax* (Table 5.1). ^{3}H-Hypoxanthine incorporation test was able to show an antitrypanosomal effect of the drug on *T. vivax* bloodstream forms. However, the results did not include those of resistant

stocks as these were not tested. Furthermore, the test is limited by the availability of sophisticated equipment.

Hence the drug-incubation survival test at 34°C and the long term viability assay at 27°C were the only *in vitro* tests which proved to be reliable, gave reproducible results and indicated potential use for future field application.

5.6.2. *In vitro* assessment of diminazene aceturate resistance in *T. vivax*

Of all the four *in vitro* tests employed in this study, none was suitable to be of value in assessing diminazene aceturate resistance in *T. vivax*. DIIT was limited by infectivity to mice and both long term viability assay and drug incubation survival test at 34°C proved to be unreliable. Results obtained with the latter 2 *in vitro* tests were quite different from the *in vivo* sensitivities (Table 5.2). Whereas, long term viability assay at 27°C showed stock IL 1392 to be very sensitive to diminazene aceturate *in vivo*, tests in mice indicated that the stock was less sensitive to diminazene aceturate (Table 4.3.). Drug incubation survival test at 34°C indicated that diminazene aceturate had antitrypanosomal activity on trypanosomes, but like the long term viability assay at 27°C, the test failed to distinguish between diminazene aceturate sensitive and resistant stocks. Stocks which were less sensitive to diminazene aceturate *in vivo* (stock IL 1392 and clone IL 2005) appeared to be very sensitive in the *in vitro* tests. Similarly, stocks which were sensitive to diminazene aceturate in cattle (CP 2171) were less sensitive in *in vitro* tests. This shows that the *in vitro* tests

were not reliable because there was no correlation between *in vivo* tests in mice and cattle and *in vitro* sensitivities. Therefore, the tests cannot be used to assess diminazene aceturate resistance in *T. vivax*.

5.6.3. Stability of drug resistance after prolonged continuous *in vitro* cultivation.

Results of experiments with CP 2171 indicated that isometamidium chloride-resistance is stable after prolonged continuous *in vitro* cultivation. Results obtained in June/Aug. 1990 (Table 4.4.2.) were similar to those obtained by Kaminsky *et al*. (1989a) in January 1988.

Thus the *in vitro* characteristics of resistance to isometamidium chloride are stable after prolonged *in vitro* cultivation, so there is no danger of basing resistance as found by culture tests.

Table 5.1. INDICATION OF ISOMETAMIDIUM CHLORIDE SENSITIVITY IN *T. VIVAX*

	Technique					
Stock Number	Cattle	Mice	DIIT at 37°C	LTVA at 27°C	^{3}H-Hypoxanthine Incoporation test at 34°C	Survival test at 34°C
IL 3185	S	S	S	S	E	S
IL 1392	S	S	S	S	E	S
IL 2005	N/D	S	S	N/A	E	S
CP 2331	R	N/A	N/A	R	N/D	N/D
CP 2171	R	N/A	N/A	R	N/D	R

S = Sensitive

R = Resistant

N/D = Not done

N/A = Not applicable

DIIT = Drug-Incubation-Infectivity-Test

LTVA = Long-Term-Viability-Assay

E = Showed antitrypanosomal effect

Table 5.2. INDICATION OF DIMINAZENE ACETURATE SENSITIVITY IN *T. VIVAX*

Stock Number	Technique				
	Cattle	Mice	DIIT at 37°C	LTVA at 27°C	Survival test at 34°C
IL 3185	S	N/D	N/D	SS	S
CP 1392	N/D	R	R	SS	S
CP 2005	N/D	R	R	N/A	S
CP 2331	S	N/A	N/A	S	N/D
CP 2171	S	N/A	N/A	S	R

S = Sensitive

R = Resistant/Less sensitive

N/D = Not done

N/A = Not applicable

DIIT = Drug-Incubation-Infectivity-Test

LTVA = Long-Term-Viability-Assay

SS = Super sensitive

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