CHARACTERIZATION OF *TRYPANOSOMA CONGOLENSE* STOCKS FROM THE WEST AND EAST AFRICAN REGIONS USING MOLECULAR KARYOTYPING

BY

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1992

DECLARATION

A. This Thesis is my original work and has not been presented for a degree in any other University.

Rebecca Waihenya

B. This Thesis has been submitted for examination with my approval as an external supervisor.

Dr. R.A. Masake

C. This Thesis has been submitted for examination with my approval as a University supervisor.

Professor G.K. Kinoti

DEDICATION

This thesis is dedicated to Dr. Rachael Asike Masake and Dr. Vinand M. Nantulya for all their efforts.

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ABSTRACT

Trypanosoma congolense, which is responsible for most of animal trypanosomiasis in Africa, has a great diversity of variants. Differences occur between stocks from different geographical regions and also between stocks within the same area. In the present work molecular karyotyping was used to investigate the differences between stocks from two geographical regions.

Trypanosoma congolense stocks from West Africa (Gambia, Burkina Faso and Nigeria) and East Africa (Kenya and Uganda) were grown and cloned in irradiated mice. The DNA of the derived clones was subjected to Orthogonal Field Alternating Gel Electrophoresis (OFAGE) in order to investigate the genomic relationships amongst the clones.

All the clones analysed displayed one overall chromosome pattern resembling that of *T. congolense* IL Nat. 3.1 (savanna type) established in similar studies. The overall pattern was characterised by the presence of relatively small mini- chromosomes (approx. 50-150kb), lack of medium-sized chromosomes (200-400kb), and a preponderance of large chromosomes (400-750kb). There was no clear-cut distinction between clones from the eastern and the western regions of Africa. Similarities in chromosome profiles were frequently observed between clones from the same stock while distinct variations were noted in the chromosome profiles of clones belonging to different trypanosome stocks. A few clones derived from different trypanosome stocks exhibited similar chromosome patterns.

Cross protection experiments, carried out in mice, demonstrated that clones exhibiting similar chromosome profiles conferred protection against each other while those with different profiles did not. Molecular karyotyping would therefore seem to be a convenient and reliable technique for identifying *T. congolense* serodemes.

The chromosome profiles of the clones were observed to remain stable following chronic infection for four months in goats. The profiles of the chromosome-sized DNA molecules in terms of the number and size also remained stable following transmission through tsetse flies.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Trypanosoma congolense is a parasitic protozoan belonging to the genus *Trypanosoma* of the order Kinetoplastida. The disease caused by members of this group is known as trypanosomiasis. In Africa, *T. congolense*, *T. vivax* and *T. brucei brucei* are the causative agents of bovine, ovine and caprine trypanosomiasis while *T. simiae* and *T. evansi* are responsible for porcine and camelid trypanosomiasis, respectively. *T. vivax* also affects cattle in South America, while *T. evansi* affects livestock in South America and South East Asia. In Africa the disease in man is known as sleeping sickness and is caused by *T. brucei gambiense* and *T. brucei rhodesiense*. In South America, human trypanosomiasis is caused by *T. cruzi*, transmitted by triatomid bugs.

Many species of the wild mammals in Africa are infected with trypanosomes but they rarely suffer any ill effects from the infection. These serve as reservoirs of infection from where trypanosomes are cyclically transmitted to domestic animals and man by biting flies belonging to the genus *Glossina*. Mechanical transmission also takes place through the haematophagous flies such as the tabanids. The latter are especially important in the transmission of *T. evansi* and *T. vivax*. In horses, the disease dourine, caused by *T. equiperdum*, is transmitted through sexual contact.

Animal trypanosomiasis is characterized by fever, fluctuating parasitaemia, anaemia, emaciation, reproductive disorders, loss of productivity and if no effective treatment is administered, death results. The clinical presentation of animal and human trypanosomiasis varies in severity, depending on the susceptibility of the host, the species,the stock and the virulence of the trypanosome. Different stocks within a single trypanosome species are capable of inducing a variety of clinical manifestations such as mild, acute and chronic infection. Furthermore an asymptomatic carrier state can be produced. Different species and breeds of animals vary greatly in their susceptibility to trypanosomiasis, for example the Boran (*Bos indicus*) cattle of Kenya Coast are more susceptible to trypanosomiasis than the N'Dama of West Africa. Other factors that

influence the pathogenesis of trypanosomiasis are physiological and enviromental factors, age, sex, pregnancy, nutritional status, previous exposure to trypanosomiasis and intercurrent infection. Shortly after a natural trypanosome infection, acute disease usually developes which lasts several weeks and terminates in death, chronic disease or spontaneous recovery (Nantulya et al., 1986). Animals which survive remain infected for several months or years, with low levels of fluctuating parasitaemia and serve as reservoirs of infection.

The principal method for the diagnosis of trypanosomiasis is the demonstration of trypanosomes in the blood of infected animals. The diagnoltic techniques used include microscopic examination of wet blood films (Lumsden et al., 1973), the buffy coat/phase contrast method (Murray et al., 1977), the capillary concentration method (Woo, 1969; Walker, 1972), the miniature anion-exchange centrifugation method (Lumsden et al., 1981), mouse sub-inoculation and blood smears stained with a Romanowsky stain such as Giemsa's stain (Lumsden et al., 1973). All these are parasitological techniques which, with the exception of the fixed smears, demonstrate the presence of live trypanosomes in a host.

Alternative diagnostic procedures include those which detect anti-trypanosome antibodies. These techniques include solid-phase radioimmunoassay (S-RIA) (Masake et al., 1983; Musoke et al., 1981), capillary tube agglutination (Ross, 1971), indirect immunofluorescence antibody tests (Katende et al., 1987), indirect haemaglutination tests (Bone and Charlier, 1975) and enzyme-labeled immunoassays (Voller, 1977). These antibody detection assays, however, do not discriminate between cured and active infection. Recently, however, an enzyme-labeled immunoassay has been developed for the diagnosis of active infections. This assay facilitates demonstration of circulating trypanosomal antigens in the blood of the infected animal (Nantulya, 1989; Nantulya and Lindqvist, 1989).

Nucleic acid hybridization has also been used in the diagnosis of trypanosomiasis. The development of species and sub-species specific DNA probes has improved the sensitivity of the technique which has recently been applied in detection of mixed infections

in cattle (Nyeko et al., 1990) and also in tsetse flies (Majiwa and Otieno, 1989; McNamara et al., 1989). The problem with this technique is that the infected blood is first inoculated into mice and then the parasites from mice are purified and identified by a DNA probe. Even though DNA hybridization is sensitive and specific, its use as a routine epidemiological tool is more of characterization than diagnosis.

Trypanosomes are transmitted from wildlife reservoir hosts to man and the domestic animals mainly by tsetse flies (*Glossina*) and other biting insects. In the tsetse fly the trypanosomes undergo cyclical development that lasts 1-3 weeks, ultimately giving rise to metacyclic trypanosomes. On entering the fly *Trypanosoma brucei* bloodstream forms undergo active division in the midgut as large procyclic trypomastigotes. These probably penetrate the peritrophic membrane (PTM) of the gut to reach the ectoperitrophic space where they migrate via the oesophagus, proboscis lumen, and hypopharynx to the vector's salivary glands. Trypomastigotes adhere to the salivary gland epithelium and transform into epimastigotes. These differentiate further to give rise to free, non-dividing, metacyclic trypomastigotes that are infective to the mammalian (definitive) host (Vickerman et al., 1988).

Trypanosoma congolense follows the same developmental pathway as *T. brucei* except that the epimastigotes multiply when attached to the chitinous wall of the labrum and the premetacyclic trypanosomes migrate to the hypopharynx where they mature into metacyclics, taking about 25 days (Vickerman et al., 1988). *T. vivax* bloodstream forms attach to the proximal third of the labrum (Jeffries, 1984) and quickly change into epimastigotes that invade the labrum. The metacyclics mature in the hypopharynx just as for *T. congolense*.

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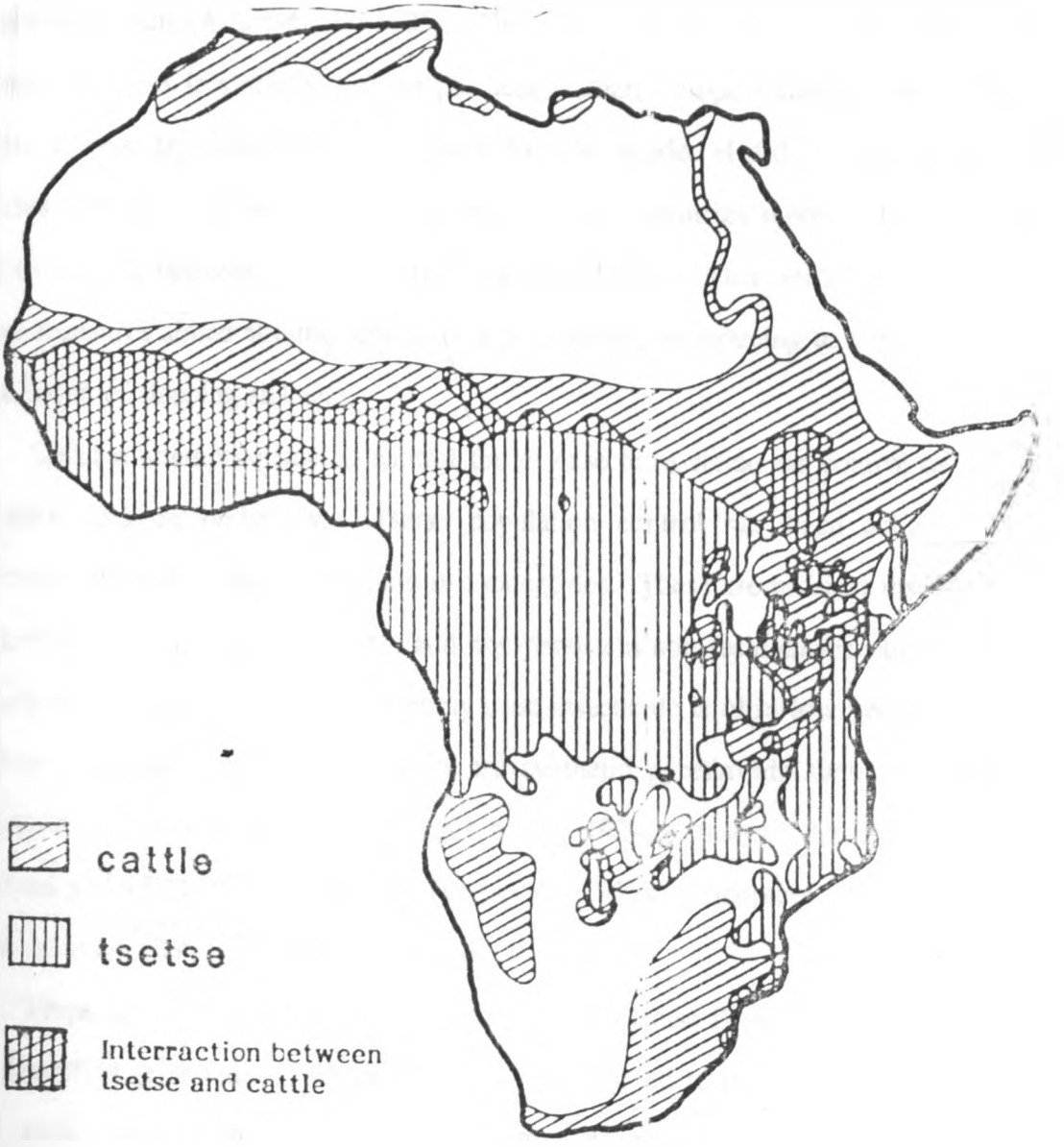
Fig. 1 : Distribution of cattle and tsetse flies in Africa



(Retrieved from ILRAD 1991 ANNUAL REPOR T Pg. 33)

In Africa the disease occurs in about 37% of the continent, covering an area of 9 - 10 million km^2. The distribution pattern of the disease correlates with that of its vector, the tsetse fly. In Africa, tsetse flies are found in a variety of habitats ranging from wooded savanna, forests to river banks. Fig.1 shows the tsetse distribution in Africa.

The available data shows that tsetse flies have continued to spread at an alarming rate (MacLenan, 1980). Approximately 50 million people and 30% of the cattle are at risk of trypanosomiasis (Allsop et al., 1985). This risk accounts in part for the fact that Africa produces 70 times less animal protein per hectare than Europe (Allsop et al., 1985). As regards human trypanosomiasis, a report by the World Health Organization (WHO) indicates that the number of cases diagnosed in nine countries more than doubled (from 5091 to 12117) between 1976 and 1983 (WHO, 1987). This situation calls for better control measures aimed at either eradicating (if possible) or reducing to a minimal level the transmission of sleeping sickness.

To date attempts made to control the disease have relied on control of the vector population and chemotherapy. Tsetse population control has been achieved through application of insecticides that fall into two categories. These are residual insecticides with long lasting effect (weeks to months) and non-persistent/non-residual insecticides requiring frequent application. The most commonly used insecticide is ultra-low volume endosulfan which is a residual insecticide. Others are synthetic pyrethroids such as deltamethrin, permethrin and cypermethrin (Spielberger et al., 1979). Insecticides are applied using motorized and knapsack hand sprayers (Lee et al., 1975), helicopters, fixed wing aircraft and can also be sprayed on traps and targets.

Traps and targets have also been used to catch tsetse flies. Targets comprising of blue electrified grids baited with an attractant have proved to be more effective than non-baited ones (Merot, 1988). Targets impregnated with deltamethrin have been used successfully in control of tsetse populations (Mawuena, 1988; Opiyo et al., 1988; Oladunmade et al., 1988). In one of such studies carried out in Togo, use of impregnated screens and targets reduced tsetse population (*Glossina morsitans centralis*) density in the experimental area from 4.6 - 0.1 flies/trap/day and the incidence of

trypanosomiasis in a cattle herd fell from 13.5% to 1.6%. The productivity in the experimental herd improved, no cases of abortion or calf mortality were recorded and the rate of parturition increased (Mawuena, 1988). Despite the effectiveness of traps and targets in reducing tsetse populations, it is apparent that complete eradication of the flies can only be achieved through an integrated programme incorporating insecticidal spraying, fly trapping and proper management of freed land.

Chemotherapy of trypanosomiasis as a means of parasite control in domestic livestock currently relies upon the use of suramin, quinapyramine, diminazene aceturate, homidium chloride and isometamidium chloride. Suramin (NAGANOL[a]) is a sulphonated compound which is ineffective in the treatment of *T. congolense* and *T. vivax* infections but effective against the infections caused by *T. b. brucei* and *T. evansi*. It is particulary useful in the treatment of equine trypanosomiasis when inoculated intravenously at a dose rate of 7-10 mg/kg body weight.

Quinapyramine dimethyl sulphate (TRYPACIDE SULPHATE[b]) is active against *T. brucei*, *T. congolense* and *T. vivax*. It is used as a curative drug for all domestic animals at a dose rate of 4.4 mg/kg body weight subcutaneously. Together with quinapyramine chloride it is used as a prophylactic agent. However, quinapyramine localises selectively in the liver and kidneys causing severe damage due to toxicity. Homidium chloride (NOVIDIUM[b]) is another therapeutic drug active against *T. vivax* infections in cattle. It is given intramuscularly at a dose rate of 1 mg/kg body weight.

Diminazene aceturate (Berenil[d]) is an aromatic compound effective for treatment of infections with *T. congolense* and *T. vivax* at a dose rate of 3.5 mg/kg body weight. But a higher dose rate of 5 mg/kg cures *T. brucei* infections. The drug is generally given via the intramuscular route even though it can be given subcutaneously. The drug is rapidly excreted through the kidneys within 24 hours, thus reducing the risk of chronic toxicity and development of resistance by the parasites following prolonged exposure to the drug.

Isometamidium chloride (SAMORIN[b]) has been used mainly for chemoprophylaxis at a dose rate of 0.25 - 0.5 mg/kg intramuscularly. It is used for protection against *T. congolense*, *T. vivax* and *T. brucei* infections in cattle.

The extensive use of trypanocidal drugs, particulary for the control of bovine trypanosomiasis, has resulted in the appearance of drug-resistant trypanosomes in many parts of Africa and elsewhere. The level of resistance encountered in the field is towards the recommended curative dose of the drug concerned. This is low compared to the degree of resistance that can be induced experimentally. The limited applicability of insecticides in high-rainfall areas and the possibility of environmental pollution by insecticides, the cost of a limited number of drugs and the development of drug resistance are some of the problems that make trypanosomiasis control difficult and expensive.

It has clearly been established that the trypomastigote possesses a characteristic electron dense coat (Vickerman, 1969; Cross, 1975) consisting of a 12 - 15 nm thick layer of tightly packed molecules which are known as the variable surface glycoproteins (VSG) and which cover the trilaminar plasma membrane. Each VSG molecule is anchored into the plasma membrane of the organism (Doyle et al., 1980).

During a trypanosome infection, an antibody response is mounted against the VSG of the trypanosome population. However, before this initial trypanosome population is cleared, some of the parasites switch off the gene controlling the initial VSG production and switch on a gene controlling the production of a different VSG (Van der Ploeg et al., 1984). This leads to the emergence of a parasite population that is unaffected by the initial antibody response. The new population is able to survive and multiply. The switching of genes can give rise to many different antigenic variants whose number has been estimated to be 100,000 in *T. brucei* (Van der Ploeg et al., 1982). This evasion of the immune response gives rise to a fluctuating parasitaemia that is characteristic of trypanosomiasis. This phenomenon of a fluctuating parasitaemia which enables the trypanosomes to survive in the immunocompetent host is referred to as antigenic variation (Doyle et al., 1980), and it curtails the efforts made towards the development of an effective vaccine based on the variant-specific antigenic epitopes on the VSG of bloodstream trypanosomes. Such a vaccine would have to be a cocktail of different surface epitopes and this is logistically impossible (Nantulya, 1986).

In view of the above constraints\ (limited number of drugs which are expensive, drug resistance, enviromental pollution due to insecticides, and the phenomenon of antigenic variation) alternative approaches for control of trypanosomiasis are being sought. One of the alternative approaches is the use of trypanotolerance. This is the ability of some breeds of livestock and several species of wild animals to survive and reproduce in tsetse infested areas in the absence of chemotherapeutic intervention (Murray et al., 1982). Amongst these are certain breeds of cattle in West and Central Africa, namely, the N'Dama (West Africa Longhorn), the West African Shorthorn cattle, and the West African dwarf sheep and goats (Trail et al., 1989).

Todate studies on trypanotolerant N'Dama cattle have concentrated on the relationship between trypanotolerance and animal performance, the association between disease resistance and major the histocompatibility complex (MHC) and common leucocyte antigens (CLA), and the development of a genetic linkage map in cattle with the prospect of using it to identify and isolate genes responsible for trypanotolerance (Trail et al., 1989). The use of advanced genetic technology, such as gene transfer, will make it possible to produce breeds that have high performance in tsetse infested areas.

Another approach to the control of trypanosomiasis is the development of more specific diagnostic tests and improved means of monitoring drug resistance to prolong the effective use of the currently available drugs. To achieve this a set of trypanosome species-specific monoclonal antibodies have been produced. Their use in diagnostic tests for trypanosome antigens in the bloodstream of infected livestock and identification of trypanosomes in tsetse is currently being assessed. Technologies are being established to quantitate the levels of chemotherapeutic agents in the blood and tissue fluids of treated animals (ILRAD Annual Scintific Report 1990).

1.2 Literature review

The sub-genus *Nannomonas* comprises the most pathogenic trypanosomes of domestic animals in Africa (Hoare, 1970). It is widely accepted that the subgenus consists of only two species. These are *Trypanosoma* (*Nannomonas*) *simiae*, associated with porcine trypanosomiasis and *T.* (*N.*) *congolense* which affects a wide range of hosts (Hoare, 1970). At the molecular level the two were found to differ significantly in that *T. simiae*-derived repetitive DNA sequence did not hybridize with DNA derived from clones of *T. congolense* (Majiwa and Webster, 1987).

T. congolense was first isolated by Broden (1904) from the blood of sheep and a donkey in the Congo (Zaire). He called it *Trypanosoma congolense*. Later in 1904 Laveran and Mesnil isolated trypanosomes from a horse in the Gambia and named them *Trypanosoma dimorphon*. This parasite was dimorphic consisting of both short and long forms and Laveran and Mesnil believed it was different from *T. congolense*. It was later renamed *T. confusum* by Montgomery and Kinghorn (1909). Laveran (1905) isolated another small *congolense*-type trypanosome from cattle in the Sudan and called it *T. nanum*. In a review by Bruce et al. (1910) all these trypanosomes were shown to be morphologically indistinguishable and were given one name, *T. pecorum*. In another review, Hoare (1959) proposed that *T. dimorphon* (15.3-17.6 μm) was distinctly different from the original form of *T. congolense* (12.2-14.4 μm). He postulated that if strains with intermediate lengths were discovered then *T. dimorphon* was to be considered a minor variant of *T. congolense*. Godfrey (1960) and Fairbairn (1962) described strains of intermediate lengths (12.98 - 13.85 μm and 12.45 - 13.85 μm, respectively). The two authors then proposed that on the basis of morphology *T. congolense* should be subdivided into 3 distinct sub-species: *T. congolense* proper, intermediate type and the long forms. For a long time there was confusion as to the existence of the three different morphological forms of *T. congolense* at different times during an infection. Nantulya et al. (1978) noted that at any point of parasitaemia there were several morphological types of the parasite, ranging from short to long forms. The authors postulated that during rising parasitaemia the short forms, considered "typical" *T. congolense*, predominated. While at peak

parasitaemia forms resembling *T. dimorphon* pre-dominated. In the stationary phase the intermediate type described by Godfrey (1960) was dominant though in low numbers. These findings led to the conclusion that *T. congolense* is polymorphic, consisting of short forms (11.3±1.09 μm), intermediate forms (14.4±0.87 μm) and long forms (17.0±1.03 μm). The parasite typically is devoid of a free flagellum and possesses a medium-sized kinetoplast, usually occupying a marginal position. The posterior end of the body is rounded in the short forms or pointed in the longer forms (Hoare, 1970).

Evidence exists however that *T. congolense* is a collection of a diversity of forms and not just a single species within the sub-genus *Nannomonas* (Godfrey, 1982). *T. congolense* stocks differ in a number of characteristics. In West Africa, the parasite causes a mild chronic infection, while in East Africa it usually causes acute or sub-acute disease (Stephen, 1970). *T. congolense* is cyclically transmitted by tsetse flies to a wide range of hosts such as cattle, horses, goats, sheep, camels, pigs as well as dogs. The difference in virulence accounts for the variability in clinical manifestations of the disease in the mammalian host. *T. congolense* is infective to all laboratory rodents. However, as in natural infections, there are differences in the infection rates of the different strains for laboratory animals (Hoare, 1970). Earlier reports described forms of *T. congolense* that were poorly infective to rodents, some of them surviving in rats for a long period at low parasitaemia (Godfrey, 1982). Such forms of low infectivity may be common in the field but are not experimentally dealt with. This is because in the laboratories experiments are carried out using strains that are highly infective in order to obtain quick results .

Host responses during an infection differ according to the isolates of *T. congolense*. For instance certain forms of *T. congolense* isolated from Kilifi along the Kenyan coast did not induce chancre (host immunological response) while isolates from Serengeti were observed to induce chancre formation (Paling et al., 1987). Variation in drug sensitivity has also been observed, for instance,by Moloo and Kutuza (1990) who demonstrated that trypanosomes isolated in the early stage of *T. congolense* infection in cattle were less sensitive to isometamidium chloride and diminazene aceturate than those isolated later in the infection.

Diversity occurs also within other trypanosome species such as *T. vivax*, another pathogenic trypanosome of cattle which occurs in West Africa, East Africa and South America. Most East African stocks produce mild infections which livestock in good condition can limit, but some forms cause an acute haemorrhagic syndrome. In West Africa the disease is often more acute, untreated cases being usually fatal (Gardiner and Wilson, 1989).

T. brucei consists of three subspecies: *T .b. brucei*, which is infective to domestic livestock, and *T. b. gambiense* and *T. b. rhodesiense* cause, respectively, chronic and acute trypanosomiasis in man. All the three subspecies are infective to laboratory rodents,but *T. b. brucei* does not infect man. *T. b. gambiense* and *T. b. rhodesiense* have some wild animals as reservoirs from where cyclical transmission by tsetse flies occurs.

Differences within single trypanosome species are quite diverse such that identification of a trypanosome as *T. congolense* or *T. brucei* is inadequate. Intraspecific variation is also common in other parasites such as *Plasmodium* and *Leishmania*. This diversity has led to the development of techniques that will help to characterize different strains and variants of a particular species.

Isoenzyme electrophoresis has been used widely in the characterization of parasitic and non-parasitic protozoa, including *Theileria*, *Leishmania*, *Plasmodium*, *Paramecium* and *Trypanosoma* (Al-Taqi and Evans, 1978; Melrose and Brown, 1979; Carter, 1973; Tait, 1969; Gibson et al., 1980).

Isoenzyme electrophoresis has been used to characterize *T. congolense*. This procedure enabled Gashumba (1986) to differentiate two stocks of *T. congolense* isolated from a sheep and a goat on the Kenya Coast from 112 other stocks of the same species using eight enzymes. In another study a total of 114 stocks of *T. congolense* originating from Kenya, Uganda, Tanzania, Zambia and Sudan and for comparison stocks from Gambia, Liberia, Cote d'Ivoire, Nigeria and Cameroon were analyzed using electrophoresis for 6 enzymes. Three different zymodemes were categorized according to both geographical and ecological zones. One zymodeme consisted of stocks from the East

Africa region only, the second had stocks from both the East and the West Africa regions and the third consisted of stocks from the Kenya Coast (Gashumba et al., 1988).

A combination of isoenzyme electrophoresis and DNA hybridization was applied by Knowles et al. (1988) in characterization of *T. congolense*. In this study, 27 stocks of *T. congolense* from Kilifi displayed completely different zymodemes from those that had been studied previously and their DNA failed to hybridize with a repetitive sequence probe (Pg NRE-372) obtained from a clone derived from a different stock of *T. congolense* which had originated from (Matuga) another location on the Kenya Coast. Several authors have observed zymodeme diversity in other trypanosome species (Gashumba, 1985; Gashumba, 1986; Gibson et al, 1980; Gashumba et al., 1986; McNamara, 1989). Comparative studies done on the pathogenicity of three zymodemes revealed marked differences in the type of disease caused by the savanna and the riverine types of *T. congolense* (Mohammed, 1989).

Hoare (1972) used the word "serodeme" to define immunologically distinct strains characterized by stability and absence of cross-immunity. Van Meirvenne et al (1975) used the word in a broader sense to mean a set of antigenic variants which can all be derived one from the other. This means that within a serodeme any antigenic variant can be used to generate several others, this is the definition applied in the present work. The number of serodemes varies from one stock to the other. Serodemes within a single stock differ in various characters such as host specificity, infectivity and host response (Luckins et al., 1980; Wellde et al., 1989; Gardiner, 1989; Milligan, 1989;Dwinger et al.,1990).

In *T. congolense* the number of serodemes is not known. Various methods have been adopted in order to identify serodemes. During a trypanosome infection, each parasitaemic wave represents a mixture of variable antigen types (VATs). All the VATs are however not expressed during infection. Moreover, the generation of bloodstream VATs is non-sequential (Masake et al., 1983). This makes it difficult to predict the antigenic repertoires (serodemes) using the bloodstream VATs. For instance,one of the techniques used involves cloning of the VATs for each repertoire and preparation of antisera against each VAT to determine whether or not any given repertoires are different. The respective VATs are then serotyped by immunolysis or indirect immunofluorescence

tests (Nantulya et al., 1980; Masake et al., 1983, 1987, 1988; Crowe et al., 1983; Frame et al., 1990).

In *T. vivax* one serodeme has been characterized from two East African stocks obtained from two seperate areas (Nantulya et al.,1986). In *T. evansi* three serodemes have been identified from fifteen stocks obtained from Sudan (Jones and McKinnell, 1985). Jones and McKinnell observed that certain varible antigen types were predominant in relapse populations and thus were important in serodeme identification.

Unlike the bloodstream VATs whose numbers are large and unpredictable, metacyclic VATs (M-VATs) have limited and predictable numbers (Nantulya et al., 1980, 1983; Crowe et al., 1983). Experimentally it has been shown that different bloodstream VATs of a serodeme of *T. congolense* revert to a specific set of metacyclic surface antigen types. However, metacyclics of different *T. congolense* serodemes have different sets of surface antigens. Thus immunization of mice against metacyclic challenge showed that the immunity acquired was serodeme specific (Nantulya et al., 1980). This observation formed the basis for characterization of *T. congolense* serodemes by metacyclic VAT typing. Using this approach 4 serodemes were identified in *T. congolense* stocks from a ranch in Kilifi district on the Kenyan Coast (Masake et al., 1987). The major drawback of serological typing of M-VATs as a basis for serodeme identificaion is that stocks have to be passaged through tsetse flies to obtain metacyclics for typing and for raising anti-M-VAT antisera. This is tedious and time consuming. Moreover, the infection rates in tsetse flies may be low, 0 - 15% (Nantulya et al., 1978).

Frame et al. (1990) grew metacyclic forms *in vitro* and raised monoclonal antibodies which they used to identify at least 7 serodemes of *T. congolense* by the indirect immunofluorescence test and by cross protection expriments. However the introduction of specific and highly sensitive monoclonal antibodies against each of the VATs, may not be efficient due to the fact that VAT repertoire is quite enormous.

African trypanosomes contain numerous variant surface glycoprotein (VSG) genes, but express only one at a time though double expressors exist. It has been speculated that for this expression to occur, there must be a single expression site into which VSG genes

move either by duplicative transposition or by a non-duplicative process (Van der Ploeg et al., 1984). At the single expression site there is intra and inter-chromosomal translocation of genes. All these speculations about the expression of a certain VSG gene can be verified by analysing the chromosome arrangements in order to investigate the expression site responsible for the various antigens exposed on the surface coat of a trypanosome. Unfortunately, trypanosome chromosomes do not condense during the cell cycle to form structures that can be analysed using cytochemical techniques. A method has however been developed that fractionates chromosome-sized DNA molecules according to size (Schwartz and Cantor, 1984). This technique was initially used to separate macromolecules of yeast cells but it has since been applied to the chromosome analysis of *Plasmodium falciparum* (Kemp et al., 1985; Babiker et al., 1991), *Leishmania* spp (Giannini et al., 1986), *T. cruzi* (Aymerich and Goldenberg, 1989), *T. brucei* (Van der Ploeg et al., 1984) and *T. congolense* (Masake et al., 1988). The technique involves resolution of chromosome-sized DNA molecules by pulsed field gradient (PFG) gel electrophoresis. The technique is based on two principles. First, the DNA molecule is stretched by a gradient field and then it is exposed to alternating pulses of current perpendicular to each other. The rate at which the molecules re-orient and migrate is dependent upon their sizes; the smaller particles move faster than the larger ones (Schwartz and Cantor, 1984). A modification of PFG electrophoresis known as Orthogonal Field Alternating Gel Electrophoresis (OFAGE) increases the resolution of large DNA molecules (Carle and Olson, 1984).

Application of the OFAGE to *T. congolense* has revealed two molecular karyotypes, namely, savanna and Kilifi types which differ genomically in their repetitive DNA sequences (Majiwa et al., 1985, 1986a and b).

Application of the OFAGE to *T. brucei* has made it possible to explore the possiblity of genetic exchange which is thought to occur in the midgut of tsetse flies due to the differences between the parental and the hybrid genetic material (Wells et al., 1987; Schweizer and Jenni 1991). Significant inter and intra-strain differences in molecular

karyotype have been shown to correlate to the high degree of phenotypic heterogeneity among *T. cruzi* strains (Engman et al., 1987).

Masake et al. (1988) analysed the chromosome profiles of *T. congolense* clones derived from 54 stocks isolated from a herd in Kilifi plantations and grouped them into 18 distinct chromosome patterns. Cross neutralization assays were conducted using pooled serum recovered from animals infected with a specific clone. The results obtained were that the pooled serum raised against a specific clone neutralized all trypanosomes with an identical chromosome pattern (karyodeme) but not those belonging to a different karyodeme. This clearly indicated that clones that exhibited a similar chromosome profile also expressed an identical VAT-repertoire (serodeme).

The chromosome profiles of the clones remained stable following infection in mice, goats, cattle and transmission through tsetse flies (Masake et al., 1988). This stability in number, size and position of chromosome-sized DNA molecules was observed at 3, 5 and 12 months post infection in goats and cattle (Masake et al., 1988). In view of the fact that these chromosome profiles would represent serodemes, and that these profiles were stable, it was felt that OFAGE technique may offer a much faster approach to serodeme identification than the commonly used serological techniques.

1.3 Objectives of the present study

In view of the fact that serodemes may be used for characterization and that chromosome profiles are serodeme specific,it has been speculated that OFAGE which reveals chromosome profiles may be a suitable technique for the characterization of *T. congolense* isolates. Such studies have been conducted using isolates from East Africa only, however the results obtained may differ from those of the isolates obtained from a different locality. The main aim of the study was to characterize *T. congolense* stocks from West Africa and some from East Africa other than those that have already been done. This will help to confirm and extend the knowledge that *T. congolense* isolates can be characterized on the basis of their molecular karyotype using the OFAGE.

The specific objectives are:

(1) To analyse the chromosome profiles of clones derived from the stocks from the two areas. The following steps were taken:

   (a) Growing and adapting the stocks in rodents.

   (b) Cloning the stocks to obtain at least two cloned populations from each stock.

   (c) Growing the cloned populations in rodents to obtain enough numbers of trypanosomes for chromosome analysis.

   (d) Chromosome analysis using the OFAGE.

(2) To confirm that the chromosome profiles can be used for serodeme identification by cross protection studies in mice. This will be done by immunizing groups of mice against specific clones and exposing them to a homologous challenge, followed by a heterologous one.

(3) To determine whether the chromosome profiles will remain stable in terms of the number and size of the chromosome-sized DNA molecules following

   (a) Transmission through tsetse flies.

   (b) Chronic infection in goats for four months.

CHAPTER TWO

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. **Trypanosomes**

Ten *Trypanosoma congolense* stocks from West Africa (Burkina Faso, Gambia and Nigeria) and five East Africa (Kenya and Uganda), were used in the present study. They were obtained from the ILRAD trypanosome bank. Table 1 gives the details.

2.1.2 **Tsetse flies**

Teneral male, one-day old *Glossina morsitans centralis* from the ILRAD tsetse colony (Moloo et al., 1985) were used. They were maintained on rabbits throughout the course of the experiment.

2.1.3 **Animals**

C_3H: He, Swiss white and Balb/c mice were used for growing trypanosome populations. The mice were between six and eleven weeks old and weighed between 20 and 28 gm. Before they were infected they were either sub-lethally or lethally irradiated (600 rads and 900 rads, respectively). Rats of the Sprague Dawley (S/D) strain were also used for growing some of the trypanosomes that were well adapted in them. They were two months old and weighed approximately 200 gm. The rats were also irradiated (900 rads) before use. Male goats (East African x Galla cross), aged 8 to 10 months and weighing about 25 kg, were used to maintain a chronic infection. All the experimental animals were obtained from the ILRAD small and large animal units and maintained there.

2.2. METHODS

2.2.1 **Growing of the trypanosome stocks**

Two capillary tubes for each stock were removed from a liquid nitrogen tank and thawed and the suspension was made up to 0.4ml in a syringe with phosphate saline glucose (38mM $Na_2 HPO_4$, 2mM $NaH_2 PO_4.2H_2O$, 29mM Nacl, 1%(w/v) D-glucose) at pH 8.0. A drop of the suspension was examined under a microscope to ascertain the viability of the organisms. It was diluted with PSG and 0.1ml containing 1x 10^4

trypanosomes was injected intraperitoneally into an irradiated Swiss white or Balb/c mouse.

At day 3 post infection parasitaemia was monitored by microscopic examination of tail blood. The parasite estimation technique used was that of Herbert and Lumsden (1976). This employs a matching technique for high parasitaemias and a count of trypanosomes in up to twenty fields is made. The lowest level of parasitaemia recorded by this method is antilog 5.4 organisms per millilitre of blood while the highest is the antilog 9.0 organisms per millilitre. At peak parasitaemia about 20 μl of blood were drawn from each mouse and diluted with 500 μl of guinea-pig serum in phosphate saline glucose. This suspension was used for cloning as described below.

2.2.2. **Cloning of trypanosomes**

Cloning means generation of a population of organisms from a single one. Cloning of trypanosomes can be carried out either *in vitro* (Hirumi et al., 1980) or *in vivo* (Cross, 1975; Barry and Gathuo, 1984). The procedure used for *in vivo* cloning was that of Barry and Gathuo, (1984).

Briefly, cloning buffer (equal volumes of PSG, pH 8.0, and inactivated fresh guinea-pig serum) was used to dilute infected blood such that there were two to three trypanosomes per 5 μl of suspension. About 10 μl of the cloning buffer were put in a cavity slide in a slide. Using the end of a paper clip single droplets of trypanosome suspension (Fig. 2a) were placed on a cover slip which was then quickly inverted over the cavity slide. The buffer in the cavity formed a moist chamber that maintained any viable trypanosome in the drop (Fig. 2b). Under an objective lens (x40) the drop was located and when only a single trypanosome was found (Fig.2c) another person was called upon to confirm. Using a needle (gauge 27½) the trypanosome was then slowly and very carefully sucked into a syringe filled with 200 μml of the cloning buffer (Fig.2d). This was then injected through a tail vein into a mouse as illustrated in Figure 3.

A preliminary cloning exercise using some of the stocks showed that clones appeared earliest on day 7. In the present work parasitaemia was monitored daily after a

prepatent period of 6 days. This gave an allowance of one day in case some clones appeared earlier than the predetermined duration. Parasite estimation was done using the method described by Herbert and Lumsden (1976). At the level of antilog 7.8 trypanosomes per millilitre of blood (an average of 16 trypanosomes per field), the mice were bled and trypanosomes preserved following the method described by Dar et al. (1972). The clones obtained were used for karyotyping as described in section 2.3.

2.2.3 KARYOTYPING

2.2.3(a). **Preparation of trypanosomes for chromosome analysis**

Trypanosoma congolense clones were retrieved from liquid nitrogen and $1x10^4$ trypanosomes/ml were inoculated intraperitoneally into each of four irradiated Swiss mice. At peak parasitaemia the mice were killed and the infected blood passaged into forty irradiated mice which were then bled, at peak parasitaemia, to obtain as much blood as possible. Sodium citrate (3%) was used as the anticoagulant. The trypanosomes were separated from infected blood using Percoll gradient (Grab and Bwayo, 1982) and further cleaning of the trypanosomes was done by column chromatography using diethyl amino-ethyl cellulose (DEAE 52) as described by Lanham and Godfrey (1970).

2.2.3(b). **Preparation of the chromosome-sized DNA molecules**

Trypanosomes freed from contamination as described in the preceding section were pelleted by centrifugation and resuspended in PSG (pH 8.0) at 50°C to a concentration of $2x10^9$ trypanosomes per millilitre. Equal volumes of trypanosome suspension and 1.3% low melting point agarose maintained at 50°C were mixed, giving a final concentration of $1x10^9$ trypanosomes per millilitre. The mixture was poured between two glass slides separated by 1mm spacers. After the gel had set (2 minutes on ice) the imbedded parasites were incubated in 20 ml of a solution prepared as follows: 17 mls of 0.5M EDTA (pH 9), 2 mls of SDS 10% and 1 ml of Pronase E (1 mg/ml). Incubation continued for 36-48 hours at 50°C in a waterbath after which the gels were washed with 10 mM EDTA. After

washing, the low melting point agarose gels were preserved in 10 mM EDTA until use. Fig 4 illustrates the procedure.

2.2.3(c). **Gel electrophoresis**

The technique used was that which employs alternating current and is known as orthogonal field alternating gel electrophoresis (Carle and Olson 1984). The apparatus used is illustrated in Figure 4(f). To run electrophoresis, 1.5% agarose gel was melted in 200mls of 0.5xTBE (TBE: 1xTBE is 90 mM tris 90 mM boric acid, 25 mM disodium ethylene diamine tetraacetic acid) in a flask . After cooling the gel was poured on a glass plate (16 cm x 15cm) whose sides were taped, a comb was placed on one side of the glass box, to make slots for the samples (trypanosome DNA imbedded in low melting point agarose). Prior to insertion of the samples, the slots were filled with the running buffer (0.5xTBE) to facilitate loading of the trypanosome samples. The gel was then transferred to the electrophoresis chamber, centred and covered with the 0.5xTBE buffer.

Electrophoresis was performed at a constant voltage of 300v,which alternated at a pulse frequency of 30 seconds. The running buffer was cooled by circulating it through water chilled at $8^{o}C$ in a waterbath. The experiment was left to run for 15 hours, after which the gel was stained in ethidium bromide (0.5ug/ml), destained in water and photographed under ultraviolet illumination.

Table 1. A list of the stocks used and some of their

STOCK	PRIMARY ISOLATION	SUSCEPTIBLE HOSTS
IL 2467	cow, natural infection Bobo Dioulasso, Burkina Faso	mouse, rat, rabbit and cow
IL 2463	cow, natural infection Date of isolation; 1983 Bobo Dioulasso, Burkina Faso	mouse, rat, rabbit and cow
IL 2975	cow, natural infection Bobo Dioulasso, Burkina Faso	mouse, rat, rabbit cow and tsetse
IL2974	cow, natural infection Date of isolation:	mouse, rat, rabbit cow and tsetse
IL2464	cow, natural infection Bobo Dioulasso	mouse, rat, rabbit and cow
IL2829	cow, natural infection Bobo Dioulasso, Burkina Faso	mouse, rat, rabbit cow
IL 3045	cow, natural infection Date of isolation: 1978 Bobo Dioulasso, Burkina Faso	mouse, rat, rabbit mouse, rat, rabbit

characteristics

DRUG SENSITIVITY	TSETSE TRANSMISABILITY
samorin resistant	-
samorin resistant	-
samorin and berenil resistant	Positive *Glossina morsitan centralis*
samorin resistant and berenil sensitive	Positive, *Glossina morsitan centralis*
samorin resistant	-
samorin and berenil resistant	-
samorin and berenil sensitive	positive, *Glossina morsitans centralis*

Table 1. continued

STOCK	PRIMARY ISOLATION	SUSCEPTIBLE HOSTS	DRUG SENSITIVITY	TSETSE TRANSMISABILITY
IL 2465	cow, natural infection Date of isolation: 16/1/1980 Bobo Dioulasso, Burkina Faso	mouse, rat	samorin sensitive	-
IL 3260	Uganda	mouse, rat, rabbit, cow, goat	-	positive
IL 3266	cow, natural infection Date ofisolation: 1967 Nigeria	mouse, rat, rabbit, cow	- -	positive for *Glossina morsitan centralis*
IL 311	cow, natural infection	mouse, rat, cow	berenil sensitive	-
IL 3411	sheep, natural infection Matuga, Coast Province	mouse, sheep	-	-
IL 3349 (C49)	cow, natural infection Date of isolation: 1966 Transmara, Southern Kenya	mouse, rat, rabbit cow, goat, sheep	berenil sensitive	positive
IL 3416	cow, natural infection Shimba Hills, Mombasa, Kenya	mouse, rat, cow sheep	-	-
IL C24	dog, natural infection Date of isolation: 29/9/1976 Kabete, Nairobi, Kenya	mouse, rabbit, rat, cow dog (Labrador)	berenil sensitive	positive

* - means that the information was not available from the records. Dates of isolation for some isolates were also not available.

Figure 2 (a) shows a drop that contains several trypanosomes on a coverslip.

(b) The viewing of a drop under a microscope to assess whether the drop contains one or several trypanosomes.

(c) A drop with only one trypanosome which in (d) is picked up using a needle gauge 27½.

Fig. 2 Diagramatic representation of cloning

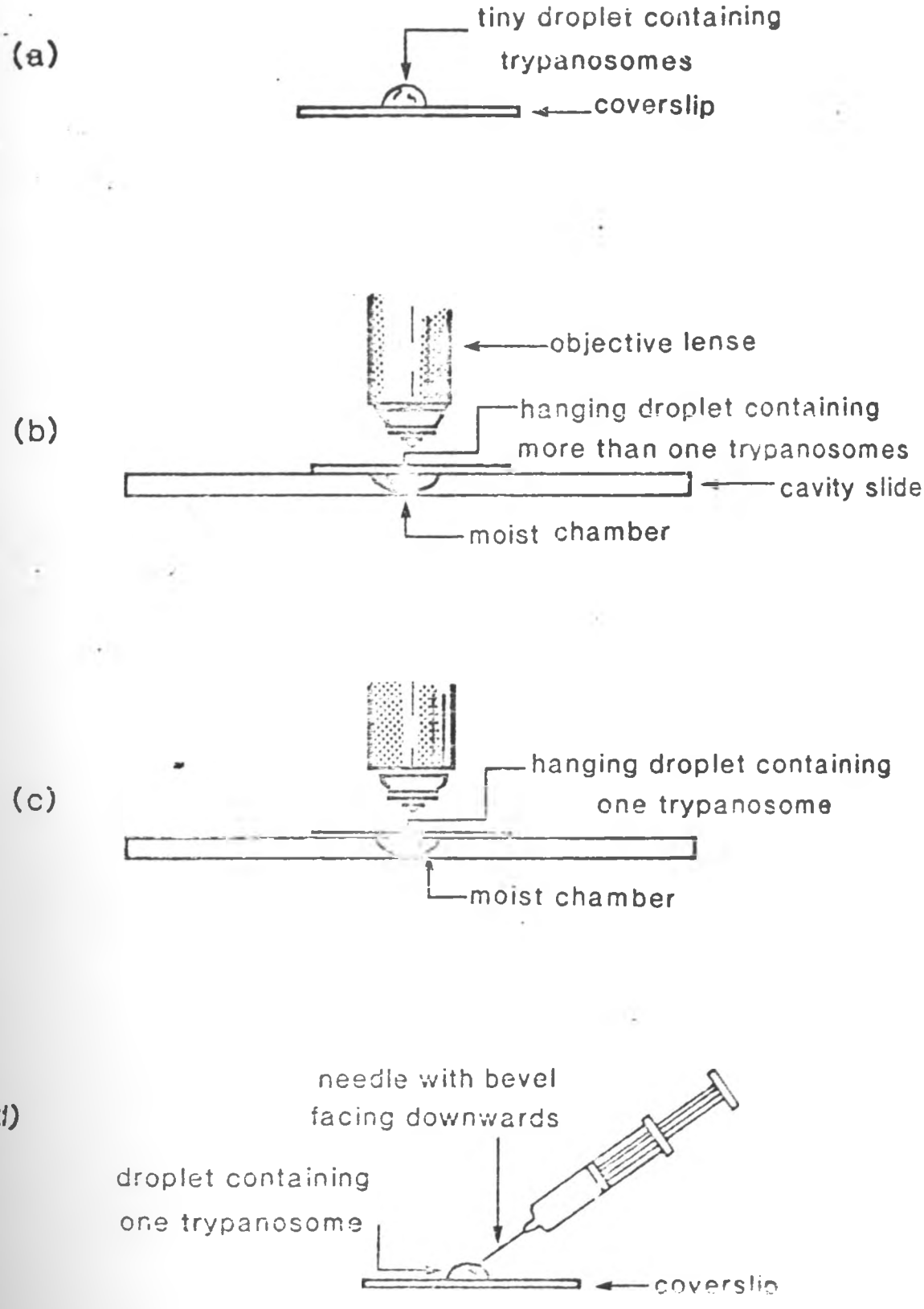


Figure 3. The diagram shows how a mouse is restrained during an intravenous injection. Before the mouse is placed into the syringe, it is exposed to a 40 watt bulb in order to dilate the vein so that it is visible during injection.

Fig.3 **Intravenous inoculation of a mouse**

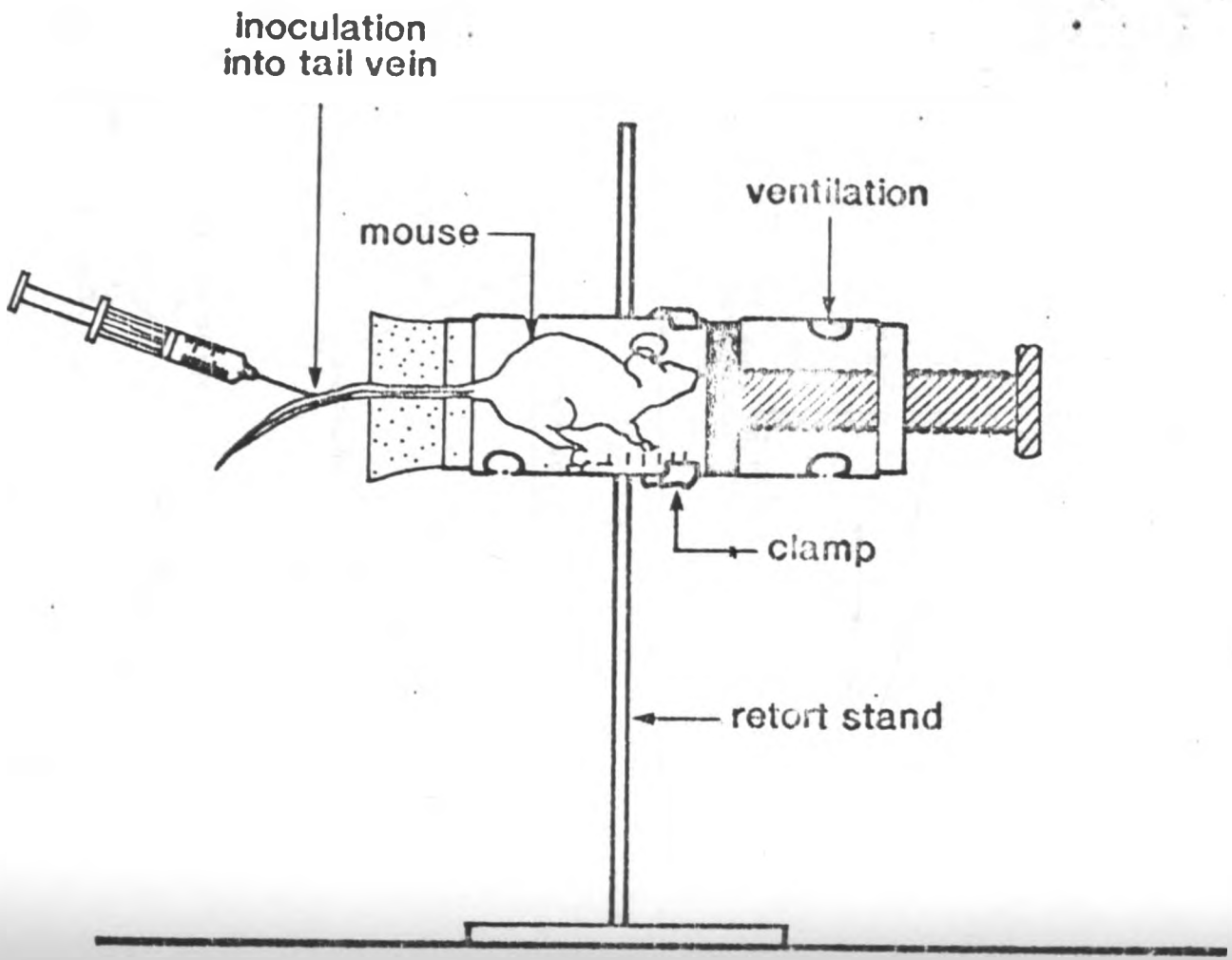
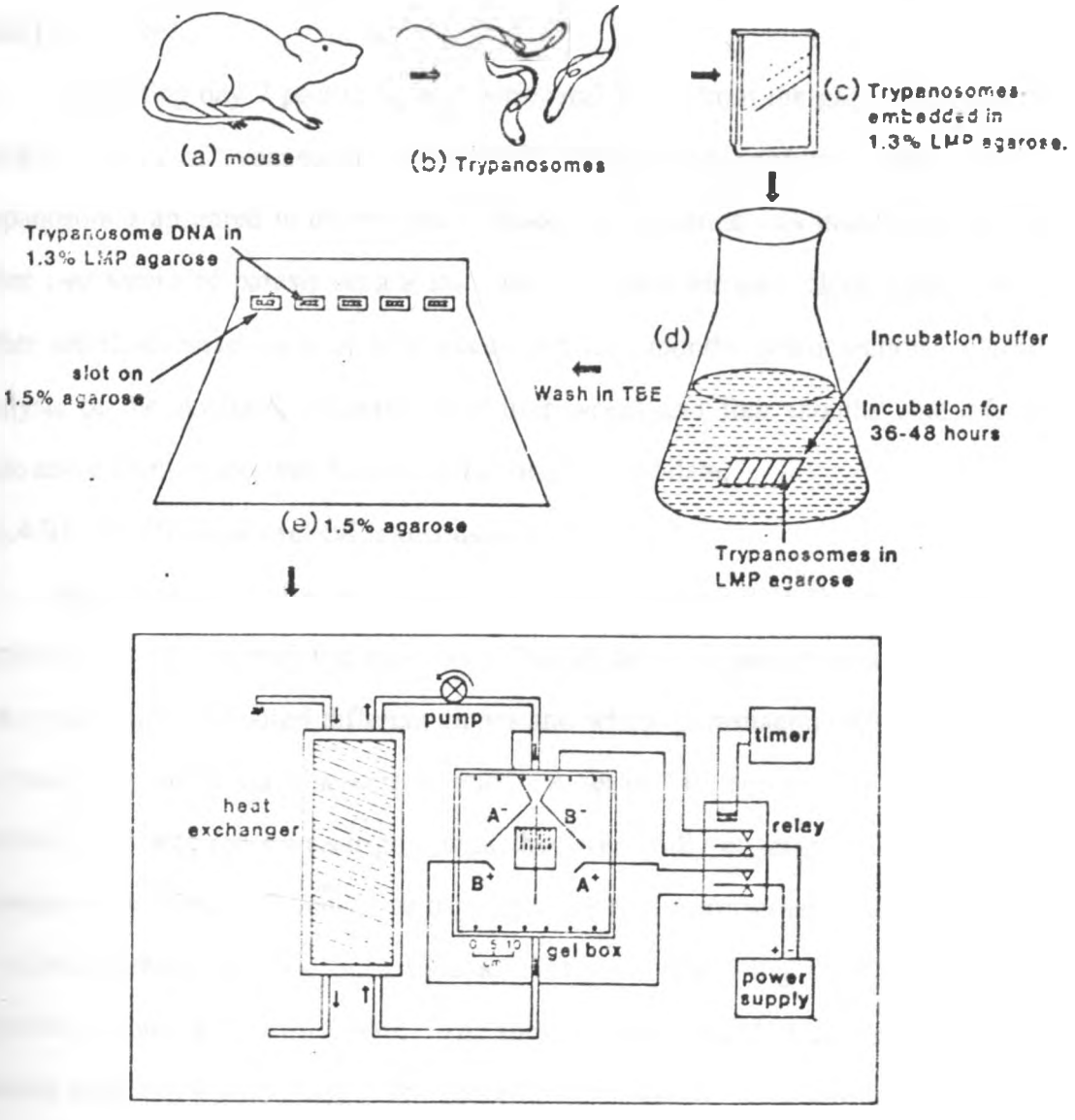


Fig. 4. PROCEDURE FOR MOLECULAR KARYOTYPING



(f) Apparatus for orthogonal field-alternating gel electrophoresis

2.2.4 STABILITY OF CHROMOSOME PROFILES

This experiment was designed to find out the effect on chromosome profiles of passaging trypanosome clones in goats for four months and of cyclical transmission of the clones.

2.2.4(a). **The effect of passaging trypanosomes clones in goats.**

Seven cloned stabilates whose profiles had already been determined were inoculated intravenously into goats. Each goat received 10^3 trypanosomes. Two goats were infected with each clone.

Beginning day 7 post-infection, peripheral blood from the goat was examined daily for the presence of trypanosomes by the buffy coat technique (Murray et al., 1977). When trypanosomes appeared in the peripheral blood, parasitaemia was monitored twice weekly. After two weeks of parasitaemia a stabilate was made for each clone (Dar et al., 1972). Other stabilates were made at four weeks and four months post-infection. Chromosome analysis of the stabilates collected after four weeks and four months post-infection was done and a comparison was made with the original stabilate.

2.2.4(b).**The effect of cyclical transmission.**

Experimentally infected goats (see under section 2.2.4(a)) were used to provide trypanosomes for cyclical transmission. During the first parasitaemic wave of infection two goats which harboured different clones and whose parasitaemia was 2-3 trypanosomes per field on a wet blood film were selected. A set of 140 teneral male *Glossina morsitans centralis* flies was fed on each goat until they were fully engorged. The flies were then maintained on rabbits. Twenty five days later the flies were screened for mature infections by allowing them to probe on a slide at 37°C and examining their saliva under a x40 objective according to Burtt (1946). The flies that were found to extrude metacyclics were isolated each into a single cage. The procedure for cyclical transmission is shown in figure 5.

The infected flies were allowed to feed on irradiated Swiss mice, each fly being allowed to engorge fully. The mice were later examined for infection. At peak parasitaemia they were bled and the blood was injected into fresh irradiated Swiss mice in

order to obtain enough numbers of trypanosomes for chromosome analysis. This procedure was repeated for another pair of clones. The chromosome-sized DNA molecules were examined and compared with those of the original infecting material.

2.2.5 **Cross Protection Experiments**

Studies were conducted in order to find out whether the chromosome profiles obtained may be used to identify antigenic repertoires (serodemes).

Each of eight clones (see Table 3. pg 35 whose chromosome profiles had been examined (IL 3669, IL 3662, IL 3633, IL 3677, IL 3593, IL 3649, IL 3612, and IL 3655) was inoculated into a group of twelve mice. 0. 2ml of a suspension containing $1x10^4$ trypanosomes per millilitre was inoculated into each irradiated mouse.

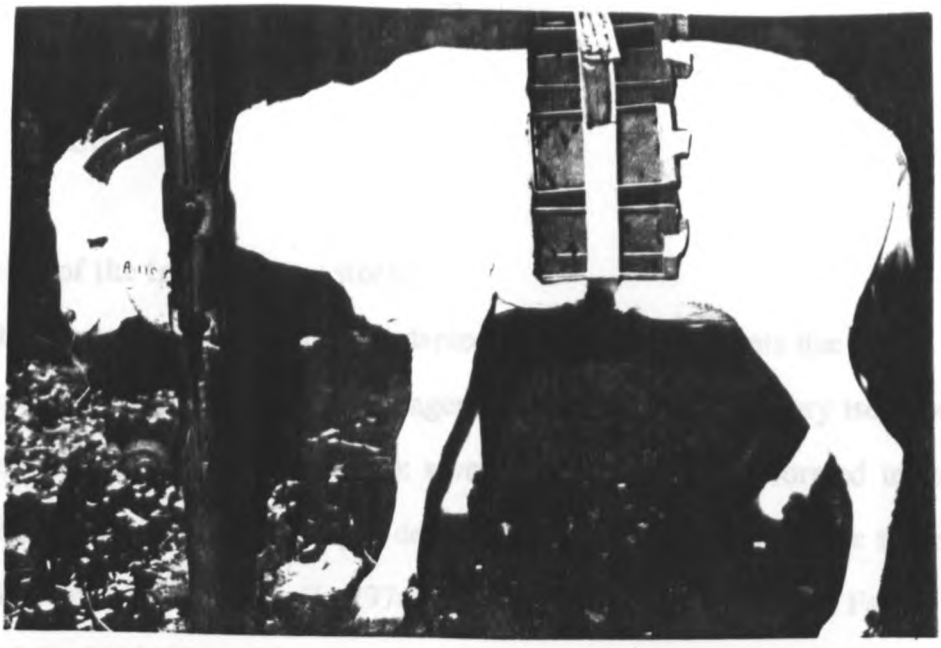
At day 3 post-infection parasitaemia was monitored and at day 5 all the mice in all groups were treated using Berenil (diminazene aceturate) at 20 mg/kg body weight. For those groups of mice that did not clear off the trypanosomes after five days of treatment another dosage of 20 mg/kg body weight of Berenil was administered. Following ten days of no detectable parasitaemia the immunized mice were challenged with 0.2 ml of $1x10^4$ trypanosomes per millilitre of a homologous clone. Parasitaemia continued to be monitored daily. At day 8 post-challenge the mice were treated with 20 mg/kg of Berenil. Five days later the mice were challenged again with a heterologous clone and parasitaemia was monitored as before. The presence or absence of trypanosomes after each challenge was used to determine whether there was protection or not.

Figure 5. Cyclical transmission of *T. congolense* clones through *Glossina morsitans centralis*

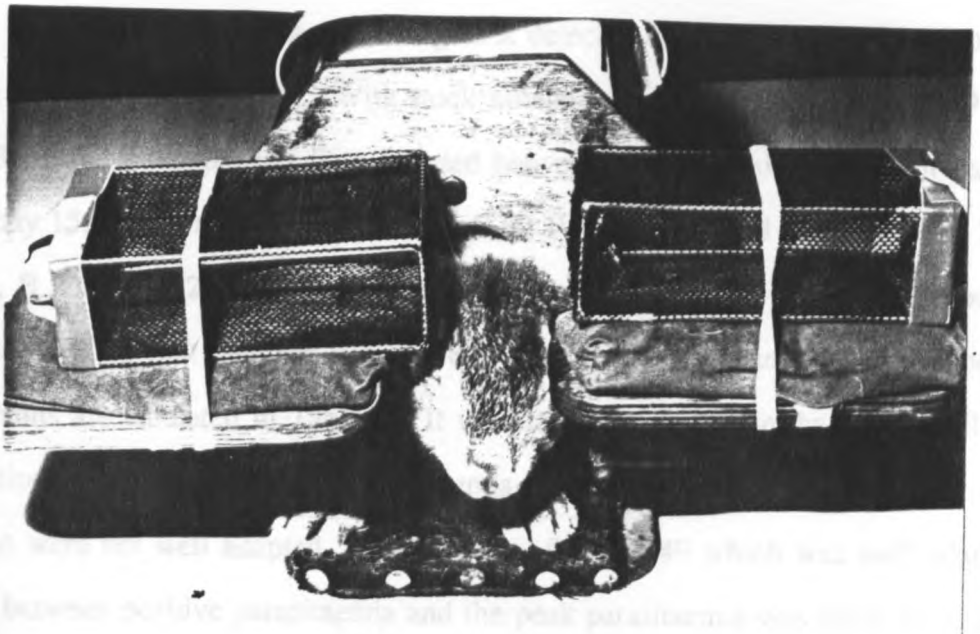
(5a) Clean flies are fed on an infected goat.

(5b) The flies are maintained by feeding them on rabbits until the infection matures.

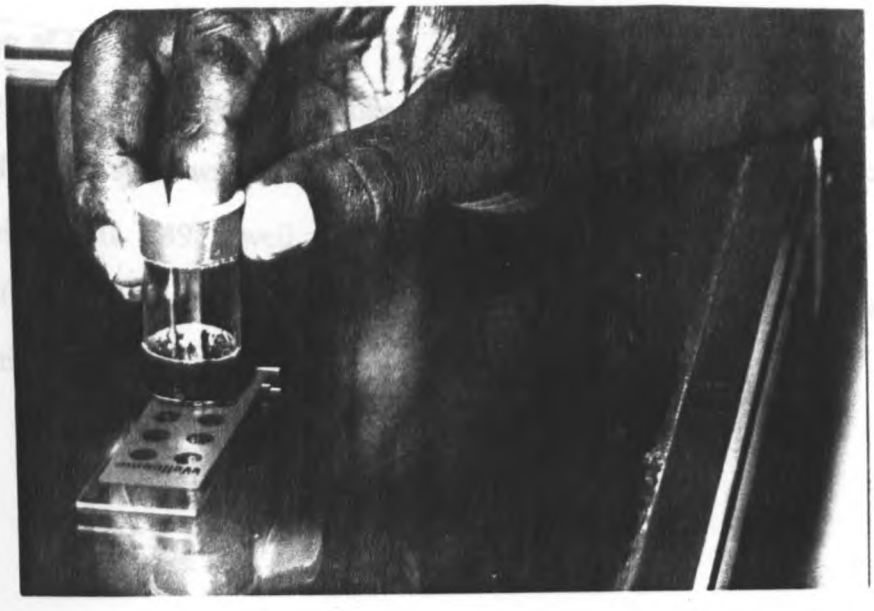
(5c) The flies are allowed to probe on a warm slide.



(5a)



(5b)



(5c)

CHAPTER THREE

RESULTS

3.1 Growing of the trypanosome stocks

Most of the stocks were not well adapted to growing in rodents due to the fact that most of them had only undergone few passages in mice from their primary isolation before they were cryopreserved. For each stock several passages were performed in mice, the number of times each stock was passaged depending on the adaptability of the stock.

Stock numbers IL 2975, IL 2974, IL 3045, IL 2829 (Burkina Faso), IL 311 (Gambia) and IL 3416 (Kenya) grew poorly in mice. IL 3045 was the least adapted to growing in mice, with parasitaemia being first detected on day 14 after infection and not reaching the peak until day 20. With stock numbers IL 2975, IL 2974, IL 2829, IL 311 and IL 3416 the infection was first detected between day 8 and day 10 and it reached its peak at day 15. Stock numbers IL C49, IL C24, IL 3260, IL 3411 (East Africa), IL 2463, IL 2467, IL 2465, IL 2464 (Burkina Faso) and IL 3266 (Nigeria) were well adapted, with parasitaemia being first detected between day 3 and day 6 and the peak at around day 8. These results are tabulated in Table 2. It was noted that for those that were well adapted the duration between detection of parasitaemia to peak parasitaemia was shorter than for those that were not well adapted. For instance, for IL C49 which was well adapted, the duration between positive parasitaemia and the peak parasitaemia was three days while for IL 3045 the duration was seven days.

3.2 Cloning of the trypanosomes

The cloning efficiency (the number of clones obtained from each stock within a given time) for each stock was directly related to its growth pattern in mice. For instance, cloning exercise for IL C49, a well adapted stock, in twenty mice yielded four clones after two weeks. In contrast IL 3045, a poorly adapted stock, yielded only one clone after two weeks of cloning.

Table 2. The growth patterns of the trypanosome stocks in mice

Stocks	Days post-infection in mice																			
	0	1	2	3	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
IL 2974								+								P				
IL 2829									+						P					
IL 2975								+								P				
IL 3045														+						P
IL 311									+					P						
IL 3416									+						P					
IL 3411					+				P											
IL 2464				+				P												
IL 2465				+				P												
IL 2463					+			P												
IL 2467						+		P												
IL 3260					+			P												
IL 3266				+				P												
IL C24					+				P											
IL C49				+			P													

\+ The day on which the infection was first detected.
P The day on which parasitaemia reached its peak.

Table 3. A list of the stocks and the clones made from each stock

STOCK	LOCATION	CLONES					
IL 2974	Burkina Faso	IL 3581	IL 3587	IL 3590	IL 3595	IL 3596	
IL 2829	"	IL 3582	IL 3585	IL 3586			
IL 2975	"	IL 3592	IL 3593	IL 3594			
IL 2463	"	IL 3610	IL 3611	IL 3612	IL 3113	IL 3614	IL 3616
IL 2464	"	IL 3624	IL 3625	IL 3626	IL 3628	IL 3629	
IL 2465	"	IL 3655	IL 3656				
IL 3045	"	IL 3609	IL 3617	IL 3618	IL 3619	IL 3620	IL 3621
		IL 3622	IL 3623				
IL 2467	"	IL 3597	IL 3598	IL 3599	IL 3601	IL 3602	IL 3603
IL 311	Gambia	IL 3672	IL 3674	IL 3677			
IL 3266	Nigeria	IL 3630	IL 3633	IL 3634	IL 3635		
IL 3260	Uganda	IL 3641	IL 3642	IL 3643	IL 3646		
IL 3416	Kenya (Shimba (Hills)	IL 3679	IL 3680	IL 3681	IL 3685		
IL 3411	Kenya	IL 3666	IL 3667	IL 3668	IL 3669	IL 3670	
IL C49	Kenya	IL 3661	IL 3662	IL 3664	IL 3665		
IL C24	Kenya	IL 3630	IL 3637	IL 3654	IL 3657	IL 3659	

Cloning was a trial experiment in that predictions could not be made as to how many clones would arise from a single trial. Each trial experiment comprised of cloning in twenty mice and sometimes no clone was obtained from a single trial. This meant that several trial experiments had to be performed to obtain at least two clones from each stock. For those stocks that were poorly adapted, three or more trials were performed to obtain two clones, but for those that were well adapted a single trial was enough to generate a good number of clones. Table 3 shows the number of clones obtained from each stock, the underlined clone(s) in each stock were analysed for their chromosome profiles.

3.3 **Chromosome analysis**

Chromosome analysis of the clones and stocks underlined in Table 3 was done using the orthogonal field alternating gel electrophoresis as described in Chapter 2. A variety of chromosome profiles was obtained, though the different patterns obtained may be put together into one basic pattern (one molecular karyotype).

The DNA in each chromosome profile separated into three clusters of chromosome-sized DNA bands dictated by their molecular weights. The smallest cluster of bands were of the size range 50-150 kb. These are referred to as minichromosomes and distinct bands were obtained when electrophoresis was performed at a pulse rate of 25 seconds. The second cluster of bands were of the size range 400-750 kb and well separated at 30 second pulse rate. The third cluster consisted of large-sized chromosomes of the range 950-1050 kb. This group of bands did not separate well into individual bands even at the pulse rate of 30 seconds. In the size range of 200-400 kb, there were no bands for most clones, one or two bands were present in a few clones.

The chromosome profiles obtained in this study are shown in Figs. 6-13. Clones from different stocks exhibited different chromosome profiles. For instance, Fig. 6 displays different chromosome profiles of clones IL 3603, IL 3618, IL 3637, IL 3582 which were all derived from different stocks. This characteristic of different profiles displayed by different clones from different stocks was a common observation throughout the chromosome analysis of various clones. Fig. 11 displays three patterns, each pattern

representing a clone from a different stock. In Fig. 12 four distinct patterns are displayed, each pattern representing a clone that was derived from a different stock. Fig. 13 also portrays the same phenomenon, where each distinct pattern represents a clone derived from a different stock.

In a few cases similarities in chromosome profile were noted between trypanosome clones from different stocks. In Fig. 7 chromosome profiles of clones IL 3669 and IL 3681 (lanes 1 and 2 respectively) are similar though the two clones were derived from different stocks. The same thing is expressed by the clones IL 3592 and IL 3612 (lanes 4 and 5) respectively. In Fig. 8, similar chromosome profiles are displayed by clones IL 3595 and IL 3655 (shown by pairs of lanes (2,3) and (4,5) respectively) the two clones were derived from different stocks.

Similarities in chromosome profile were frequently noted amongst clones derived from the same stock. For instance, in Fig. 6, IL 3599 and IL 3603 (lanes 1 and 2 respectively) display similar patterns, the two clones were derived from the same stock. Figure 9 displays similar chromosome profiles; clones IL 3677 and IL 3672 (lanes 1 and 3 respectively) were derived from the same stock. In fig. 10, IL 3664 displays similar profile to IL 3662 (lanes 1 and 3 respectively) though IL 3662 (lane 3) has one band missing at the size of 400 kb.

Comparison between the chromosome profile of a stock and its clones showed that a stock may have a completely different chromosome profile from its clones, as shown in Fig. 9, where the chromosome profile of the stock, IL 311 (in lane 2) is different in terms of the number and size of the chromosome-sized DNA molecules of its clones (lanes 1 and 3). Another possibility is that a stock may portray a chromosome profile that are partially or completely similar to those of its clones. This is displayed in Fig. 10, where the stock IL C49 (in lane 2) is identical to the clone IL 3664 (in lane 1) while the other clone IL 3662 (in lane 3) lacks one band the size of 400 kb.

Fig. 6. Orthogonal field alternating gel electrophoresis of chromosome-sized DNA molecules from clones of bloodstream *Trypanosoma congolense*. Clone numbers IL 3599, IL 3603, IL 3618, IL 3637 and IL 3582 in lanes 1, 2, 3, 4 and 5, respectively. The first two clones were derived from one stock,the rest from different stocks.

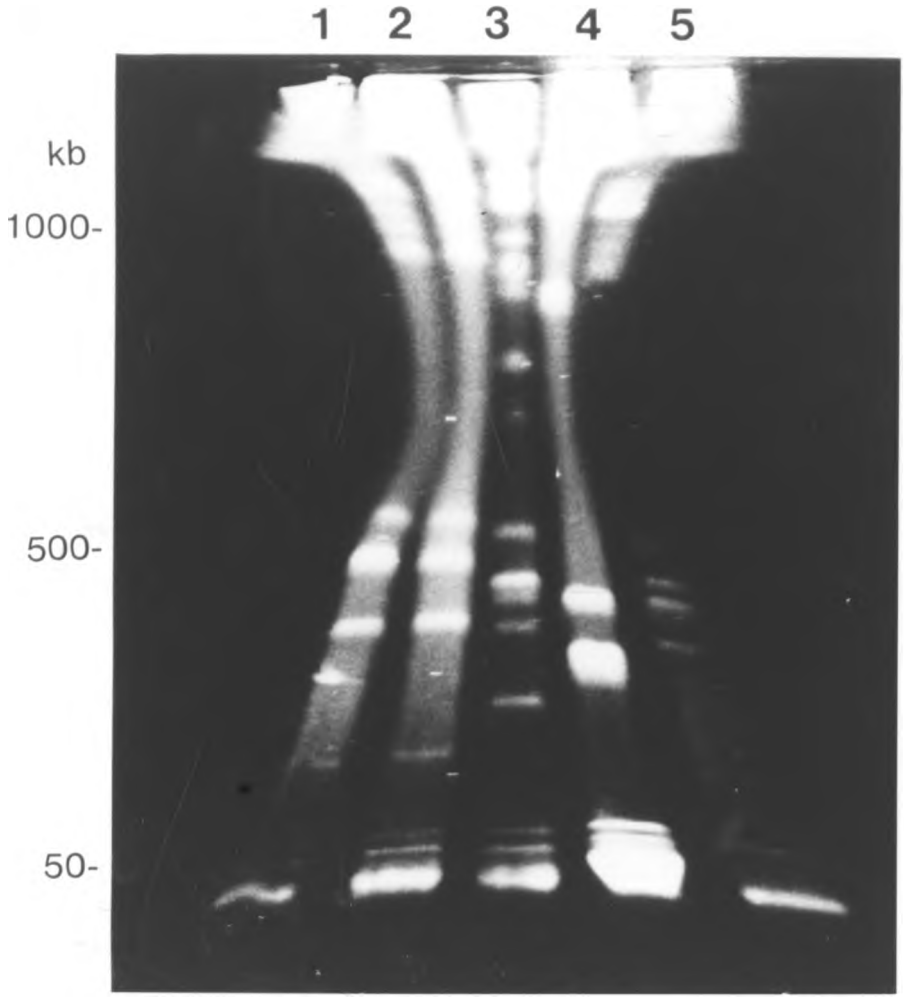

1 2 3 4 5
kb
1000-
500-
50-

Fig. 7. Orthogonal field alternating gel electrophoresis of chromosome-sized DNA molecules from clones of bloodstream *Trypanosoma congolense*. Clone numbers IL 3669 and IL 3681, represented in lanes 1 and 2, display similar patterns. IL 3592 and IL 3612 in lane 4 and 5 respectively display another pattern. The four clones were derived from different stocks. Lane 3 is IL Nat. 3.1 previously characterized as a typical savanna *congolense*.

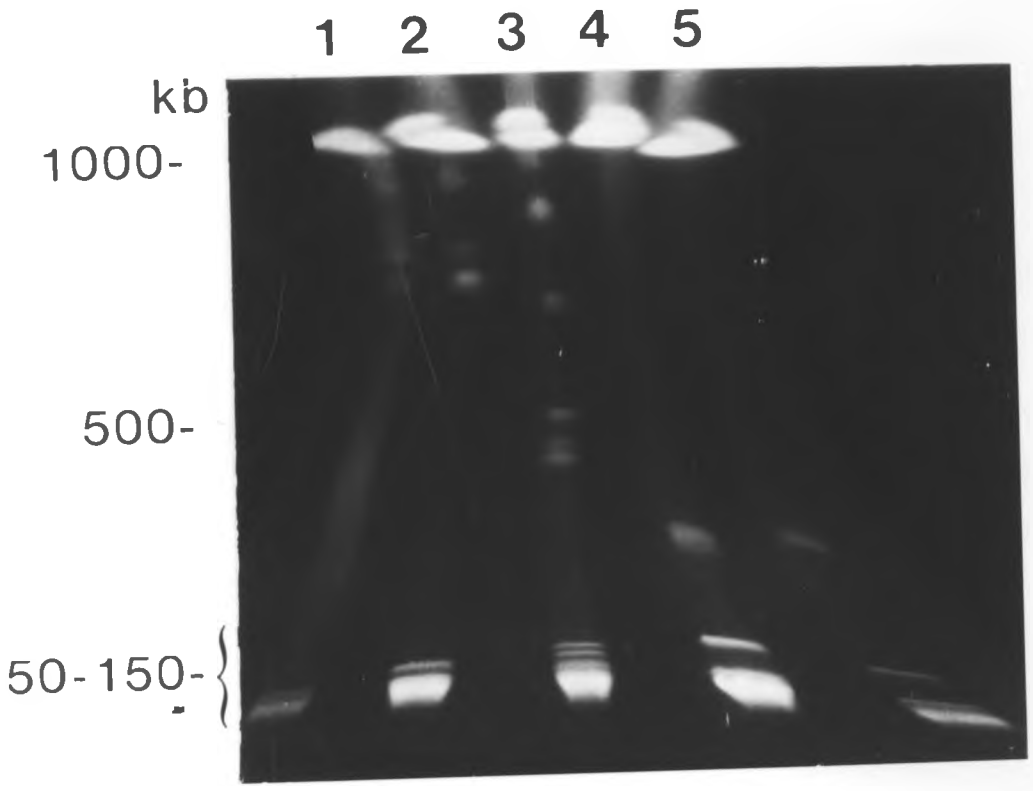

1 2 3 4 5
kb
1000-
500-
50-150-

Fig. 8. Orthogonal field alternating gel electrophoresis of chromosome-sized DNA molecules from bloodstream *Trypanosoma congolense*. Clone numbers IL 3599, IL 3595, IL 3655, IL3592 are represented in lanes 1, (2, 3), (4, 5), and (6, 7) respectively. All the clones were derived from different stocks.

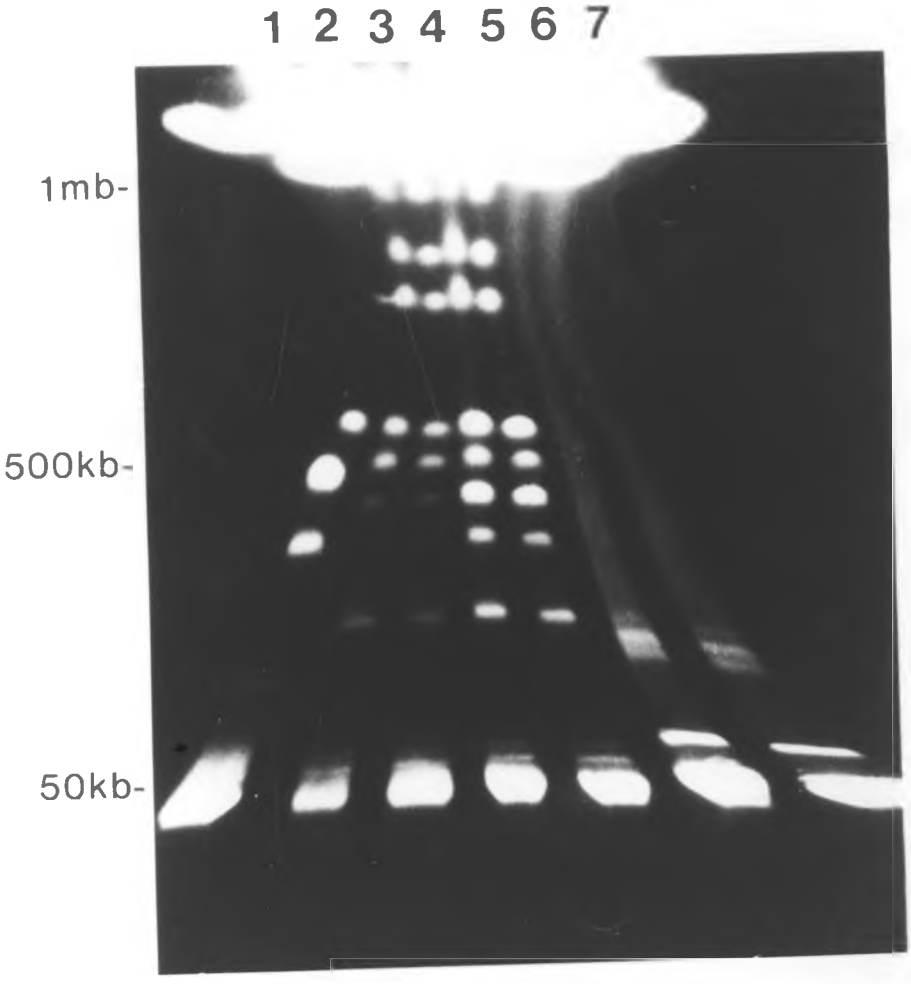

1 2 3 4 5 6 7
1mb-
500kb-
50kb-

Fig. 9. Orthogonal field alternating gel electrophoresis of chromosome-sized DNA molecules from clones of bloodstream *Trypanosoma congolense*. Clone numbers IL 3677 and IL 3672 are represented in lanes 1 and 3 respectively. IL 311 in lane 2 is the stock from which the clones were derived. Note the similarities between the two clones and yet the sizes of their chromosomes are distinctly different from those of the parent stock.

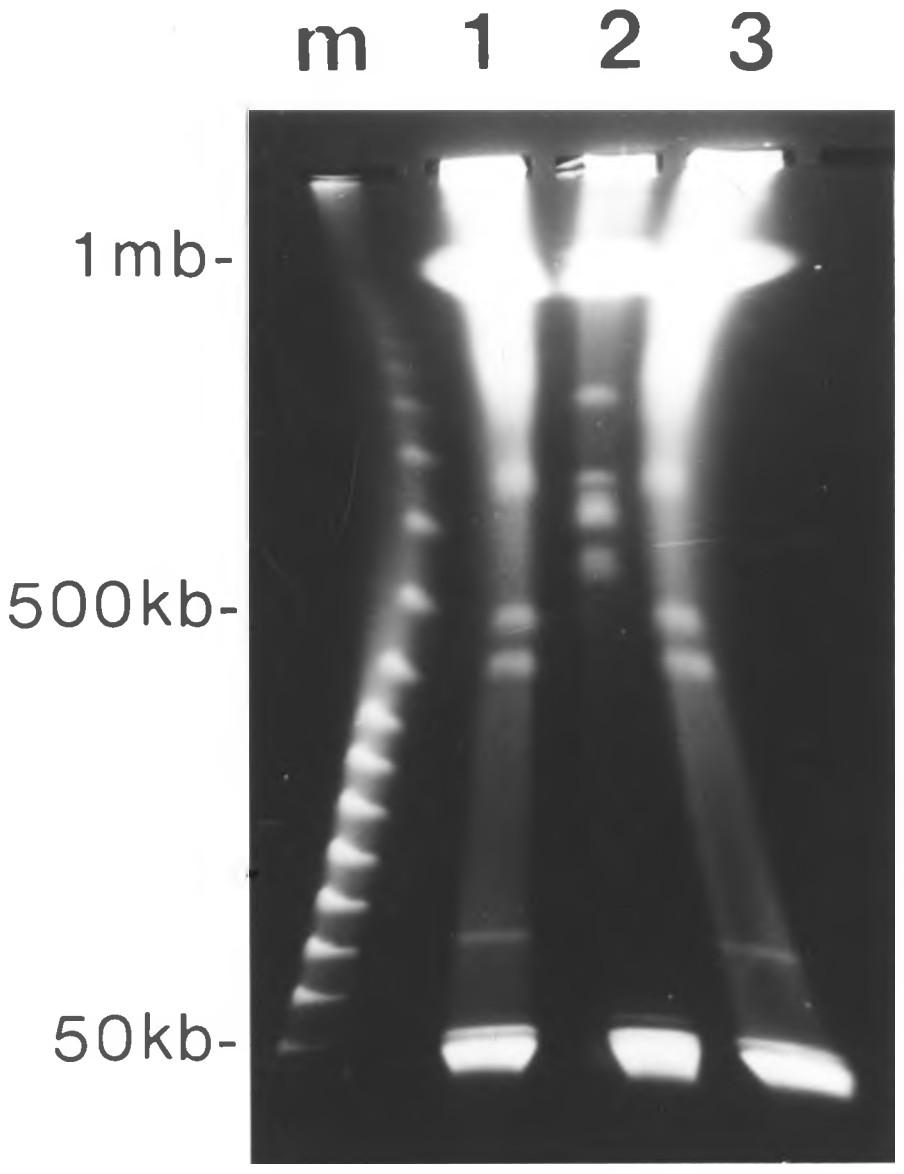
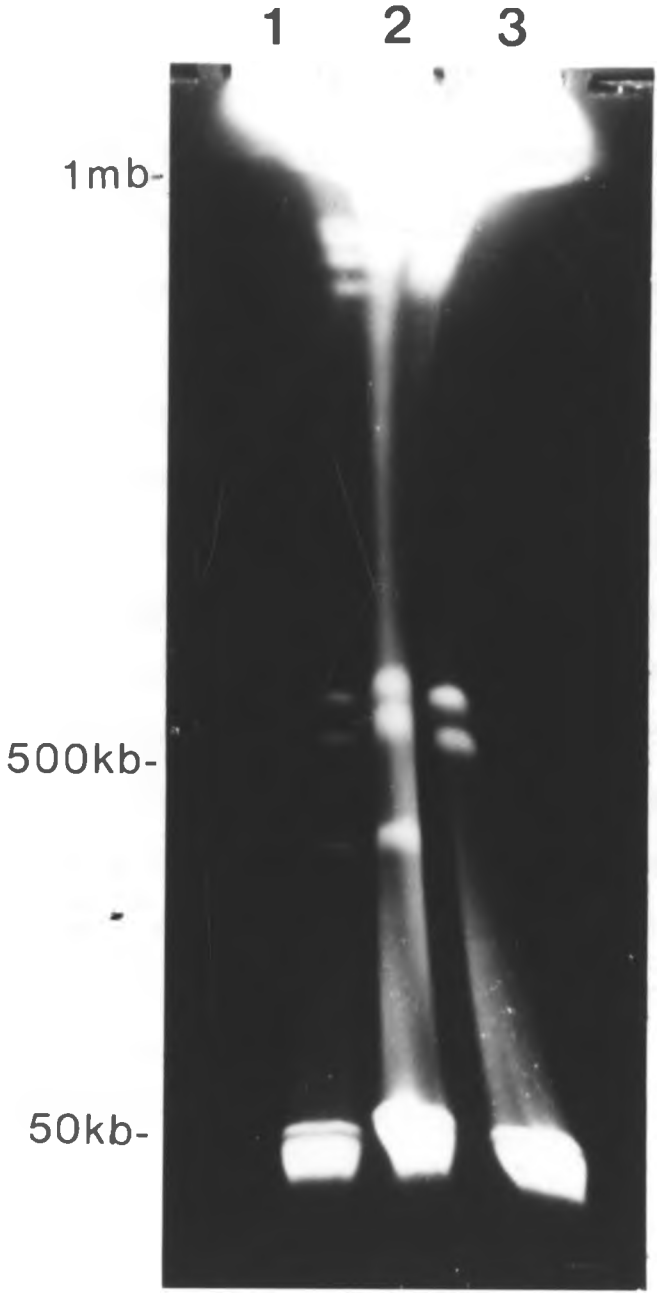

m
1
2
3
1mb-
500kb-
50kb-

Fig. 10. Orthogonal field alternating gel electrophoresis of chromosome-sized DNA molecules from clones of bloodstream *Trypanosoma congolense*. Clone numbers IL 3664 and IL 3662 are represented in lanes 1 and 3 respectively. IL C49 in lane 2 is the stock from which the clones were derived. Clone IL 3662 (lane 3) differs slightly from C49 and IL 3664 by the absence of a chromosome-sized DNA molecule of approximately 350 Kb.


1
2
3
1mb-
500kb-
50kb-

3.4.1 The effect of passaging trypanosomes in goats for four months on the chromosome profiles.

Goats were infected with *Trypanosoma congolense* clones exhibiting different chromosome profiles. The goats were left to run a chronic infection for four months. Trypanosome stabilates were made at various intervals during the course of infection. The stabilates were subsequently expanded in mice, trypanosomes harvested and imbedded in low melting point agrose for use in OFAGE. Chromosome profiles of the original and subsequent parasite stabilates were compared. No obvious changes in the size,intensity or position of the chromosome-sized DNA molecules were observed over a period of 4 months (Fig. 11).

3.4.2 The effect of cyclical transmission on the stability of chromosome profiles

Batches of 140 one-day old male teneral *Glossina morsitans centralis* were fed on goats infected with clones IL 3662, IL 3633, IL 3612 and IL 3649. After feeding the flies on the infected goats, they were maintained by feeding them on rabbits. At day 25, the flies were screened for the presence of metacyclics and those found infected were isolated from the rest. The infection rate obtained by probing the flies (flies with mature infections only) ranged from 15 - 30%. The infected flies were fed singly onto irradiated Swiss mice. At peak parasitaemia, the mice were bled and trypanosomes karyotyped. The chromosome profiles obtained were compared with those of the original infecting clone. For all the clones, the profiles remained stable in terms of the number and position of the chromosome -sized DNA bands (Figs. 12 and 13).

Fig. 11. Orthogonal field alternating gel electrophoresis of chromosome-sized DNA molecules from clones of bloodstream *Trypanosoma congolense*. Clone numbers IL 3633, IL 3662 and IL 3672 are represented in lanes 1, 3 and 5 while lanes 2, 4 and 6 represent the same clones respectively after a period of four months passage in goats. M is the standard molecular weight marker.

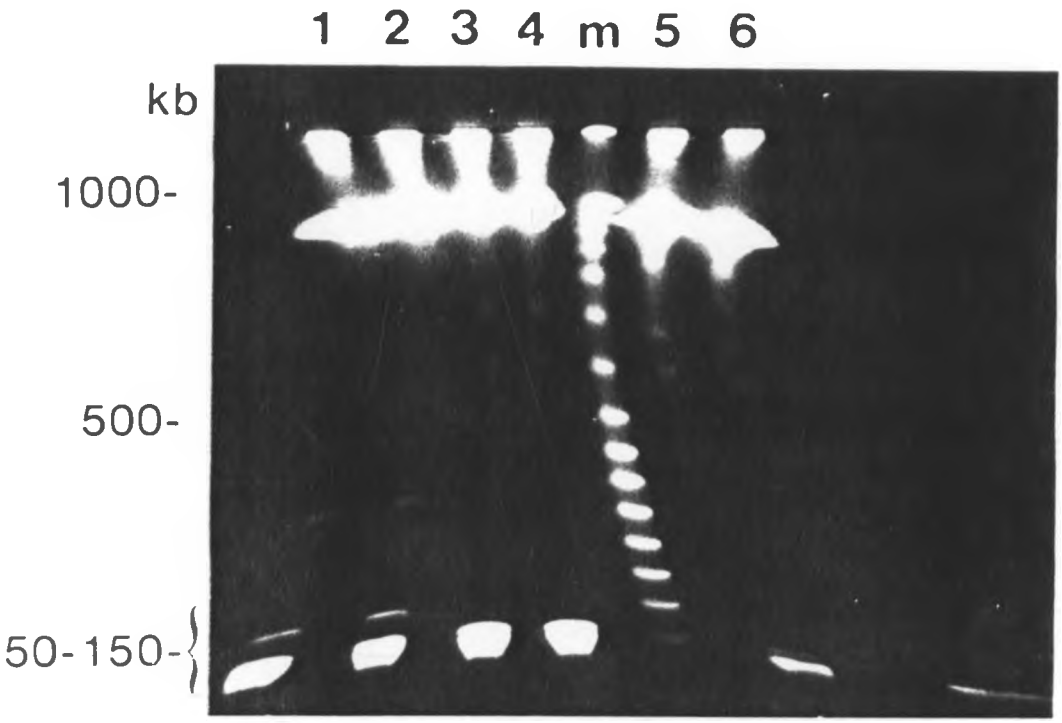

1 2 3 4 m 5 6
kb
1000-
500-
50-150-

Fig. 12. Orthogonal field alternating gel electrophoresis of chromosome-sized DNA molecules from clones of bloodstream *Trypanosoma congolense*. Clone numbers IL 3593, IL 3649 and IL 3612 are represented in lanes 1, 3 and 6 while lanes 2, 4 and 7 represent the same clones after cyclial transmission respectively. Lanes 6, 7 and 8 represent the same clone (IL 3612) before transmission, after transmission and also after four months infection respectively. Lane 5 presents clone IL3662 after four months chronic infection in flies.

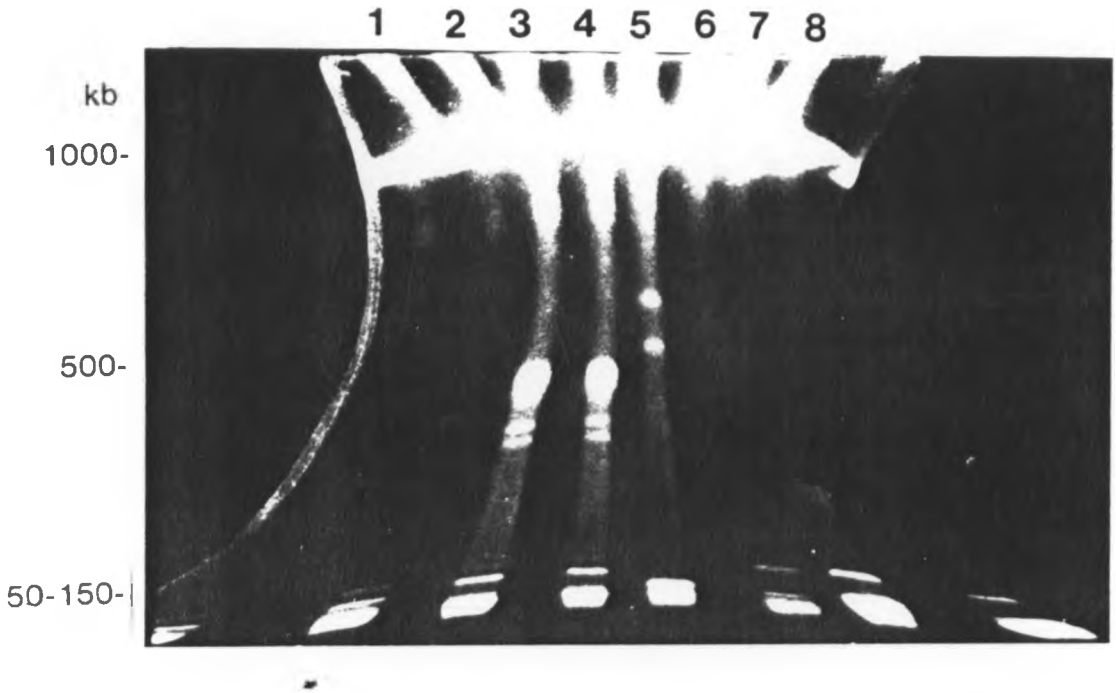
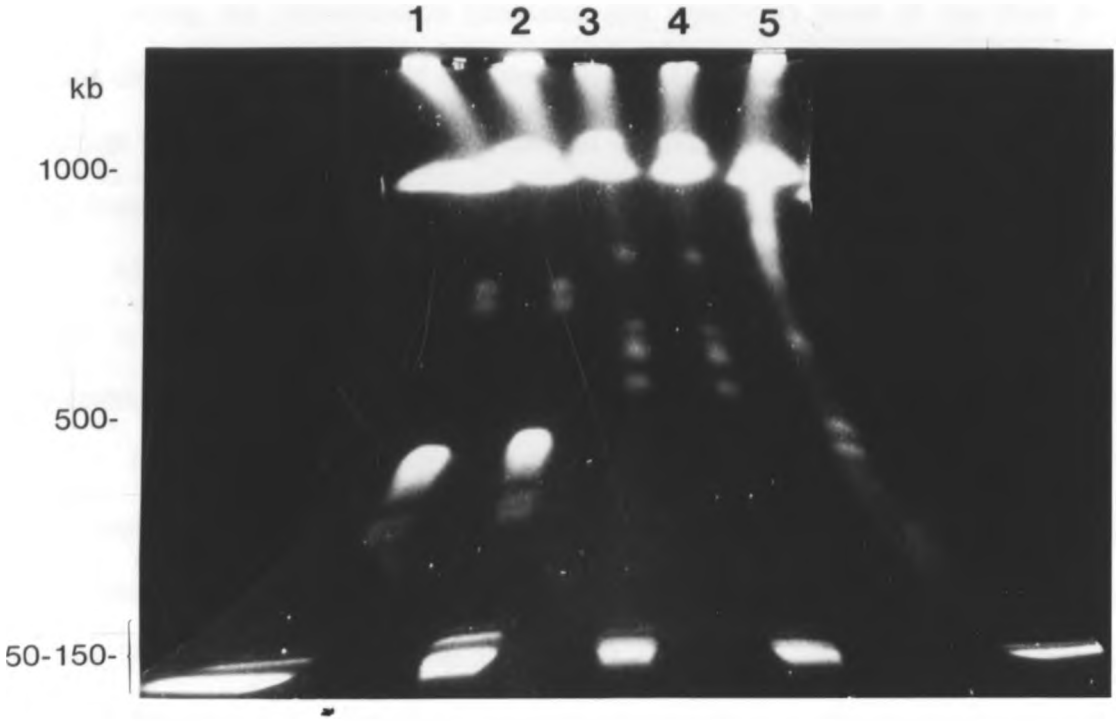

1 2 3 4 5 6 7 8
kb
1000-
500-
50-150-

Fig.13. Orthogonal field alternating gel electrophoresis of chromosome-sized DNA molecules of bloodstream *Trypanosoma congolense*. Clone numbers IL 3649 and IL 3669 are represented in lanes 1 and 3 respectively, while lanes 2 and 4 represent the same clones after cyclical transmission. Lane 5 displays the chromosome profile of a different clone IL 3672.


1
2
3
4
5
kb
1000-
500-
50-150-

3.5 Cross Protection Experiments

Cloned stabilate numbers IL 3593, IL 3612, IL 3649, IL 3655, IL 3662, IL 3669 and IL 3672, all from different stocks, were used for this experiment. Each clone was inoculated into a group of twelve mice intra-peritoneally, each mouse receiving 10^3 trypanosomes. The chromosome profiles of these clones had been determined before the cross protection experiments commenced as described in the Materials and Methods in Chapter 3.

During the immunization (infection and treatment) some of the mice in various groups died and only those that survived were given a challenge infection. Each challenge infection was given five days after treatment in order to allow complete elimination of the drug from the body systems.

All the clones protected against a homologous challenge, except for one group where one mouse was not protected. Protection was confirmed by the absence of detectable parasitaemia from the peripheral circulation for a period of not less than two weeks. Mice that were protected survived after a challenge infection with no detectable parasitaemia. Protection was conferred by clones with similar though not identical chromosome profiles. For instance, mice infected with clone number IL 3662 (represented in lane 3 of Fig. 10) were protected against challenge with the clone number IL 3664 (represented in lane 1). For identical clones, mice infected with the clone number IL 3672 (Lane 1 of Fig. 9) offered protection against a challenge with the identical clone number IL 3677 (Lane 3of Fig. 9). Clones that displayed different chromosome profiles did not offer protection for challenge infections with each other. This indicates that heterologous immunity was not acquired except in two cases where one mouse each survived the challenge infection with a heterologous clone. Results of the cross protection experiment are summarised in Table 4.

Table 4. A summary of the results of the cross protection experiments.

Immunizing Clone	Homologous challenge	No. of mice protected / No. of mice challenged	Heterologous challenge	No. of mice protected / No. of mice challenged
IL 3621	IL3611	6/6	IL 3672	1/6
IL 3672	IL 3677	6/6	IL 3611	0/6
IL 3662	IL 3664	11/12	IL 3668	1/12
IL 3649	-	-	IL 3664	0/10
IL 3669	-	-	IL 3649	0/10
IL 3655	IL 3595	10/10	-	-
IL 3593	-	-	IL 3592	0/10

CHAPTER FOUR

DISCUSSION

4.1 The behaviour of the trypanosome stocks in mice

Although all the stocks were confirmed to be *T. congolense* by examination of wet preparations, differences were observed in their growth patterns in mice. Some of the stocks were fast growers and became quite virulent within a short time. Other stocks were slow growers and survived in the experimental mice for a long period, even at high parasitaemia before killing them. Some other stocks survived at very low parasitaemia for long periods in some strains of experimental animals such as the Sprague Dawley rats, while they were fast growers in other strains of experimental animals such as the Swiss white mice. This variation reflects the diversity of *T. congolense* described by Godfrey (1982).

4.2 Cloning of the trypanosome stocks

The variation observed in the growth patterns of the stocks in mice affected the cloning procedure. The number of clones and the duration within which the clones were obtained from each stock depended on the adaptability of the stocks in mice. For instance, those stocks that were well adapted produced more clones in a shorter period than stocks that were poorly adapted.

Cloning of the trypanosome stocks assured that each population analysed was of similar genetic composition. This was due to the fact that each population was derived from only a single trypanosome and this suited experiments such as cross protection where the immunity induced was clone specific. However, this was a selection procedure whereby within a stock that could have a large number of clones only one or two clones were analysed. This was a limitation because all the clones derived from a single stock may not necessarily have displayed the same characteristics. The results of chromosome analysis showed that there was not much difference between the chromosome profiles of some stocks and those of their clones. Therefore the clones represented the stocks from which they were derived.

4.3 Chromosome analysis.

The molecular karyotyping of *T. congolense* has revealed the existence of two karyotypic groups (Majiwa et al., 1985) which are referred to as the Kilifi and the savanna types. Each karyotype is composed of characteristic chromosome profiles that can be distinguished from those of other trypanosome species on the basis of the molecular weights of the chromosome-sized DNA molecules. The Kilifi type has got mini-chromosomes in the range 75-150kb, numerous medium-sized chromosomes in the range 200-450kb and relatively few above this region (Majiwa et al., 1985; Masake et al., 1988). The savanna type has mini-chromosomes of a lower molecular weight ,50-150kb. The medium-sized chromosomes in this type of *congolense* fall within the region 450-900kb (Majiwa et al., 1986a). In the range 200-400kb there are very few chromosomes.

Chromosome profiles of *T. brucei* resemble those of the Kilifi type except that *T. brucei* has fewer medium-sized chromosomes within the region 200-400kb and the mini-chromosomes are of lower molecular weight than those of the Kilifi type *T. congolense* (Masake et al., 1988). *T. vivax* has chromosome-sized DNA molecules not less than 2000kb (Majiwa et al., 1985). *T. simiae* has two distinct bands, one at the size of approximately 50kb and the other one at 1000kb (Majiwa and Webster, 1987).

Chromosome profiles obtained in the present work presented a pattern that was characterized by very small mini-chromosomes of sizes < 50-150kb, which appeared as a thick band in all profiles. At the region 200-400kb there were very few bands. This contrasts with the Kilifi type where there are numerous medium-sized DNA bands in this region. Most of the medium-sized DNA bands in the present work were within the region 400-750kb. Above this region some DNA remained unseparated at 1000kb while another batch of DNA remained in the slots. It is clear therefore that the clones described in the present work are savanna-like on the basis of their molecular karyotype.

4.4 Serodeme identification

Chromosome analysis has in the past revealed that each chromosome pattern is characteristic for each antigenic repertoire or serodeme (Masake et al., 1988). The

correlation between chromosome patterns and serodemes was investigated using *in vitro* neutralization tests. Sera obtained from chronically infected goats that had undergone spontaneous recovery neutralized all trypanosome clones that had a similar chromosome profile, but not clones that had different chromosome profiles. In the present work the relationship between the chromosome profile and serodeme was investigated by cross-protection experiments in mice, where mice that were immunized against a certain clone were challenged first with a homologous clone (similar chromosome profiles). Results obtained were that the challenged mice aquired protection against the homologous clones and survived. In one case there was no protection, probably indicating normal biological variability.

The homologous challenge (where mice immunized with a certain clone were challenged with a clone of similar profile) was followed by a heterologous one, where the immunized mice were challenged with a clone that had a different chromosome profile. In this case the mice did not survive since they did not acquire protection. However in two experiments some mice survived a heterologous challenge, indicating a partial immunity. This may have been due to the presence of some shared VATs between the two heterologous clones where similar antibodies were produced that offered some immune protection. The results conform to those obtained by Majiwa et al. (1986b) where five clones that had distinct chromosome profiles were also seen to belong to different serodemes and this was confirmed by immunological examination of their respective metacyclic VATs. The five clones had been obtained from different parts of East Africa.

4.5 **Stability of the chromosome profiles.**

After establishing the fact that the chromosome profiles could be used for serodeme identification, the next step was to find out whether these profiles remain stable when the trypanosomes are cyclically transmitted and when the infection run a chronic course in a host. The results would indicate what may be happening in the field where the two processes occur. Masake et al. (1988) confirmed that for a period of up to one year the chromosome profiles remained stable in terms of the size, position and number of

chromosome-sized DNA molecules. Stability was also maintained after a cyclical transmission except that in one clone, one of the chromosome-sized DNA molecules shifted to a lower molecular weight (Masake et al., 1988). In the present work chromosome profiles of the clones studied remained stable in terms of the size and number of chromosome sized-DNA molecules.

4.6 **Conclusions**

(1) The clones derived from the various stocks displayed different chromosome profiles, though these profiles can be put into one basic pattern.

(2) These profiles may be used to identify serodemes, due to the fact that the immunity acquired during cross protection experiments was specific for a particular pattern.

(3) If each profile represents a serodeme it is clear from the various chromosome profiles obtained that a large number of serodemes exist in *T. congolense*.

(4) Neither cyclical transmission nor chronic infection in a goat affects these profiles.

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