DECLARATION

This is my original work and has not been submitted for a degree at any other university or institution

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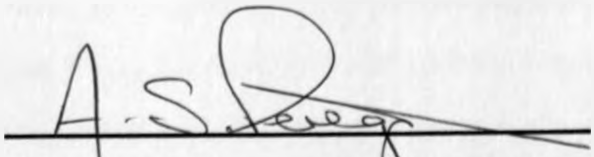

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To my father Jotham Burudi and my mother Dainah Indomboh, who like Joshua endeavoured to nurture a God-fearing family

Acknowledgements

I am very grateful to DAAD for providing me with a scholarship that enabled me to cover my tuition and subsistence expenses conveniently.

Special thanks go to ILRAD for giving me the sponsorship for this research project. In this regard, I am greatly indebted to Dr. A.S. Peregrine for his supervisory prowess and brotherly support during my research and write-up of the thesis. He was a teacher indeed.

I owe a lot of thanks to Prof. S.M. Mbiuki for the guidence he offered to me on the one hand as my university supervisor, and on the other like a father. He was a great source of inspiration to me.

Much gratitude go to Dr. N. Murphy for supervising me in molecular biology work. Dr. P. Majiwa is highly complimented for providing some of the very crucial molecular biology materials for the project, and for his willingness to help whenever requested to. The help I got from Dr. J. Waitumbi in molecular biology is highly appreciated.

The technical assistance provided by Mr. S. Wasike and Mr. S. Kemei of Unit one is amply appreciated. The ILRAD farm staff are highly commended for the management of the experimental animals and for their assistance during the goat experiment.

Much thanks to Mr D. Elsworth, Mr. F. Shikhubari and Mr. J. Maenga, of graphic arts, for offering photographic and art services efficiently and promptly.

Great regards for the ILRAD library staff for being very helpful in providing literature materials and photocopy services promptly whenever they were required.

The help of Miss Maria Mulindi in typing of the thesis is sincerely appreciated.

Drs. E. Taracha and M. Mamman were friends indeed during the whole course of this work.

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ABBREVIATIONS

AP-PCR -	arbitrarily-primed polymerase chain reaction
bp -	base pair
b.w. -	body weight
cDNA -	complementary deoxyribonucleic acid
cpm -	counts per minute
dATP -	deoxyadenosine triphosphate
dCTP -	deoxycytidine triphosphate
CD_{50} -	dose of drug that cures 50% of infected population of animals
DEAE -	diethyl-amino-ethyl
DFMO -	DL-α-difluoromethylornithine
dGTP -	deoxyguanosine triphosphate
DNA -	deoxyribonucleic acid
dNTP -	deoxynucleotide triphosphate
dTTP -	deoxythymidine triphosphate
EDTA -	ethylene diamine tetra-acetic acid
ELISA -	enzyme-linked immunosorbent assay
HCl -	hydrochloric acid
hr -	hour
ICI -	Imperial Chemical Institute
ILNar 3 -	ILRAD *Nannomonas* antigenic repertoire 3
ILNat 3.1 -	ILRAD *Nannomonas* antigenic type, serodeme 3, VAT 1
ILNat 3.2 -	ILRAD *Nannomonas* antigenic type, serodeme 3, VAT 2
ILNat 3.3 -	ILRAD *Nannomonas* antigenic type, serodeme 3, VAT 3
ILO -	ILRAD oligonucleotide

ILRAD -	International Laboratory for Research on Animal Diseases
i/m -	intramuscularly
i/p -	intraperitoneally
iu -	international units
i/v -	intravenously
KCl -	potassium chloride
kDNA -	kinetoplast DNA
kg -	kilogramme
M & B -	May and Baker (U.K.)
MCHC -	mean corpuscular haemoglobin concentration
MCV -	mean corpuscular volume
mg -	milligramme
$MgCl_2$ -	magnesium chloride
min -	minute
ml -	millilitre
mm -	millimetre
mM -	millimolar
ng -	nanogramme
NP40 -	Nonidet p-40
nt -	nucleotide
O.D -	optical density
PCR -	polymerase chain reaction
PCV -	packed cell volume
pg -	picogramme
PSG -	phosphate saline glucose
R -	trade mark
RAPD	randomly amplified polymorphic DNA
RF -	revised formula

RNA -	ribonucleic acid
RNase A -	enzyme for digesting ribonucleic acid
rpm -	revolutions per minute
SDS -	sodium dodecyl sulphate
sec -	seconds
SSC -	standard sodium citrate, sodium chloride solution
TAE -	tris-acetate
Taq DNA polymerase -	*Thermus aquaticus* deoxyribonucleic acid polymerase
TDS -	triple-distilled, deionised sterile
TE -	tris EDTA
TNE -	tris sodium EDTA
UK -	United Kingdom
USA -	United States of America
U V -	ultraviolet light
VSG -	varriable surface glycoprotein
WHO -	World Health Organization

ABSTRACT

The research described in this thesis was carried out to investigate the phenotypic basis of resistance to diminazene (Berenil^{R}) in *Trypanosoma congolense*.

Earlier work has shown that, in both goats and mice, the majority of *T. congolense* trypanosomes which reappear following treatment with diminazene aceturate are sensitive to the dosage that was used. Such a phenomenon could be due to the ability of sensitive trypanosomes to survive treatment when mixed with resistant trypanosomes. Thus, the work described here was carried out to establish whether a diminazene-sensitive clone of *T. congolense* could survive treatment with diminazene aceturate at a dose of 7.0 mg/kg b.w. when mixed with a diminazene-resistant clone in goats. Since the study used two Savannah-type clones of *T. congolense*, the work necessitated the development of a polymerase chain reaction (PCR) technique that could differentiate the two clones of *T. congolense*.

The 2 clones of *T. congolense* that were used were *T. congolense* IL 1180 (a derivative of STIB 212 which is sensitive in goats to intramuscular [i/m] treatment with diminazene aceturate at a dose of 7.0 mg/kg b.w.) and *T. congolense* IL 3274 (a derivative of IL 2865, isolated from a cow in Burkina Faso, which is resistant in goats to i/m treatment with diminazene aceturate at a dose of 7.0 mg/kg b.w.).

In order to distinguish the 2 clones, a PCR technique was developed which utilised a DNA sequence that is present in IL 1180, but not in IL 3274. A pair of 20 nt primers were developed on the basis of DNA sequence information for the ends of the cloned DNA sequence. The primers were then shown to amplify a 900 bp sequence from the plasmid in which the gene was cloned (p1616/5), and from genomic DNA of IL 1180. However, a similar product was not produced with IL 3274 genomic DNA. In further work, the 900 bp product

amplified by PCR from p1616/5 was purified and labelled with ^{32}P, in order to generate a probe specific for the trypanosome-specific PCR product.

Using the above reagents, the first study endeavoured to establish the minimum amount of IL 1180 DNA that could be detected when mixed with 25 ng of IL 3274 DNA. When using ethidium bromide-staining of PCR products in an agarose gel, the minimum level of detection was 100 pg of IL 1180 DNA. However, when such a gel was blotted and the filter hybridized with the [^{32}P]-labelled 900 bp product, the sensitivity was increased by 100-fold (i.e., to 1 pg).

In an experiment to determine whether the diminazene-sensitive clone (IL 1180) could survive treatment with diminazene aceturate when mixed with a resistant clone (IL 3274), 24 goats were randomised into 3 groups of 5 goats each (Groups A, B and C) and 3 groups of 3 animals each (Groups D, E and F). Groups A and D were infected with IL 1180; Groups B and E were infected with IL 3274; and Groups C and F were infected with both clones simultaneously. All animals were infected intravenously, via the jugular vein, and animals that were treated were administered diminazene aceturate i/m at a dose of 7.0 mg/kg b.w. Animals in Groups A and B were treated after all the goats in the respective groups had been detected parasitaemic. In contrast, goats in Group C were not treated until all the animals in both Groups A and B had been detected parasitaemic, thereby ensuring that both trypanosome clones in these animals had fully developed by the time of treatment. Groups D, E and F served as non-treatment controls and also facilitated a comparison of the pathogenicity of the 2 clones individually and when mixed. Following treatment, all goats in all groups were monitored 3 times a week for 84 days for their levels of anaemia and parasitaemia. During the entire experiment, trypanosome stabilates were collected as follows from all animals; stabilates of goat blood, once a week; parasitaemic mouse blood as a result of inoculation with goat blood, once every 2 weeks; buffy-coat preparations of parasitaemic goat blood, twice a week.

All 5 goats infected with IL 1180 and treated with diminazene aceturate (Group A) did not develop a relapse infection for the entire 84 days following treatment. This was in contrast to goats infected with IL 3274 (Group B) and those infected with both clones (Group C) in which 5 out of 5 and 4 out of 5 relapses occurred, respectively. All the non-treatment control goats infected with IL 3274 developed a severe anaemia (Packed Cell Volume [PCV]< 12%), along with a high level of parasitaemia, and were therefore removed from the experiment since this level of anaemia was deemed fatal. In contrast, all non-treatment goats infected with IL 1180 maintained their PCV between 15% and 20% throughout the entire experiment. Finally, of the 3 non-treatment control goats infected with both clones, one developed a severe anaemia and was removed from the experiment; the remaining 2 maintained their PCV above 12% during the entire experimental period. Thus, on the basis of ability to maintain PCV, it can be concluded that the drug-resistant clone was more pathogenic than the drug-sensitive clone.

In order to determine that IL 1180 was present in goats with mixed infections at the time of treatment (Group C), goat blood stabilates collected 3 days before treatment were expanded in irradiated mice. Goat buffy-coat preparations taken on the day of treatment, were also examined. However, while trypanosomes in goat blood were expanded in mice, trypanosomes in buffy-coats were examined directly. Goat blood stabilates of relapse trypanosome populations occurring in the same animals on days 18, 32, 46 and 60 following treatment were also examined; each stabilate was expanded in irradiated mice. The DNA from all the aforementioned samples were screened for the presence of IL 1180 using the PCR-technique described above, and the [^{32}P]-labelled 900 bp probe. For each reaction, 25 ng of genomic DNA was used as the template. The results demonstrated that IL 1180 was present in all mixed infections prior to treatment, but was absent in all relapse populations following treatment at the level of detection of the radiolabelled probe (i.e., 1 pg/25 ng total DNA). The

data therefore indicated that a diminazene-sensitive trypanosome population is unable to survive treatment with diminazene when mixed with a diminazene-resistant population.

CHAPTER ONE INTRODUCTION

Trypanosomiasis is a systemic infection of animals and man caused by parasites in the genus *Trypanosoma* (Hoare, 1970). In Africa, the disease in animals is caused mainly by 3 species which fall in the salivaria group; namely, *Trypanosoma brucei*, *T. congolense* and *T. vivax*. The salivaria group of trypanosomes develop in the mouth parts of tsetse flies and are primarily transmitted from host to host subsequent to cyclical development through the tsetse fly. However, *T. vivax* is also capable of mechanical transmission via the mouth parts of biting diptera such as Tabanus sp. (Hoare, 1970).

The geographical distribution of trypanosomiasis in Africa parallels that of the vector. The most affected region is the area of sub-Saharan Africa between latitudes 15^{o}N and 30^{o}S; an area that has suffered considerably both economically and socially due to the disease (Soulsby, 1987).

The severity of the disease due to trypanosomiasis is dependent on a number of factors, including the methods used to control the disease. Thus, vector control, keeping and development of trypanotolerant animals, and the use of drugs (chemotherapy) can alter the relationship between the host and the parasite (Killick-Kendrick and Godfrey, 1963; Ford, 1971). Whilst chemotherapy removes the trypanosomes from the host, long-term chemotherapy may also result in the development of drug resistance (Whiteside, 1960a).

Drug resistance is manifested as the occurrence of a relapse infection in an infected animal following administration of a drug at the recommended dose rate. A considerable amount of research has been carried out on drug resistance and the findings indicate that not all relapse infections occur due to drug resistance *per se* (Jennings *et al.*, 1977; Haase *et al.*, 1981; Mamman *et al.*, 1991). The possible explanations for such a conclusion are that;

1) The parasites could be localising in privileged parts of the body that are not well perfused by the drug. Such areas include the brain (Jennings *et al.*, 1979; Haase *et al.*, 1981), and possibly the chancre (Luckins and Gray, 1978) and blood vessels in capillary beds where there may be limited perfusion (Banks, 1978, 1980). 2) An alteration in the sensitivity of the parasite to the drug during a certain stage of development in the definitive host, as suggested by Goodwin and Rollo (1955).

In a study conducted in mice, Jennings *et al.* (1977) investigated the response of *T. brucei* infections to treatment with diminazene aceturate (Berenil) at various intervals following initiation of infection. When treatment was initiated 3 days after infection all animals were cured. This compared to almost 100% relapses when treatment was delayed to 14 days post-infection. Since relapse strains were also completely susceptible to treatment early in infection, this suggested that the phenomenon observed was not due to increase in drug resistance. Rather, it seemed to be a result of trypanosomes having escaped the action of the chemical, either because they were in an inaccessible situation in the body or in an unsusceptible stage of development. A possible explanation for the type of relapse described in this case could be the persistence of the "amastigote phase" of *T. brucei* described by Ormerod and Venkatesan (1971a,b). There are also several reports in which the existence of "occult" or "tissue" forms in bovine trypanosomiasis, caused by *T. congolense* and *T. vivax*, has been postulated as being responsible for similar observations in ruminants (Goodwin and Rollo, 1955). This suggestion is consistent with the finding that the sensitivity of a certain clone of trypanosome to diminazene varies with the time between infection and treatment in ruminants (Mamman *et al.*, 1993; Silayo *et al.*, 1992). Thus, Silayo *et al.* (1992) demonstrated that treatment of goats infected with *T. congolense* IL 3274 with diminazene aceturate at a dose of 7.0 mg/kg b.w. 24 hours post-tsetse challenge, resulted in complete cure. In contrast, when treatment was given after detection of trypanosomes, relapse infections occurred.

Mamman *et al.* (1993) demonstrated the same phenomenon. In other work, Mamman *et al.* (1991) demonstrated that populations of *T. congolense* IL 3274 relapsed in mice when treated intraperitoneally with diminazene aceturate at a dose of 25.0 mg/kg b.w.. In further work in mice to study the sensitivity of the relapse populations to the latter drug dosage, it was revealed that the frequency of resistant trypanosomes within relapse populations was very low (less than 1%). Thus, it was concluded, most of the trypanosomes which relapsed following treatment with diminazene were apparently sensitive to the drug dosage that was used. Furthermore, it was also concluded that the level of resistance expressed by a trypanosome population *in vivo* was dependent on the population size. On this basis, it could be concluded that the phenomenon of resistance to diminazene in *T. congolense* appears to be multifactorial. If this is true, then it would imply that although drug resistance exists *per se,* the drug-resistance phenotype is controlled by a number of factors. In the aforementioned studies, greater than 99% of trypanosomes relapsing following treatment with diminazene were shown to be sensitive to the treatment used. It is therefore important to determine whether the sensitive trypanosomes arise following treatment or whether they are able to survive treatment when mixed with resistant trypanosomes.

OBJECTIVES

1: To determine whether a population of *T. congolense* that is sensitive to treatment with diminazene aceturate at a dose of 7.0 mg/kg b.w (IL 1180) can survive treatment with diminazene aceturate when it is mixed with a population of *T. congolense* that is resistant to diminazene aceturate at the same dosage (IL 3274).

2: To test the feasibility of using DNA amplification by PCR to distinguish between the 2 Savannah-type *T. congolense* clones; *T. congolense* IL 1180 and *T. congolense* IL 3274.

3: To establish the minimum proportion and concentration of *T. congolense* IL 1180 that can be detected by the polymerase chain reaction in a mixed population of *T. congolense* IL 1180 and IL 3274.

4: To compare the haematological and parasitological changes occurring in goats when infected with either of the 2 strains of *T. congolense*, or when infected with both. The resultant data will be used to compare the pathogenicity of the 2 strains in goats and, in the process, try to establish whether there is a correlation between drug resistance and pathogenicity.

JUSTIFICATION

The work to be undertaken here is a follow-up to the findings of Mamman *et al.* (1991). In that work they showed that populations of *T. congolense* IL 3274 relapsed in goats when treated intramuscularly with 7.0 mg diminazene aceturate/kg b.w. and in mice when treated intraperitoneally with 25.0 mg diminazene aceturate/kg b.w. In the study conducted in mice, they observed that the frequency of diminazene-resistant trypanosomes in relapse populations was very low. However, their study did not demonstrate whether the diminazene-sensitive trypanosomes in relapse populations were present before treatment or arose following treatment.

Currently, it is not known what makes a trypanosome resistant to a drug. However, some trypanosome clones of the same species have been shown to vary in their resistance to a given drug. For instance, in mice the diminazene aceturate CD_{50} value (dose of drug required to cure 50% of an infected population) of *T. congolense* IL 1180 is 2.3 mg/kg b.w. whereas that of *T. congolense* IL 3274 is 12.4 mg/kg b.w. (Peregrine *et al.*, 1991).

This study is designed to determine whether the diminazene-sensitive trypanosomes that occur in relapse populations following treatment actually evade the effect of the drug or arise following treatment. The study will be carried out using 2 clones of *T.congolense*; namely, *T. congolense* IL 1180 and *T. congolense* IL 3274. Three groups of goats will be infected; with *T. congolense* IL 1180, *T. congolense* IL 3274 or both clones (IL 1180 and IL 3274) simultaneously. The aim is to find out whether *T. congolense* IL 1180, sensitive to diminazene aceturate at 7.0 mg/kg b.w., can survive treatment with the same drug dosage when mixed with a resistant population (IL 3274). Since a diagnostic tool is required to distinguish the 2 clones with a high level of sensitivity, this justifies the need for the development of a DNA probe.

A final aspect of the work is to compare the pathogenicity of the 2 trypanosome clones in goats. This will be carried out to determine whether drug-resistant trypanosomes are less pathogenic than drug-sensitive ones, as suggested by Leach and Roberts (1981).

In conclusion, it is envisaged that the findings from this work will enable a clearer understanding of the mechanisms involved in the development of the drug-resistance phenotype in African trypanosomes, thereby helping to improve strategies for treatment and control of the disease. Secondly, it is also hoped that a tool will be developed to distinguish between the 2 Savannah-type *T. congolense* used in this experiment, at the molecular level. This tool will be of benefit to further studies in chemotherapy of African trypanosomiasis, especially in areas related to drug-resistant and drug-sensitive parasites. Finally, it is hoped that a comparison could be made between the ability of drug-resistant and drug-sensitive parasites to cause disease in goats.

CHAPTER TWO LITERATURE REVIEW

2.1 IMPORTANCE OF TRYPANOSOMIASIS

In sub-Saharan Africa, trypanosomiasis in domestic livestock is caused mainly by 3 salivarian trypanosomes; *T. vivax*, *T. brucei* and *T. congolense* (Whiteside, 1958; Omuse 1973). However, clinical disease caused by *T. simiae*, *T. evansi* and *T. equiperdum* may be occasionally encountered in some parts of Africa. The disease caused by the first three trypanosomes occurs over an area of 10 million square kilometers in Africa between latitudes 15^{o}N and 30^{o}S (Soulsby, 1987) and the trypanosomes are transmitted to domestic livestock via the bites of infected tsetse flies. The distribution of the tsetse-transmitted trypanosomes is therefore essentially dictated by the distribution of the vector, the tsetse fly. However, *T. vivax* can be transmitted to livestock by mechanical transmission (Leach and Roberts, 1981) and therefore occurs over a greater area.

The presence of trypanosomiasis precludes the rearing of livestock in many areas. Where cattle are maintained in trypanosomiasis endemic areas, the main economic losses associated with the disease emanate from death of infected livestock, emaciation due to chronicity of the disease (Whiteside, 1958) and reduced meat, milk, hide and skin production. The importance of the disease is highlighted by the fact that the low daily per capita consumption of animal protein in sub-Saharan Africa, as per a 1979 report, is thought to be partly due to trypanosomiasis (Griffin, 1979).

Trypanosomiasis occurs in cattle, sheep and goats. However, trypanosomiasis in sheep and goats has been dismissed by several researchers as being an insignificant disease (Kramer, 1966; MacLennan, 1970; Stephen, 1970). This is inspite of the scarce information on the epidemiology, distribution, prevalence and economic impact of the disease in these livestock species. In general, infected goats and sheep tend to develop a chronic progressive infection

when infected with either *T. congolense* or *T. vivax* (Stephen, 1970). However, goats are said to be mildly susceptible to *T. vivax* (Ilemobade *et al.*, 1975). In contrast to cattle, *T. simiae* infections in goats have an acute or subacute progression, which more often than not terminate in death (Ilemobade *et al.*, 1975).

Experimental work has established that exotic breeds of goats are more susceptible to trypanosomiasis than indigenous small East African breeds, while crosses between the two show an intermediate susceptibility (Griffin and Allonby, 1979). Furthermore, MacLennan (1970) reported that the larger varieties of sheep and goats kept by nomadic pastoralists are more susceptible to trypanosomiasis than the smaller varieties owned by sedentary villagers. In other work, the possible economic importance of the disease in small ruminants and the cost-benefit of utilizing cross-breeding programme to produce highly productive, and yet trypanosome-resistant breeds has been described (Griffin and Allonby, 1979)

2.2 CLASSIFICATION AND STRUCTURE OF TRYPANOSOMES

Attempts have been made over a long time to classify trypanosomes on the basis of various criteria. Currently, trypanosomes are categorized in the phylum protozoa and genus trypanosoma (Hoare, 1970). The mammalian trypanosomes fall naturally into two major divisions; stercoraria and salivaria groups, which differ mainly in the course of development in their vectors (Hoare, 1970).

The Stercoraria section encompasses all the trypanosomes whose development is completed in the posterior station of the vector and whose transmission is by contamination (mechanical). This section includes the

subgenus hepertosoma, schizotrypanum, and megatrypanum under which *T. theileri* falls.

The Salivaria section comprises trypanosome species whose development is completed in the anterior station of the vector and whose transmission is by inoculation. Many of the trypanosomes in this section are transmitted by the tsetse fly and undergo cyclical development in this insect. However, *T. vivax* and *T. evansi*, which fall in this section, may be mechanically transmitted by biting flies (Tabanidae). In contrast, *T. equiperdum* is only transmitted during coitus in horses. The pathogenic trypanosomes in this section include the following (Hoare, 1970);

Subgenus Duttonella, under which fall *T. vivax* and *T. uniforme*.

Subgenus Nannomonas, which includes *T. congolense* and *T. simiae*.

Subgenus Trypanozoon, which includes *T. brucei*, *T. rhodesiense*, *T. gambiense* and *T. evansi*.

Subgenus Pycnomonas, represented by *T. suis*.

Mammalian trypanosomes typically have a spindle-shaped flattened body which is usually curved. However, the shape varies with different species. It also depends on the stage of development. There is a single nucleus, of the vesicular type, containing a large chromatic karyosome and peripheral chromatin lining the membrane (Hoare, 1970). The organ of locomotion is the flagellum, the base of which originates from the posterior part of the body, and runs along the outer margin of a frilled fold of the pellicle to form the undulating membrane. The base of the flagellum is formed by the basal body (blepharoplast). Adjacent to the basal body is the kinetoplast which accompanies the base of the flagellum whenever the position of the latter shifts in the course of development (Hoare, 1970).

2.3 PATHOGENESIS OF AFRICAN TRYPANOSOMIASIS

2.3.1 Clinical signs

Progression of trypanosomiasis in individual animals is complex and variable, depending upon the nature and stage of the infection studied. Infections with the 3 most common trypanosome species (*T. b. brucei*, *T. congolense* and *T. vivax*) may run anything from an acute to a chronic course. A fatal termination is indicative of failure of the host's immune mechanism; complete recovery denotes the host's ability to overcome the infection; an intermediate condition is indicative of an equilibrium between the host and the parasite (Mulligan, 1970).

In naturally transmitted cases of African animal trypanosomiasis in goats, the disease manifests itself initially with the development of a chancre at the site of the tsetse bite. Subsequently, enlargement of the lymph node draining the site of the testse-fly bite occurs. Pyrexia, anaemia and debilitation are common subsequent occurrences.

The development of a local skin reaction at the site of inoculation of metacyclic trypanosomes (chancre) follows the successful bite of an infected tsetse fly. The chancre is a local skin reaction that occurs in association with establishment of metacyclic trypanosomes in the dermis of the skin (Emery *et al.*, 1980). Thus, when metacyclic trypanosomes are inoculated subcutaneously or intravenously, the development of a chancre does not occur. In goats, chancres have been described following tsetse transmission of *T. congolense*, *T. vivax* (Emery and Moloo, 1981) and *T. brucei* (Emery and Moloo, 1980). Subsequent to initiation of infection at the level of the skin, the draining lymph node enlarges. This occurs as a result of proliferation of polymorphonuclear leucocytes, in response to the presence of trypanosomal antigens (Barry and Emery, 1984).

The occurrence of pyrexia during *T. congolense* infections in goats has been described by many authors (Stephen, 1970; Kaaya, 1975). The magnitude and duration of pyrexia is said to depend on the duration of infection and the

level of parasitaemia (Fiennes, 1970; Kaaya, 1975). Peaks of pyrexia tend to coincide with peaks of parasitaemia, and normal temperatures tend to occur during aparasitaemic phases.

2.3.2 Parasitaemia

The susceptibility of an animal to African trypanosomiasis appears to be directly related to its ability to limit, reduce or control the level of parasitaemia. This has been demonstrated by Murray and Morrison (1979) who carried out a series of studies in cattle and mice, where breeds of cattle and strains of mice showed a range of susceptibility to the disease. The trypanotolerant N'dama cattle breed showed a superior capacity to control parasitaemia as compared to the more susceptible Zebu. Similarly, in in-bred strains of mice there was a correlation between the susceptibility of individual strains to the disease and the levels of parasitaemia that were observed.

2.3.3 Anaemia

The major clinical sign associated with *T. congolense* infections in animals is anaemia. However, as mentioned, other lesions such as leucopaenia, emaciation, lymphadenomegally, and oedema of the face and submandibular regions also occur (Kaaya, 1975; Tizard, 1985). As far as anaemia is concerned, the available literature indicates that there is a significant drop in the red blood cell count and hemoglobin levels which starts during the second week postinfection. It then persists at a low level throughout the infection. Kaaya (1975) also reported changes in MCV and MCHC, and established that non-infected animals have a higher capacity to regenerate reticulocytes than infected counterparts.

Anaemia is typified by haemolysis, sequestration of injured erythrocytes by the spleen, increased blood and plasma volumes, and the anaemia of chronic disorders (Kaaya, 1975; Tizard, 1985). Haemolysis occurring due to direct

trauma of erythrocytes by parasites is likely to be greater for *T. congolense* than other trypanosomes because of its adherence property to erythrocytes (Murray *et al.*, 1977).

The degree of anaemia is said to vary directly with the level of parasitaemia (Kaaya, 1975). In ruminants, anaemia is the major disease-promoting factor (Ugochukwu, 1983). Furthermore, it has also been established that in domestic ruminants the severity of anaemia, and hence the severity of the disease, is directly related to the level of parasitaemia, at least in the early phases of the disease (Murray and Morrison, 1979). Therefore, the degree of parasitaemia and the level of anemia in trypanosome-infected ruminants can be used as parameters to compare the pathogenicity of various species or strains of trypanosomes in the same breed of host.

2.3.4 Interference between trypanosome infections

Interference as a phenomenon in trypanosome infections is inhibition of establishment or progression of one particular trypanosome population in a definitive host as a result of the presence of another. Generally, the population that is already established (the primary infection) inhibits the development of a superinfection (secondary infection). Such an interference phenomenon has been described in goats (Dwinger *et al.*, 1986, 1989) in association with populations of both *T. congolense* and *T. brucei*. In these studies, primary infections with the former species delayed chancre development when animals were superinfected with the latter trypanosome species. In contrast, a primary infection with *T. congolense* had no effect on the establishment of a secondary infection with *T. vivax* (Dwinger *et al.*, 1989).

In similar work, Sones *et al.* (1989) carried out a study in goats with two unrelated stocks of *T. congolense*, one of which was highly sensitive to isometamidium chloride and the other which was highly resistant to the drug. Superinfection with the sensitive stock resulted in establishment of the infection

and suppression of the resistant stock to below the limit of detection of the method used. These observations suggested that isometamidium-resistant stocks may be less viable than sensitive ones and could explain the relative scarcity of isometamidium-resistance in the field. The finding also lends credence to the belief that drug-resistant strains are less adapted to life in the absence of the drug than unmodified parental populations (Sones *et al.*, 1989).

Regardless of the drug sensitivity of the trypanosome serodemes or species involved, the significance of the interference phenomenon is that it might result in a limitation of the number of serodemes of *T. congolense* or *T. brucei* that an animal can be infected with at any one time in the field.

The mechanism(s) by which the interference phenomenon occurs is/are not known. It may be due to the presence or absence of a factor in the skin, a situation induced shortly after initiation of a trypanosome infection in goats, which delays parasite development when metacyclic trypanosomes are deposited by tsetse flies (Dwinger *et al.*, 1986). However, the identity of this factor has not been determined. In studies conducted in cattle, Morrison *et al.* (1982) demonstrated that interference in the establishment of a superinfection, between different serodemes of *T. congolense*, was dependent on an active infection. It did not occur as a result of specific immunity. Finally, Hawking (1963b) suggested that the observed differences in viability of 2 strains of trypanosomes in mixed infections was a reflection of the slower replication rate of drug-resistant stocks.

A number of hypotheses have therefore been advanced to explain the interference phenomenon. The role of differences in replication rate as a contributory factor to the phenomenon has been put forward by Seed (1978). In a study carried out in mice infected with *T. b. gambiense*, it was shown that different antigenic variants compete with each other and that one clone can replace another. Furthermore, it was also demonstrated that there was a positive correlation between the ability to compete *in vivo* and the survival times of mice, thus suggesting that the fastest-growing clone gradually replaced the slower

growing ones. However, the rate at which one clone out-competed another by far surpassed the ratio of their doubling times. Therefore, it was suggested that one clone may actually inhibit the growth of another, possibly through the production of some metabolic products. As to whether this phenomenon applies to different clones of *T. congolense* is not known, although the interference observed between drug-resistant and drug-susceptible populations of *T. congolense* would indicate that it may (Sones *et al.*, 1989). However, these latter authors used stocks and not cloned populations, thereby making interpretation of their data a little difficult.

2.3.5 Relapse infections

As previously mentioned, the occurrence of relapse infections following trypanocidal drug treatment is usually either attributed to drug resistance on the part of the parasite, or to underdosage with a given drug. However, relapse infections have been demonstrated in goats infected with *T. vivax* that appear not to be due to either of these phenomenons but due to invasion of sites within the host by parasites which are then inaccessible to the drug (Whitelaw *et al.*, 1988). The same phenomenon has also been documented by Jennings *et al.* (1977) for *T. brucei* infections in mice; the timing of treatment relative to initiation of infection appeared to alter the diminazene sensitivity of trypanosome populations. Subsequently, this observation was shown not to be associated with alteration in drug sensitivity of the trypanosome population, but due to invasion of the central nervous system by the trypanosomes. Thus, when treatment was delayed, the parasites had the time to invade a part of the body where there was either a very low level of the drug or non at all.

Since *T. congolense* is essentially a bloodstream-restricted parasite, invasion of the central nervous system would not appear to be a factor contributing to the relapse infection phenotype with this trypanosome species.

However, adherence of the parasites to the walls of the microvasculature by their anterior ends (Banks, 1978) may be a factor contributing to relapse infections if there is poor perfusion of such capillary beds, exposing trypanosomes to low drug concentrations. This theory appears to be supported by the occurrence of both drug-sensitive and drug-resistant populations of *T. congolense* in relapse infections (Mamman *et al.*, 1991). However, definitive proof of such a hypothesis is lacking. It should be noted that although *T. congolense* is said to be only an intravascular parasite, extravascular occurrence of *T. congolense* has been described by Roberts *et al.* (1969), Luckins and Gray (1978) and Masake *et al.* (1984). These sites may therefore act as a source of relapse infections following treatment of infected animals.

2.4 CONTROL OF TRYPANOSOMIASIS

In the control of trypanosomiasis in Africa, there are 3 main methods employed. These are: tsetse control, keeping of trypanotolerant animals, and chemotherapy. Whenever control methods for a given area are being designed, the economic cost and returns from the program have to be thoroughly considered, especially for high potential areas (Finelle, 1974).

2.4.1 Tsetse control

Currently, control of tsetse populations depends on bush clearing, application of insecticides, the sterile male technique, the use of fly traps and impregnated targets. Although tsetse flies are apparently sensitive to low levels of modern insecticides, application of some of the compounds over large areas can be environmentally damaging (Koeman *et al.*, 1971). The sterile male technique has produced promising results in some areas but is relatively expensive and needs alot of expertise (Dame and Schmidt, 1970). The destruction of forests and the clearance of bush where tsetse live effectively rids areas of tsetse habitats (Jordan, 1974). However, in some areas such destruction is unacceptable. Thus,

many types of tsetse fly traps have been developed. Although they require maintenance and can be damaged by animals and harsh climatic conditions, they have been shown to be effective for controlling some tsetse species, such as *Glossina tachinoides* and *G. palpalis* (Kupper, 1987). Of the traps and targets developed, the use of insecticide-impregnated biconical traps with attractants has been shown to be by far the least expensive approach to tsetse control, and certainly the least damaging to the environment (Kupper, 1987)

2.4.2 Keeping trypanotolerant livestock

Trypanotolerance is the ability of an animal to resist or control infection caused by parasites in the genus *Trypanosoma* (Murray and Morrison, 1979). This is a relative attribute in that non of the animals that are said to be trypanotolerant are absolutely tolerant.

In the desire to overcome the costs associated with trypanosomiasis, development of trypanotolerant animals is one means by which one can maintain livestock in tsetse-infested areas. Rather than eradicating the disease, it allows one to live with the disease. Currently, there are two breeds of cattle from west Africa, the N'dama and Muturu, which have proved to have a significant degree of trypanotolerance. A few breeds of goats and sheep have also been identified in east Africa with an appreciable level of trypanotolerance (Griffin and Allonby, 1979). Whilst there are therefore different trypanotolerant breeds in different parts of Africa, the dependence on trypanotolerant livestock has 2 main drawbacks;

1) Many farmers believe that trypanotolerant animals are unproductive because of their small size. This applies to cattle, sheep and goats. However, work done at Mushie Ranch, Zaire, indicates that reproductive performance, calf growth and cow productivity are greater for trypanotolerant cattle than the susceptible ones, when both are infected with the disease (ILCA, 1989). Thus, d'Ieteren and Trail

(1987) have also reported that trypanotolerant animals have an economic advantage over susceptible breeds when both are kept in tsetse-infested areas.

2) There are relatively few such animals. For instance, the N'dama constitute approximately 50% of all trypanotolerant cattle in Africa, yet account for only 3-4% of the total cattle population in the tsetse-infested areas. A similar situation applies to sheep and goats. This therefore limits the access of farmers to the benefits inherent in these breeds, such as upgrading of the breeds for trypanotolerance (Griffin and Allonby, 1979).

2.4.3 Chemotherapy and chemoprophylaxis

The use of drugs in the control of trypanosomiasis in domestic livestock is the method most widely used in sub-Saharan Africa (Ilemobade, 1987). Currently, the drugs on the market have varying degrees of efficacy, based on the species and strain of parasites involved.

2.4.4 Limiting factors in the control of trypanosomiasis

Despite the aforementioned control strategies, control of trypanosomiasis by such methods has been limited by;

i) Damaging side effects that insecticides have on the environment.

ii) Wildlife acting as reservoirs of trypanosomes.

iii) Poor availability of trypanotolerant livestock.

iv) Time consuming and expensive diagnostic techniques.

v) A narrow therapeutic index for the trypanocides.

vi) Development of resistance to the drugs being used.

The above hinderances therefore need to be alleviated to ensure that long-lasting efficacious control methods are established.

2.5 CHEMOTHERAPY OF TRYPANOSOMIASIS

Currently, chemotherapy is the most practical and most commonly applied method in the control of animal trypanosomiasis in areas where the disease is endemic (Ilemobade, 1987).

Chemotherapy is reliant on drugs which fall into 5 main categories based on their chemical structures. These are the quinaldines, represented by quinapyramine (Austin *et al.*, 1957); the phenanthridine derivatives introduced by Morgan and Walls (1931) and represented by homidium and isometamidium; naphthalene derivatives, represented by suramin (Williamson, 1970); diamidines, represented by diminazene (Bauer, 1958); and organic arsenicals, developed by Friedheim (1949).

Chemotherapy is dependent on the fact that there are specific anabolic, catabolic and regulatory pathways in parasitic protozoa which can be targets for drug action (Fairlamb, 1989). Thus, suramin acts by interfering with carbohydrate metabolism and energy production in bloodstream-forms of African trypanosomes (Opperdoes, 1987). Nuclear and kinetoplast DNA-dependent processes are inhibited by trypanocides that intercalate (phenanthridines) or bind externally to DNA (diamidines) (Williamson, 1979). Drugs such as diminazene are also thought to interfere with polyamine biosynthesis (Bitonti *et al.*, 1986). Finally, the drug uptake mechanism which involves transport, intracellular metabolism, binding and distribution is also thought to be critical for selective toxicity (Hawking, 1963a; Frommel and Balber, 1987).

2.5.1 Quinaldines

Quinapyramine was first introduced onto the market in the early 1950s (Davey, 1950). It is currently marketed under the trade name Trypacide$^{(R)}$ (May & Baker, UK) but used to be marketed as Antrycide$^{(R)}$ (ICI, UK). It is available in 2 forms; quinapyramine sulphate (Trypacide sulphate$^{(R)}$) for curative treatment, and quinapyramine prosalt for combined curative and prophylactic

treatment. Originally, quinapyramine prosalt was a mixture formed from 3 parts by weight of quinapyramine dimethyl-sulphate and 4 parts by weight of quinapyramine chloride. However, this formulation was subsequently changed and quinapyramine prosalt RF (revised formula), or Trypacide prosalt (May & Baker, UK), now contains the sulphate and chloride salts in proportions of 3:2. The prosalt RF formulation is used for prophylaxis at a recommended dose of 7.4 mg/kg b.w, and is said to give protection for 3 months in areas of low tsetse challenge, and 2 months in areas of medium tsetse challenge in Kenya (Whiteside, 1960a). Besides giving protection to cattle against *T. congolense* and *T. vivax*, it has also been reported to be prophylactic against *T. evansi* in camels for up to 10 months (WHO, 1962)

Quinapyramine dimethyl-sulphate is a crystalline odourless powder with a white, cream or very pale yellow colour and is readily soluble in water (1:2 at 20°C). In addition to activity against the aforementioned trypanosomes, it is also said to be efficacious against infections with *T. equiperdum* (Hawking and Thurston, 1955). The drug sometimes causes toxic reactions, especially in equidae, but this can be prevented by dividing the dose and injecting the second half 5 or 6 hours after the first one. The dimethyl sulphate formulation is said to provide some degree of prophylaxis (Davey, 1957). This has proved disadvantageous as it extends the period during which reinfection might occur in the presence of waning concentrations of the drug, thereby facilitating development of resistance. Since both formulations of quinapyramine have prophylactic activity, quinapyramine appears to be particularly associated with development of drug resistance.

Generally, quinapyramine is said to localise selectively in the liver and kidney, remaining in the sites for more than 4 weeks. Such deposition may cause kidney and liver damage (Davey, 1957).

2.5.2 Phenanthridine derivatives

This group of drugs includes homidium and isometamidium.

a) **Homidium** is marketed as a bromide salt under the trade name Ethidium bromide(R) (CAMCO, UK.) and as the chloride salt under the trade name Novidium(R) (May & Baker, UK). It is an almost odourless, dark-purple, crystalline or amorphous powder with a solubility in water of 1:29 at 20°C, which increases to 1:10 at 100°C. Five percent solutions are stable for several days at 20°C.

Homidium is mainly used as a curative drug, although its prophylactic effects may last for 95-110 days (Mwambu, 1971). The prophylactic activity may account for the occurrence of resistant strains which have developed over time, probably due to its persistence in the host for long periods at low concentrations (Stephen, 1963; Gray and Roberts, 1971b). This appears to be particularly true with *T. congolense*, and to a lesser extent with *T. vivax* (Ilemobade and Buys, 1970). The development of such resistant strains appears to be enhanced in areas of high tsetse challenge (Scott and Pegram, 1974).

b) **Isometamidium chloride** is marketed as Samorin(R) (May & Baker, UK) or Trypamidium(R) (Rhone Merieux, Lyon, France) and is a phenanthridine derivative (Wragg *et al.*, 1958).

Isometamidium chloride is a dark reddish brown powder with solubility of 6% (w/v) in water at 20°C. It is used as 1%, 2% or 4% (w/v) solutions depending on the dosage rate employed, and is indicated for therapy and prophylaxis of *T. congolense* and *T. vivax* infections in cattle, and other animals (Robson, 1962). In cattle, it is used for treatment of overt disease, to prevent spread of a disease in a herd, to prevent outbreaks of the disease in high risk areas, and to maintain the economic value of stock trekked through tsetse-fly belts (Na'Isa, 1969)

In a study conducted in cattle on the use of intravenous isometamidium in the control of bovine trypanosomiasis, it was established that intravenous administration of isometamidium, as a therapeutic drug, improved herd health and resulted in a marked increase in calving rates (Dowler *et al.*, 1989). However, the high standard of management required to implement this regime appeared to limit its widespread use. On the basis of these field observations a laboratory-based experiment was conducted in Boran cattle to elucidate the prophylactic and therapeutic activity of isometamidium against a clone of *T. congolense* when the drug was given intravenously (Sutherland *et al.*, 1991). In this work, it was found that although intravenous administration of isometamidium chloride eliminated a fully sensitive infection, treatment by this route appeared not to enhance the therapeutic efficacy of the drug in the treatment of a *T. congolense* clone which expressed a high level of resistance. It was also demonstrated that repeat intravenous treatment of cattle infected with a highly resistant trypanosome was not significantly more efficacious than a single intramuscular dose of the drug. Thus, the significance of the aforementioned field observations was questioned.

In addition to activity in cattle, isometamidium has also shown some efficacy against *T. brucei* infections in dogs (Toure, 1970), *T. evansi* in cattle, buffalo, camels, donkeys and dogs (Raghavan and Khan, 1970; Petrovskij, 1974), and in the treatment of *T. vivax* and *T. congolense* infections in the horse (Srivastava and Malhotra, 1967; Chand and Singh, 1970).

Isometamidium chloride is administered to cattle via the deep intramuscular route in the neck muscles, thigh muscles or the rump, at a dosage rate of 0.25-0.5 mg/kg b.w. when given as a therapeutic agent for *T. congolense* and *T. vivax* infections. When given as a prophylactic agent, it is given at a dose of 0.5-1.0 mg/kg b.w. and may give protection for up to 6 months (Abwao, 1981). However, 2-6 treatments may be necessary per year depending on the level of challenge. For *T. evansi* infections in camels, isometamidium chloride is

given at a dose of 1-2 mg/kg b.w. by deep intramuscular injection. In cattle, isometamidium chloride is well tolerated. However, when it is given at dosages above 0.5 mg/kg b.w. muscular swellings may occur at the site of injection (Abwao, 1981). Despite this, the incidence of systemic disturbances are low when given at dosages below 1 mg/kg b.w. However, isometamidium should not be used concurrently with other trypanocidal drugs and should not be given to cattle more frequently than once a month (Fairclough, 1963c).

2.5.3 Naphthalene derivatives

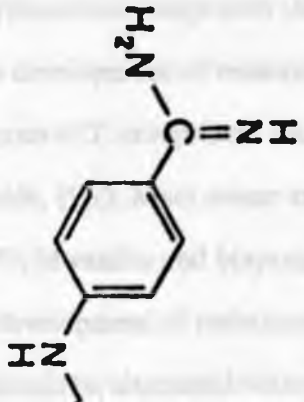
Of the trypanocides currently in use, this group is represented by suramin. Suramin was one of the first compounds to be used for treatment of trypanosomiasis in man (sleeping sickness) and *T. equiperdum* infection in equidae (dourine). It was first marketed in 1925, and was found to have prophylactic activity against many trypanosomes (Leach and Roberts, 1981). It is said to have appreciable activity against *T. vivax* and *T. congolense* infections in cattle and therapeutic activity against *T. simiae* infections in pigs (Stephen, 1966). However, its main use is in the treatment of *T. evansi* infections in camels.

In light of suramin's anionic nature, it has been combined with cationic trypanocides to form sparingly soluble salts called suraminates. Such salts have been used both in man and in animals and were generally produced by combining suramin with metamidium (2 isomers resulting from combination of the diazotized p-aminobenzamide moiety of diminazene with homidium chloride [Wragg *et al.*, 1958]) or diminazene (Williamson and Desowitz, 1956; Austin *et al.*, 1957; Williamson, 1958; Wragg *et al.*, 1958). These complexes are said to have lower toxicity than the parent compounds (Desowitz, 1957; Williamson, 1957). Furthermore, their prophylactic activity has been noted in cattle (Desowitz, 1957) and is said to be due to depot formation of the complex at the site of injection. For treatment of Zebu cattle, quinapyramine suraminate has been given at a dosage of 40 mg/kg b.w., whereas homidium suraminate has been

given at a dose of 10 mg/kg b.w.. Such formulations conferred prophylaxis to cattle against *T. vivax* for 5 1/2 and 13 months, respectively. Unfortunately, suraminates proved to be very costly and hence were never marketed.

2.5.4 Diamidines

The diamidine group of compounds is represented by diminazene aceturate and is marketed as Berenil (R) (Hoechst Veterinar ambH, Germany) or Veriben (R) (Sanofi Animal Health Ltd, UK). Diminazene aceturate is a trypanocide, babesiocide and bacteriocide (Alexander, 1969). Its chemical name is 4,4-diazoaminobenzene-di-acetamidoacetate (Fig. 2.5.4) and is prepared as a 7% (w/v) aqueous solution. Aqueous solutions of diminazene aceturate are stable when in contact with air for only 2-3 days. Thus, since it was introduced onto the market the manufacturers have added phenyldimethyl pyrazolone (antipyrine) as a stabiliser, enabling solutions to be used for up to 10-15 days when stored at room temperature. Berenil(R), or Veriben(R), is an odourless yellow powder with a solubility of 1:14 in water at 20°C. A dose of 3.5 mg/kg b.w. is recommended for treating animals infected with *T. congolense* and *T. vivax*. However, a dose of 5 mg/kg b.w. may be required for *T. brucei* infections (Davey, 1957). The maximum tolerated dose in mice is approximately 80 mg/kg b.w. However, in cattle, if the causative organism is particularly resistant, the dose may be increased to 8 mg/kg b.w., although subcutaneous doses in sheep and cattle as high as 21 mg/kg b.w. have been found to be safe (Fairclough, 1963a,b). It is advisable, however, not to exceed 4 g total dose (Hoechst). The drug can be given by the subcutaneous route, but the deep intramuscular route is preferable to reduce local pain. If the quantity to be injected is very large, then it is preferable to use 2 injection sites. It should be noted, however, that although cattle can safely tolerate high dosages, severe toxic reactions can occur in camels given 7 mg/kg b.w when treated for *T. evansi* infections.



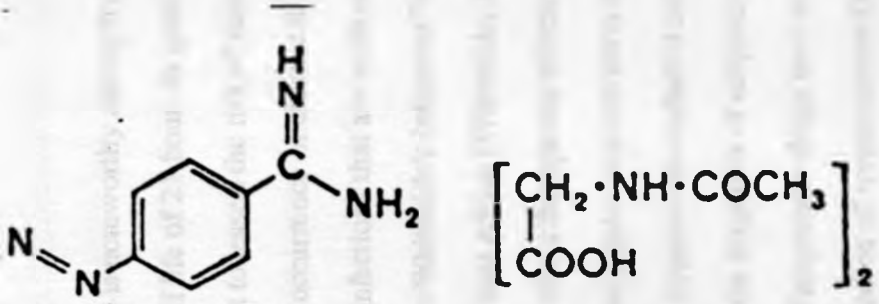


Fig. 2.5.4

DIMINAZENE ACETURATE

Diminazene aceturate is very effective at eliminating infections with *T. congolense*, *T. vivax*, *T. brucei* (Cunningham *et al.*, 1964; Cunningham, 1968) and *T. equinum* (Boehringer *et al.*, 1959). After considerable clinical experience there is now general consensus that 3.5 mg/kg b.w. will cure *T. congolense* and *T. vivax* infections in cattle that are sensitive to the drug (Fussganger and Bauer, 1958).

Of the trypanocidal compounds that have been described, diminazene is least prone to the development of resistance (Fiennes, 1958; Whiteside, 1960a,b), although a few cases of *T. congolense* and *T. vivax* resistant to it have have been reported (Whiteside, 1963; MacLennan and Na'Isa, 1970; Gray and Roberts, 1971b; Hull, 1971; Mwambu and Mayende, 1971; Jones-Davies, 1967). To avoid or minimise the development of resistance to diminazene it is recommended that use of the drug should be alternated with other drugs such as isometamidium (Whiteside, 1960a)

Diminazene is noteworthy among trypanocidal drugs in that it is rapidly excreted, with a half life of 21 hours in goats (Aliu, 1989). This property of fast clearance is thought to lessen the risk of resistance developing and also reduces the opportunity for occurrence of chronic toxicity. Such properties, coupled with its activity against infections that are resistant to isometamidium or homidium formed the basis for Whiteside's infamous "sanative pair" system of control for trypanosomiasis in East Africa (Whiteside, 1960a,b), as explained later.

Besides the direct therapeutic effects of diamidines, they have also been found to increase the jugular concentration of trypanosomes in blood following administration to a trypanosome-infected animal (Kalu, 1983). This property may be of value in the diagnosis of subpatent infections, especially those caused by *T. congolense*. Vasopressor drugs have also been found to cause an increase in the jugular concentration of trypanosomes (Kalu and Haruna, 1985). In an experiment conducted in cattle and sheep, the drugs ephedrine, adrenaline and insulin increased the jugular concentration of parasites in *T. congolense*-infected

animals. On this basis, it was suggested that one of the mechanisms by which trypanocides increase the jugular blood concentration of *T. congolense* may be by vasoconstriction of capillary walls. It has also been suggested that the duration and amplitude of the increase in parasitaemia following treatment is related to the therapeutic efficacy of the trypanocide used (Kalu and Aina, 1983).

Finally, Maxie and Losos (1977) have suggested that since a direct trypanocidal effect of diminazene was not observed *in vitro*, diminazene may act indirectly, by making trypanosomes available to the host defence mechanisms, such as the reticuloendothelial system.

2.5.5 Organic arsenicals

This group of drugs was introduced into the market many years ago (Friedheim, 1949). However, their efficacy against trypanosomes has generally been negated by their high central and peripheral nerve toxicity (Williamson, 1970). The compound within this group that is currently marketed for use in animals is Cymelarsan (Mel Cy) and its use is restricted to camels.

Cymelarsan is a combination of melarsen oxide and cysteamine. It is a water-soluble trivalent arsenical that was patented in 1985 (Rhone Merieux, France) and occurs as a white powder. The solution in water is stable and biologically fully effective for several hours under field conditions (Raynaud *et al.*, 1989). It is administered intramuscularly, subcutaneously or intravenously and is very effective against trypanosomes belonging to the *T. brucei* group (Raynaud *et al.*, 1989). It is usually given as a single curative dose of 0.25-5.0 mg/kg b.w.

Cymelarsan eliminates *T. evansi* and other parasites in the *T. brucei* group (even when resistant to other drugs) in acute, sub-acute or chronic infections in camels, horses and cattle (Raynaud *et al.*, 1989). It has a very short half life in animals, with low toxicity and minimal tissue residues. More work, however, has to be done in future to find out whether the drug can be used for treatment of *T.*

evansi infection in other domestic animal species, and to establish the minimum curative dose for various field stocks or strains of *T. evansi* in camels.

2.6 CHEMORESISTANCE

Drug resistance is fastness of pathogens to the effect of a drug at the recommended dose rate. However, amongst trypanosome strains or species, there is considerable variation in their drug sensitivity. Drug resistance is thought to be mainly caused by injudicious use of the drugs in question. Although, as given below, other factors may be responsible. The level of drug resistance encountered in the field is usually resistance to the normal curative dose of the drug concerned. This level of resistance is often lower than that that can be induced in the laboratory. Nevertheless, resistance to the curative dose is the level of resistance that is of practical importance in the field (Hawking, 1963b), especially since the curative dose is near the maximum tolerated dose for many trypanocidal drugs (that is to say, the compounds have a narrow therapeutic index).

As stated, drug resistance is the ability of a trypanosome population to withstand treatment. It covers a wide range of phenomena, including host-related and parasite-related factors. Host-related factors that contribute to the resistance phenotype include:

1. Differences in the bioavailability of a drug to different tissues. For example, poor distribution to infected tissues or intracellular sites, or variation in drug metabolism between individuals (Croft, 1989).
2. Immunological status. For example, diminished activity of suramin and quinapyramine in animals with suppressed immune systems (Bitonti *et al.*, 1986).

Parasite-related factors that are known to contribute to drug resistance in trypanosomes include:

1. Reduced drug accumulation, as has been described for organic arsenicals (Hawking, 1963b), diamidines, phenanthridines and acriflavine (Frommel and Balber, 1987).

2. Increased metabolite production or metabolite retention, as reported in *T. brucei* with increased levels of ornithine in resistant parasites competing with DFMO for ornithine decarboxylase (Bellofatto *et al.*, 1987).

Other undescribed modes of resistance that may also play a role include:

1. An alteration in drug metabolism by trypanosomes.

2. A change in the enzyme target, through either an increase in intracellular enzyme levels or a change in enzyme affinity.

3. Use of alternative biochemical pathways by trypanosomes to enable them to bypass the site of inhibition (Croft, 1989).

2.6.1 Natural resistance (Tolerance) is a phenomenon in which expression of drug resistance by a trypanosome strain or species is not dependent on previous exposure to the drug in question. This is thought to be exemplified by *T. congolense* field isolates which are only mildly sensitive to homidium (Killick-Kendrick and Godfrey, 1963). The same is also thought to apply to West African strains of *T. vivax* which tend to have a higher level of resistance to diminazene than *T. congolense* populations from the same area (Williamson, 1960). However, it should be noted that some of the apparent natural resistance may be due to persistent cross-resistance, induced by drugs used previously, such as quinapyramine (Gray and Roberts, 1971a).

2.6.2 Acquired drug resistance is resistance that arises as a result of prior exposure to the drug. Most cases of drug resistance in the field are thought to have arisen in this manner, and primarily occur as a result of underdosing. Underdosing can arise in several ways (Whiteside, 1960a,b):

-incorrect estimation of body weights

-wrong dose rates

-irregular use of drugs or termination of their usage before the trypanosomes are eliminated.

It should be noted that there is a critical period after drug administration when the therapeutic levels of the drug are not attained in the bloodstream or tissues but the levels are sufficiently high to induce resistance. This period is longest with long-acting prophylactic drugs that are slowly excreted (e.g., quinapyramine) and shortest with drugs that are rapidly excreted (e.g., diminazene aceturate).

2.6.3 Cross-resistance

Cross resistance, in its broadest sense, is the development of resistance to a particular compound as a result of using another (Williamson, 1962). Williamson (1962) states that structural similarities between the different trypanocidal drugs account for some of the cross-resistance that is observed, though not all. Furthermore, he also concludes that common receptor sites on trypanosomes, and modification of these sites during the development of resistance, may be important factors in the development of cross-resistance (Williamson, 1970).

It is the concept of cross-resistance amongst trypanocides that led to the development of Whiteside's sanative drug pairs (Whiteside, 1960a). These pairs include homidium and diminazene, and isometamidium and diminazene; induction of resistance to either of the drugs in the drug pair does not result in cross-resistance to the other. Thus, if resistance develops to one of the drugs in the pair, the other drug can be used to eliminate such trypanosomes (see Table 2.6.3).

Whilst induction of resistance to homidium, isometamidium and quinapyramine appears to occur relatively easily, development of resistance to diminazene is very difficult to produce experimentally, and is probably unstable during repeated passage from animal to animal (Whiteside, 1962). It should

TABLE: 2.6.3

Cross-resistance patterns of drugs used in cattle trypanosomiasis (After Whiteside, 1960a)

Trypanosomes resistant to	Response of Trypanosomes to:			
	Quinapyramine	Homidium	Metamidium	Diminazene
Quinapyramine	R	++	+	++
Homidium	+	R	+	0
Metamidium	+	++	R	0
Diminazene	0	0	0	R

R : direct resistance

+ : cross-resistance to curative dose

++ : cross-resistance to higher dose

0 : no cross-resistance

be noted that trypanosome field isolates that are resistant to diminazene are generally also resistant to quinapyramine. Thus, as a result of Whiteside's work (Whiteside, 1960a), these strains could be directly resistant to quinapyramine and cross-resistant to diminazene (Gray and Roberts, 1971a). This is in contrast to direct resistance, whereby a parasite population develops the ability to withstand treatment with a particular drug as a result of prior exposure to that drug.

2.6.4 Stability of drug resistance

Some evidence has been adduced to the effect that drug resistance in trypanosomes is stable during both passage from one animal to another and when trypanosomes undergo tsetse transmission (Fiennes, 1953; Whiteside, 1962; Jones-Davies, 1968). Experimental evidence described by Gray and Roberts (1971a,b) is in line with this view for both *T. congolense* and *T. vivax* in cattle. They further showed that the immune response of the host does not affect the drug-resistance phenotype of a trypanosome strain. The authors concluded that disappearance of drug-resistant trypanosomes in the field does not result from reduced infectivity for animals or tsetse flies, or from loss of drug resistance. Instead, they suggested that it may be due to a reduction in the proportion of drug-resistant trypanosomes in the total trypanosome population in the reservoir hosts, to an insignificant proportion. In contrast to these observations, Peregrine *et al.* (1991) established that expression of a high level of resistance to isometamidium in mice is unstable, and that at least a component of the genetic determinant(s) for this drug-resistance phenotype is (are) likely to be unstable. Thus, the above hypothesis may not hold true.

Finally, some workers have concluded that drug-resistant trypanosomes disappear in infected animals in the absence of the drug pressure (Whiteside, 1960a; Finelle and Yvore, 1963). Whether this was because the resistance was unstable or because it was associated with overgrowth of a sensitive subpopulation was not clear.

2.7 MOLECULAR BIOLOGY OF TRYPANOSOMES

Trypanosomes are protozoan parasites which, unlike mammalian cells, have genetic material not only in the nucleus but also in the mitochondrion, known as kinetoplast deoxyribonucleic acid (kDNA). In studying the total genomic DNA of a trypanosome it is imperative that both these forms of DNA are considered. The kDNA consists of thousands of circular DNA elements which are located within a single mitochondrion and are interlocked together to form a single massive network (Englund *et al.*, 1982). Two kinds of circles are present; 25-50 large circles (maxicircles) containing genes for mitochondrial biogenesis, and five to ten thousand small circles (minicircles) whose function is not yet known. The total chromosome profiles of all the DNA of an organism can enable one to differentiate the organism species. Furthermore, arbitrary priming of DNA can enable one to differenciate organisms belonging to the same strain (Gomez-Eichelmann *et al.*, 1988; Welsh *et al.*, 1991)

2.7.1 Polymerase chain reaction

The polymerase chain reaction (PCR) is a chemical process by which DNA can be replicated (amplified) *in vitro*. It involves cycling DNA samples between temperatures of about 40°C and 94°C along with a DNA polymerase. At the end of each cycle the DNA chains are doubled in number, thus achieving an exponential increase in the amount of DNA at every step (Mullis, 1990).

2.7.2 Clone/strain identification using PCR

Organisms within the same species tend to have similar morphological characteristics. In the case of trypanosomes, their identity in terms of species is most commonly determined by parasitological methods such as determination of parasite motility, morphology, host specificity, virulence and site of development

in the vector, all of which are physical characteristics common to organisms in the same species (Nyeko *et al.*, 1990). More recently, DNA-probes have been developed which can identify a trypanosome in many cases up to the sub-species level. A DNA-probe has a DNA sequence that is specific for only a certain genome. Thus, it cannot hybridize to any other sequence other than the one from which it was developed. The technique has thus been applied to the field situation and has been found to be a useful epidemiological tool (Nyeko *et al.*, 1990). Having said this, the current repertoire of DNA-probes do not allow one to distinguish organisms falling within the same sub-species. In this case, a tool is required that is more specific than the current hybridization DNA-probes. The use of arbitrary priming and the polymerase chain reaction (AP-PCR) may provide such a possibility (Welsh *et al.*, 1991). An AP-PCR fingerprint is generated by subjecting a small amount of template DNA to PCR with a randomly selected PCR primer. Divergence of even a fraction of a percent between 2 genomes often results in a different fingerprint pattern because of different sets of sites in the genome which have the best matches with the primer. Each primer thus gives a different pattern of AP-PCR products, each with the potential of detecting polymorphisms between strains (Welsh *et al.*, 1991). With such data, one is able to differentiate even closely related strains of the same species.

However, in the case of a mixed population of trypanosomes, AP-PCR may not be effective in detecting, with high sensitivity, a population that is present at low proportions, especially if the populations are very closely related genetically. Such was the case in the experiment described here. The problem occurs because most of the DNA building materials (nucleotides) and the DNA Taq polymerase are utilised in amplifying sequences of DNA from the population that is present in greatest amount, thus leaving little for the minor population. To ensure a high degree of sensitivity and specificity of the PCR for such a minor population, it is therefore necessary to use a primer that is specific for the meagre

population. The primer need not be exclusively specific for the clone, strain or species in question, so long as it has greater specificity for the population in question relative to the other populations in the mixture.

2.7.3 DNA-probes

A DNA-probe is a diagnostic tool that enables one to distinguish between organisms that differ to some degree in their genomic base sequences. The specificity of a DNA probe depends on the level of homology of the DNA sequences from which it was developed with that in other organisms. The sensitivity of a DNA probe is dependent on the copy number of the sequence onto which the probe hybridizes.

DNA probes have been increasingly used to identify both pathogenic and non-pathogenic species, strains and clones of parasites affecting animals and man (Chen *et al.*, 1991). In African trypanosomiasis, DNA-probes have been developed to the level of identifying the parasites at species and subspecies levels (Gibson *et al.*, 1988). Thus, Gibson *et al.* (1988) developed DNA probes specific for 3 of the major groups of *T. congolense*; namely, Savannah, Forest and Kilifi types. The probes were developed from repeat sequences (satellite DNA) of each subspecies and could detect as few as 100 trypanosomes by dot-blot hybridization.

Currently no probe has been developed to distinguish trypanosomes at the intra-subspecies level. Given that the trypanosome variable surface glycoprotein is the most variable part of the parasite, VSG genes were deemed the most appropriate for developing an intra-subspecies-specific probe. Thus, in work done by Majiwa *et al.* (1986) it was shown that there are 2 *T. congolense* VSG genes restricted in occurrence to their respective serodemes. These findings were suggestive of such variant specific genes being useful as hybridization probes in discriminating trypanosome clones of a homologous serodeme from those that belong to different serodemes.

CHAPTER THREE MATERIALS AND METHODS

3.1 INTRODUCTION

The experiment was conducted at the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi and commenced in December 1991.

3.2 EXPERIMENTAL ANIMALS

3.2.1 Goats

Twenty four goats of an East African breed (East Africa x Galla) were obtained from Nanyuki, an area devoid of both trypanosomiasis and tsetse flies. They were aged between one and two years on arrival at ILRAD and, up to this point, had been kept extensively on a diet of natural pastures. No supplementation had been provided.

On arrival, all goats were dewormed using Ranizol$^{(R)}$ (MSD AGVET, Netherlands). Ranizol$^{(R)}$ consists of rafoxanide and thiabendazole and the two compounds were given at dose rates of 7.5 mg/kg b.w. and 44.0 mg/kg b.w. *per os*, respectively. Animals were vaccinated against foot and mouth disease using 2 ml of an inactivated trivalent vaccine (O/A/Sat 2; KEVEVAPI, Kenya). Animals were also given an intramuscular injection of long-acting oxytetracycline (Pfizer International, USA) at a dose of 20.0 mg/kg b.w. as a prophylactic against contagious caprine pleuropneumonia, and amprolium in their drinking water; 240 mg/litre of 20% amprolium, powder was given (Amprol$^{(R)}$, MSD AGVET, Netherlands) was given for 5 days, as a coccidiostat.

Before start of the experiment, feaces were collected from all animals and examined for gastrointestinal parasites. Serum samples were also collected and tested for anti-trypanosomal antibodies with the aid of an antibody-ELISA (Katende *et al.*, 1987). The latter test showed that all animals were negative for anti-trypanosomal antibodies. However, the former test showed a low level of

infection with Coccidia species. In some animals these infections flared up at a later date. However, this occurred prior to the experiment. The infections were therefore controlled by mass treatment with amprolium at the same dose rate as before, for 3 days.

Prior to experimental work, all the goats were weighed and distributed into 3 groups of 5 animals each, and 3 groups of 3 animals each. At the time of experimental infection, they weighed between 15 and 31 kg, the wide range arising as a result of the transient coccidia infections that occurred in some animals. Prior to commencement of the experiment the animals were bled and the PCV ascertained. Blood samples were also screened for the level of parasitaemia. Finally, the temperature was also taken. All these parameters were measured for baseline data.

3.2.2 Mice

Mice were used for expansion of trypanosome populations to provide sufficient trypanosomes to infect goats, and for isolation of trypanosomal DNA. For all experimental work, outbred male Swiss mice were used which were obtained from ILRAD's small animal unit, and which weighed between 20 and 30 g. Trypanosomes were inoculated intraperitoneally into sublethally irradiated mice in 0.2 ml of PSG (pH 8) using a 1 ml syringe and a 26 gauge needle (Green Cross Medical Corp, Korea). After inoculation, the mice were first checked for parasitaemia on the sixth day post infection and subsequently, on a daily basis. This was done by cutting off the tip of the tail and making a wet smear of the blood on a slide. This was then examined under a light microscope at x250 magnification. Mice were only exsanguinated for collection of trypanosomes after achieving a parasitaemia of more than 100 trypanosomes per field. Prior to exsanguination, mice were anaesthetised by placing them in ether vapour in a closed bottle. They were then bled directly from the heart using a 23 gauge

needle and a 2 ml syringe. The 2 ml syringe contained sodium citrate anticoagulant; 0.1 ml of 3% (w/v) sodium citrate per ml of blood.

3.3 FEEDING AND HOUSING

During experimental work, goats were kept in a well-ventilated fly-proof isolation unit that had a concrete floor and was bedded with wood shavings. During experimental work, animals were maintained on a diet of lucern hay mix, and were supplemented with Young Stock feed pellets (Belfast millers, Kenya Ltd.) at the rate of 250 g/goat per day. Water was freely available via an automatic tap system.

Mice were kept in plastic cages with wood shavings as bedding material. Both maize-based mice feed pencils (Unga Feeds Ltd., Nairobi) and water were supplied *ad libitum*.

3.4 TRYPANOSOMES

Two clones of trypanosomes were used:

T. congolense IL 1180 was derived after one passage in mice from clone IL 968, a derivative of STIB 212 (Nantulya *et al.*, 1984) which was isolated from a lion in the Serengeti region of Tanzania (Geigy and Kauffmann, 1973) (see Fig. 3.4a). Previous work has shown it to be sensitive to diminazene aceturate at a dose of 3.5 mg/kg b.w. in goats (Peregrine *et al.*, 1991).

T. congolense IL 3274 is a derivative of isolate Banankeledaga/83/CRTA/67 which was collected from a cow in Burkina Faso in 1984 (Pinder and Authie, 1984). The clone's immediate parental stock, IL 2856, was passaged in rats and mice and cloned in irradiated mice to produce this clone (IL 3274) (Peregrine *et al.*, 1991) (see Fig. 3.4b). Earlier studies to characterise the sensitivity of the

clone to diminazene and isometamidium in mice have shown that it has a higher degree of sensitivity to both compounds as compared to the parental stock. However, it is considerably more resistant to both compounds compared to IL 1180 (Peregrine *et al.*, 1991).

Pedigree of T. congolense IL 1180

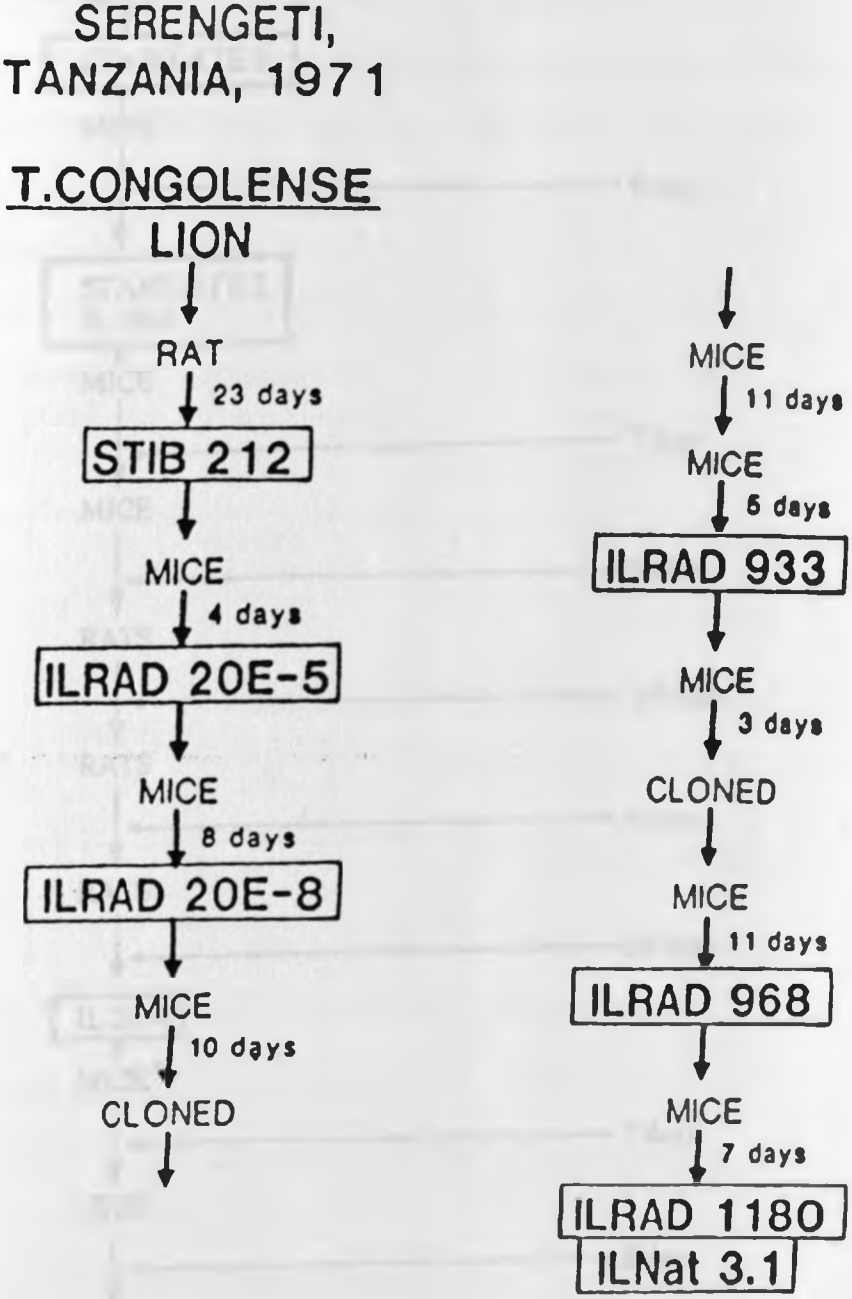
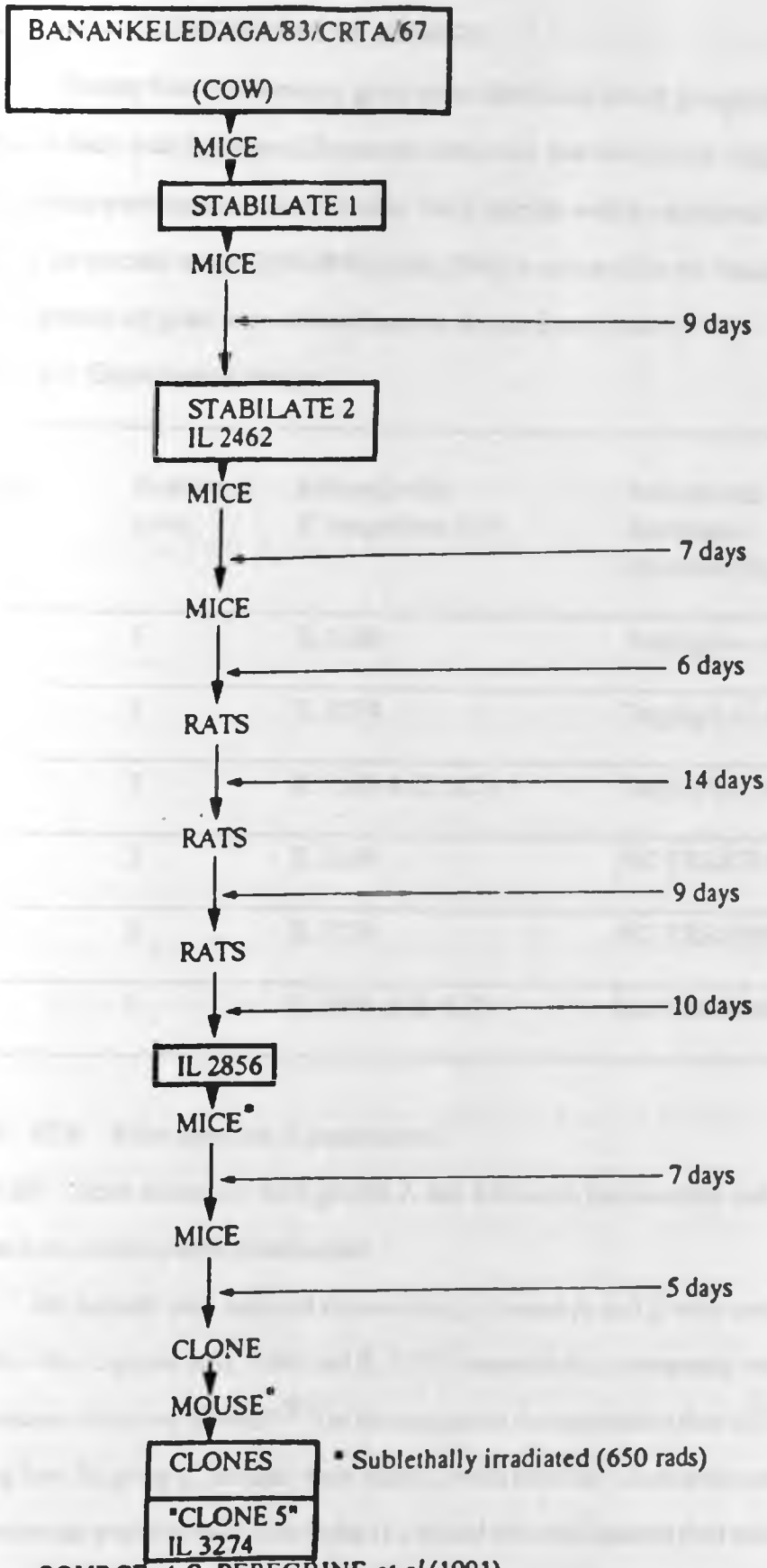


FIG. 3.4a

Pedigree of *T. congolense* IL 3274



SOURCE: A.S. PEREGRINE *et al* (1991)

FIG. 3.4.b.

3.5 EXPERIMENTAL DESIGN

Twenty four experimental goats were distributed into 3 groups of 5 animals each, and 3 groups of 3 animals each, such that mean body weights in each group were approximately similar. Only animals with a packed cell volume within the normal range (22%-38%) (Jain, 1986) were used for the experiment. The 6 groups of goats were infected and treated as shown below:

Table 3.5: Experimental design

Group	Number of goats	Infected with *T. congolense* (i/v)	Treated with diminazene aceturate (i/m)
A	5	IL 1180	7mg/kg b.w. ADP
B	5	IL 3274	7mg/kg b.w. ADP
C	5	IL 1180 & IL 3274	7mg/kg b.w. ABP
D	3	IL 1180	NO TREATMENT
E	3	IL 3274	NO TREATMENT
F	3	IL 1180 & IL 3274	NO TREATMENT

KEY: ADP : After detection of parasitaemia

ABP : After animals in both groups A and B became parasitaemic and the animals in question were parasitaemic.

All animals were infected intravenously. Groups A and B were used to monitor the response of IL 1180 and IL 3274, respectively, to treatment with diminazene aceturate (Berenil$^{(R)}$) at the maximum recommended dose of 7 mg/kg b.w. In group C, animals were infected with both the aforementioned trypanosome populations to determine if a mixed infection altered their resistance phenotype. In these animals inoculation of the different trypanosome clones was

done simultaneously, and on opposite sides of each animal, to avoid any chance of interference between the clones (Dwinger *et al.*, 1986, 1989; Sones *et al.*, 1989); the interference phenomenon is only said to occur in cases where the initial infection has already established itself in the host (Dwinger *et al.*, 1989).

Groups D, E and F served as non-treatment controls for the first 3 groups, and also facilitated a comparison of the pathogenicity of the 2 clones individually and when mixed.

3.6 INFECTION AND TREATMENT OF GOATS

Goats were infected using parasitaemic blood obtained from mice, after quantifying the level of parasitaemia in a Neubauer haemocytometer and diluting the blood with sterile PSG, pH 8. Approximately 1.0×10^6 trypanosomes were inoculated into the jugular vein of each goat. IL 1180 was inoculated into the left jugular vein whereas IL 3274 was inoculated into the right jugular vein. Thereafter, the goats were examined daily for the presence of trypanosomes using the buffy-coat phase-contrast technique (Murray *et al.*, 1977). All goats in groups A, B and C were treated i/m with diminazene aceturate at a dose of 7 mg/kg b.w. on day 18 post infection, when all the animals had become parasitaemic, thus ensuring that both clones in group C should have fully developed by the time of treatment. Thereafter, blood samples were collected 3 times per week from the treated goats and examined for the presence of trypanosomes using the buffy-coat phase-contrast technique (Murray *et al.*, 1977). Goat blood was also inoculated into mice once every 2 weeks in an attempt to detect subpatent infections (Paris *et al.*, 1982). Parasitaemic goat blood samples were also collected once every 2 weeks and stabilated as described in section 3.8. These were later expanded in mice for trypanosome identification (see 3.9).

3.7. PARAMETERS MEASURED

3.7.1 Haematology

Goats were bled directly from the jugular vein into EDTA-coated 5 ml vacutainer tubes (Monoject sterile, non-silicon coated, Sherwood Medical, Ballymoney, N. Ireland). Blood samples were collected between 8.30 a.m. and 9.30 a.m., 3 times a week. The blood was analysed within one hour of collection to determine the PCV. For this purpose, microhaematocrit heparinised capillary tubes with an internal diameter of 1.1-1.2 mm, an external diameter of 1.5-1.6 mm and a length of 75 mm (Superior, West Germany), were filled with blood. The base was then sealed with plasticine and the capillary tubes centrifuged at 12,000 rpm for 5 min in a microhematocrit centrifuge (IEC MB, UK). The PCV was then read off directly with the aid of a microhaematocrit reader and the PCV expressed as a percentage of the total blood volume.

3.7.2 Parasitaemia

Blood collected as described (3.7.1) was used for determining the level of parasitaemia with the aid of the buffy-coat phase-contrast technique (Murray *et al.*, 1977). The blood was analysed within 1 hr of collection. The aforementioned microhaematocrit heparinised capillary tubes were filled with blood, as described, and centrifuged at 12,000 rpm for 5 min using a microhaematocrit centrifuge (IEC MB, UK). After reading the PCV, the tubes were cut with the aid of a diamond-tipped pen, 1 mm below the buffy-coat interface to include some red blood cells. The buffy-coat interface was then expressed onto a glass slide and covered with a 22 x 22 mm cover slip. The preparation was then examined under a light microscope (Leitz Wetzler, SM Lux, Germany) at a magnification of x250, and the concentration of trypanosomes determined by counting the number of trypanosomes per field. Twenty fields were examined per sample. The

level of parasitaemia in each animal was then determined using the conversion table given in Table. 3.7.

Table 3.7: Parasitaemia estimation

Number of Trypanosomes per field	Score	Estimated level of parasitaemia (trypanosomes/ml)
Swarming>100/field	6+	$>5 \times 10^6$
> 10 per field	5+	$>5 \times 10^5$
1-10 per field	4+	$10^4 - 5 \times 10^5$
1 per 2 fields-1 per 10 fields	3+	5×10^3 5×10^4
1-10 per preparation	2+	$10^3 - 10^4$
1 per preparation	1+	$10^2 - 10^3$

Source: Paris *et al.* (1982)

3.8 STABILATES PREPARED

3.8.1 Parasitaemic blood from goats

Goats were bled weekly from the jugular vein into 5 ml vacutainer tubes coated with EDTA (Sherwood Medical, Ballymoney, Ireland). The blood was then mixed with 20% glycerol in PSG, at the ratio of 1:1 (v/v) to produce 10% (v/v) glycerol in the stabilate preparation (Murray *et al.*, 1983). The presence of viable trypanosomes was confirmed at this stage with a light microscope and the concentration of trypanosomes was quantified. The stabilate preparation was then left on ice to cool down for about 10 min. Thereafter, capillary tubes were filled with the preparation and sealed with plasticine. The tubes were then packed into 5 ml Greiner tubes which were perforated to allow access of liquid nitrogen. The tubes were then suspended in liquid nitrogen vapour within an insulated jacket for 24 hours, to enable gradual cooling, before placing them into liquid nitrogen for storage.

3.8.2 Parasitaemic blood from mice

Parasitaemic mice were anaesthetised in ether vapour and bled directly from the heart using a 1 ml syringe and a 23 gauge needle. Blood was collected into sodium citrate anticoagulant at the rate of 0.1 ml of 3% (w/v) sodium citrate per ml of blood. This blood was then mixed with 20% glycerol at the ratio of 1:1 (v/v) and further manipulations were as those described for goat blood (section 3.8.1) (Murray *et al.*, 1983).

3.8.3 Goat buffy-coat preparation

Goat blood was collected into EDTA and centrifuged as described for Parasitological Examination (see 3.7.2). The capillary tube was then cut 1 mm below the buffy coat using a diamond-tipped pen. The buffy coat and plasma

contents were then expressed into an eppendorf tube (one per animal), labelled and preserved at -70°C until required for molecular biology studies.

3.9 TRYPANOSOME CLONE IDENTIFICATION

3.9.1 Isolation of trypanosomes

Trypanosomes were separated from blood cellular components by the anion exchange method (Murray *et al.*, 1983). This method is based on the principle that host blood cells are more negatively charged than trypanosomes, such that they adsorb onto diethyl-amino-ethyl cellulose (DEAE-cellulose) while the trypanosomes pass through the column, still retaining their viability and infectivity.

DEAE columns were prepared by adding 500 g DE52 (Whatman Laboratory Division, Maidstone, England) to 2 litres of PSG, pH 8, and adjusting the pH to 8 with the help of a pH meter, using concentrated hydrochloric acid and 5 M sodium hydroxide. After achieving a homogeneous suspension and the correct pH, the PSG was removed using filtration. The remaining DEAE was then resuspended in 2 litres of PSG, pH 8, and poured onto a cintered filter funnel. Subsequently, the settled DEAE-cellulose was overlayed with a piece of wetted filter paper. The PSG was then allowed to drain off until the surface of the buffer reached the surface of the DEAE-cellulose. At this point, parasitaemic blood was added and allowed to flow through the column. The eluate was then checked regularly for the presence of trypanosomes by examining wet films under the light microscope. Collection of trypanosomes only commenced when trypanosomes were detected. At this stage, the eluate was placed in 50 ml Falcon tubes and centrifuged at 3,000 rpm for 15 min. The supernatant was then discarded leaving a pellet of trypanosomes. The pellet was then resuspended in a small amount of PSG, pH 8, and placed in a 10 ml Sterilin tube (Bibby Sterilin Ltd, Stone, Staffs, UK). Thereafter, the trypanosome concentration was

determined before the suspension was centrifuged at 3,000 rpm for 15 min. The pellet was then either used immediately for experimental purposes or stored at -80°C for later use.

3.9.2 Isolation of DNA

The trypanosome pellet, prepared above, was resuspended in 0.5 ml TNE. Thereafter, 50 µl of 10% (w/v) sodium dodecyl sulphate (SDS) in distilled water was added and gently mixed. After achieving a viscous solution, RNase A was added to give a final concentration of 100 µg/ml. The solution was then mixed gently and incubated at 37°C for 1 hr. Proteinase K was then added to give a final concentration of 100 µg/ml and the solution mixed gently before incubation at 50°C for 4-24 hours. After this incubation, an equal volume of phenol, mixed 1:1 (v/v) with TE to equilibrate, was added and the solution mixed gently to achieve an emulsion. It was then centrifuged at 14,000 rpm for 10 min at room temperature. The aqueous top layer containing DNA was then removed gently using a pasteur pipette and placed into a sterile 1 ml eppendorf tube. Care was taken to ensure that this did not include interphase material or phenol. Absolute ethanol (2.5 volumes), chilled to -70°C on dry ice, was then added to 1.0 volume of the aqueous interphase, to precipitate the DNA. The solution was centrifuged at 14,000 rpm for 10 min at 0°C and the DNA pellet washed twice with room temperature 70% ethanol; each time centrifuging at 14,000 rpm for 5 min. The DNA was then dissolved in 100 µl TE by incubating it at 37°C for 5 minutes, then at 4°C for 4-24 hours to ensure complete dissolution. The resultant DNA solution in TE could then be stored at 4°C until required.

3.9.3 Quantification of isolated DNA

Quantification of DNA was carried out with the spectrophotometric method described by Maniatis et al. (1982). The method is based on the principle that the ability of a sample to absorb ultraviolet light (UV) irradiation is

dependent on the quantity of nucleotide bases in the sample. Measurements were carried out at 260 nm and 280 nm on samples diluted 100 times in 1 x TE. The conversion formula 1.000 O.D = 50 μg/ml at 260 nm was used for determining the concentration of the double-stranded DNA in the sample. Dilution factors were also taken into consideration. The ratio $O.D_{260}/O.D_{280}$ was used for estimating the purity of the nucleic acid. Ratios falling within the range 1.8-1.2 were considered to be sufficiently pure; a smaller value was considered indicative of contamination of the sample by either phenol or protein; a higher value was indicative of the presence of RNA or DNA degradation (Maniatis et al., 1982).

3.9.4 Extraction of DNA for PCR from buffy coat preparations

On the understanding that PCR is a method of amplifying small quantities of relatively short target sequences of DNA using sequence-specific oligonucleotide primers and a thermostable DNA polymerase, a simple, rapid and efficient method for the purification of DNA template from buffy coat preparations was paramount. This was achieved by the use of Chelex 100 (Bio-Rad, Richmond, CA) (Walsh et al., 1991). It is not certain what the role of Chelex is in DNA preparation for PCR. However, Singer-Sam *et al.* (1989) suggested that Chelex plays a protective role by sequestering divalent heavy metals that would otherwise introduce DNA damage.

The procedure for extracting DNA for PCR from buffy-coat preparations was carried out as follows: To 50 μl of thawed buffy coat in a 1.5 ml eppendorf tube was added 200 μl of Dulbecco's PBS buffer. The suspension was then centrifuged for 1-2 minutes at 15,000 rpm at room temperature. The supernatant was removed and discarded. The cells were then resuspended in 100 μl of triple distilled and sterile (TDS) water and to this was added 200 μl of 5% (w/v) Chelex solution . The mixture was mixed gently. It was then incubated at 56°C for 30 min and then vortexed for 5-10 sec. The sample was then incubated in a boiling water bath for 8-10 min. Thereafter, it was cooled rapidly on ice before vortexing

again for 5-10 sec. The sample was then spun in a microfuge for 2-3 minutes at 15,000 rpm. At this stage, the sample could be used for the PCR by taking 5-10 µl of the supernatant and adding it to a PCR buffer mix (1 x PCR buffer = 10mM Tris HCl, 50 mM KCl, 0.05% NP40 and 0.05% Tween 20) (see 3.9.6) to make up to 25 µl reaction volume. The remainder was stored at 4°C until needed. To re-use the sample it was vortexed at high speed for 5-10 sec and spun for 2-3 min at 15,000 rpm before taking an aliquot for PCR.

In the procedure used in this experiment, the boiling treatment served to release DNA from low numbers of cells (Walsh et al., 1991) and Chelex protected the DNA from the effects of boiling. The final spinning step was essential to prevent mixture of Chelex beads with the PCR mixture lest they chelate magnesium ions that are essential for the activity of *Taq* DNA polymerase.

3.9.5 DNA oligonucleotides (primers)

Oligonucleotides ILO1044 and ILO1045 are 20 nucleotide sequences designed from sequence information (Provided by P. Pandit and N. B. Murphy) from plasmid p1616/5. The p1616/5 cDNA clone, partial nucleotide sequences of which were used to design oligonucleotide primers ILO 1044 and ILO 1045, was provided by Dr. P. A. O. Majiwa. The gene which encodes the transcript cloned in this recombinant plasmid has been shown previously to be present in ILNaR3 trypanosomes and not in the trypanosomes derived from ILNaR1, ILNaR2, IL 2462 and IL 2468 (Majiwa, personal communication). Plasmid p1616/5 contains a sequence inserted at the *Pst* 1 site of pUC 19. This sequence encodes part of a specific IL Nat 3.3 gene. IL Nat 3.3 is a trypanosome clone developed from IL Nat 3.1 (IL 1180). Thus, oligonucleotides ILO1044 and ILO1045, forward and reverse primers respectively, were designed to amplify a sequence encoded in the plasmid p1616/5. Since the primers resulted in amplification of a 900 bp product from p1616/5 we expect the same set of primers to amplify a 900 bp sequence in the genomic DNA of any trypanosome within the IL Nar 3 serodeme.

A. Sequences of oligonucleotides

ILO1044

Number of bases : 20

Bases used: A=2, C=4, G=8, T=6

Sequence:

5' TGC CGA TGG TGG AGG TTT CC3'

ILO1045

Number of bases: 20

Bases used: A=3, C=4, G=7, T=6

Sequence:

5' TGC TCA GCA TGG TGA TGC TG 3'

Finally, *T. congolense* Savannah group-specific oligonucleotides (c-oligos) are primers developed from a repetetive sequence specific for this group of trypanosomes (Moser et al., 1989). When used in PCR, the pair of primers amplify a sequence of approximately 400 bp from all isolates of *T. congolense* Savannah group (Moser *et al.*, 1989). Due to the occurrence of the sequence in these organisms at a very high copy number, the primers were used in the work described here as a positive control for the reaction. Thus, on attainment of the 400 bp product from either IL 1180 or IL 3274 using the c-oligos, monitoring of the efficiency and components of the PCR could be carried out.

3.9.6 The Polymerase Chain Reaction

In initial studies, randomly amplified DNA polymorphisms were used to distinguish between the two clones of *T. congolense*; IL 1180 and IL 3274. In this technique, random DNA segments are amplified with single primers of arbitrary nucleotide sequences to produce a "fingerprint" which is characteristic of the template DNA and primer used. The amplification reactions were performed in volumes of 25 µl containing 10 mM Tris HCl, pH 8, 50 mM KCl, 2 mM $MgCl_2$,

0.05% NP40, 0.05% Tween 20, 200 μM of each of the four dNTPs, 50 ng of primer, 25 ng of genomic DNA and 0.5 units of *Taq* DNA polymerase. The programmable thermal cycler was set for 30 cycles as described below, except that annealing was at 40°C. This techique was used to demonstrate the presence of *T. congolense* IL 3274 in relapse populationss following treatment of mixed infections in goats.

In subsequent studies using specific PCR primers designed earlier (see 3.9.5), polymerase chain reaction was carried out in accordance with the methods of Maniatis *et al.* (1982), Saiki (1990) and Welsh *et al.* (1991). The protocol was carried out on a total reaction volume of 25 μl or 50 μl for each sample. The solutions that were used were prepared as follows:

A. PCR Buffer

Stock solution	Final concentration per sample	Amount taken from stock solution (µl)
10 x PCR buffer	1 x PCR buffer*	5.0
100 mM MgCl2	1.5 mM	1.5
5 mM dNTP	200 µM	2.0
Taq polymerase**	1.25 units/50 µl	0.25
Total per sample		8.75

*10 x PCR buffer contains:

100 mM Tris HCl, pH 8.0
500 mM KCl
0.5% NP40
0.5% Tween 20

** Taq polymerase was added last, after all the other ingredients.

B. PCR priming

Ingredients	Amount (µl)
PCR buffer (prepared in A)	8.75
DNA template (sample)***	1.00
Primer (25 ng each)ı	2.00
Water (distilled and deionised)	38.25
Total volume	50.00

***1 µl contained 25ng DNA

ı Each µl contained 25 ng of primer

The PCR preparation was then overlayed with 1 drop of mineral oil and amplified in a Programmable Thermal Controller (MJ Research Inc., UK) at 94°C for 45 sec., 60°C for 1 min and 72°C for 1.5 min, for 40 cycles. At the end of the 40th cycle, the extension reaction at 72°C was allowed to continue for 5 min to ensure that all the product DNA was double stranded.

3.9.7 Determination of the minimum quantity of *T. congolense* IL 1180 DNA detectable by PCR

Total genomic DNA of *T. congolense* IL 1180 was prepared at the following concentrations per microlitre: 25 ng, 10 ng, 5 ng, 1 ng, 500 pg, 100 pg, 10 pg and 1 pg. Each dilution was then mixed with *T. congolense* IL 3274 total genomic DNA (25 ng/ml) at a ratio of 1:1 (v/v) for the PCR as shown below:

Concentration of *T. congolense* IL 1180 DNA (per µl) mixed with 1 µl of 25 ng/µl *T. congolense* IL 3274 DNA

Quantity/µl	25 ng	10 ng	5 ng	1 ng	500 pg	100 pg	10 pg	1 pg
Amount of IL 1180 DNA (µl):	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Amount of IL 3274 DNA 25 ng/µl (µl)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
PRIMERS 25ng/µl ILO1044 (µl)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
ILO1045 (µl)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
TDS water	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5
TOTAL VOLUME (µl)	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5

All preparations were then boiled in a water bath for 5 minutes before mixing with the PCR buffer.

PCR buffer was prepared in one eppendorf tube, as shown below, and 12.5 µl aliquots added to each tube.

Stock solution	Final concentration in the reaction	Amount taken per sample (µl)
10 x PCR Buffer	1 x PCR buffer	2.500
25 mM MgCl2	1.5 mM	1.500
5 mM dNTP	200 mM	1.000
Taq DNA polymerase	0.725 iu	0.125
TDS		
water		7.375
Total volume (µl)		12.500

The following were used as controls in the experiment;

IL 1180 DNA (25 ng) with ILO1044/ILO1045 primers (positive control)

IL 3274 DNA (25 ng) with ILO1044/ILO1045 primers (negative control)

Plasmid p1616/5 (50 pg) with ILO1044/ILO1045 primers (positive control)

IL 1180 DNA (25 ng) with *Nannomonas* subgenus-specific oligonucleotides (c-oligonucleotides) as primers (positive control).

The 25 µl reaction volumes were then run in an automatic thermal cycler at 94°C for 45 seconds, 60°C for 60 seconds, 72°C for 90 seconds, for 40 cyles and extension for 5 minutes at 72°C. The PCR products were electrophoresed on a 2% agarose gel, alongside an *Ase* 1 size marker.

3.9.8 Agarose gel electrophoresis

Agarose gel-electrophoresis was carried out as described by Maniatis *et al.* (1982), except for some minor alterations: To 1.1 litres of 1 x TAE (TAE = 0.04M Tris acetate, 0.001M EDTA in TDS water) was added 10 µl of 1% (w/v) ethidium bromide, for staining of DNA. Powdered agarose, 2 g, was then added to 100 ml of this solution. The suspension was then boiled in a microwave heater to ensure complete dissolution, then cooled to 50°C. The liquid agarose was poured onto a medium-sized tray (10 cm x 14 cm) which had been sealed at both ends with paper tape. A comb was then inserted before the gel set. After setting, the comb was removed and the gel loaded into a medium-sized electrophoresis tank filled with 1.0 litres 1 x TAE buffer, prepared as above. 18 µl and 10 µl of each PCR product, for 50 µl and 25 µl reactions, respectively, was then mixed with 2 µl of loading buffer (appendix I) and loaded into individual wells. The loaded gel was then subjected to 100 V, 37 mA for 2 hours.

3.9.9 Gel photography

Following electrophoresis of the gel, the current was turned off and the gel was viewed on an ultraviolet transluminator (Mighty Bright(R), Hoefer, Hoefer scientific instruments, San Francisco). Photographs were taken on polaroid coatless black and white instant pack films (Polaroid(R), OPXW3031A, UK). One minute was allowed for the films to develop after exposure. This was carried out at room temperature.

3.9.10 Southern transfer of PCR products to nylon filter

When IL 1180 genomic DNA was used as template in PCR with ILO1044/ILO1045 set of primers a 900 bp DNA fragment was amplified. To determine whether this was the same DNA fragment as that amplified with the positive control plasmid p1616/5, the DNA from the ethidium bromide-stained gel (see 3.9.8) was transferred to a nylon membrane filter by Southern blotting

(Southern, 1975; Ausubel, 1988) for hybridization analyses. The ethidium bromide-stained gel was placed in a tray containing 1.5 litres of 0.5 M sodium hydroxide and 1.5 M sodium chloride and rocked continuously for 1 hr to denature the double-stranded DNA fragments. Thereafter, a piece of nylon membrane filter (Nytran, Schleicher and Schvell, Germany) was cut such that it was 3 mm smaller in both dimensions than the gel. The filter was then placed in water for 1 min followed by 20 x SSC for 5 min. Whatman 3 mm paper was cut into sheets, approximately 7 mm smaller in both dimensions than the piece of nylon filter, to form a stack of 2-3 cm. A wick was prepared by cutting 2 pieces of Whatman 3 mm paper, approximately 2 cm wider than the width of the gel and 10 to 20 cm longer than the gel. A tray was then filled with 500 ml of 20 x SSC. A sponge wider and longer than the gel was thoroughly soaked in 20 x SSC in the tray and the cut wick put on top of the sponge such that it hung over it into the 20 x SSC. Air bubbles were carefully removed from between the wick and the sponge by rolling a 10 ml pipette back and forth over the wick. Thereafter, the gel was lifted out of the denaturising solution and most of the liquid allowed to drip off. It was then laid on top of the Whatman 3 mm wick and air bubbles removed from between the gel and the wick by rolling a pipette over the gel as above. The nylon filter was then removed from the 20 x SSC and laid on top of the gel, ensuring that the nylon filter did not overhang the gel. Air bubbles were again gently removed from between the gel and the filter, as previously described. One piece of the stack of cut Whatman 3 mm paper was then wetted in 20 x SSC, excessive 20 x SSC was removed by brief blotting on dry Whatman 3 mm paper, and then placed on top of the nylon filter, ensuring that the 3 mm paper did not overhang the filter. Air bubbles were removed as above. The remaining stack of cut Whatman 3 mm paper was then placed on top of the first to form a stack of about 3 cm. To encourage soaking of the 20 x SSC through the gel and nylon filter to the Whatman paper, a 300 g weight was put on top of the stack (see Fig. 3.9.10). The ends of the tray were then covered with plastic rap

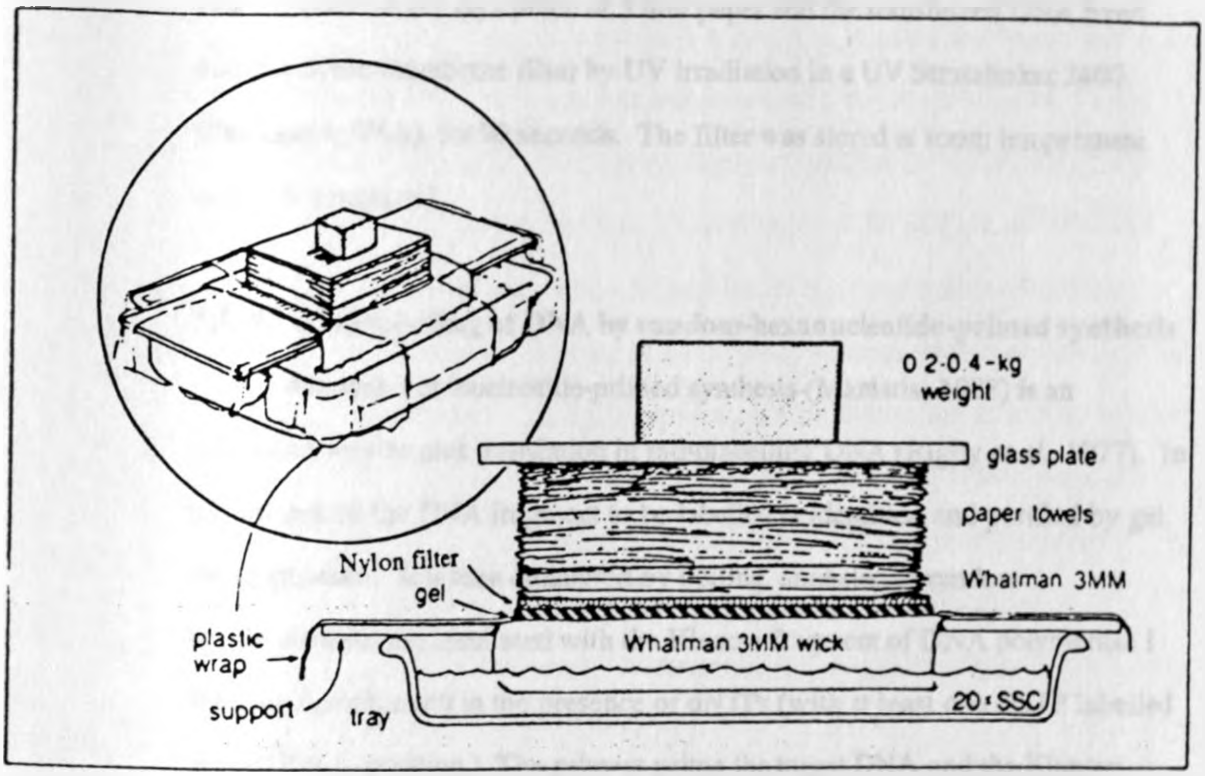


FIG. 3.9.10 A PYRAMID STACK FOR SOUTHERN TRANSFER OF DNA

paper to minimise evaporation during the transfer and to avoid short circuting of the blotting by overhanging paper. The transfer was allowed to proceed for 12-24 hr. After completion of the transfer, the stack was taken apart and the nylon membrane filter marked with a ball pen at the location of the slots.The filter was then removed using blunt forceps and placed in 20 x SSC to wash away debris. It was then blotted dry on a piece of 3 mm paper and the transferred DNA fixed onto the nylon membrane filter by UV irradiation in a UV Stratalinker 2400 (Stratagene, USA), for 90 seconds. The filter was stored at room temperature until when required.

3.9.11 Radiolabelling of DNA by random-hexanucleotide-primed synthesis

Random-hexanucleotide-primed synthesis (Maniatis, 1988) is an alternative way to nick translation in radiolabelling DNA (Rigby *et al.*, 1977). In this procedure the DNA fragment to be labelled is identified and purified by gel electrophoresis. It is then denatured by boiling, annealed to random hexanucleotide and incubated with the Klenow fragment of DNA polymerase I from *Escherichia coli* in the presence of dNTPs (with at least one dNTP labelled with ^{32}P at α-position). The primers prime the target DNA and the Klenow fragment incorporates the dNTPs, including the ^{32}P labelled dNTP, into DNA products synthesised (Ausubel, 1988).

The 900 bp fragment amplified from plasmid p1616/5 was identified on an ethidium bromide-stained gel under UV light and the relevant section of the gel carefully cut out. The fragment was then put into a sterile 1.5 ml eppendorf tube packed with a small amount of glass wool and perforated at the bottom with an 18 gauge needle. The eppendorf was then spun at 6,000 rpm in a microfuge and the eluate collected into another sterile 1.5 ml eppendorf tube that was attached at the base of the former. To ensure complete elution, the piece of gel was checked under UV light and spinning was only terminated after ensuring complete absence of fluorescence in the piece of gel. Phenol-chloroform

extractions and ethanol precipitations were then carried out on the eluate to purify the DNA (see 3.9.2). The resultant pellet was then dissolved in 50 µl 1 x TE, ready for labelling.

The 900 bp product isolated from the gel, as previously described, was then used as the template for preparation of a radiolabelled probe. To 5 µl (50 ng) of this template was added 18 µl of TDS water. The mixture was boiled in a water bath for 5 minutes. It was then cooled rapidly on ice and 5 µl of random primers (Amersham, UK) and 10 µl of oligonucleotide mix (dTTP, dATP and GTP; Amersham, UK.) were added. Thereafter, 5 µl (50 µCi) of α-^{32}P-dCTP (3000 Ci/mmol, Amersham, UK) was addded using recommended handling procedures for radioactive isotopes. 2 µl (2 units) of the *Escherichia coli* Klenow fragment of DNA polymerase I (Amersham, UK) was then added and the mixture incubated at 37°C for 30-60 minutes for labelling to proceed. The labelled DNA was then separated from the unincorporated ^{32}P-dCTP by the spin-column method, as described by Ausubel (1988), on a Sephadex G-50 column. Quantification of the radioactivity of the probe was performed by scintillation in a Beckman scintilatin counter, USA.

3.9.12 Prehybridizing, hybridizing and autoradiography of the filters

The nylon membrane filter with bound DNA was prehybridized* in a bottle containing 15-20 ml overnight at 65°C in a Hybaid oven (UK)bottle on a rotating clamp. To this was added 20 µl of the labelled after boiling to denature it (equivalent of 10 million cpm) probe prepared in 3.9.11. Hybridization was allowed to proceed for 15 hours at 65°C. The filter was then carefully removed from the hybrization bottle and washed twice for 30 min at 37°C in a solution consisting of 0.1 x SSC and 0.1 x SDS (in a shaking water bath). Following the washing step, the filter was wrapped in light a polythene paper and loaded into a film casette. Then, in a dark room, a sheet of X-ray film (Fuji, Japan) was placed between the filter and an intensifying screen (Dupont, Lightening plus) and the

sandwich clamped into a film casette. Exposure of the filter to the X-ray film was at -80°C for between 30 min and 1 week (see Fig. 3.9.12). After this period of exposure, the film was developed by an automatic developer (Fuji photo film Co. Ltd, Japan).

* Prehybridization solution contained:
20 x Denhardt's solution
4 x SSC
0.1% SDS

NB. 50 x Denhardt's solution contains:
Ficoll 5g
Polyvinylpyrrolidone 5g
0.1% Sodium pyrophosphate
Bovine serum albumin 5g
In 500 ml of TDS water

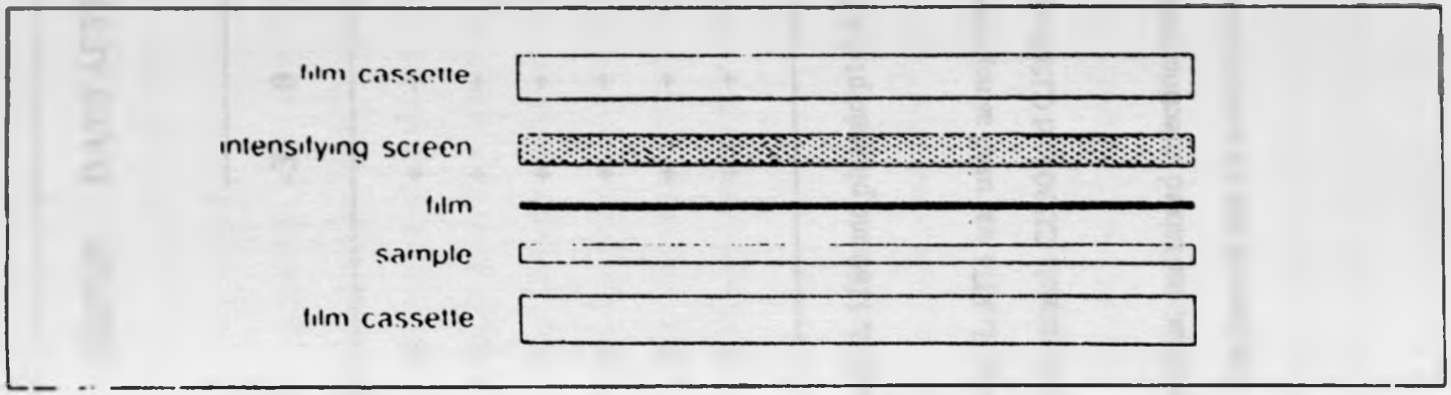


FIG. 3.9.12 Autoradiography setup: Intensifying screen, film, and sample in film cassette.

3.10 SAMPLES EXAMINED

Table 3.10: Samples examined

GOAT NUMBER	GROUP	DAYS AFTER TREATMENT					
		-3	0	18	32	46	60
CJ517	C	+	+	+	+	+	+
CJ522	C	+	+	+	+	+	+
CJ532	C	+	+	+	+	+	+
CJ537	C	+	+	+	+	+	+
CJ530	D	+	+	+	+	+	+
CJ565	F	+	+	+	+	+	+

NB: *T. congolense IL 1180 and plasmid p1616/5 were used as positive controls.

*T. congolense IL 3274 was used as negative control.

*Samples from animals CJ530 and CJ565 were used as positive controls for single

IL 1180 infections, and mixed infections respectively.

*Treatment was done on day 18 postinfection.

CHAPTER FOUR RESULTS

4.0. DEVELOPMENT OF PARASITAEMIA

On the basis of detection of trypanosomes by the buffy-coat phase-contrast technique (Murray *et al.*, 1977), animals infected with only *T. congolense* IL 1180 (Groups A and D) had an average parasitaemia prepatent period of 3 days following infection, with a standard deviation of 1.1 days. Animals in Group A were treated on day 18 following infection, after all animals in this group had been detected parasitaemic. Following treatment, all 5 animals became aparasitaemic within 24 hours. Furthermore, no relapse infections were detected in any of the goats that were infected and treated for the entire experimental period of 84 days following treatment. In contrast, the untreated control animals (Group D) remained parasitaemic for the entire experimental period; none of the animals were removed from the experiment because of low PCV.

Animals that were infected with only *T. congolense* IL 3274 (Groups B and E) had an average prepatent period following infection of 14 days, with a standard deviation of 4.9 days. Treatment of animals in Group B was done 18 days post-infection, after all animals in both groups had been detected parasitaemic. Following treatment of animals in Group B, trypanosomes were undetectable in all animals within 48 hours of treatment. However, relapse infections occurred in all animals within 14 days of treatment and persisted for the entire experimental period; none of the animals were removed from the experiment because of low PCV. The untreated control animals (Group E) remained persistently parasitaemic and were all removed from the experiment at various intervals following infection (25, 57 and 57 days) due to low PCV.

Animals infected with both *T. congolense* IL 1180 and *T. congolense* IL 3274 (Groups C and F) had an average parasitaemia prepatent period following infection of 3.3 days, with a standard deviation of 1.2 days. However, since

animals in Group B were not detected parasitaemic until 14 days following infection, animals in Group C were not treated until all animals in both Groups A and B had been detected parasitaemic. They were therefore treated on day 18 following infection. Aparasitaemia developed in all the animals by 24 hours post-treatment. However, infections in 4 of the 5 treated animals (Group C) relapsed within 17 days of treatment and persisted for the entire experimental period; none of the animals were removed from the experiment because of low PCV. One animal remained aparasitaemic for the entire experimental period following treatment. Of the three non-treatment control animals (Group F), one was removed from the experiment on day 32 post-infection due to low PCV; the other two remained parasitaemic until the termination of the experiment (day 102) since they were able to maintain their PCV above 12%.

4.1 LEVEL OF PARASITAEMIA

The level of parasitaemia associated with either or both of the *T. congolense* clones used in this study was assessed in untreated animals (i.e., Groups D, E and F). For the purpose of having a linear comparison of the levels of parasitaemia between the 3 groups of goats, the scoring system adopted for the levels of parasitaemia was the number of trypanosomes per field at x250 magnification (see Tables 4.1a and b), using a buffy-coat preparation.

Goats infected with *T. congolense* IL 1180 (Group D) attained their highest mean level of parasitaemia of 1.3 trypanosomes/field over the first 60 days post infection. This level reduced to 1.0 trypanosomes/field for the first 100 days following infection (see Table 4.1b).

Of the 3 goats infected with *T. congolense* IL 3274 (Group E), 2 were on experiment for only 56 days; over this period their mean level of parasitaemia was 19.6 trypanosomes/field. One animal in this group (CJ 518) was on the experiment for only 25 days. It was removed from the experiment on this day due to low PCV (see Table 4.1b).

Table. 4.1a
LEVEL OF PARASITAEMIA IN GOATS WITH RELAPSE INFECTIONS FOLLOWING TREATMENT

GROUP	ANIMAL NUMBER	DAY OF RELAPSE POST-TREATMENT	NO. DAYS ON EXPT.ι	MEAN PARASITAEMIA IN THE FIRST** 20	40	60	80
	CJ524	10	102	40.6	27.7	21.3	22.1
	CJ538	14	60	10.0	16.7	*	*
	CJ541	10	102	5.8	5.0	5.0	4.6
B	CJ544	10	102	10.5	7.7	6.9	6.0
	CJ577	11	102	14.6	17.0	15.3	13.3
	Mean	11	94	16.3	14.8	12.1	11.5
	STDEV			13.9	9.0	7.6	8.0
	CJ517	17	102	23.5	14.5	12.4	11.0
	CJ521	+	102	-	-	-	-
	CJ522	14	102	5.4	8.5	7.5	6.6
C	CJ532	10	102	17.8	13.2	11.2	9.7
	CJ537	10	102	27.5	22.3	17.8	15.4
	Mean	12.9	102	8.6	14.6	12.2	10.7
	STDEV			9.7	5.8	4.3	3.6

* Animal did not survive to this day
** Days following infection; data = number of trypanosomes/field at x250 magnification
+ Relapse infection did not occur for the whole experimental period
- No parasitaemia detected
STDEV -Standard deviation
ι Day 0 = Day of infection

Table. 4.1b

LEVEL OF PARASITAEMIA IN GOATS WITHOUT TREATMENT

GROUP	ANIMAL NUMBER	NO. DAYS ON EXPT.ᵃ	MEAN PARASITAEMIA IN THE FIRST** 20	40	60	80	100
D	CJ519	102	0.5	0.4	0.3	0.3	0.3
	CJ530	102	1.6	1.3	1.1	1.0	0.9
	CJ575	102	0.4	0.4	2.4	1.9	1.7
	Mean		0.8	0.7	1.3	1.1	1.0
	STDEV		0.7	0.5	1.0	0.8	0.7
E	CJ513	56	19.1	20.4	19.3	*	*
	CJ518	25	*	*	*	*	*
	CJ568	56	20.8	18.4	19.8	*	*
	Mean		19.9	19.4	19.6		
	STDEV		1.2	1.4	0.4		
F	CJ514	32	0.2	0.5	*	*	*
	CJ515	102	0.5	0.7	5.4	4.8	4.3
	CJ565	102	2.4	4.8	7.5	6.8	7.6
	Mean		1.0	2.0	6.4	5.8	5.9
	STDEV		1.2	2.4	1.5	1.4	2.3

* Animal did not survive to this day

** Days following infection; data = number of trypanosomes/field at x250 magnification

STDEV - Standard deviation

ᵃ Day 0 = Day of infection

One of the 3 goats infected with both *T. congolense* IL 1180 and *T. congolense* IL 3274 (CJ 514; Group F) was removed from the experiment on day 32 following infection due to a low PCV. The other 2 goats remained on the experiment for the whole experimental period of 102 days. The highest mean level of parasitaemia attained by animals in this group was 6.4 trypanosomes/field, over the first 60 days following infection. However, this level reduced to 5.9 trypanosomes/field when the 102 experimental days were considered (see Table 4.1b).

Comparing the mean levels of parasitaemia attained by animals in the 3 groups over the first 60 days following infection, Group E had the highest followed by Group F. Group D had the lowest. These differences were significant at the 0.05 level of significance. A graph of the mean levels of parasitaemia in the 3 groups, based on the scoring system by Murray *et al.* (1977), is shown in Fig. 4.1.1.

4.2. HAEMATOLOGY

The haematological parameter used in this experiment as a measure of the degree of anaemia was the PCV. Like the level of parasitaemia, the PCV associated with either or both of the *T. congolense* clones used in this study was assessed in untreated animals (i.e., Groups D, E and F)

Goats infected with only *T. congolense* IL 1180 (Group D) had a 20% drop in PCV within 11 days following infection. Animals CJ 519 and CJ 575 developed a 40% reduction in PCV within 46 and 53 days post-infection, respectively. However, they were able to maintain their PCV above 12% for the entire experimental period. The lowest PCV attained by animal CJ 530 over the entire experimental period (102 days) was equivalent to a 20% reduction from the pre-infection level (see Table 4.2). All animals in this group survived until the end of the experiment.

Fig. 4.LI

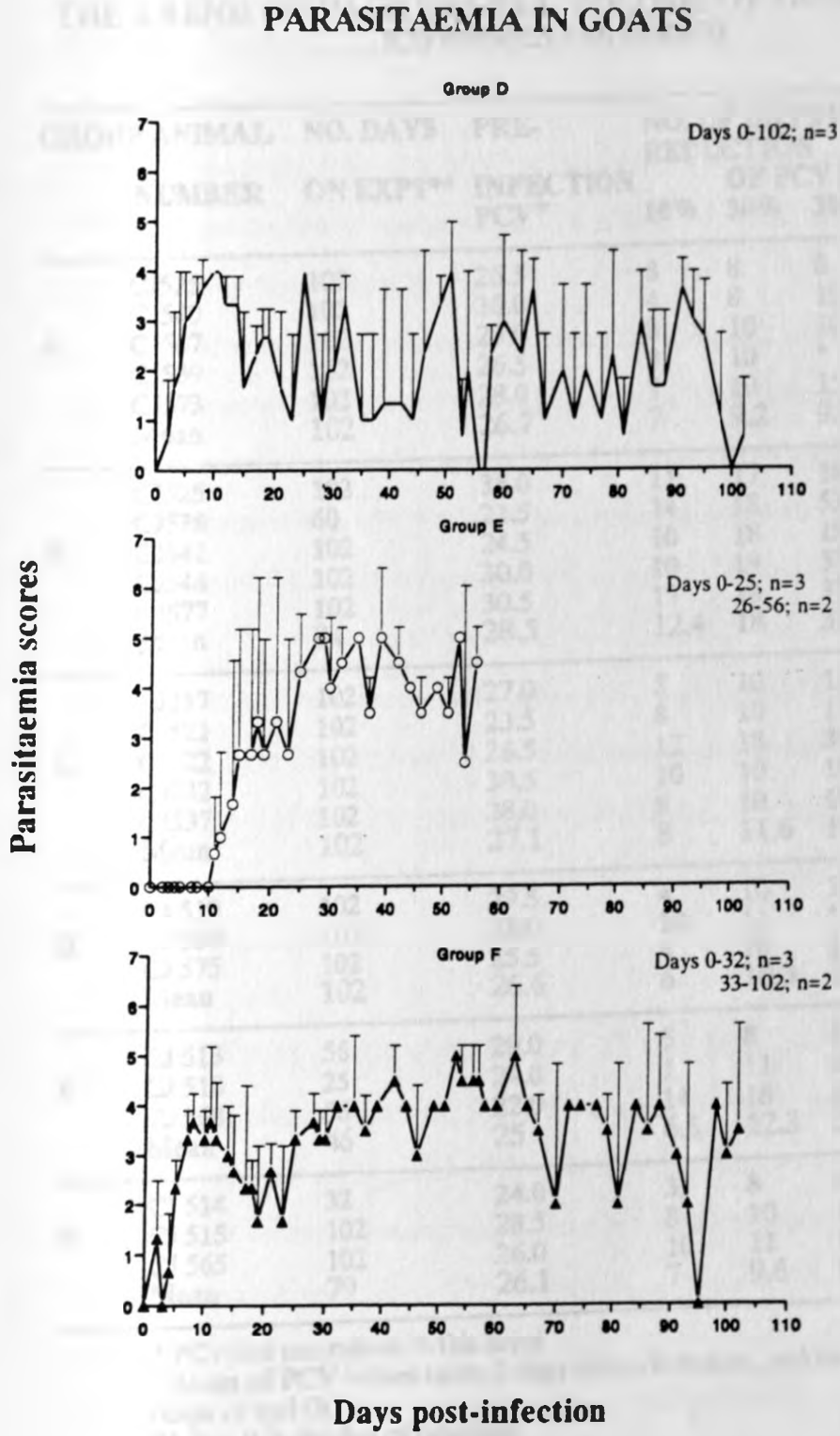

PARASITAEMIA IN GOATS
Group D
Days 0-102; n=3
Group E
Days 0-25; n=3
26-56; n=2
Group F
Days 0-32; n=3
33-102; n=2
Parasitaemia scores
Days post-infection

Table 4.2

THE TREND OF PACKED CELL VOLUME OF GOATS OVER THE EXPERIMENTAL PERIOD

GROUP	ANIMAL NUMBER	NO. DAYS ON EXPT**	PRE-INFECTION PCV+	NO. OF DAYS FOR REDUCTION OF PCV BY 10%	20%	30%	40%	50%
A	CJ525	102	26.5	8	8	8	*	*
	CJ540	102	30.0	4	8	10	12	*
	CJ567	102	23.0	8	10	10	*	*
	CJ569	102	26.5	8	10	*	*	*
	CJ573	102	28.0	7	10	11	*	*
	Mean	102	26.7	7	9.2	9.75	12	*
B	CJ525	102	35.0	11	17	18	70	91
	CJ538	60	21.5	14	18	53	60	*
	CJ541	102	24.5	10	18	19	*	*
	CJ544	102	30.0	10	19	37	*	*
	CJ577	102	30.5	17	18	37	46	100
	Mean	94	28.5	12.4	18	32.8	58.6	95.5
C	CJ517	102	27.0	5	10	11	12	15
	CJ521	102	23.5	8	10	11	*	*
	CJ522	102	26.5	12	18	39	*	*
	CJ532	102	30.5	10	10	19	*	*
	CJ537	102	28.0	8	10	19	54	*
	Mean	102	27.1	8	11.6	19.8	33	15
D	CJ 519	102	29.5	4	10	11	46	*
	CJ 530	102	25.0	10	11	*	*	*
	CJ 575	102	25.5	4	10	11	53	*
	Mean	102	26.6	6	10.3	11	49.5	*
E	CJ 513	56	29.0	5	8	8	51	53
	CJ 518	25	24.0	1	11	18	19	28
	CJ 568	56	22.0	14	18	19	19	56
	Mean	46	25	6.6	12.3	15	13	45.6
F	CJ 514	32	24.0	3	8	10	10	32
	CJ 515	102	28.5	8	10	11	11	*
	CJ 565	102	26.0	10	11	11	14	*
	Mean	79	26.1	7	9.6	10.6	11.6	32

* PCV did not reduce to this level

\+ Mean of PCV values taken 3 days before infection, and on the day of infection (days -3 and 0)

** Day 0 is the day of infection

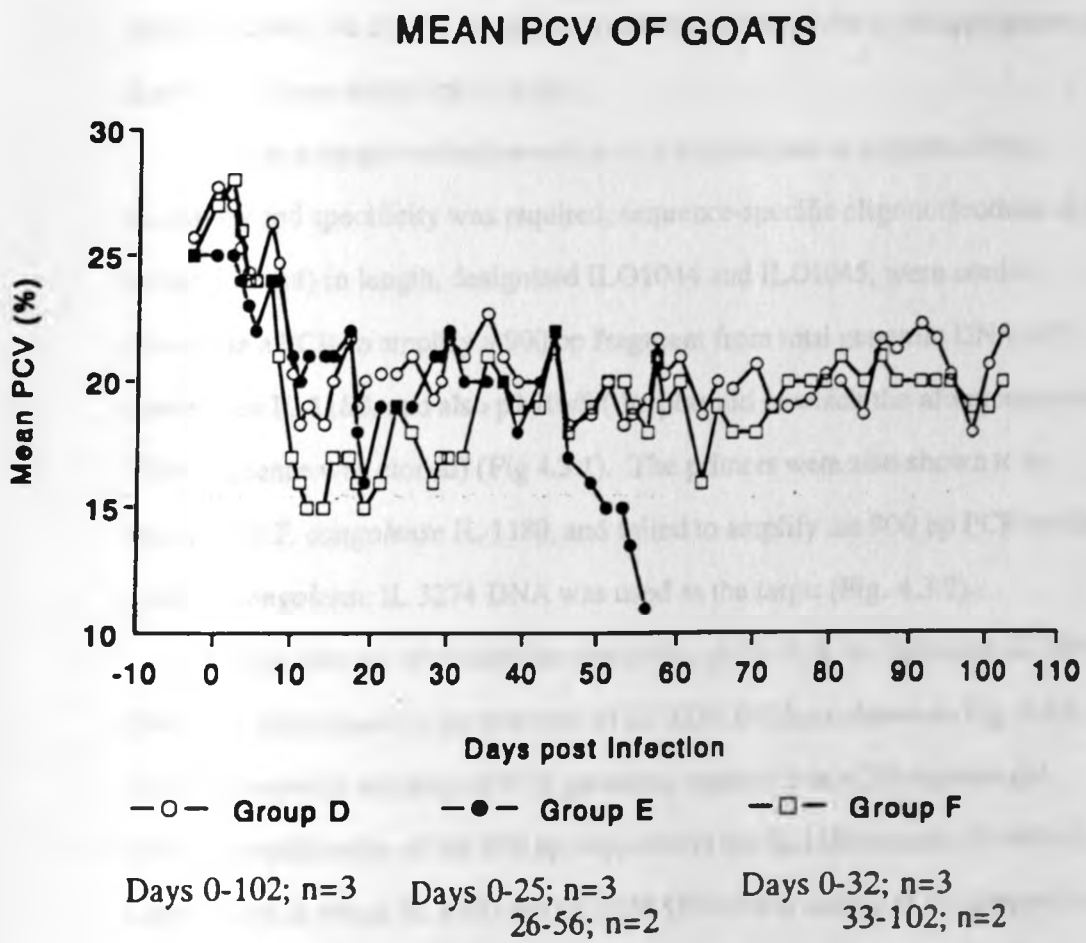
Animals infected with only *T. congolense* IL 3274 (CJ 513, CJ 518 and CJ 568; Group E) had a 20% reduction in PCV within 8, 11 and 18 days, respectively, following infection. Furthermore, all animals exhibited pronounced reductions in PCV, to less than 50% of the pre-infection levels, within 53, 28 and 56 days, respectively, following infection. All these animals were removed from the experiment because of attaining a PCV of 12% or below on days 56, 28 and 56, respectively (see Table 4.2).

In a similar manner to animals in Group D, all animals infected with both clones of trypanosomes (Group F) had a 20% drop in PCV within 11 days of infection. One of the animals in this group (CJ 514) developed a 50% reduction in PCV (coincident with a PCV of 12%) on day 32 following infection. It was therefore removed from the experiment on this day. The remaining two animals in this group (CJ 515 and CJ 565) attained a minimum PCV, that was equivalent to a 40% reduction from the pre-infection levels, within 11 and 14 days, respectively, following infection. However, since these two animals maintained their PCV above 12% for the entire experimental period, they were not removed from the experiment (see Table 4.2). A graph of PCV in the 3 groups of animals is shown in Fig. 4.2.1.

4.3. SENSITIVITY AND SPECIFICITY OF THE PCR

In preliminary work using RAPDs, a number of random primers used in the PCR on IL 1180 total genomic DNA, IL 3274 total genomic DNA, and their mixtures gave various polymorphisms. Some of the polymorphisms could be used to distinquish the 2 clones (Fig. 4.3.0a). Using primer ILO524, a 0.1 kb PCR product was produced from IL 1180, but not IL 3274; and a 0.4 kb PCR product was produced from IL 3274 but not IL 1180. Furthermore, 0.8 and 0.9 kb PCR products were generated from IL 1180 but not IL 3274. It was therefore thought that these differences could be used as distinguishing markers for the 2 parasite

Fig. 4.2.1


MEAN PCV OF GOATS
Mean PCV (%)
30
25
20
15
10
-10
0
10
20
30
40
50
60
70
80
90
100
110
Days post Infection
—○— Group D
—●— Group E
—□— Group F
Days 0-102; n=3
Days 0-25; n=3
26-56; n=2
Days 0-32; n=3
33-102; n=2

clones. In subsequent analyses, primer ILO525 was used since it gave the most reproducible results. RAPD analyses carried out on goat relapse trypanosome populations using primer ILO525 showed that IL 3274 was present in all relapse infections in animals in Group C on day 18 following treatment (on the basis of the 183 bp PCR product as the marker for IL 3274), and IL 1180 was absent (on the basis of the 861 bp PCR product as the marker for IL 1180) (Fig. 4.3.0b). However, because the technique lacked sensitivity, and because of problems with reproducibility, the RAPD technique was not considered the most appropriate for analysing relapse infections in goats.

Since a simple technique with a very high degree of reproducibility, sensitivity and specificity was required, sequence-specific oligonucleotides of 20 nucleotides (nt) in length, designated ILO1044 and ILO1045, were used as primers in a PCR to amplify a 900 bp fragment from total genomic DNA of *T. congolense* IL 1180, and also p1616/5 (the plasmid in which the aforementioned DNA sequence was cloned) (Fig 4.3.1). The primers were also shown to be specific for *T. congolense* IL 1180, and failed to amplify the 900 bp PCR product when *T. congolense* IL 3274 DNA was used as the target (Fig. 4.3.2).

In the first set of studies the sensitivity of the PCR for detecting IL 1180 DNA was determined in the presence of IL 3274 DNA, as shown in Fig. 4.3.2. Ethidium bromide staining of PCR products, separated on a 2% agarose gel, showed amplification of the 900 bp sequence in the IL 1180 sample. However, in experiments in which IL 1180 and IL 3274 DNA were mixed, as the proportion of IL 1180 to IL 3274 DNA decreased there was an increase in non-specific amplification of an IL 3274 sequence, which gave a PCR product of approximately 600 bp in size. On the basis of the 900 bp product, ethidium bromide-staining could detect the presence of IL 1180 DNA in a sample down to the level of 100 pg in a 25 µl reaction. A product of 700 bp, possibly a dimer of the tandemly repetitive satelite, was also amplified with IL 1180 and IL 3274 DNA when using c-oligos which are specific for the *T. congolense* (Savannah)

species. Using the 900 bp ^{32}P-labelled probe, it was demonstrated that the 900 bp ^{32}P-labelled sequence hybridized to the 900 bp PCR products of IL 1180 and p1616/5, but not to the smaller PCR product from IL 3274 (Fig. 4.3.3). In studies to determine the sensitivity of the radiolabelled PCR technique, the sensitivity was found to be appreciably increased by using the 900 bp ^{32}P-labelled probe; as little as 1 pg IL 1180 DNA could be detected (Fig 4.3.3.) when mixed with 25 ng IL 3274 total genomic DNA.

Finally, goat DNA was also used as template in PCR, and it was demonstrated that a 900 bp product could not be amplified in its sequence. The probe developed from the 900 bp product of the plasmid was shown to be specific for IL 1180, to the exclusion of both IL 3274 DNA and goat DNA (Fig. 4.4.1b). Thus, these results showed that the PCR-technique could be used directly on goat blood buffy-coat preparations to identify low numbers of *T. congolense* IL 1180 parasites.

4.4. ANALYSIS OF TRYPANOSOME SAMPLES FROM GOATS WITH PCR TECHNIQUE

Examination of parasitaemic blood samples collected from all goats before and after treatment with diminazene aceturate (see Table 4.4.1; Groups C, D and F) showed that IL 1180 was present in animals with mixed infections on the day of treatment (day 0), when buffy-coat preparations (see 3.8.3) were used to generate template DNA for the PCR, as described earlier (3.9.4). These results thus indicate that prior to treatment trypanosome populations in all animals in Groups C, D and F contained *T. congolense* IL 1180 (Fig 4.4.1a,b).

As previously mentioned, of the 5 goats in Group C that were infected with both *T. congolense* IL 1180 and *T. congolense* IL 3274, 4 relapsed following treatment; all were parasitaemic by day 18 following treatment. In the work reported here, the relapse populations occurring in these 4 animals were examined and the results, as elucidated by ethidium bromide staining,

demonstrated the absence of *T. congolense* IL 1180 in relapse populations of mixed infections in any of the 4 animals in Group C when sampled on days 18, 32, 46 and 60 following treatment (Fig. 4.4.2). However, IL 1180 was shown to be present in both single IL 1180 infections (Group D) and non-treated mixed infections of *T. congolense* IL 1180 and *T. congolense* IL 3274 (Group F) in samples taken on the same days as for the above mixed infections. These results established that separation of the PCR products on agarose gels followed by ethidium bromide-staining was sufficiently sensitive to detect IL 1180 parasites in the untreated mixed infections (Fig. 4.4.2). In Fig. 4.4.2 it should be noted that lane 4 (lower set), containing relapse parasites from animal CJ 537 (Group C), showed a PCR product with an approximate size of 600 bp. This occurred as a result of non-specific amplification of an IL 3274 DNA sequence.

Using the 900 bp ^{32}P-labelled probe to examine a Southern blot of the gel on which the above PCR products had been separated (i.e., Fig. 4.4.2), the findings were identical with those revealed by ethidium bromide-staining (see Fig 4.4.3). Furthermore, the ^{32}P-labelled probe hybridized to only the 900 bp PCR products. Despite extended exposure of the film to the hybridized filter (14 days), no hybridization signal was detectable in lanes loaded with samples from Group C goats with relapse infections. This therefore indicated that at the level of detection of the radiolabelled probe (i.e. 1 pg), IL 1180 was not present in relapse trypanosome populations occurring in animals that had been infected with both IL 1180 and IL 3274.

Fig. 4.3.0a

Randomly amplified polymorphic DNA (RAPD), using primers ILO101, and ILO524. The PCR parameters used were; 94°C for 1 min, 40°C for 1.5 min, 72°C for 2 min, for 30 cycles, and finally an extention at 72°C for 5 min. The size markers used were *Hae* III and *Hind* III restriction products of *phi*X174 and *lambda* DNA, respectively. An ethidium bromide-stained agarose gel of the PCR products is shown.

The lanes contain;

1	Size markers	
2	Blank	
3	IL 3274	→ Total genomic DNA
4	IL 1180	
5	IL 3274 + IL 1180	
6	Blank	
7	IL 3274	→ PCR products, primer ILO101
8	IL 1180	
9	IL 3274 + IL 1180	
10	Blank	
11	IL 3274	→ PCR products, primer ILO524
12	IL 1180	
13	IL3274 + IL 1180	

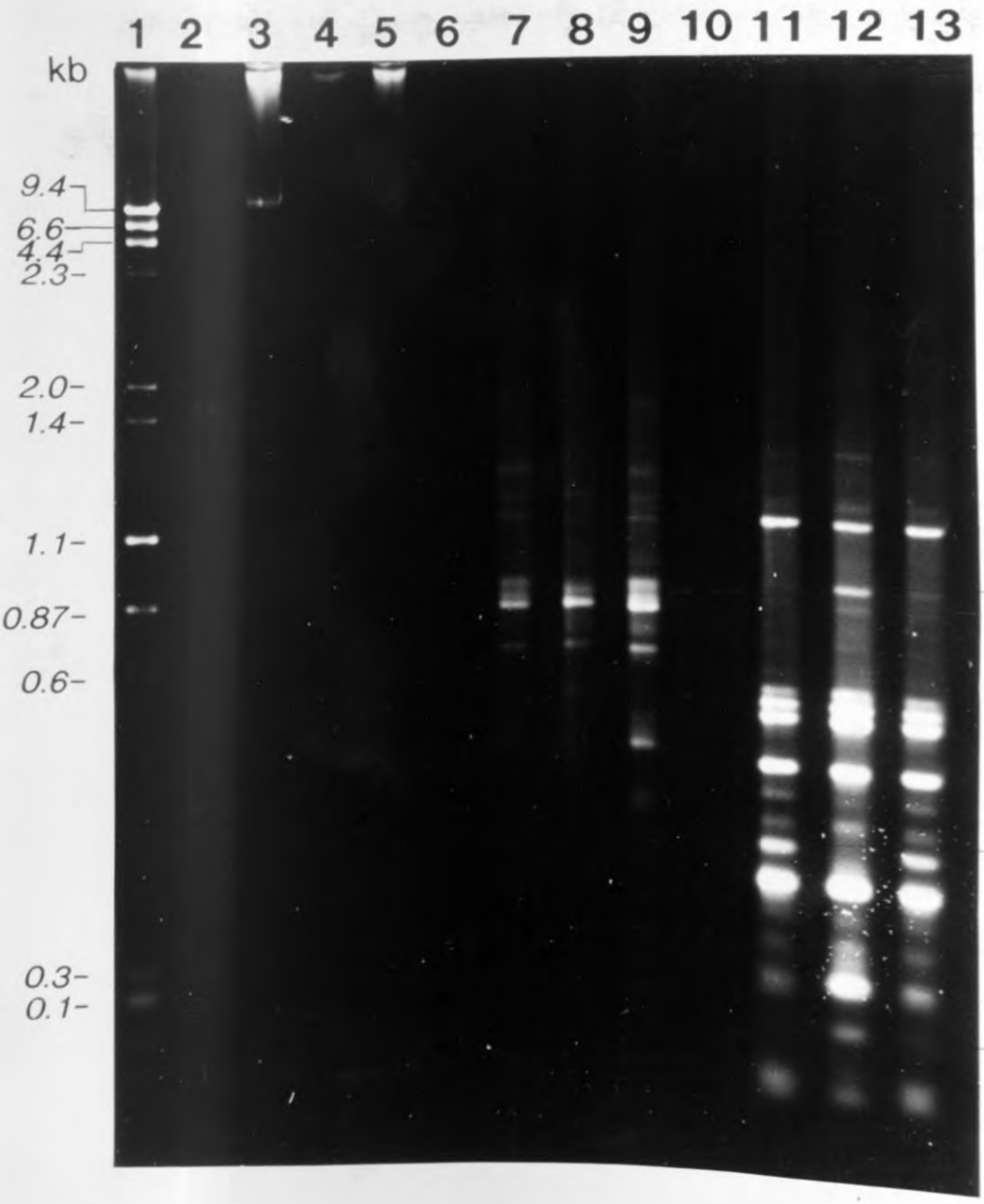

1 2 3 4 5 6 7 8 9 10 11 12 13
kb
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.87
0.6
0.3
0.1

Fig. 4.3.0b

RAPD products for trypanosome populations from goats. The samples were taken 18 days following treatment. The primer used was ILO525. The PCR parameters were as for 4.3.0a. The size marker was a ladder marker (BRL, UK). A polyacrylamide gel of the PCR products is shown.

The lanes contain;

		Group	Details
1	Size marker		
2	IL 1180		Postive control
3	IL 3274		Negative control
4	Blank		
5	CJ 515	F	MC
6	CJ 517	C	R
7	CJ 522	C	R
8	CJ 532	C	R
9	CJ 537	C	R
10	CJ 565	F	MC

MC- Mixed infection without treatment

R- Relapse infection following treatment of animals with a mixed infection

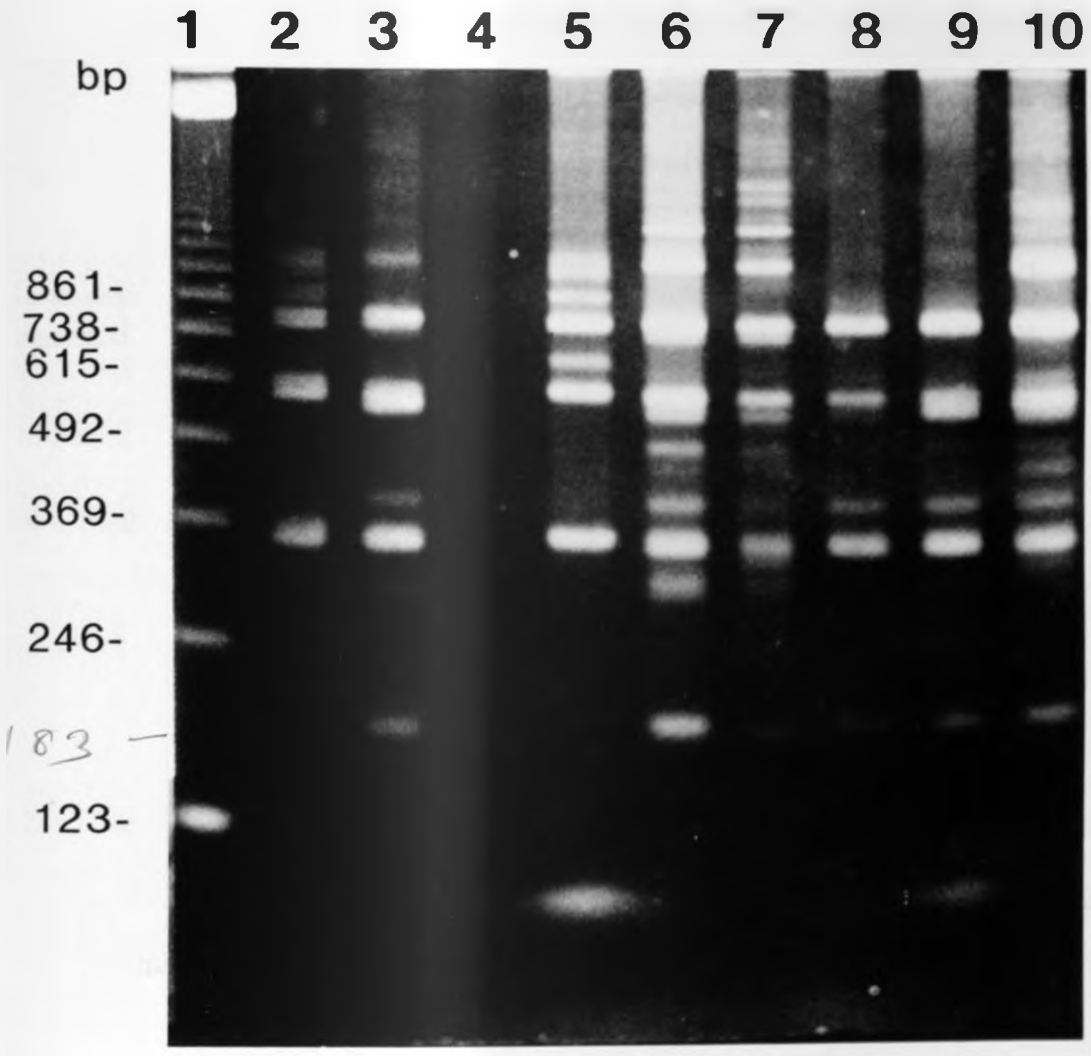

1 2 3 4 5 6 7 8 9 10
bp
861-
738-
615-
492-
369-
246-
183
123-

Fig. 4.3.1

PCR amplification of a 900 bp *T. congolense* sequence using primers ILO1044 and ILO1045. The PCR parameters were 94°C for 45 sec, 60°C for 1 min, 72°C for 1.5 min, for 40 cycles followed by 72°C for 5 min. The 900 bp fragment amplified from the plasmid containing the cloned *T. congolense* repeat sequence, p1616/5, (Lane 2) was labelled and used as a probe in subsequent experiments.

The following target DNAs were used in the PCR:

1. Purified *T. congolense* IL 1180 total DNA
2. Plasmid p1616/5
3. DNA extracted from trypanosome samples from goats with mixed infections (IL 1180 and IL 3274) before treatment.

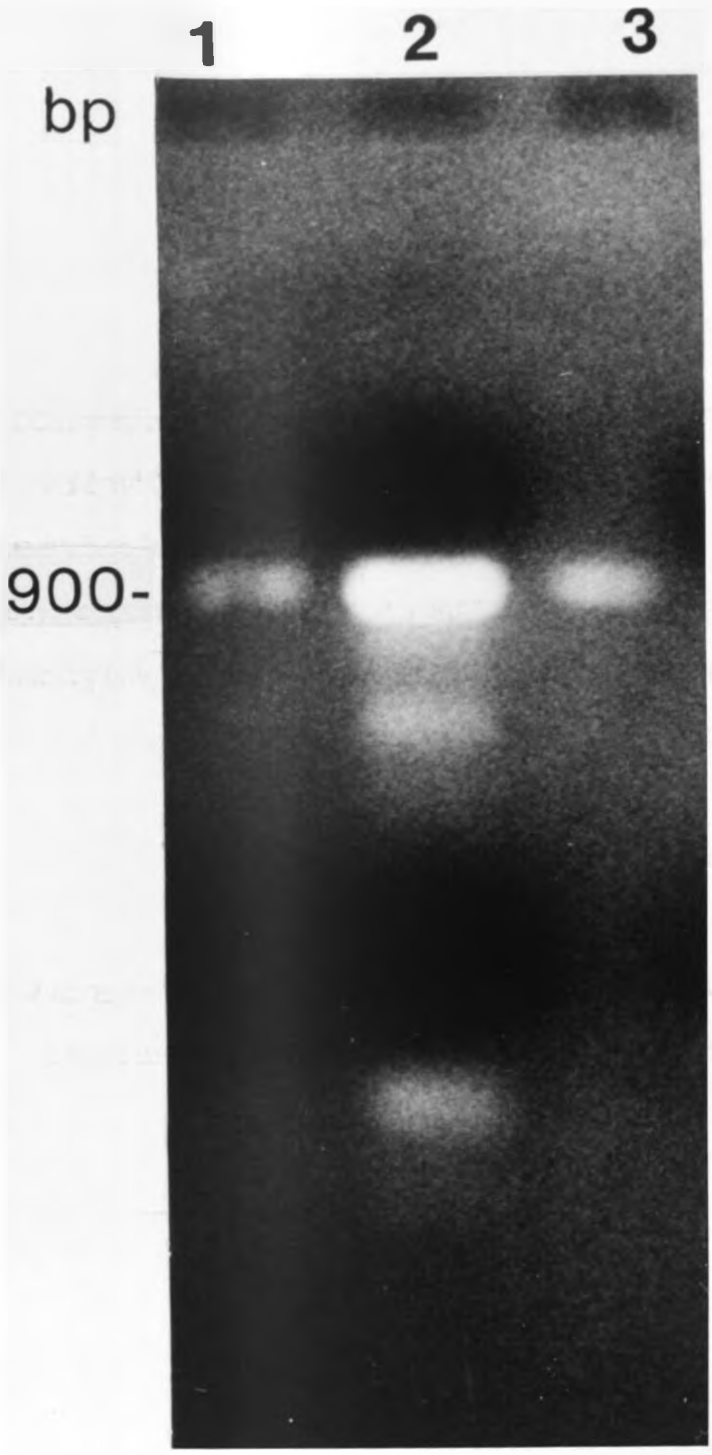

1
2
3
bp
900-

Fig 4.3.2.

T. congolense (IL 1180) DNA titration in a PCR using oligonucleotides ILO1044 and ILO1045 as primers. The amplification parameters were as described in 4.3.1. Following the PCR, samples were run on a 2% agarose gel which was stained with ethidium bromide and photographed under U.V. light. The size marker was prepared by digesting lamda DNA with *Ase* I restriction enzyme.

Lane	Sample	Primers
1	Size Marker	-
2	Blank	-
3	IL 1180	ILO1044/ILO1045
4	Plasmid p1616/5	ILO1044/ILO1045
5	Blank	-
6	IL 3274	ILO1044/ILO1045
7	IL 1180	T. congolense (Savannah)-specific oligonucleotides
8	IL 3274	
9	25ng IL 1180 + 25ng IL 3274	ILO1044/ILO1045
10	10ng IL 1180 + 25ng IL 3274	ILO1044/ILO1045
11	5ng IL 1180 + 25ng IL 3274	ILO1044/ILO1045
12	1ng IL 1180 + 25ng IL 3274	ILO1044/ILO1045
13	500 pg IL 1180 + 25ng IL 3274	ILO1044/ILO1045
14	100 pg IL 1180 + 25ng IL 3274	ILO1044/ILO1045
15	10 pg IL 1180 + 25ng IL 3274	ILO1044/ILO1045
16	1pg IL 1180 + 25ng IL 3274	ILO1044/ILO1045

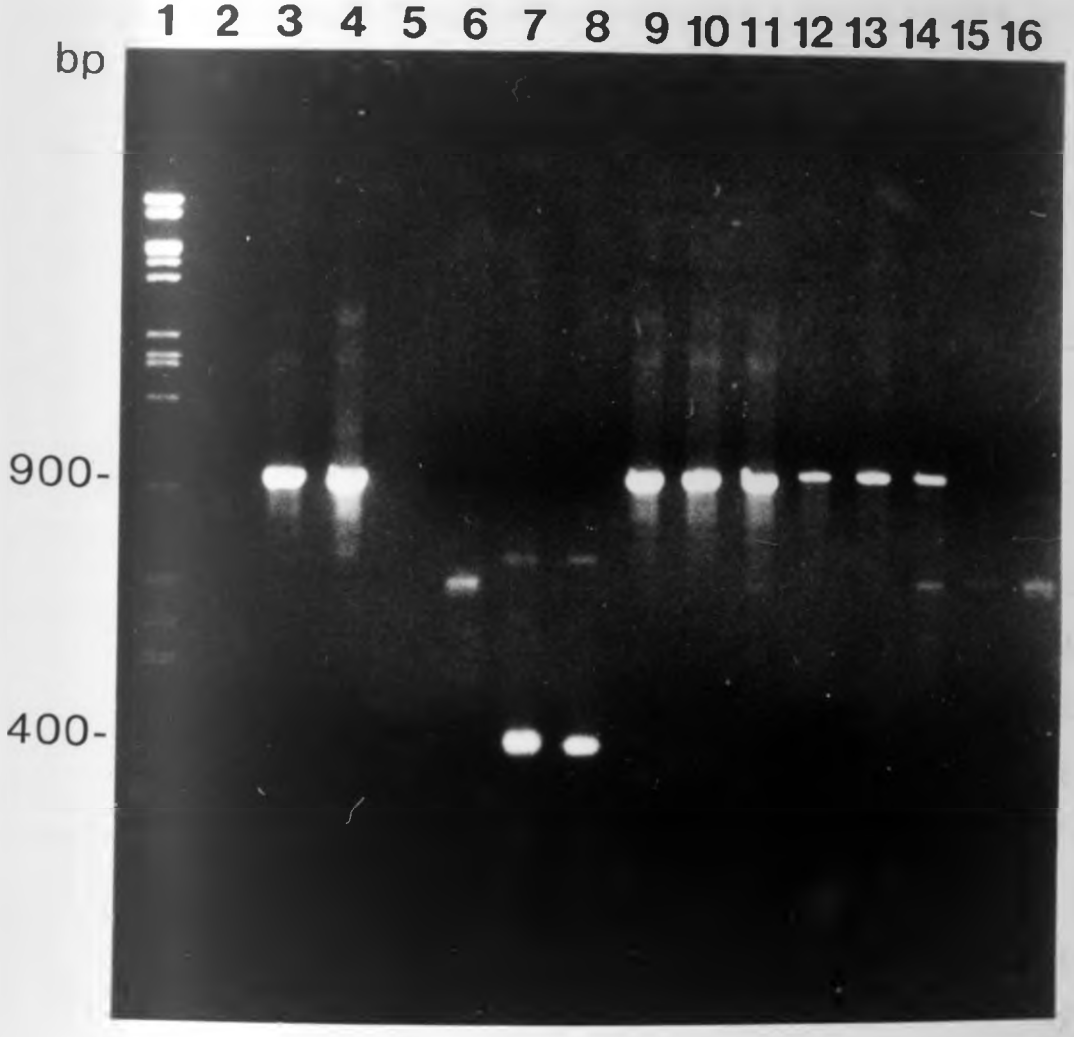

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
bp
900-
400-

Fig. 4.3.3

DNA in the gel shown in Fig. 4.3.2. was transferred to a nylon filter and hybridized with the ^{32}P-labelled 900 bp probe. Washing of the filter post-hybridization was in 0.1xSSC, 0.1% SDS for two washes of 30 min each at 37°C. Exposure was carried out at -70°C for 1 week with Fuji X-ray film and an intensifying screen (Dupont lightening plus). An autoradiograph of the hybridization is shown.

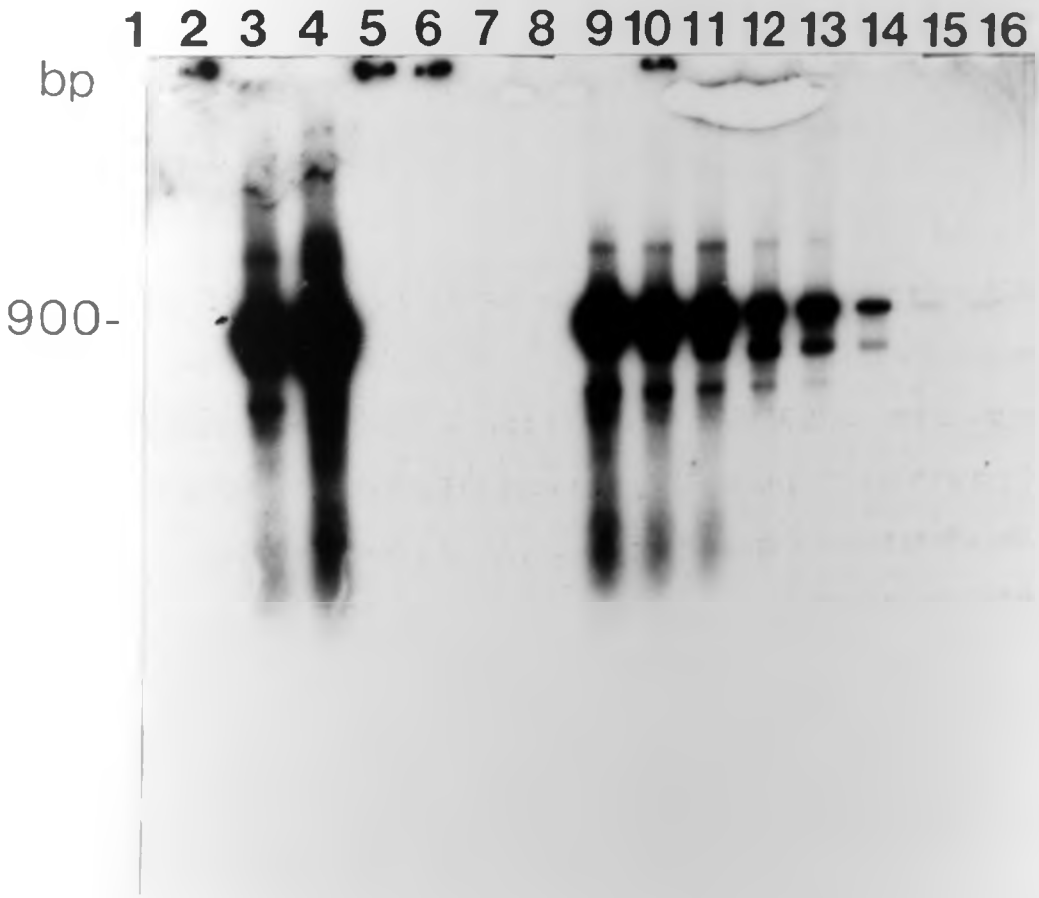

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
bp
900-

Fig: 4.4.1a

PCR amplification of trypanosome populations taken from parasitaemic goats 3 days before treatment using primers ILO1044 and ILO1045 under the same conditions as in 4.3.1. Trypanosome DNA was purified and used as the target (template) in the PCR. The resultant products were run on a 2% agarose gel, stained with ethidium bromide and photographed under U.V. light.

The lanes contain the following samples:

Lane	Sample	Group	Details
1	Size Marker	-	Lambda DNA digested with *Ase* I
2	Blank		
3	IL 1180	-	POSITIVE CONTROL (Primers ILO1044 and ILO1040)
4	IL 1180	-	POSITIVE CONTROL (Primers c-oligos)
5	IL 3274	-	NEGATIVE CONTROL (Primers ILO1044 and ILO1045)
6	CJ 517	C	MIXED INFECTION
7	CJ 522	C	MIXED INFECTION
8	CJ 530	F	MIXED INFECTION
9	CJ 532	C	MIXED INFECTION
10	CJ 537	C	MIXED INFECTION
11	CJ 565	E	SINGLE INFECTION (IL 1180)

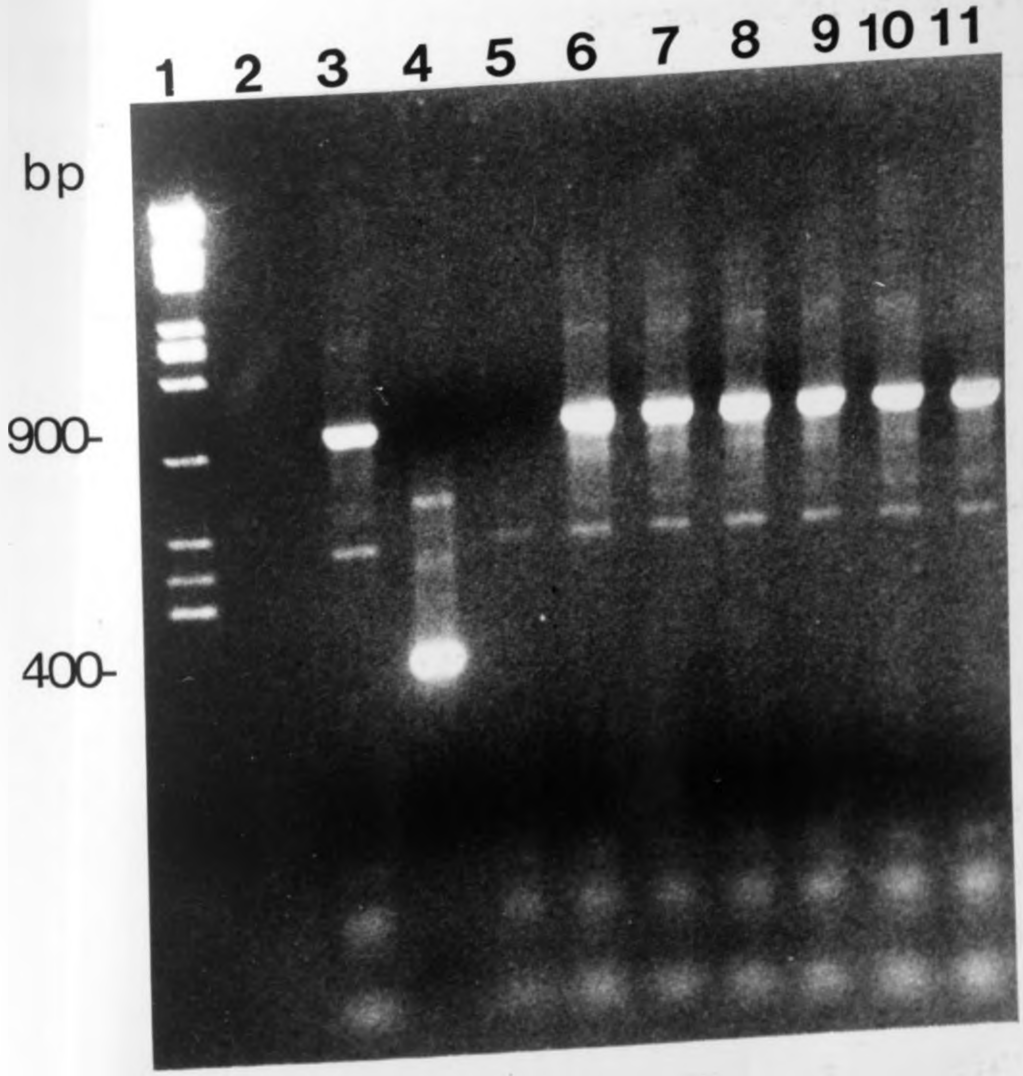

1 2 3 4 5 6 7 8 9 10 11
bp
900-
400-

Fig: 4.4.1b

A:
PCR amplification of trypanosome populations in buffy coats taken from parasitaemic goats on the day of treatment using primers ILO1044 and ILO1045. PCR was carried out on these samples as previously described and the products were run on a 2% agarose gel, then blotted onto a nylon filter. The filter was then hybridized with the 900 bp ^{32}P-labelled probe. The stringency for washing was as for 4.3.3. Exposure to X-ray film was carried out at -80°C for 1 week. The resultant autoradiograph is shown. Target samples in the PCR were as follows:

Lane	Sample	Details
1	Size Marker	Lambda DNA digested with *Ase* I
2	Blank	
3	IL 1180	POSITIVE CONTROL (Primers ILO1044 and ILO1040)
4	IL 1180	POSITIVE CONTROL (Primers c-oligos)
5	IL 3274	NEGATIVE CONTROL (Primers ILO1044 and ILO1045)
6	CJ 517	MIXED INFECTION
7	CJ 522	MIXED INFECTION
8	CJ 530	MIXED INFECTION
9	CJ 532	MIXED INFECTION
10	CJ 537	MIXED INFECTION
11	CJ 565	SINGLE INFECTION (IL 1180)

B:
PCR amplification of goat DNA using primers ILO1044 and ILO1045. Non-infected goat blood buffy-coat was used as template in a PCR under the conditions described in 4.3.1. The gel was blotted onto a nylon filter and hybridized with the ^{32}P-labelled probe. The size marker used was *phi*X174 DNA digested with the restriction enzyme *Hae* III. A photograph of the ethidium bromide-stained gel (I), and an autoradiograph of the same gel (II), are shown. Stringency for washing of the filter was as for 4.3.3. A 900 bp product was only obtained with IL 1180 DNA (positive control) and not with goat DNA. Target samples in the PCR were as follows:

Lanes:
1:- Size marker (*phi*X174 DNA digested with *Hae* III)
2:- Blank
3:- IL 1180 DNA; primers, c-oligos
4:- IL 1180 DNA; primers, ILO1044 and ILO1045
5:- IL 3274 DNA; primers, ILO1044 and ILO1045. Negative control
6:- Blank
7:- Goat A buffy coat; primers, ILO1044 and ILO1045
8:- Goat B buffy coat; primers, ILO1044 and ILO1045

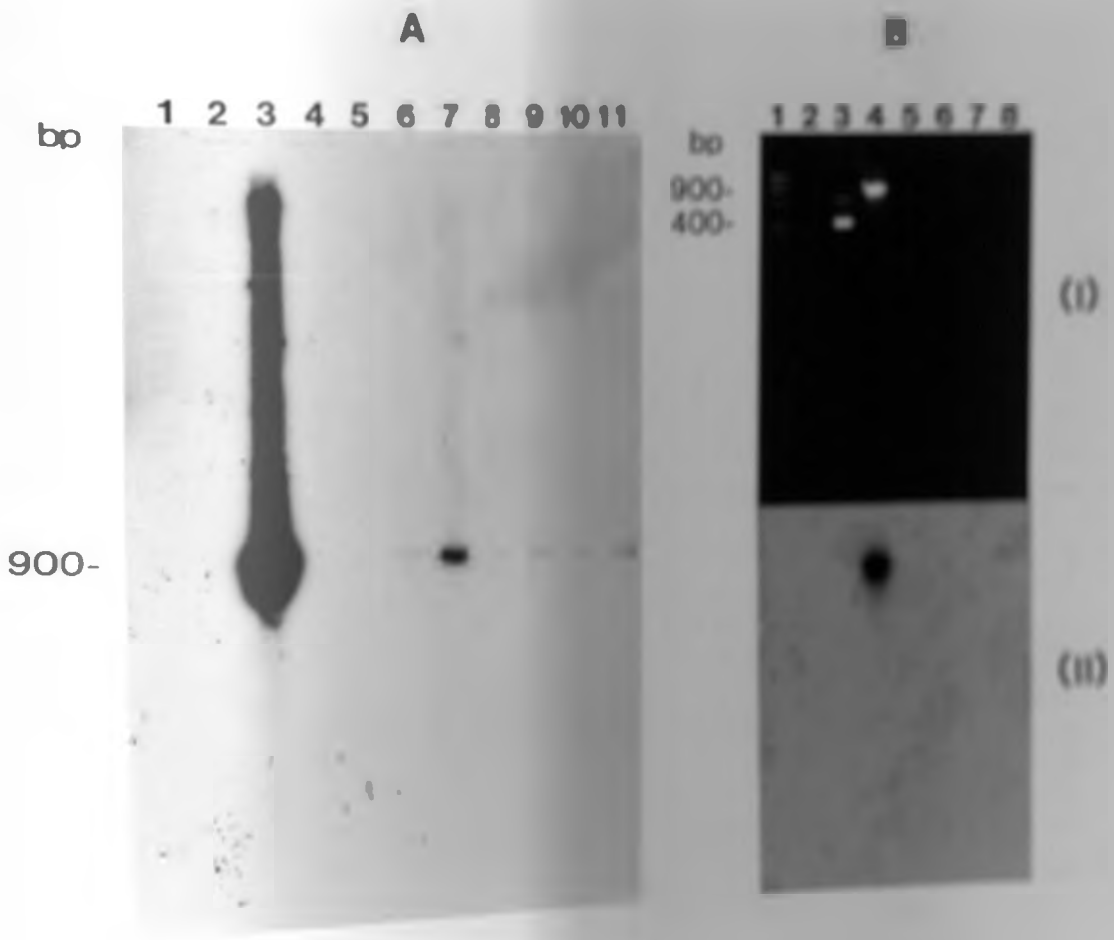

A
B
1 2 3 4 5 6 7 8 9 10 11
bp
900-
1 2 3 4 5 6 7 8
bp
900-
400-
(i)
(ii)

Fig: 4.4.2

PCR amplification products of goat relapse populations using primers ILO1044 and ILO1045. Samples in Lanes 13 and 14 (lower set) were amplified using c-oligo primers.

Lane	Sample	Day sample was taken (Days post infection	Details
UPPER SET			
1	Size Marker		
2	Blank		
3	CJ 517	36	MR
4	CJ 517	50	MR
5	CJ 517	64	MR
6	CJ 517	78	MR
7	CJ 522	50	MR
8	CJ 522	64	MR
9	CJ 522	78	MR
10	CJ 530	50	MC
11	CJ 530	64	MC
12	CJ 530	78	MC
13	CJ 532	36	MR
14	CJ 532	64	MR
LOWER SET			
1	Size Marker	-	-
2	Blank	-	-
3	CJ 532	7	MR
4	CJ 537	36	MR
5	CJ 537	50	MR
6	CJ 537	6	MR
7	CJ 537	8	MR
8	CJ 565	36	IL1180
9	CJ 565	50	IL1180
10	CJ 565	64	IL1180
11	IL 1180		POSITIVE CONTROL
12	IL 3274		NEGATIVE CONTRO
13	IL 1180		PCR POSITIVE CONTROL
14	IL 3274 POSITIVE CONTROL		PCR

Key : MR= mixed infection with IL 1180 and IL 3274; relapse population following treatment.
: MC = mixed infection with IL 1180 and IL 3274 without treatment (control)
: Single infection with IL 1180, without treatment.

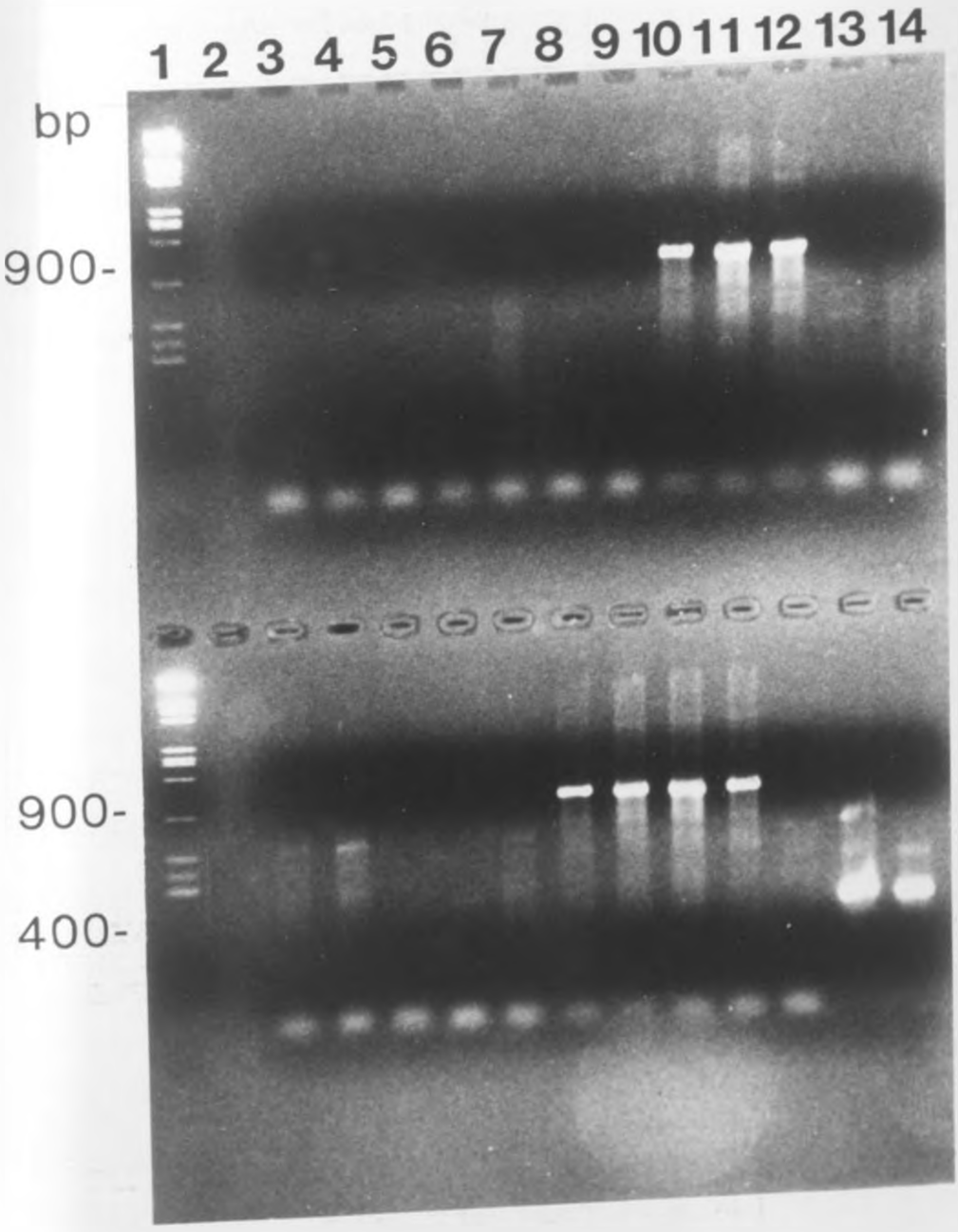
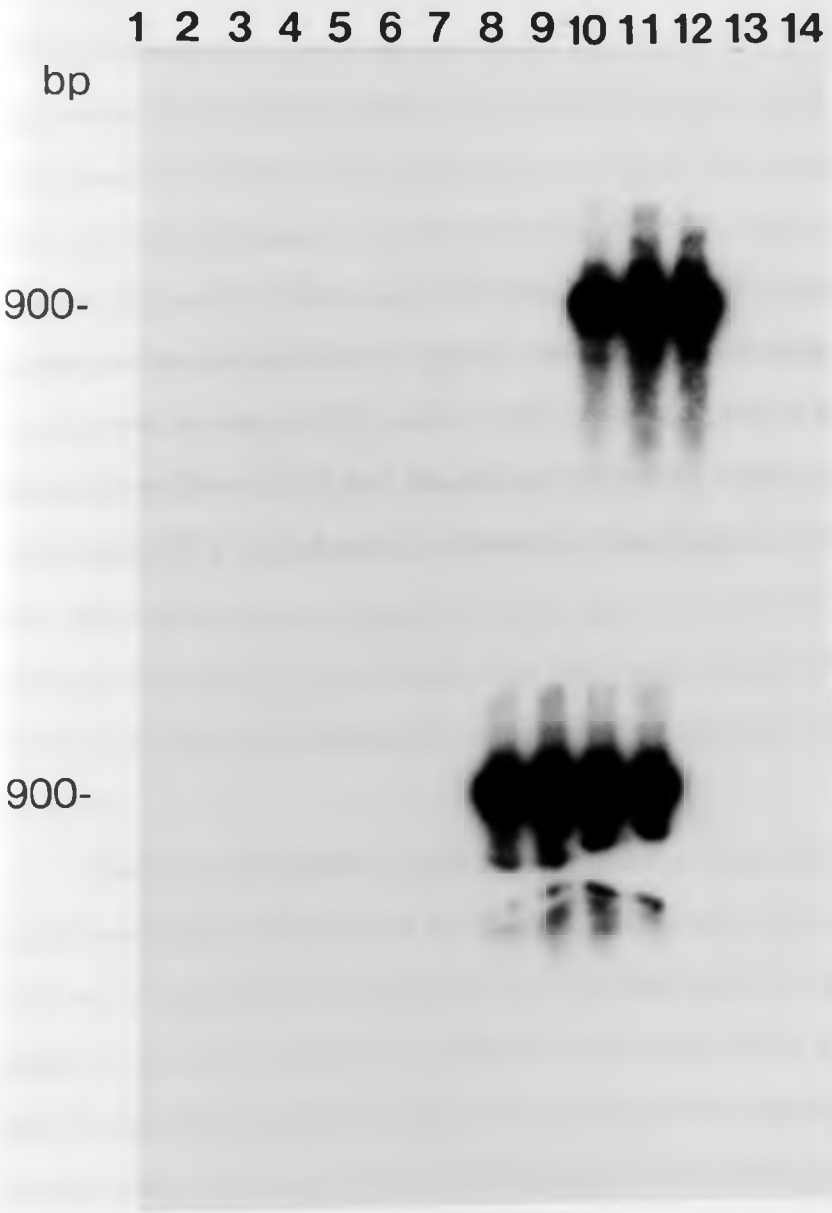

1 2 3 4 5 6 7 8 9 10 11 12 13 14
bp
900-
900-
400-

Fig 4.4.3

Hybridization of PCR products from relapse trypanosome populations. The gel shown in Fig 4.4.2 was blotted onto a nylon filter sheet and hybridized with a ^{32}P-labelled 900 bp probe as described earlier. Washing was carried out in 0.1xSSC, 0.1% SDS for two washes of 30 min each at 37°C . Exposure to X-ray film was at -80°C for 1 week with an intensifying screen. An autoradiograph is shown.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
bp
900-
900-

CHAPTER FIVE DISCUSSION

Since the 2 clones of *T. congolense* used in this experiment are very similar genetically, there was a need to develop a technique that could facilitate the distinction of the two clones at the molecular level. In initial studies, RAPDs were evaluated for use in distinguishing between the 2 clones of *T. congolense* and were subsequently used to demonstrate the presence of IL 3274 in relapse trypanosome populations of mixed infections. However, the technique proved to be relatively insensitive in distinguishing the two populations in mixed infections where the minor population represented less than 10% of the total parasite numbers. The need for a technique with higher sensitivity and reproducibility necessitated the development of a specific PCR amplification technique. A PCR technique was developed which utilised a gene sequence that is present in *T. congolense* IL 1180, but not in *T. congolense* IL 3274. The technique was shown to be specific for *T. congolense* IL 1180 but not *T. congolense* IL 3274 or goat DNA. Since there was a requirement for a high degree of sensitivity for the PCR technique at detecting *T. congolense* IL 1180 DNA when mixed with other DNA, the 900 bp PCR product from p1616/5 was purified and labelled with ^{32}P, to act as a probe.

Studies on the sensitivity of the PCR showed that, using ethidium bromide-staining of PCR products, and a radiolabelled probe, a minimum of 1,300 and 13 trypanosomes, respectively, could be detected. It was therefore not possible to detect *T. congolense* IL 1180 DNA when it occurred at quantities less than 100 pg (1,300 trypanosomes) in a total of 25 ng of total trypanosome genomic DNA, when using ethidium bromide-stained gels. Using probes developed from repetitive DNA sequences of trypanosomes, some workers have been able to detect as little as 100 trypanosomes by the direct dot-blot technique (Gibson *et al.*, 1988). The relatively low sensitivity of the PCR technique described in this study could be due to the small copy number of the sequence in

the genome (only 2 copies; J. Waitumbi, personal communication). It could also be due to inhibition by *T. congolense* IL 3274 DNA in the PCR, as previously found by Diaz *et al.* (1992). The latter possibility occurs as a result of physical obstruction of primers by the non-template DNA at the priming sites. Thus, as the ratio of template DNA versus non-template DNA decreases, the efficiency of primer interaction with specific sites on the template diminishes. Finally, the observation that there was increased amplification of non-specific sequences from *T. congolense* IL 3274 total genomic DNA as the proportion of *T. congolense* IL 1180 to *T. congolense* IL 3274 DNA decreased could have been due to similarities between the priming sites of different sequences in the 2 clones. However, since the PCR products amplified by the primers in the 2 genomes were of different sizes, and there was no cross-hybridization between the 900 bp PCR product amplified in p1616/5 with *T. congolense* IL 3274, it was demonstrated that the PCR products from the 2 clones did not have the same sequences.

In order to determine whether the diminazene-susceptible clone, *T. congolense* IL 1180, could survive treatment with diminazene aceturate when mixed with a diminazene-resistant clone, *T. congolense* IL 3274, goats were simultaneously infected i/v with both clones in order to evade the effects of the interference phenomenon described previously (Dwinger *et al.*, 1986, 1989; Sones *et al.*, 1989). Since the 2 clones had different growth rates, treatment of animals with mixed infections was carried out at a time when it was extrapolated from animals with single infections that both clones would have developed to a detectable level of parasitaemia. Using both the PCR technique and the radiolabelled probe it was demonstrated that *T. congolense* IL 1180 was present in all mixed infections prior to treatment. Since relapse infections in 4 of the animals in group C were detected by day 17 following treatment, relapse samples from these animals were examined beginning on day 18 following treatment. *T. congolense* IL 1180 could not be detected in any of the relapse populations using

the PCR technique and the radiolabelled probe. Thus, on the basis of the sensitivity of the detection techniques used, it can be concluded that although *T. congolense* IL 1180 was present in mixed infections before treatment, the population was cleared when the animals were treated with diminazene aceturate at a dose of 7.0 mg/kg b.w. This therefore discounts the hypothesis that the drug-sensitive population, *T. congolense* IL 1180, might be refractory to treatment when mixed with a resistant population. It thus suggests that the sensitive parasites that were observed by Mamman *et al.* (1991) following treatment arise from the population that survives treatment.

Considering the sensitivity of the 2 techniques used in the study described here, it is possible that *T. congolense* IL 1180 did occur in relapse populations, but at levels below the minimum level of detection of the techniques used. However, even if *T. congolense* IL 1180 did occur in the relapse populations, the fact that it would have comprised such a small percentage of the relapse populations would further suggest that the majority of the sensitive trypanosomes which occur in relapse populations are derived from trypanosomes which are refractory to treatment. These findings, therefore, could suggest that expression of resistance to diminazene by *T. congolense* is unstable in goats. Alternatively, they could also suggest that the parasites occur in different physiological states in the host, with some in a state that is refractory to treatment.

In view of the findings in this study, that diminazene-sensitive trypanosomes do not survive treatment with diminazene aceturate when mixed with a resistant population, there is need for further investigation into the phenomenon of drug-sensitive relapse infections which occur with *T. congolense*, a trypanosome species not known to be tissue invasive (Losos and Ikede, 1972). Further work is required to understand why some of the parasites within the relapse population are apparently sensitive to treatment.

In an investigation carried out to compare the pathogenicity of the two *T. congolense* clones (IL 1180 and IL 3274) in goats, all the goats infected with *T.*

congolense IL 1180 and not treated remained parasitaemic and exhibited progressive anaemia until termination of the experiment. In contrast, 5 goats that were infected with the same clone were apparently cured when diminazene aceturate was administered after detection of parasites. This therefore indicated that clearance of *T. congolense* IL 1180 in the treated goats was due to the effect of treatment. In contrast to goats infected with *T. congolense* IL 1180, goats infected with *T. congolense* IL 3274 relapsed following treatment, and non-treatment controls developed a fatal anaemia within the experimental period. Thus, two conclusions can be made; firstly, that *T. congolense* IL 3274 is indeed refractory to treatment with diminazene aceturate at a dose of 7.0 mg/kg b.w. Secondly, that on the basis of ability to induce fatal anaemia, *T. congolense* IL 3274 is more pathogenic for goats than *T. congolense* IL 1180. This is therefore a deviation from the earlier contention that drug-resistant trypanosomes are less pathogenic than drug-sensitive trypanosomes (Leach and Roberts, 1981).

In addition to work described here the pathogenicity of mixed infections was investigated in animals in Groups C and F. It was shown that 4 of the 5 animals in Group C remained parasitaemic following treatment. The apparent cure observed in one animal could have been due to the inability of *T. congolense* IL 3274 to become established in the presence of *T. congolense* IL 1180. In contrast to goats in group E, all of which were removed from the experiment due to low PCV, one of the 3 untreated control goats (Group F) was removed from the experiment on day 35 following infection due to a PCV less than 12%. The other 2 animals in Group F remained parasitaemic but maintained their PCV in excess of 12% until termination of the experiment.

One phenomenon that was consistently observed in this study was that untreated animals infected with *T. congolense* IL 1180 were detected parasitaemic before those infected with equal numbers of *T. congolense* IL 3274. This could imply that the doubling time of *T. congolense* IL 1180 is shorter than that of *T. congolense* IL 3274. Alternatively, it could be associated with greater

attachment of IL 3274 to capillary linings of the host as compared with IL 1180, as described by Banks (1978). If the former possibility is true, then *T. congolense* IL 1180 would overgrow *T. congolense* IL 3274 if it behaved the same way in a mixed infection as it does in a single infection (Seed, 1978; Dwinger *et al.*, 1986, 1989). It is, however, interesting to note that the level of parasitaemia attained and maintained in animals infected with only *T. conglense* IL 3274 was significantly higher than in animals infected with only *T. congolense* IL 1180. If the argument that *T. congolense* IL 1180 overgrew *T. congolense* IL 3274 is true, then given that the level of resistance expressed by a trypanosome population *in vivo* is dependent on the trypanosome population size (Mamman *et al.*, 1991) the drug resistance phenotype expressed by low numbers of *T. congolense* IL 3274 might account for clearance of this population by the drug. This therefore might explain why one out of 5 animals with mixed infections was cured.

When the pathogenicity of the 2 single infections was compared with the pathogenicity of the mixed infections, it appeared that the pathogenicity of *T. congolense* IL 3274 was reduced when mixed with *T. congolense* IL 1180. This could be due to interaction between the 2 parasites *in vivo*. To date, the only mechanism of interaction described *in vivo* between different trypanosome populations is interference (Dwinger *et al.*, 1986, 1989). However, in these studies animals were infected with a given trypanosome species via the bites of infected tsetse flies. When animals were subsequently detected parasitaemic and challenged with a second trypanosome species in the same manner, the presence of the primary infection appeared to impede development of the secondary infection. Thus, although animals were infected with 2 populations, a mixed infection manifested itself like a single infection with the clone that is able to outgrow the counterpart clone. As mentioned, the interference phenomenon has to date been described when animals are infected with 2 trypanosome species via the bites of infected tsetse flies. However, unlike the study described here, animals were not infected simultaneously. Thus, interference has not previously

been shown to occur when animals are simultaneously infected with 2 trypanosome populations via the intravenous route. However, since *T. congolense* IL 1180 appears to grow much faster in single infections than *T. congolense* IL 3274, it is possible that as a result of a relatively greater growth rate, the former clone is able to impede the development of the latter, thereby reducing the pathogenicity of the infection. This hypothesis deserves further investigation.

5.2 CONCLUSIONS

(a) When goats were infected with both *T. congolense* IL 1180 and *T. congolense* IL 3274, treatment with diminazene aceturate at a dose of 7.0 mg/kg b.w. eliminated the drug-sensitive population. Thus, the presence of *T. congolense* IL 3274 did not alter the drug sensitivity phenotype of *T. congolense* IL 1180.

(b) A clone-specific PCR technique was developed that could distinguish IL 1180 from IL 3274. The technique could also discriminate against goat DNA when buffy coat preparations were used as a template in PCR.

(c) The detection limit of the technique for the diminazene-sensitive clone (IL 1180), in mixed populations with IL 3274 in an ethidium bromide-stained 2% agarose gel, was 100 pg. Use of a radiolabelled probe increased the sensitivity by 100-fold.

(d) The diminazene-resistant clone *T. congolense* IL 3274 was more pathogenic for goats than the diminazene-sensitive clone, IL 1180 on the basis of ability to reduce the PCV.

REFERENCES

Abwao, S.M. (1981). A study on the use of Berenil and Samorin for treatment and control of bovine trypanosomiasis in a medium challenge area. MSc. thesis, University of Nairobi, 1981, 99 pages.

Alexander, F. (1969). *Introduction to veterinary pharmacology*. Second edition. Edinburgh, Livingstone, 1969. pp. 223-228 and pp. 253-271.

Aliu, Y.O. (1989). Pharmacokinetics and therapeutic efficacy of diminazene in *Trypanosoma congolense* infected cattle. In: Proceedings of a Workshop held at ILRAD, Nairobi, Kenya, August, 1989, on Chemotherapy for Trypanosomiasis. Published by ILRAD, Nairobi, Kenya, pp.161-162.

Aspinall, K.W., Ford, J., Steel, W.S., Steele, B. and Swan, J.F.C. (1961). The control of Trypanosomiasis in the Federal Republic of Rhodesia and Nyasaland. Eighth meeting, International Scientific Council for Trypanosomiasis Research, Jos, Nigeria. Publication No. 62, Commission for Technical Cooperation South of the Sahara, pp. 77-96.

Ausubel, F.M. (1988). Preparation and analysis of DNA. In: Current protocols in molecular biology. Volume 1. Eds. Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman J.G. Smith, J.A. and Struhl, K. Published by Greene publishing associates and Wiley - Interscience pp. 2.0.1.-2.10.3.

Austin, W.C., Collier, H.O.J., Porter M.D., Smith, G.K.A. and Taylor, E.D. (1957). A new compound active against *Trypanosoma congolense* and *T. vivax*.
Nature (London) **179** pp. 143-144.

Banks, K.L. (1978). Binding of *Trypanosoma congolense* to the walls of small blood vessels.
J. Protozool. **25** pp. 241-245.

Banks, K.L. (1980). Injury induced by *Trypanosoma congolense* adhesion to cell membranes.
J. Parasitol. **66** pp. 34-37.

Barry, J.D. and Emery, D.L. (1984). Parasite development and host responses during the establishment of *T. brucei* infection transmitted by tsetse fly.
Parasitology. **88** pp. 67-84.

Bauer, F. (1958). Uber den wirkungsmechanismus des Berenil (4, 4'-diamidino-diazoaminobenzol) bei *T. congolense*.
Zetralbl. Bakteriol. Abt. 1 Orig. B Hyg. Krankenhaushyg. Betriebshyg. Praev. Med. **172** pp. 605-620. (Abstract)

Bellofatto, V., Fairlamb, A.H., Henderson, G.B. and Cross, G.A.M. (1987). Biochemical changes associated with alpha-difluoromethylornithine uptake and resistance in *Trypanosoma brucei*.
Mol. Biochem. Parasitol. **25** pp. 227-238.

Bitonti A.J., Durmont, J.A. and McCann, P.P. (1986). Characterization of *T. brucei* S-adenosyl-L-methionine decarboxylase and its inhibition by Berenil, pentamidine and methylglyoxal bis(guanylhydrazone) *Biochem. J.* **237** pp. 685-689.

Boehringer, E.G., Fornari, O.E, and Boerhinger, I.K. (1959). Terapeutical del "Mal de caderas" Ensayo de una nuera droga primera communicacion *Rev. Investig. Ganad.* No. 6, pp. 139-150.(Abstract)

Chand, K. and Singh, R.P. (1970). Therapeutic effect of samorin in donkeys and dogs experimentally infected with *T. evansi.* *Indian Vet. J.* **47** p. 475.

Chen, P.P, Conrad, P.A., Ole-Moi Yoi, O.K., Brown, W.C and Dolan, T.T. (1991). DNA probes detect *Theileria parva* in salivary glands of *Rhipicephalus appendiculatus* ticks. *Parasitol. Res.* **77** pp. 550-554.

Croft, S.L. (1989). Recent developments in the chemotherapy of African Trypanosomiasis. In: Proceedings of a workshop held at ILRAD, Nairobi, Kenya, August, 1989, on Chemotherapy for Trypanosomiasis. Workshop overview. Published by ILRAD, Nairobi, Kenya, pp. 9-14.

Cunningham, M.P. (1968). Vaccination of cattle against trypanosomiasis by infection and treatment. In*: Isotopes and radiation in parasitology.* International Atomic Energy Agency, pp. 79-81.

Cunningham, M.P., Van Hoeve, K and Lumsden, W.H.R. (1964). A bio-assay technique for the determination of trypanocide drug levels and its application in estimating the duration of activity of Berenil in treated cattle. Proceedings of the First International Congress of Parasitology, Rome, 1964, pp. 301-303.

Dame, D.A and Schmidt, C.H. (1970). The sterile-male technique against tsetse flies, Glossina spp. *Bull. Entomol. Soc. Amer.* **16** pp. 24-30.

Davey, D.G. (1950). Experiments with "Antrycide" in the Sudan and East Africa. *Trans. R. Soc. Trop. Med. Hyg.* **43** pp. 583-616.

Davey, D.G. (1957). The chemotherapy of animal trypanosomiasis with particular reference to the trypanosomal disease of domestic animals. *Vet. Rev. Annot.* **3** pp. 15-36.

Desowitz, R.S. (1957). Suramin complexes II: prophylactic activity against *T. vivax* in cattle. *Ann. Trop. Med. Parasit.* **51** pp. 457-463.

Diaz, C., Mussenzweig, V. and Gonzalez, A. (1992). An improved polymerase chain reaction assay to detect *Trypanosoma cruzi* in blood. *Am. J. Trop. Med. Hyg.* **46** pp. 616-623.

d'Ieteren, G.D.M. and Trail, J.C.M. (1987). An overview of the African trypanotolerant livestock network. In: The African trypanotolerant livestock network: Livestock production in tsetse affected areas of Africa. Proceedings of a meeting held 23-27 November, 1987, Nairobi, Kenya. Ed. ILCA/ILRAD, 1988, pp. 31-34.

Dowler, M.E., Schillinger, D. and Connor, R.J. (1989). Notes on the routine intravenous use of isometamidium in the control of bovine trypanosomiasis on the Kenya coast.
Trop. Anim. Hlth. Prod. **21** pp. 4-10.

Dwinger, R.H., Murray, M., Luckins, A.G., Rae, P.F. and Moloo, S.K. (1986). Interference between different serodemes of *T. congolense* in the establishment of superinfections in goats following transmission by tsetse.
Parasit. Immunol. **8** pp. 293-305.

Dwinger, R.H., Murray, M., Luckins, A.G., Rae. P.F. and Moloo, S.K. (1989). Interference in the establishment of tsetse-transmitted *Trypanosoma congolense*, *T. brucei* or *T. vivax* superinfections in goats already infected with *T. congolense* or *T. vivax*.
Vet. Parasitol. 30 pp. 177-189.

Emery, D.L., Akol, G.W.O., Murray, M., Morrison, W.I. and Moloo, S.K. (1980). The chancre: early events in the pathogenesis of African trypanosomiasis in domestic livestock. In: The Host-Invader Interplay. Ed. H. Van den Bossche, Elsevier/North Holland biomedical press, Amsterdam, pp. 345-356.

Emery, D.L. and Moloo, S.K. (1980). The sequential cellular changes in the local skin reaction produced in goats by *Glossina morsitans morsitans* infected with *T. brucei*.
Acta Trop. **37** pp. 137-149.

Emery, D.L. and Moloo, S.K. (1981). The dynamics of the cellular reactions elicited in the skin of goats by *Glossina morsitans morsitans* infected with *T. congolense* or *T. vivax*.
Acta Trop. **38** pp. 15-28.

Englund, P.T., Hajduk, S.L. and Marini, J.C. (1982). The molecular biology of trypanosomes.
Ann. Rev. Biochem. **51** pp. 695-726.

Fairclough, R. (1963a). A summary on the use of Berenil in Kenya. Ninth meeting, International Scientific Council for Trypanosomiasis Research and Control, Conakry, 1972. Publication No. 88. Commission for Technical Cooperation South of the Sahara, London. pp. 81-86.

Fairclough, R. (1963b). Observation on the use of Berenil against trypanosomiasis in Kenya.
Vet. Rec. **75** pp. 1107-1112.

Fairclough, R. (1963c). A comparison of metamidium, "Samorin", "Berenil" and ethidium bromide under field conditions in Kenya.
Vet. Rec. **75** p. 855.

Fairlamb, A.H. (1989). Novel biochemical pathways in parasitic protozoa.
Parasitology **99** pp. S93-S112.

Fiennes, R.N.T.W. (1953). The therapeutic and prophylactic properties of Antrycide in trypanosomiasis of cattle.
Brit. Vet. J. 109 pp. 280-295 and 330-334.

Fiennes, R.N.T.W. (1958). Animal trypanosomiasis. Control measures by means of drugs. Eighth meeting, International Scientific Council for Trypanosomiasis Research and Control, Jos, Nigeria. Publication No. 62, Commission for Technical Cooperation South of Sahara, London, pp. 117-122.

Fiennes, R.N.T.W. (1970). Pathogenesis and pathology of animal trypanosomiasis. In: The African trypanosomiases. Ed. H.W. Mulligan. Allen and Unwin, London, pp. 729-773.

Finelle, P. (1974). African animal trypanosomiasis part III: Control of vectors. *World Anim. Rev.* **10**. pp. 39-43.

Finelle, P. and Yvore, P. (1963). Quelques observations sur la chimioresistance. Ninth meeting, International Scientific Council for Trypanosomiasis Research and Control, Conakry. Publication No. 88. Commission for Technical Cooperation South of Sahara, London. pp. 107-110.

Ford, J. (1971). The role of trypanosomiases in African ecology. A study of the tsetse problem. Clarendon press, Oxford. 568 pages.

Friedheim, E.A.H. (1949). Mel B in the treatment of human trypanosomiasis. *Am. J. Trop. Med.* **29** p. 173

Frommel, T.O. and Balber, A.E. (1987). Flow cytofluorometric analysis of drug accumulation by multidrug-resistant *Trypanosoma brucei brucei* and *T. b. rhodesiense*.
Mol. Biochem. Parasitol. **26** pp. 183-192.

Fussganger, R. and Bauer, F. (1958). Berenil: a new chemotherapeutic agent in veterinary medicine.
Med. U. Chem. **6** pp. 504-531.

Geigy, R. and Kauffmann, N.M. (1973). Sleeping sickness survey in the Serengeti area (Tanzania) 1971 part I: Examination of large mammals for trypanosomes.
Acta Trop. **30** pp. 12-23.

Gibson, W.C., Dukes, P. and Gashumba, J.K. (1988). Species-specific DNA probes for the identification of African trypanosomes in tsetse flies. *Parasitology* **97** pp. 63-73.

Gomez-Eichelmann, M.C., Holz, G., Beach, D., Simpson, A.M. and Simpson, L. (1988). Comparison of several lizard *Leishmania* species and strains in terms of kinetoplast minicircle and maxicircle DNA sequences, nuclear chromosomes, and membrane lipids.
Mol. Biochem. Parasitol. **27** pp. 143-158.

Goodwin, L.G. and Rollo, I.M. (1955). Bovine trypanosomiasis in Africa. In: Biochemistry and physiology of protozoa, Vol. 2, Eds. S.H. Hutner and A. Lwoff, New York and London, Academic press, pp. 255-263.

Gray, A.R. and Roberts, C.J. (1971a). The cyclical transmission of strains of *T. congolense* and *T. vivax* resistant to normal therapeutic doses of trypanocidal drugs.
Parasitology **63** pp. 67-89.

Gray, A.R. and Roberts, C.J. (1971b). The stability of resistance to diminazene aceturate and quinapyramine sulphate in a strain of *T. vivax* during cyclical transmission through antelope.
Parasitology **63** pp. 163-168.

Griffin, L. (1979). African trypanosomiasis in sheep and goats.
Vet. Bull. **48** pp. 819-824.

Griffin L. and Allonby, E. W. (1979). Studies on the epidemiology of trypanosomiasis in sheep and goats in Kenya.
Trop. Anim. Hlth. Prod. **11** pp. 133-142.

Haase, M., Bernard, S. and Guidot, G. (1981). Trypanosomiasis in Zebu cattle. Reappearance of *Trypanosoma congolense* in brain tissue after treatment with Berenil.
Rev. Elev. Med. Vet. Pays. Trop. **34** pp. 149-154.

Hawking, F. (1963a). Chemotherapy of trypanosomiasis. In: *Experimental Chemotherapy* Vol. 1. Eds. Schnitzer, R.J. and Hawking, F. Academic Press, NewYork, London, pp. 129-256.

Hawking, F. (1963b). Drug resistance of *T. congolense* and other trypanosomes to quinapyramine, phenanthridines, Berenil and other compounds in mice.
Ann. Trop. Med. Parasit. **57** pp. 262-282.

Hawking, F. and Thurston, J.P. (1955). The action of Antrycide on trypanosomes *in vitro*.
Brit. J. Pharm. **10** pp. 454-455.

Hoare, C.A. (1970). The mammalian trypanosomes of Africa. In: African Trypanosomiasis. Ed. H. W. Mulligan. London, George Allen and Unwin, Ministry of Overseas Development. pp. 3-19.

Hull, R.M. (1971). Laboratory studies on a South American strain of *T. vivax*
Trans. R. Soc. Trop. Med. Hyg. **65**. p. 258.

ILCA (1989). Trypanotolerance Thrust. In: ILCA Annual Report, 1989, Ed. ILCA, Addis Ababa, Ethiopia, pp. 91-102.

Ilemobade, A.A. (1987). Chemotherapy against African animal trypanosomiasis: It's strengths and limitations. In: African trypanotolerant livestock network: Livestock production in tsetse affected areas of Africa. Proceedings of a meeting held 23-27 November 1992, Nairobi, Kenya. ILCA/ILRAD pp. 265-273.

Ilemobade, A.A. and Buys J. (1970). The isolation of a strain of *T. vivax* resistant to Novidium from cattle in northern Nigeria.
Vet Rec. **87** pp. 761-762.

Ilemobade A.A., Leeflang, P., Buys, J. and Blotkamp, J. (1975). Studies on isolation and drug sensitivity of *Trypanosoma vivax* in Northern Nigeria.
Ann. Trop. Med. Parasit. **69** pp. 13-18.

Jain, C.N. (1986). The Goat: normal haematology with comments on response to disease. In: *Schalms veterinary hematology*, pp. 225-239, 4th Edition, Lea and Febiger, Philadelphia, 1986.

Jennings, F.W, Whitelaw, D.D, Holmes, P.H., Chizyuka, H.G.B. and Urquhart, G.M. (1979). The brain as a source of relapsing *Trypanosoma brucei* infection in mice after chemotherapy.
Int. J. Parasitol. **9** pp. 381-384.

Jennings, F.W., Whitelaw, D.D. and Urquhart, G.M. (1977). The relationship between duration of infection with *T. brucei* in mice and the efficacy of chemotherapy.
Parasitology **75** pp. 143-153.

Jones-Davies, W.J. (1967). A berenil resistant strain of *T. vivax* in cattle.
Vet. Rec. **81** pp. 567-568.

Jones-Davies, W.J. (1968). Diminazene aceturate and homidium chloride resistance in tsetse fly transmitted trypanosomes of cattle in Nothern Nigeria.
Vet. Rec. **83** pp. 433-437.

Jordan, A.M. (1974). Recent developments in the ecology and methods of control of tsetse flies (Glossina spp) (Dipt. Glossinidae) - a review.
Bull. Entomol. Res. **63** pp. 362-399.

Kaaya, G.P. (1975). Experimental trypanosomiasis: a study of the pathology of *T. congolense* in the goat with a special emphasis on the pathogenesis of anaemia.
MSc. Thesis, University of Nairobi, 1975, pp. 9-86.

Kalu, A.U. (1983). Effects of trypanocides on the jugular concentration of *T. congolense* in the West African dwarf sheep.
Vet. Parasitol. **13** pp. 299-309.

Kalu, A.U. and Aina, A. (1983). *Trypanosoma congolense* in calves: parasite concentration during treatment and its relation to the efficacy of the trypanocides.
Bull. Anim. Hlth. Prod. Africa. **31** pp. 197-206.

Kalu A.U. and Haruna, E. (1985). Effects of vasopressor drugs on the number of *Trypanosoma congolense* in ruminant blood.
Vet. Parasitol. **17** pp. 287-294.

Katende, J.M., Nantulya, V.M. and Musoke, A.J. (1987). Comparison between bloodstream and procyclic forms of trypanosomes for serodiagnosis of African human trypanosomiasis.
Trans. R. Soc. Trop. Med. Hyg. **81** pp. 607-608.

Killick-Kendrick, R.R. and Godfrey, D.G. (1963). Bovine trypanosomiasis in Nigeria. II: The incidence among some migrating cattle with observations on the examination of wet blood preparations as a method of survey.
Ann. Trop. Med. Parasit. **57** pp. 177-186.

Koeman, J.H., Rojksen, H.D., Smies, M., Na'Isa, B.K. and MacLennan, K.J.R. (1971). Faunal changes in a swampy habitat in Nigeria sprayed with insecticide to exterminate Glossina.
Netherlands J. Zool. **21** pp. 443-463.

Kramer, J. (1966). Incidence of trypanosomiasis in West African dwarf sheep and goats in Nsukka, Eastern Nigeria.
Bull. Epizoot. Dis. Afr. **14** pp. 423-428.

Kupper, W. (1987). Tsetse control by means of insecticide impregnated bioconical traps. Results of 5 consecutive campaigns in Cote d'Ivoire. In: The African Trypanotolerant Livestock Network: Livestock production in tsetse affected areas of Africa. Proceedings of a meeting held 23-27 November, 1987, Nairobi, Kenya. ILCA/ILRAD, 1988, pp. 63-69.

Leach, T.M. and Roberts, C.J. (1981). Present status of chemotherapy and chemoprophylaxis of animal trypanosomiasis in the Eastern hemisphere.
Pharm. Ther. **13** pp. 91-147.

Losos, G.J. and Ikede, B.O. (1972). Review of pathology of diseases in domestic and laboratory animals caused by *T. vivax*, *T. congolense*, *T. brucei*, *T. rhodesiense* and *T. gambiense*.
Vet. Path. **9** (Supplement) pp. 1-71.

Luckins, A.G. and Gray, A.R. (1978). An extravascular site of development of *Trypanosoma congolense*.
Nature (London) **272** pp. 613-614.

MacLennan, K.J.R. (1970). The epizootiology of trypanosomiasis in livestock in West Africa. In: *The African Trypanosomiasis:* Ed. H. W. Mulligan. London, George Allen and Unwin, Ministry of Overseas Development, pp. 751-765.

MacLennan, K.J.R. and Na'Isa, B.K. (1970). Relapsing *T. vivax* infections in Nigerian Zebu cattle treated with diminazene aceturate.
Trop Anim. Hlth. Prod. **2** pp. 189-195.

Majiwa, P.A.O., Young, J.R., Hammers, R. and Matthyssens, G. (1986). Minichromosomal variable surface glycoprotein genes and molecular karyotypes of *Trypanosoma (Nannomonas) congolense.*
Gene. **41** pp. 183-192.

Mamman, M., Katende, J., Moloo, S.K. and Peregrine, A.S. (1993). Variation in sensitivity of *Trypanosoma congolense* to diminazene during the early phase of tsetse-transmitted infection in goats.
Vet. Parasitol. (submitted)

Mamman, M., Murphy, N.B., Kemei, S. and Peregrine, A.S. (1991). Sensitivity to diminazene and frequency of diminazene-resistant trypanosomes in populations of *Trypanosoma congolense* arising in animals following treatment with diminazene. Paper presented in Tanzanian Veterinary Association Scientific meeting, December, 1991, Arusha, Tanzania, 5 pages.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Volume I and II.

Masake, R.A., Nantulya, V.M., Akol, G.W.O. and Musoke, A.J. (1984). Cerebral trypanosomiasis in cattle with mixed *Trypanosoma congolense* and *T. b. brucei* infections.
Acta Trop. **41** pp. 237-246.

Maxie, M.G. and Losos, G.J. (1977). Release of *Trypanosoma congolense* from the microcirculation of cattle by Berenil.
Vet. Parasitol. **3** pp. 277-281.

Morgan, C.T. and Walls, L.P. (1931). Researches in the phenanthridine series. I: A new synthesis of phenanthridine homologues and derivatives.
J. Chem. Soc. pp. 2447-2456

Morrison, W.I., Wells, P.W., Moloo, S.K., Paris, J. and Murray, M. (1982). Interference in the establishment of superinfections with *Trypanosoma congolense* in cattle.
J. Parasitol. **68** pp. 755-764.

Moser, D.R., Cook, G.A., Ochs, D. E., Bailey, C.P., McKane, M.R. and Donelson, J.E. (1989). Detection of *Trypanosoma congolense* and *T. brucei* subspecies by DNA amplification using the polymerase chain reaction.
Parasitology **99** pp. 57-66.

Mulligan, H.W. (1970). *The African trypanosomiasis.* Ed. H.W. Mulligan. Allen and Unwin, London, 950 pages.

Mullis, B.K. (1990). The unusual origin of the polymerase chain reaction.
Sci. Am. April 1990, pp. 56-65.

Murray, M. and Morrison, W.I. (1979). Parasitaemia and host susceptibility to African trypanosomiasis. In: *Pathogenicity of trypanosomes.* Eds. Losos, G. and Chorinad, A. Ottawa, International Development Research Centre. pp. 71-81.

Murray, M., Murray, P.K. and McIntyre, W.I.M. (1977). An improved parasitological technique for the diagnosis of African trypanosomiasis.
Trans. R. Soc. Trop. Med. Hyg. **71** pp. 325-326.

Murray, M., Trail, J.C.M., Turner, D.A. and Wissocq, Y. (1983). Livestock productivity and Trypanotolerance. Network Training Manual, ILCA, 510 pages.

Mwambu, P.M. (1971). The effect of block-treatment regime using ethidium on cattle trypanosomiasis in endemic areas.
East Afr. Agric. J. **36** pp. 414-418.

Mwambu, P.M. and Mayende, J.S.P. (1971). Occurrence of Berenil resistant strains of *T. vivax.*
Trans. R. Soc. Trop. Med. Hyg. **65** pp. 254-255.

Na'Isa K. (1969). The protection of 60 Nigerian trade cattle from trypanosomiasis using Samorin.
Bull. Epizoot. Dis. Afr. **17** pp. 45-54.

Nantulya, V.M., Musoke, A.J., Rurangirwa, F.R. and Moloo, S.K. (1984). Resistance of cattle to tsetse-transmitted challenge with *Trypanosoma brucei*, or *T. congolense* after spontaneous recovery from syringe-passaged infections.
Inf. Immun. **43** pp. 735-738.

Nyeko, J.H.P., ole MoiYoi, O.K., Majiwa, P.A.O., Otieno, L.H. and Ociba, P.M. (1990). Characterisation of trypanosome isolates from cattle in Uganda using species-specific DNA-probes reveals predominance of mixed infections.
Insect. Sci. Applic. **11** pp. 271-280.

Omuse, J.K. (1973). Pathogenesis of *Trypanosoma congolense* infection in the bovine.
MSc. Thesis, University of Nairobi, 100 pages.

Opperdoes, F.R. (1987). Compartmentation of carbohydrate metabolism in trypanosomes.
Ann. Rev. Microbio. **41** pp. 127-151.

Ormerod, W.E. and Venkatesan, S. (1971a). An amastigote phase of the sleeping sickness trypanosome.
Trans. R. Soc. Trop. Med. Hyg. **65** pp. 736-741.

Ormerod, W.E. and Venkatesan, S. (1971b). The occult visceral phase of mammalian trypanosomes with special reference to the life cycle of *Trypanosoma brucei.*
Trans. R. Soc. Trop. Med. Hyg. **65** pp. 722-736.

Paris, J., Murray, M. and McOdimba, F. (1982). A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle.
Acta Trop. **39** pp. 307-316.

Peregrine, A.S., Knowles, G., Ibitayo, A.I., Scott, J.R., Moloo, S.K. and Murphy, N.B. (1991). Variation in resistance to isometamidium chloride and diminazene aceturate by clones derived from a stock of *Trypanosoma congolense.*
Parasitology **102** pp. 93-100.

Petrovskij, V.V. (1974). Problemes actuels de l'eradication des trypanosomes.
Veterinaya **5** pp. 68-70.(Abstract)

Pinder, M. and Authie, E. (1984). The appearance of isometamidium resistant *Trypanosoma congolense* in West Africa.
Acta Trop. **41** pp. 247-252.

Raghavan, R.S. and Khan, N.A. (1970). Bovine trypanosomiasis control with Samorin.
Indian Vet. J. **47** pp. 187-188.

Raynaud, J.P., Sones, K.R. and Friedheim, E.A.H. (1989). A review of Cymelarsan(R) - A new treatment proposed for animal trypanosomiasis due to *T. evansi* and other trypanosomes of the *T. brucei* group. Twentieth meeting, International Scientific Council for Trypanosomiasis Research and Control, Mombasa, Kenya.
Publication No. 115, OAU/STRC, Nairobi, Kenya, pp. 334-338.

Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977). Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase 1
J. Mol. Biol. **113** pp. 237-251.

Roberts, C.J., Gray, M.A. and Gray, A.R. (1969). Local skin reactions in cattle at the site of infection with *T. congolense* by *Glossina morsitans* and *Glossina tachinoides*.
Trans. R. Soc. Trop. Med. Hyg. **63** pp. 620-624.

Robson, J. (1962). Prophylaxis against trypanosomiasis in Zebu cattle -IV. A field trial of metamidium and isometamidium.
Vet. Rec. **74** pp. 913-917.

Saiki, K.R. (1990). Amplification of genomic DNA. In: PCR protocols: A guide to methods and applications. Eds. Innis M.A., Gelfand D.H., Sninsky, J.J. and White T.J. Academic Press. Inc., pp. 13-20.

Scott, M. and Pegram, R.G. (1974). A high incidence of *T. congolense* strains resistant to homidium bromide in Ethiopia.
Trop. Anim. Hlth. Prod. **6** pp. 215-221.

Seed, R.J. (1978). Competition among serologically different clones of *Trypanosoma brucei gambiense in vivo*.
J. Protozool. **25** pp. 526-529.

Silayo, R.S., Mamman, M., Moloo, S.K., Aliu, Y.O., Gray, M.A. and Peregrine, A.S. (1992). Response of *Trypanosoma congolense* in goats to single and double treatment with diminazene aceturate.
Res. Vet. Sci. **53** pp. 98-105.

Singer-Sam, J., Tanguay, R.L. and Riggs, A.D. (1989). Use of Chelex to improve the PCR signal from a small number of cells. Amplifications: a forum for PCR users, issue 3 (September): 11.

Sones, K.R., Holmes, P.H. and Urquhart, G.M. (1989). Interference between drug-resistant and drug-sensitive stocks of *T. congolense* in goats.
Res. Vet. Sci. **47** pp. 75-77.

Soulsby, E.J.L. (1987) African Trypanosomiasis. In: Immune responses in parasitic infections: immunolgy, immunopathology and immunoprophylaxis.Vol.III, Protozoa. CRC-press Inc. BucaRatan, Florida, pp. 2-18.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis
J. Mol. Biol. **98** pp. 503-517.

Srivastava, R.V.N. and Malhotra, M.N. (1967). Efficacy of Mel W-trimelarsan-melarsonyl potassium and Samorin-isometamidium chloride against *T. evansi* in experimental dogs.
Indian J. Anim. Hlth. **6** p. 291.

Stephen, L.E. (1963). An attempt to produce resistance to homidium in a strain of *T. vivax* transmitted by tsetse fly.
J. Comp. Path. **73** pp. 76-83.

Stephen, L.E. (1966). Pig trypanosomiasis in Africa. Review series No. 8, Commonwealth Agricultural Bureaux, Farnham Royal, 65 pages.

Stephen, L.E. (1970). Clinical manifestations of trypanosomiasis in livestock and other domestic animals. In: *The African trypanosomiasis*; Ed. Mulligan H.W. Allen and Unwin, London, pp. 774-794.

Sutherland, I. A., Moloo, S.K., Holmes, P.H. and Peregrine, A.S. (1991). Therepeutic and prophylactic activity of isometamidium chloride against a tsetse-transmitted drug-resistant clone of *Trypanosoma congolense* in Boran cattle. *Acta Trop.* **49** pp. 57-64.

Tizard, I. (1985). Immunology and pathogenesis of trypanosomiasis. Ed. I. Tizard, Boca Raton, CRC Printers, 237 pages.

Toure, S.M. (1970). Prothidium and isometamidium in the treatment of trypanosomiasis in a dog due to *Trypanosoma brucei*. *Rev. Elev. Med. Vet. Pays. Trop.* **23** p. 321.

Ugochukwu, E.I. (1983). Haematological observations in goats with natural infection of trypanosomiasis. *Bull. Anim. Hlth. Prod. Afr.* **31** pp. 391-395.

Walsh, P.S., Metzger, D.A. and Higuchi, R. (1991). Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques **10** pp. 506-513.

Welsh, J., Peterson, C. and McClelland, M. (1991). Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. *Nucleic Acid Res.* **19** pp. 203-206.

Whitelaw, D.D., Gardiner, P.R. and Murray, M. (1988). Extravascular foci of *Trypanosoma vivax* in goats: the central nervous system and the aqueous humour of the eye as potential sources of relapse infections after chemotherapy. *Parasitology* **97** pp. 51-61.

Whiteside, E.F. (1958). The control of animal trypanosomiasis. In: Symposium on Animal trypanosomiasis, Commission for Technical Cooperation in Africa South of the Sahara, London, Publication No. 45, pp. 81-116.

Whiteside, E.F. (1960a). Recent work in Kenya on the control of drug-resistant cattle trypanosomiasis. Eighth meeting of the International Scientific Council for Trypanosomiasis Research and Control, Jos, Nigeria. Publication No. 62, Commission for Technical Cooperation South of the Sahara. pp. 141-154.

Whiteside E.F. (1960b). The maintenance of cattle in tsetse infested country. Seventh meeting of the International Scientific Council for Trypanosomiasis Research and Control, Brussels. Publication No. 41. Commission for Technical Cooperation South of the Sahara, London, pp. 83-90.

Whiteside E.F. (1962). Interaction between drugs, trypanosomes and cattle in the field. In: *Drugs, parasites and hosts.* Eds. Goodwin, L.G. and Nimmo-Smith, R.H., Churchill, London, pp. 116-141.

Whiteside, E.F. (1963). A strain of *T. congolense* directly resistant to Berenil. *J. Comp. Path.* **73** pp. 167-175.

WHO (1962). Expert committee on trypanosomiasis. WHO Technical Report, Serial Number 247, 57 pages.

Williamson, J. (1957). Suramin complexes, I: Prophylactic activity against *Trypanosoma congolense* in small animals. *Ann. Trop. Med. Parasit.* **51** pp. 440-456.

Williamson, J. (1958). Progress with suramin complexes in the treatment of animal trypanosomiasis. Seventh meeting of the International Scientific Council for Trypanosomiasis Research. Publication No. 41. Commission for Technical Cooperation South of the Sahara, Brussels, 1958, pp. 47-50.

Williamson, J. (1960). Some problems in trypanocidal drug resistance. In: Eighth meeting of the International Scientific Council for Trypanosomiasis Research and Control, Jos, Nigeria. Publication No. 62, Commission for Technical Cooperation in Africa South of the Sahara, London, pp. 162-169.

Williamson, J. (1962). Chemotherapy and chemoprophylaxis of African trypanosomiasis. *Exp. Parasitol.* **12** pp. 354-367.

Williamson, J. (1970). Review of chemotherapeutic and chemoprophylactic agents. In: *The African trypanosomiasis*. Ed. Mulligan H.W., Allen and Unwin, London, pp. 125-221.

Williamson, J. (1979). Effects of trypanocides on the fine structure of target organisms. *Pharmacol. Ther.* **7** pp. 445-512.

Williamson, J. and Desowitz, R.S. (1956). Prophylactic activity of suramin complexes in animal trypanosomiasis *Nature* (London) **177** pp. 1074-1075.

Wragg, W.R., Washbourne, K., Brown, K.N., and Hill, J. (1958). Metamidium: a new trypanocidal drug. *Nature* (London) **182** pp. 1005-1006.

APPENDICES

APPENDIX I

Details on some chemicals used

DNA loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol in TAE.

TAE: 0.04 M Tris acetate, 0.001 M EDTA in TDS water.

TNE: 25 mM Tris HCl, 100 mM NaCl, 5 mM EDTA, pH 8.0.

TE: 10 mM Tris HCl, 0.1 mM EDTA, pH 8.0 (autoclaved)

RNase: RNase A in 10 mM Tris HCl, pH 7.5, 15 mM NaCl (boiled for 15 minutes to destroy DNase)

20 x SSC: 175 g NaCl, 88.2 g sodium citrate in 1 litre distilled water, pH 7.0.

APPENDIX II

PCV for animals with *T.congolense* IL 1180 single infection (GROUP D)

DAYS POST INFECTION	ANIMAL NUMBER			MEAN	STAND. DEV.
	CJ519	CJ530	CJ575		
-3	29.0	24.0	24.0	25.7	2.9
0	30.0	26.0	27.0	27.7	2.1
2	30.0	26.0	25.0	27.0	2.6
3	28.0	25.0	23.0	25.3	2.5
4	26.0	25.0	22.0	24.3	2.1
5	27.0	23.0	22.0	24.0	2.6
7	30.0	27.0	22.0	26.3	4.0
8	28.0	24.0	22.0	24.7	3.1
10	22.0	21.0	18.0	20.3	2.1
11	20.0	19.0	16.0	18.3	2.1
12	20.0	19.0	18.0	19.0	1.0
14	20.0	19.0	16.0	18.3	2.1
15	22.0	20.0	18.0	20.0	2.0
17	25.0	21.0	19.0	21.7	3.1
18	21.0	19.0	17.0	19.0	2.0
19	23.0	19.0	18.0	20.0	2.6
21	24.0	19.0	18.0	20.3	3.2
23	24.0	18.0	19.0	20.3	3.2
25	25.0	18.0	20.0	21.0	3.6
28	23.0	19.0	16.0	19.3	3.5
29	24.0	18.0	18.0	20.0	3.5
30	24.0	20.0	21.0	21.7	2.1
32	21.0	21.0	21.0	21.0	0.0
35	22.0	21.0	25.0	22.7	2.1
37	23.0	22.0	18.0	21.0	2.6
39	18.0	22.0	20.0	20.0	2.0
42	18.0	23.0	19.0	20.0	2.6
44	20.0	24.0	20.0	21.3	2.3
46	16.0	21.0	18.0	18.3	2.5
49	18.0	21.0	17.0	18.7	2.1
51	20.0	23.0	17.0	20.0	3.0
53	19.0	21.0	15.0	18.3	3.1
54	19.0	21.0	16.0	18.7	2.5
56	20.0	21.0	17.0	19.3	2.1
57	24.0	22.0	19.0	21.7	2.5
58	22.0	22.0	17.0	20.3	2.9
60	24.0	23.0	16.0	21.0	4.4
63	20.0	21.0	15.0	18.7	3.2
65	21.0	22.0	17.0	20.0	2.6
67	19.0	24.0	16.0	19.7	4.0
70	21.0	24.0	17.0	20.7	3.5
72	20.0	21.0	16.0	19.0	2.6
74	20.0	21.0	16.0	19.0	2.6
77	19.0	23.0	16.0	19.3	3.5
79	22.0	22.0	17.0	20.3	2.9
81	20.0	22.0	18.0	20.0	2.0
84	17.0	22.0	17.0	18.7	2.9
86	21.0	26.0	18.0	21.7	4.0
88	23.0	23.0	18.0	21.3	2.9

APPENDIX II (continued)

91	24.0	22.0	21.0	22.3	1.5
93	23.0	25.0	17.0	21.7	4.2
95	20.0	23.0	18.0	20.3	2.5
98	18.0	21.0	15.0	18.0	3.0
100	21.0	23.0	18.0	20.7	2.5
102	25.0	23.0	18.0	22.0	3.6

STAND. DEV.- Standard Deviation

APPENDIX III

PCV for animals with *T. congolense* IL 3274 single infection (GROUP E)

DAYS POST INFECTION	ANIMAL NUMBER			MEAN	STD. DEV.
	CJ513	CJ518	CJ568		
-3	28	27	21	25	4
0	30	21	23	25	5
2	31	22	23	25	5
3	27	22	22	24	3
4	27	20	21	23	4
5	25	20	20	22	3
7	30	21	22	24	5
8	20	22	22	24	3
10	22	20	20	21	1
11	25	18	21	20	2
12	24	18	20	21	3
14	27	17	19	21	5
15	25	17	21	21	4
17	27	17	21	22	5
18	20	15	16	18	4
19	21	14	13	16	4
21	26	15	15	19	6
23	25	16	16	19	5
25	23	15	20	19	4
28	23	-	19	21	3
29	22	-	19	21	2
30	24	-	19	22	4
32	21	-	18	20	2
35	22	-	17	20	4
37	21	-	18	20	2
39	21	-	15	18	4
42	22	-	17	20	4
44	25	-	19	22	4
46	20	-	14	17	4
49	18	-	14	16	3
51	15	-	15	15	0
53	13	-	16	15	2
54	13	-	13	13	0
56	10	-	12	11	1

STD. DEV.- Standard Deviation
- = Removed from experiment

APPENDIX IV

PCV for animals with *T. congolense* IL 1180 & IL 3274 mixed infections (GROUP F)

DAYS POST INFECTION	ANIMAL NUMBER			MEAN	STAND. DEV.
	CJ514	CJ515	CJ565		
-3	24	27	25	25	2
0	24	30	27	27	3
2	23	31	30	28	4
3	21	28	28	26	4
4	21	26	26	24	3
5	21	26	26	24	3
7	20	26	25	24	3
8	17	23	24	21	4
10	14	20	16	17	3
11	15	17	17	16	1
12	14	16	16	15	1
14	14	16	15	15	1
15	13	19	18	17	3
17	13	20	17	17	4
18	13	18	17	16	3
19	13	17	16	15	2
21	12	19	18	16	4
23	14	22	21	19	4
25	13	21	21	18	5
28	13	19	17	16	4
29	13	20	19	17	4
30	13	22	18	17	5
32	11	22	17	17	6
35	-	22	19	21	2
37	-	21	18	20	2
39	-	18	19	19	1
42	-	18	20	19	1
44	-	21	22	22	1
46	-	17	19	18	1
49	-	18	20	19	1
51	-	19	20	20	1
53	-	18	22	20	3
54	-	18	19	19	1
56	-	19	17	18	1
57	-	21	21	21	0
58	-	19	19	19	0
60	-	20	19	20	1
63	-	16	16	16	0
65	-	21	16	19	4
67	-	18	18	18	0
70	-	18	17	18	1
72	-	21	17	19	3
74	-	21	18	20	2
77	-	21	19	20	1
79	-	19	20	20	1
81	-	20	21	21	1
84	-	19	20	20	1
86	-	20	22	21	1

APPENDIX IV (continued)

88	-	19	20	20	1
91	-	20	19	20	1
93	-	21	19	20	1
95	-	21	18	20	2
98	-	20	18	19	1
100	-	20	18	19	1
102	-	22	17	20	4

STAND. DEV.- Standard Deviation
- = Removed from experiment

APPENDIX V

Level of parasitaemia for *T. congolense* IL 1180 single infections (Group D)

DAYS POST INFECTION	ANIMAL NUMBER			MEAN	STD. DEV.
	CJ519	CJ530	CJ575		
0	0.00	0.00	0.00	0.00	0.00
2	0.05	0.00	0.00	0.02	0.03
3	0.00	0.05	0.20	0.08	0.10
4	0.05	0.00	1.00	0.35	0.56
5	0.30	0.05	1.00	0.45	0.49
7	0.20	5.00	0.20	1.80	2.77
8	5.00	0.50	1.00	2.17	2.47
10	1.00	4.00	1.00	2.00	1.73
11	1.00	10.00	0.15	3.72	5.46
12	0.25	3.00	0.35	1.20	1.56
14	0.25	1.00	0.15	0.47	0.46
15	0.05	0.15	0.00	0.07	0.08
17	0.05	0.25	0.05	0.12	0.12
18	0.15	0.15	0.05	0.12	0.06
19	0.10	0.05	0.20	0.12	0.08
21	0.00	0.05	0.20	0.08	0.10
23	0.00	0.15	0.00	0.05	0.09
25	1.00	1.00	4.00	2.00	1.73
28	0.00	0.10	0.00	0.03	0.06
29	0.10	0.10	0.00	0.07	0.06
30	0.10	0.15	0.00	0.08	0.08
32	0.20	0.50	1.00	0.57	0.40
35	0.00	0.00	0.10	0.03	0.06
37	0.10	0.00	0.00	0.03	0.06
39	0.00	5.00	0.00	1.67	2.89
42	0.00	0.25	0.00	0.08	0.14
44	0.15	0.00	0.00	0.05	0.09
46	0.00	3.00	0.35	1.12	1.64
49	0.25	1.00	0.50	0.58	0.38
51	1.00	0.50	20.00	7.17	11.12
53	0.00	0.05	0.00	0.02	0.03
54	0.00	1.00	0.05	0.35	0.56
56	0.00	0.00	0.00	0.00	0.00
57	0.00	0.00	0.00	0.00	0.00
58	0.15	0.05	0.10	0.10	0.05
60	0.05	0.05	50.00	16.70	28.84
63	0.30	0.00	2.00	0.77	1.08
65	2.00	1.00	0.15	1.05	0.93
67	0.00	0.00	0.15	0.05	0.09
70	0.10	0.15	0.00	0.08	0.08
72	0.15	0.00	0.00	0.05	0.09
74	0.10	0.00	0.05	0.05	0.05
77	0.00	0.00	0.10	0.03	0.06
79	0.00	1.00	0.20	0.40	0.53
81	0.05	0.00	0.00	0.02	0.03
84	0.05	3.00	0.10	1.05	1.69
86	0.05	0.10	0.00	0.05	0.05
88	0.00	4.00	0.05	1.35	2.30
91	0.15	1.00	1.00	0.72	0.49
93	0.05	1.00	0.10	0.38	0.53
95	0.05	0.05	5.00	1.70	2.86

Appendix V (continued)

98	0.00	0.00	0.25	0.08	0.14
100	0.00	0.00	0.00	0.00	0.00
102	0.00	0.00	0.05	0.02	0.03

* Trypanosomes per field (Magnification x250)

STD. DEV.- Standard Deviation

APPENDIX VI

Level of parasitaemia for *T. congolense* IL 3274 single infections (Group E)

DAYS POST INFECTION	ANIMAL NUMBER			MEAN	STD. DEV.
	CJ513	CJ518	CJ568		
0	0.00*	0.00	0.00	0.00	0.00
2	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00
7	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.00
11	0.05	0.00	0.00	0.02	0.03
12	0.10	0.00	0.00	0.03	0.06
14	30.00	0.00	0.00	10.00	17.32
15	30.00	0.00	0.10	10.03	17.29
17	0.50	0.00	30.00	10.17	17.18
18	50.00	0.00	30.00	26.67	25.17
19	6.00	0.00	10.00	5.33	5.03
21	50.00	0.00	20.00	23.33	25.17
23	1.00	0.00	5.00	2.00	2.65
25	30.00	0.20	30.00	20.07	17.21
28	50.00	-	15.00	32.50	24.75
29	15.00	-	30.00	22.50	10.61
30	0.30	-	30.00	15.15	21.00
32	4.00	-	30.00	17.00	18.38
35	20.00	-	30.00	25.00	7.07
37	0.50	-	10.00	5.25	6.72
39	100.00	-	7.00	53.50	65.76
42	50.00	-	2.00	26.00	33.94
44	10.00	-	10.00	10.00	0.00
46	0.15	-	2.00	1.08	1.31
49	1.00	-	5.00	3.00	2.83
51	0.40	-	10.00	5.20	6.79
53	30.00	-	70.00	0.00	28.28
54	0.00	-	10.00	5.00	7.07
56	3.00	-	50.00	6.50	33.23

* Trypanosomes per field (Magnification x250)

STD. DEV.- Standard Deviation
- = Animal removed from experiment
N.B.: None of the animals in this group survived beyond day 56 post-infection

APPENDIX VII

Level of parasitaemia for *T. congolense* IL 1180 and IL 3274 mixed infections (Group F)

DAYS POST INFECTION	ANIMAL NUMBER			MEAN	STD. DEV.
	CJ514	CJ575	CJ565		
0	0.00*	0.00	0.00	0.00	0.00
2	0.05	0.05	0.00	0.03	0.03
3	0.00	0.00	0.00	0.00	0.00
4	0.00	0.05	0.00	0.02	0.03
5	0.05	0.15	0.05	0.08	0.06
7	0.15	3.00	0.30	1.15	1.60
8	1.00	1.00	0.20	0.73	0.46
10	0.15	0.15	10.00	3.43	5.69
11	0.05	2.00	12.00	4.68	6.41
12	0.50	1.00	6.00	2.50	3.04
14	0.05	0.15	3.00	1.07	1.68
15	0.05	0.05	1.00	0.37	0.55
17	1.00	0.00	1.00	0.67	0.58
18	0.05	0.05	0.10	0.07	0.03
19	0.05	0.10	0.00	0.05	0.05
21	0.05	0.10	0.10	0.08	0.03
23	4.00	0.05	0.00	1.35	2.30
25	1.00	0.35	0.35	0.57	0.38
28	1.00	0.10	10.00	3.70	5.47
29	0.10	0.10	10.00	3.40	5.72
30	0.25	0.15	1.00	0.47	0.46
32	5.00	4.00	8.00	5.67	2.08
35	-	0.20	15.00	7.60	10.47
37	-	0.15	3.00	1.58	2.02
39	-	2.00	4.00	3.00	1.41
42	-	3.00	20.00	11.50	12.02
44	-	1.00	10.00	5.50	6.36
46	-	3.00	0.05	1.53	2.09
49	-	4.00	7.00	5.50	2.12
51	-	5.00	1.00	3.00	2.83
53	-	20.00	80.00	50.00	42.43
54	-	5.00	15.00	10.00	7.07
56	-	20.00	7.00	13.50	9.19
57	-	10.00	20.00	15.00	7.07
58	-	6.00	3.00	4.50	2.12
60	-	2.00	2.00	2.00	0.00
63	-	100.00	3.00	51.50	68.59
65	-	10.00	1.00	5.50	6.36
67	-	0.20	5.00	2.60	3.39
70	-	0.00	1.00	0.50	0.71
72	-	1.00	3.00	2.00	1.41
74	-	1.00	2.00	1.50	0.71
77	-	1.00	2.00	1.50	0.71
79	-	0.15	4.00	2.08	2.72
81	-	0.00	1.00	0.50	0.71
84	-	10.00	4.00	7.00	4.24
86	-	0.05	15.00	7.53	10.57

Appendix VII (continued)

88	-	0.25	50.00	25.13	35.18
91	-	0.10	0.50	0.30	0.28
93	-	0.00	6.00	3.00	4.24
95	-	0.00	0.00	0.00	0.00
98	-	8.00	1.00	4.50	4.95
100	-	0.05	10.00	5.03	7.04
102	-	0.05	20.00	10.03	14.11

* Trypanosomes per field (Magnification x250)

STD. DEV.- Standard Deviation
- = Animal removed from experiment