PREVALENCE OF *TRYPANOSOMA CONGOLENSE, T. VIVAX* AND *T. BRUCEI* INFECTIONS IN CATTLE IN PETAUKE DISTRICT OF ZAMBIA.

By

Clement Lubinga

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Declaration

I declare that the work presented in this dissertation was carried out by myself and has not been previously submitted to this or any other university.

Signature 

Dedication

I dedicate this work to my wife, Enala, *and sons*, Chabota and Javan, *for tolerating my absence from home, their encouragement and love.*

APPROVAL

This dissertation of Dr. Clement Lubinga is approved as fulfilling part of the requirements for the award of the degree of Master of Veterinary Medicine by the University of Zambia

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ABSTRACT

One hundred Trypanosomiasis infected cattle were selected from a sample of 240 cattle located in Petauke district using the haematocrit woo method. This gave an overall trypanosomiasis prevalence of 41.7% in the area. These selected animals were further examined by the stained thin blood smear(TBS), Antigen detecting ELISA(Ag-ELISA) and the Polymerase Chain Reaction(PCR) techniques. The actual infection rates were determined by pooling the results from the respective diagnostic techniques used for each individual animal. The overall Net infection rates were 96% *Trypanosoma congolense*, 81% *T. vivax* and 76% *T. b. brucei* giving an infection ratio of 1.3:1.1:1 respectively.

All the diagnostic techniques used have shown certain limitations in their ability to detect the three trypanosome species. Parasitological methods(Haematocrit and TBS) have shown higher sensitivity for *Trypanosoma congolense* than for *T. vivax* and *T. brucei* giving infection ratios of 29:3:1 by the Haematocrit and 4:2:1 by the TBS for *T. congolense*, *T. vivax* and *T. brucei* species respectively. Although, more mixed infections were detected by the Ag-ELISA and the PCR techniques, their sensitivity for detecting the trypanosome species, especially *T. vivax* and *T. b. brucei* was lower than was anticipated. Both Ag-ELISA and the PCR methods gave infection ratios of about 2:1:1 for *T. congolense*, *T. vivax* and *T. brucei* respectively.

Statistical analysis (ANOVA) of the results showed no significant differences in the established statistical means of the Net infection rates between the three trypanosome species. These results also indicate a high rate of mixed infections of *T. congolense, T. vivax* and *T. b. brucei* in cattle suffering from trypanosomiasis in Petauke and the location of sampling(crush pen) did not show any statistical effect on the results.

It is clear that the apparent predominance of *Trypanosoma congolense* reported in Zambia could be a result of the low sensitivity of the routine parasitological methods in use particularly, in detection of *T. vivax* and *T. brucei.* A combination of the parasitological examination with Ag- ELISA and/or PCR in the epidemiological surveys of trypanosome species is therefore highly recommended. To improve on the diagnostic performance of the Ag-ELISA and the PCR, it is also recommended that attempts to prepare monoclonal antibodies and primer sets using the local strains should be considered in order to avoid false negative results which may be originating from some possible regional variations in the trypanosome species.

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Chapter one

1.0 INTRODUCTION

1.1 Trypanosomes and trypanosomiasis

Trypanosomes are protozoan parasites which belong to the order Kinetoplastida in the family Trypanosomatidae. Important in Africa, are salivarian trypanosomes which belong to the Subgenera Nannomonas (*congolense*), Trypanozoon (*brucei*), Duttonella (*vivax*) and Pycnomonas(*suis*) (Hoare, 1964; Kirchoff and Donelson, 1993).

Trypanosomes are digenic flagellates whose life cycle alternates between two hosts; (i) the invertebrate host, representing the vector and, (ii) the mammalian host. The main vectors of salivarian trypanosomes are tsetse flies of the genus *Glossina* in which trypanosomes undergo cyclic development (Hoare, 1970a; Luckins, 1992). Mechanical transmission by other haemophagous flies such as the Tabanids and *Stomoxys*(Stable flies) has also been indicated but this is considered to play a minor role in the transmission of trypanosomes in Sub Saharan Africa (Jahnke, 1975; Wells, 1984)

Most wild animals are the typical mammalian hosts of these trypanosomes but they do not seem to suffer their pathogenic effects. This sylvatic cycle between wild animals and tsetse flies has existed for a long period. The problem occurs when domestic animals intrude into this tsetsefly - wild animal cycle as they become hosts to trypanosomes whose pathogenic effects they are susceptible to and develop the disease called trypanosomiasis (Jordan, 1986). Trypanosomiasis in tropical Africa is of great social and economic importance

(Jahnke, 1975). In cattle, the disease is characterised by superficial lymph node enlargement, anaemia and generalised loss of condition.

1.2 Trypanosomiasis in Zambia

The most important trypanosomes affecting cattle in Zambia and in many other tropical African countries are *Trypanosoma congolense*, *T. vivax* and *T. b. brucei* (Hoare, 1970a; Chizyuka, 1980; Chitambo and Arakawa, 1991). In Zambia trypanosomiasis is ranked as one of the most economically important diseases affecting livestock production. Over a third of the total habitable land of Zambia is tsetse infested (Mumba and Chizyuka, 1987) and over 60% of the Zambian cattle are reportedly exposed to trypanosomiasis (Akafekwa and Awan 1975). The main tsetse fly species which act as vectors in Zambia are *Glossina morsitans subspecies, G. pallidipes, G. brevipalpis* and *G. fuscipes* in that descending order of importance(Clarke, 1969)

The reported prevalence of trypanosomiasis in cattle in Zambia and elsewhere in the sub-Saharan Africa, show higher rates for *Trypanosoma congelense,* followed by *T. vivax* and *T. b. brucei* (Akafekwa and Awan, 1975; Awan and Sawchuck, 1976; Ford, 1964; Keymer, 1969, DVTCS, 1988-92). In contrast, the reported prevalence of tsetsefly infection show higher rates for *Trypanosoma vivax*, intermediate for *T. congolense* and least for *T. b. brucei* (Clarke, 1969; Keymer, 1969; Okimelu, 1977; RTTCP, 93).

1.3 Diagnosis of Trypanosomiasis in Zambia.

In Zambia, the main laboratory diagnostic tests for the detection of trypanosomes in animals are the conventional parasitological examinations using ordinary light microscopy

of blood samples collected and processed as wet or stained thin and/or thick dry smears. Other parasitological methods such as the haematocrit concentration (Woo method) are widely used in surveys by the Regional Tsetse and Trypanosomiasis Control programme whilst the mouse inoculation are only used to a limited extent due to logistic limitations.

Parasitological diagnosis of trypanosome species, by thin blood smears, is determined by studying the morphology of stained bloodstream forms which show characteristics upon which trypanosome species can be confirmed (Hoare, 1970a). The main differential features used are: a) the general body size and shape, b) the shape of the posterior end, c) the kinetoplast size and position, d) the nucleus size, shape and position, e) the development of the undulating membrane (where a well developed undulating membrane is convoluted and conspicuous while a poorly developed one is flattened and inconspicuous) and f) the absence or presence and length of the free flagella(Hoare, 1970a). The absence or presence of cytoplasmic vacuoles and granules and their location in the body can be another useful characteristic for identification of trypanosome species(Stephen, 1986). The parasitological methods are generally associated with problems of low and varying sensitivities (Ashkar and Ochilo, 1972; Masake and Nantulya, 1990). This diagnostic approach often fails to detect the trypanosomes when the parasitaemia is too low such as during early / or chronic infections.

There are other diagnostic techniques for trypanosomiasis such as the serological tests which include the Complement Fixation Tests (CFT), Immunofluorescense Antibody test (IFAT) and the Antibody detecting Enzyme Linked Immuno Sorbent Assay (Ab-ELISA).

These serological assays are indirect diagnostic techniques as they detect the host anti trypanosome antibodies in circulation. They are of higher sensitivity but of low specificity due to cross reactions between different trypanosome species (Ashkar and Ochilo, 1972; Luckins, 1993; Wilson, 1969). These serological assays are mainly used in Zambia for trypanosomiasis surveys but not in routine diagnosis because of logistic limitations.

More advanced and direct *in vitro* methods for detection and identification of trypanosome species are being introduced in Zambia. These include the Antigen detecting ELISA(Ag-ELISA), which is already undergoing extensive field validation trials at the Central Veterinary Research Institute, and the nucleic acid based Polymerase Chain Reaction (PCR), which was used for the first time in this study.

The Monoclonal Antibody based antigen detecting ELISA (Ag-ELISA) test detects invariant species specific antigens on surface coat of trypanosomes (Jongejan *et al*, 1988; Luckins 1993). It is reported that Ag-ELISA has both high sensitivity and specificity and can detect trypanosomes within 14 days of infection and about 14 days after treatment (Nantulya and Lindvist, 1989). Ag- ELISA has had no cross reactions reported but false negative results may be observed during the early stages of infection, possibly when the blood Ag levels are below the detection limits of the assay(Nantulya and Lindqvist, 1989). Recent developments in nucleic acid isolation, separation and amplification (Swaminathan and Mataar 1993) offers yet another attractive approach in the specific species detection of trypanosomes(Majiwa and Webster, 1987). The most popular of these techniques is the

Polymerase Chain Reaction (PCR), an *in vitro* target amplification system for enzymatic replication of a target DNA molecule to levels at which it can be readily detected (Iniss and Gelfand, 1990; Saiki, 1990; Mullis and Faloona, 1987). The PCR principle is based on the identification and inclusion of a specific nucleic acid sequence (Primer) that can serve as a faithful surrogate for the identification of the whole organism (Persing, 1993a). The running protocol involves repeated cycles of high heat denaturation of the target DNA, annealing of the primers to their complementary sequences on the target DNA strands and the extension of the annealed primers with DNA Polymerase (Saiki 1990, Persing, 1993a). The PCR is highly sensitive that a even single DNA molecule can be amplified to produce several copies of genes which can then be visualised as a distinct band on agarose gel (Innis and Gelfand, 1990). The PCR is also reported to be so specific that it can differentiate between the morphologically similar *Trypanosoma congolense* and *T. simiae*(Moser *et al,* 1989).

1.4 Research hypothesis

Reports on trypanosomiasis epidemiology in Zambia indicate higher *Trypanosoma vivax* infection rates in tsetse flies where as *T. congolense* infection rates are higher in cattle reared in the same area and infected under natural challenge. Given that tsetse flies are the main vectors of trypanosomes to cattle and that mechanical transmission is negligible (Jordan, 1986; Wells, 1984), it is surprising how *T. congolense* should be predominant in cattle. Does *Trypanosoma congolense* suppress the establishment of *T. vivax* and *T. b. brucei*(Clarke, 1969; Ford, 1964) or do all the three trypanosome species actually co-exist

in cattle but are simply not being detected because of the limitations in the diagnostic methods which are routinely used to detect trypanosomes(Losos, 1986; Stephen, 1986)?

1.5 Research objective

The main objective in this study was an attempt to establish the actual infection rates of *Trypanosoma congolense, T. vivax* and *T. b. brucei* in naturally infected cattle in areas of Petauke district of Zambia. Since most diagnostic methods have certain limitations in their abilities to detect the trypanosome species, several methods were employed in order to arrive at the actual infection rates of the trypanosome species infecting a particular host (Luckins, 1993). The actual infection rates were determined by combining results from the Parasitological examinations and those obtained from Ag- ELISA and the PCR diagnostic techniques for each respective animal. These results were statistically compared using Analysis of Variance (ANOVA) in order to test whether or not there was any significant difference in the distribution of *T. congolense, T. vivax* and *T. b. brucei* in cattle under natural challenge.

Chapter Two

2.0 LITERATURE REVIEW

2.1 Historical review

African animal trypanosomiasis is not a new disease. It has been reported by early explorers and missionaries such as David Livingstone (1857) who identified the disease with respect to the tsetse fly bite. However, according to Stephen's review(1986) investigations on trypanosomes and trypanosomiasis date back to the 17th century, following the invention of the light microscope by Anthony Van Leuwen Hock. Among the notable contributors he cited are Gruby (1843) who provided the genus name *Trypanosoma,* Timothy Lewis (1878) who reported the presence of trypanosomes in rats in India, which were named *Trypanosoma lewis* and Anthony Evans (1880) who showed that trypanosomes were pathogenic when he found trypanosomes (*T. evansi)* in horses dying of Surra disease in India.

In Africa, Bruce (1895) discovered that trypanosomes, similar to *T. evansi,* were responsible for the fatal disease of cattle (Nagana), in Natal province of South Africa. He later indicated that tsetse flies played a role in the transmission of the disease when he demonstrated identical trypanosomes in cattle suffering from Livingstone's 'Tsetse fly disease.' Later Klein (1909) showed that trypanosomes were actually transmitted cyclically by tsetseflies.

In Zambia, trypanosomiasis, locally known as Kaodzera, meaning to nod(Ngoni) and Luuka by name of the fly(Tonga) (Chizyuka, 1980) was first investigated by Montgomery and

Kinghorn(1908). The disease gave problems to the early European settlers and has continued to be to the Department of Veterinary and Tsetse control Services (Shaw, 1960; Chizyuka, 1980).

2.2 Scientific review

2.2.1 Trypanosome anatomy and immunology

Generally, the mammalian trypanosomes are elongated and slightly curved flagellate organisms. They have one nucleus in the centre and a kinetoplast at the posterior end. An axoneme arises from the basal body located in the posterior end and runs anteriorly along the body margin forming an undulating membrane. The axoneme terminates anteriorly either as an attached or free flagellum (Jordan, 1986).

Bloodstream forms of trypanosomes possess variant antigens on their surface coat (Vickerman, 1969). These enable trypanosomes to evade the immune response of the host by producing relapse strains that are antigenically different (antigenic variation), a phenomenon that results in successive parasitaemic waves (Doyle, 1977; Vickerman and Tertley, 1978). Each parasitaemic peak of the wave indicates the multiplication of trypanosomes of a particular population with a different surface antigen from that of the previous wave (Vickerman and Tertley, 1978; Lumsden, 1972). Parasitaemic remission indicates the destruction of the respective trypanosome population (Vickerman *et al*, 1976). This phenomenon can be explained by the sequential expression of cell surface glycoproteins (Cross, 1978) leading to the expression of alternative antigens to produce a particular

variable antigenic type (VAT). The number of alternative antigens which can be expressed has not been determined (Williams, 1978).

2.2.2 Trypanosomiasis epidemiology

All *Glossina species* where ever they occur are almost invariably infected with trypanosomes which cause African animal trypanosomiasis (Jordan, 1986). Clarke (1969) reported that all *Glossina species* except *G. fuscipes* are vectors for African trypanosomes but the highest infection rates are achieved in *G morsitans subspecies*.

Glossina morsitans are more predominant than any other species of tsetse flies in Zambia. Their highest prevalence is reported in the Eastern province especially in areas around Petauke district and the Luangwa valley. Other areas infested by these tsetse flies include the Kafue National park, extending downwards to Kalomo and upwards to the North-Western, Copperbelt and Central provinces. Isolated pockets of *G. morsitans* are present in the Western, Northern, Lusaka and Luapula provinces. *Glossina Pallidipes* are present in the Eastern province, Gwembe valley and the Kafue national park (Clarke, 1969; DVTCS, 1988). *G brevipalpis* are found in the Eastern, Lusaka and Luapula provinces while, *G .fuscipes* are found mainly in Luapula province especially around lake Tanganyika.

In Zambia, *T vivax* is reported to be the most predominant trypanosome species in tsetse flies followed by *T congolense* and *T. b. brucei* respectively (Keymer 1969 and RTTCP, 1993). Clarke (1969) found that *T. vivax* infection rates in tsetse flies were five times more than that observed for *T congolense* while cattle in the same area exhibited more *T*

congolense infection rates than *T. vivax* or *T. b. brucei.* Field surveys in cattle from all over Zambia indicate that *T. congolense* is a more predominant trypanosome species. For instance, infection rates of cattle in Namwala district were as follows: *T. congolense,* 72.3 %, *T. vivax*, 18.6 % and *T. b brucei* 9.1 % (Awan and Sawchuck, 1975). Similar surveys conducted in Petauke district, gave the following results; *T. congolense* 87.5 % and *T. vivax* 12.5 % (DVTCS, 1988). In Katete district *T congolense* was reported to be 85 % over *T. viv*ax with 15 % (DVTCS, 1988). However, mixed infection with any of the three *Trypanosome species* is not uncommon in the field. Surveys conducted in Kabwe indicated infection rates of 77.5 % for *T. congolense,* 8.5 % for *T. vivax,* 3.7 % for *T. b. brucei* while 10.3% were mixed infections (Njobvu, 1989). Monthly reports compiled by DVTCS (July, 1988) in Petauke district had the following rates; *T. congolense* 85.6 %, *T. vivax* 9 %, *T. b. brucei* 4.1 % and 1.3 % mixed infections. Similar reports from Katete district showed the following rates; *T. congolense* 85.8 %, *T. vivax* 8.32 %, *T. b. brucei* 4.6 % and 1.3 % mixed infections. In the Western Province, *T congolense* was 46 %, *T. vivax* 34.4 %, *T. b. brucei* 8.0 % and 15.6 % were mixed infections. The above results are in conformity with observations made by Ford and Leggate (1961) who noted that in spite of higher *T. vivax* infection rates in the fly, cattle were more frequently infected with *T. congolense.*

Transmission of trypanosomiasis is mainly cyclical through tsetse fly bites. The establishment of the trypanosome from a mammalian form to a tsetse fly form involves its metabolic and environmental adaptation(Stephen, 1986). This ability in the trypanosome metabolism switch is invested in its kinetoplast, which contains the mitochondria, whose

inner invaginations, the cristae, increase in number when trypanosomes are in the fly to permit for the synthesis of ATP which provides for energy (Vickerman, 1971; Bowman *et al.,* 1972; Brown *et al*, 1973).

Infection in the tsetse fly is affected by various factors, which include the complexity of the developmental cycle in the tsetse fly. For instance, *T. vivax* develops only in the proboscis (Losos, 1986; Tertley et al, 1981; Vickerman, 1969), *T. congolense*, develops in the proboscis and mid-gut (Jordan, 1976; Okimelu, 1977), while *T. b. brucei* develops in the proboscis, mid-gut, and salivary glands (Moloo, *et al*, 1991 and Jordan, 1986). Surveys on naturally infected wild tsetse flies indicate that *T. vivax* tends to achieve higher infection rates than *T. congolense* and *T. b. brucei* probably because of its direct development cycle in the tsetse fly (Moloo *et al,* 1991; Stephen, 1986; Clarke, 1969; Harley and Wilson, 1968). For *T. b. brucei* and, to some extent, *T. congolense*, tsetse fly infection can only take place at certain developmental phase of the trypanosomes. For example, it is reported that in *T. b. brucei* infections, only the stumpy forms of *T. b. brucei* can be established and mature in the tsetse fly (Stephen, 1986).

The species, sex and age of the tsetse flies can also influence their susceptibility to infection by trypanosomes. For instance, *G. morsitans* attains higher infection rates than *G. brevipalpis, G. pallidipes* and *G. fuscipes* respectively (Moloo *et al*, 1991; Clarke, 1969) while, male flies tend to achieve higher infection rates than female flies (Moloo *et al.,* 1991; Clarke, 1969). Generally, tsetse flies are readily infected when they feed as teneral

flies (Stephen, 1986). The age of a fly at its first feeding on an infected blood is, therefore, an important factor for establishment of trypanosome infection in tsetse flies especially so for *T. b. brucei.* The peritrophic membrane in the teneral tsetse fly gut lining is readily penetrated by trypanosomes and this permeability declines with age (Jordan, 1976; Stephen, 1986). However, the frequency of feeding by tsetse flies can also contribute to the variations in tsetse infectivity. For example, a high feeding frequency can lead to higher infectivity by *T. vivax* but not for *T. congolense* and quiet insignificant for *T. b. brucei* (Stephen, 1986). Normally, infection by *T. congolense* and *T. b. brucei* in tsetse flies mainly occurs when the fly takes its first feed on infected blood (Vale, 1976; Stephen, 1986). Therefore, a tsetse population with a higher average age group are more likely to have higher trypanosome infection rates with all species than a population with young average age group which will tend to have more *T. vivax* infection (Harley, 1971). On the other hand tsetseflies with feeding preferences to mammals which are refractile to trypanosome infection tend to have lower infection rates (Stephen, 1986).

Mechanical transmission by Tabanids and other haemophagous diptera flies, which is more efficient for *T. vivax* transmission, has been indicated in non-tsetse fly infested areas (Roeder *et al*, 1984) as evidenced by the presence of *T. vivax* in non-tsetse infested habitants of S. America (Losos, 1986). This mode of transmission may attribute to the observed predominance of *T. vivax* in the marginal areas of the tsetse fly zones (Ford, 1964; Losos, 1986). However, mechanical transmission is difficult to study and much of the evidence for its occurrence is circumstantial (Jordan, 1986). One example of the

circumstantial evidence of mechanical transmission is the change in predominance to *T. vivax* from *T. congolense* in infected cattle as distance increases from the centre of tsetse fly zone towards the marginal areas, a phenomenon known as the vivax ratio (Ford, 1964). In some localities, however, the vivax ratio may also be attributed to a lowering in the mean age of the tsetse populations near the limits of the tsetse fly zones, because the flies are under stress, which, in turn, precludes the development of *T. congolense* and *T. b. brucei* in the flies and, thus, allowing only the quick maturing *T. vivax* to establish (Rogers *et al*, 1984).

Other factors which may lead to the presence of trypanosomiasis in apparently non-tsetse infested areas include; 1) presence of very low population of the tsetse flies which are otherwise difficult to detect (especially those not easily attracted to man); 2) tsetse flies which are attracted to moving objects (man, vehicles, animals etc.) can reach non-tsetse infested areas where they feed on and infect domestic animals before they die; 3) undetected movements of infected live stock from infested areas to tsetse free areas which may manifest the disease, etc. (Wells, 1972). Therefore, while mechanical transmission may play a role in spreading trypanosomiasis, especially in the abundance of the biting flies, it does not seem capable of maintaining the normally tsetse transmitted trypanosomes in the absence of the *Glossina species*. For instance trypanosomiasis was eliminated in areas of Northern Nigeria following tsetse fly eradication (Putt et al, 1980). It may be concluded, therefore that mechanical transmission of trypanosomes by diptera flies is insignificant in tsetse infested areas although it favours *T. vivax* transmission (Wells, 1984).

Cyclical transmission is, therefore, the major source of bovine trypanosomiasis infection in Africa (Jahnke, 1975). The infection rates observed in the bovine host will depend on many factors such as; 1) the vector infection rates, 2) the vector ability to transmit trypanosomes to their respective host and 3) the subsequent ability of these trypanosomes to establish themselves in that host. The incidence and infection rates of trypanosomes in bovine will also depend on the prevailing climatic conditions and the availability of the hosts to feed on, which in turn determines how long the tsetse fly population can survive in a given area. Tsetse flies become cyclically infective after the procyclic trypanosomes complete their development cycle and transform into mature metacyclics that are infective for bovines (Stephen, 1986).

2.2.3 Comparative pathogenesis

a) Prepatent period

Infection with pathogenic African animal trypanosomes is initiated by the deposition of the metacyclic stages into the subcutaneous tissues of the mammal (Vickerman and Tetley, 1978; Losos, 1978; Stephen, 1986). Trypanosomes then multiply locally in the connective tissues to form a chancre - like lesion (Losos, 1978; Stephen, 1986), which is not prominent in cattle (Luckins and Gray, 1978).

From the chancre, the trypanosomes enter the blood stream either directly or via the lymphantics establishing parasitaemia, which disseminates the trypanosomes to the rest of the circulatory system and, in some species, to various solid organs (Losos, 1978). This period between the introduction of infective metacyclics and the appearance of parasitaemia is called the prepatent period (Stephen, 1986).

The prepatent period is affected by many factors which may relate to;

1) host factors such as innate resistance, antibody levels, nutritional state, age, sex, intercurrent infections, hormonal levels, etc.

2) vector fly factors such as the ability to probe the mouth parts deeply and extensively, suitability of the saliva to support cyclical development, density of metacyclics in the saliva and presence of mixed infections, and

3) the trypanosome factors, such as the species, strain, possible residence in abnormal host etc.(Stephen, 1986).

Trypanosoma vivax metacyclic forms initially develop at the site of the bite before entering the regional lymph nodes and the central lymph stream to finally appear in the peripheral blood (Emery *et al*, 1980; Emery and Moloo, 1981). The duration of the prepatent period is not well documented but in cattle it is estimated at 8- 17 days (Stephen, 1986).

T. congolense multiplies extensively at the site of the bite(Luckins and Gray, 1978, Gray and Luckins, 1979). The trypanosomes have been detected in the local lymph nodes before appearing in the peripheral blood (Gray and Luckins, 1980) suggesting involvement of lymphatic drainage in the dissemination of *T. congolense* from the chancre. The prepatent period of 13 days has been noted in *T. congolense* infections (Gray and Luckins, 1980).

During *T. brucei* development in the chancre, metacyclics transform from short stumpy forms into long slender forms which invade the local lymphatic vessels and then multiply in the blood vessels. From the blood vessels, they may invade the connective tissues (Vickerman and Tetley, 1978).

b) Tissue tropism

Losos and Ikede (1972) described two groups of animal Trypanosomiasis, the haematic and the humoral groups. The haematic group, comprising *T. congolense* and *T. vivax* are mainly confined to the plasma of the blood vessels (Losos, 1978) where as the humoral group of the Trypanozoon (*T. b. brucei*) are mainly found in the extravascular tissues such as loose connective tissues and body cavity fluids.

T. congolense, is strictly a 'plasma parasite' (Banks, 1978; Ssenyonga and Adam, 1975) while *Trypanosoma vivax* is occasionally found outside the blood vessels suggesting that it has some humoral group properties (Stephen, 1986). The continuous presence of *T. vivax* in the lymph nodes after resolution of the chancre may suggest that the trypanosomes leave the bloodstream and are re-cycled through the lymphatic system or that there is continued proliferation of trypanosomes within the lymph nodes (Stephen, 1986). However, more *T. vivax* trypanosomes tend to be found more in the blood vessels than in the connective tissues (Losos, 1978).

c) Parasitaemia.

T. b. brucei infections in cattle tend to produce lower parasitaemia than *T. congolense* or *T. vivax.* and are characterised by periodic absence of trypanosomes in the circulating blood. Another typical feature of *T. brucei* parasitaemias is the transformation from dividing long slender forms towards the non-dividing short stumpy forms during the successive parasitaemic peaks (Robertson, 1912; Vickerman and Tetley, 1978). The short stumpy

forms, unlike their progenator long forms, are less susceptible to the host immune response and are reported to be only infective to the tsetse fly (Vickerman and Tetley, 1978; Vickerman, 1971; Doyle *et al*, 1978). The few slender forms which survive the host immune responses are regarded to continue with the replication leading to the characteristic chronic course of infections. It is suggested that even in *T. congolense* and *T. vivax* infections, pleomorphism of distinct dividing and non-dividing forms may be present (Nantulya, *et al,* 1978).

2.2.4. Diagnostic methods

A) Parasitological Examination.

1) Direct methods

a) Wet blood film examination WBF);

Trypanosomes are motile organisms(Soulsby, 1982) and by carefully studying their movements, an educated guess can be made of the trypanosome species involved (Stephen, 1986). The following criteria is often used in wet films to determine the trypanosome species:

Trypanosoma vivax in fresh blood is very motile, it is seen moving rapidly across the field in a straight line, pushing the red blood cells aside as it goes (Urquhart *et al*, 1987).

T. b. brucei movements are wriggling or snake like, moving rapidly only within small areas of the microscopic field (Soulsby, 1982; Urquhart *et al*, 1987). The organism characteristically makes a cyclic path in its movements.

On the other hand, *T. congolense* makes active but non-progressive movements, and is often seen to be apparently attached the red blood cells.

Wet blood film examination is quick and, therefore, convenient for screening a large number of cattle in the field (Nantulya, 1990).

b) Stained blood smear examination.

Confirmatory parasitological diagnosis of trypanosomes is achieved by studying the morphology of stained bloodstream forms which gives distinct criteria upon which *Trypanosome species* can be determined (Hoare, 1970a; Masake and Nantulya, 1990; Nantulya et al, 1987).

Trypanosoma vivax is club shaped, typically swollen with a rounded posterior end. It is about 20-31 μm long and about 3.7μm at its widest part posterior to the nucleus from which it tapers anteriorly in a rather regular fashion. The kinetoplast is characteristically large and terminal while the undulating membrane is inconspicuous and terminates into a free flagellum measuring about 3.6 μm long (Hoare, 1970b; Stephen, 1986). The nucleus is elongate oval , approximately 3.7μm long and 2.5 μm wide and occupies nearly the whole transverse diameter at its location (Stephen, 1986). The cytoplasm stains homogeneously but vacuoles and granules are seldomly seen. There is no evidence of dimorphism in *T. vivax* (Stephen, 1986).

T. congolense is a smaller trypanosome about 8-18 μm long, with a posterior end which is either tapered or blunt (Urquhart *et al*, 1987). Its body tapers uniformly from the posterior end. The undulating membrane is not well developed and the free flagellum is either absent or short while the kinetoplast is medium sized and marginal. The nucleus is round and centrally placed (Hoare, 1970b; Stephen, 1986).

T. b. brucei exhibits polymorphism with three major forms; The long and slender form has a long and thin curved body, more than 25 μm long with a long free flagellum about 6 μm long ((Hoare, 1970b; Stephen, 1986). The undulating membrane is conspicuous with 3 prominent loops. The posterior end is narrow and pointed whilst the kinetoplast is small and subterminally located. The nucleus is elongated. The short and stumpy form, usually 17-22 μm long and 3.5 μm at its widest part has broad and squat body with a rigid appearance. The nucleus is round and oval while the kinetoplast is usually small and subterminal. The undulating membrane is prominent with the free flagellum either absent or short. The intermediate form of *T. b. brucei* has characteristics which lie between the two forms mentioned above. The posterior end tends to be more blunt than the long forms, they posses a free flagellum and a moderately well developed undulating membrane.

2) Concentration techniques

This approach increases the sensitivity of the direct examination by concentrating trypanosomes through centrifugation (Paris et al, 1982). In the Hematocrit Centrifugation Technique (HCT), whole blood is spun in a haematocrit centrifuge to concentrate the trypanosomes at the buffy coat layer (Nantulya, 1990). This method, initially used to detect avian trypanosomes has undergone wide modifications and applications (Woo, 1969; Murray et al, 1977; Kelly and Schillinger, 1983) and it has an added advantage of determining anaemia by measuring the Packed Cell Volume (PCV). Field application of this method is limited because of its requirement for electricity and centrifuges which are normally not available in most diagnostic laboratories in Zambia. The miniature Anion-

Exchange Centrifuge Technique (mAECT) increases the sensitivity by separating the trypanosomes from blood cells by passing them through DEAE cellulose before concentrating them by centrifugation for examination (Stephen, 1986). This method is also not used for routine diagnosis in Zambia.

3) Sub inoculation

Suspected blood is inoculated into susceptible laboratory rodents (mice or rats), which are later examined by demonstrating the presence of trypanosomes using the direct method (Paris et al, 1983). This method is considered to be very sensitive and the trypanosome isolates can be collected for other uses in the laboratory (Nantulya, 1990).

However, this method may be limited in that diagnosis is not immediate and the cost of maintaining laboratory animals is prohibitively expensive for routine field diagnosis. Further more, *T. vivax* does not readily infect the laboratory rodents (Masake and Nantulya, 1990; Nantulya, 1990).

Factors affecting parasitological examination

Parasitological examination is based on the demonstration of the parasite(Hoare, 1970a; Luckins, 1993) which, in turn, will depend on factors which may relate to i) parasitaemia, ii) collection and handling of the specimens and iii) the examination technique.

i) Parasitaemia; Antigenic variation in trypanosomes (Vickerman, 1969) leads to intermittent parasitaemia which can affect parasitological examination. For instance, during the peak parasitaemic phases the parasites are readily detected while during the remission phases only a few or no parasites may be detected (Masake and Nantulya, 1990; Nantulya, 1990). The tissue tropism of the trypanosomes (Losos and Ikede, 1972) can also affect

parasitological examination in that the haematic group (*T. congolense* and *T. vivax*) are readily detected in the peripheral blood than the humoral group (*T. b. brucei*) which invades mainly the extravascular tissues and body cavity fluids (Losos and Ikede, 1978). *T. b. brucei* is, therefore, not easily detected in the peripheral blood even when it may actually be abundant in the internal organs (Masake and Nantulya, 1990).

The course of infection also has an effect on the parasitaemia. No parasites are detected during the prepatent period while the initial peak of the parasitaemic wave is normally very high especially for the haematic group. In succeeding waves, the peaks become more reduced such that in chronic infections, the peaks are very much reduced (Murray and Morrison, 1978). On the other hand, the humoral group, become more parasitaemic in the terminal stages of infection (Soulsby, 1982).

The time of sampling, may affect the availability of the trypanosomes in the peripheral blood vessels probably due to the changes in the ambient temperature (Stephen, 1986). Trypanosomes are readily detected in the morning sampling hours than later in the day. The physical activity subjected to the animal prior to the sampling may also affect the outcome of parasitological examination in that samples from animals which are excited before the sampling are likely to give false negative results (Stephen, 1986).

ii) Collection and handling of specimens;

The type of sample collected can affect the out come of a parasitological examination. For example, *Trypanosoma vivax* is more readily detectable from lymph node biopsy smears (Soulsby, 1982), whilst *T. congolense* is easily found in the peripheral blood. *T. b. brucei* is

readily detected from the body cavity fluids (e.g. Cerebral spinal fluid), a technique which is rarely used in cattle due to the obvious limitations (Baker, 1970).

Success in wet smear examination, relies on the detection of motile organisms (Baker, 1970). This ability is reduced by factors that are detrimental to the survival of trypanosomes such as heat, long exposure to sunlight, drying and prolonged storage period of the sample before examination. Reduced motility of the trypanosomes can lead to a false identification whilst death of the trypanosomes will lead to a false negative result.

In thin smear preparations, it is important to ensure a thin spread of the blood film. Very thick smears may become too dense for light to pass through making it difficulty to appreciate the differential morphological features of the trypanosomes. Stained blood smears may also be adversely affected by delays in fixing and inadequate staining procedures.

iii) Examination techniques

The experience of the examiner is very important in the identification of trypanosome species. In wet smear preparations, the examiner should be able to differentiate between the agitation of red blood cells caused by trypanosome movement from that caused by the Brownian movement. It is also important to differentiate pathogenic trypanosomes from non-pathogenic *T. theileri* and other extra cellular blood parasites (e.g. Microfilaria worms). The examiner should further appreciate the different movement patterns of the trypanosomes in order to give a more probable identification (Stephen, 1986).

When examining thin blood smears, the examiner should appreciate the differential morphological features of the different *Trypanosome species*. The sensitivity of the

diagnostic technique also affects parasitological examination. Concentration techniques, for example, increase the number of trypanosomes in a microscopic field (Nantulya, 1990).

B) ANTIBODY DETECTION

Limitations in parasitological diagnostic techniques have led to research in alternative methods to indirectly detect trypanosome infections such as the serological techniques (Luckins, 1977). The serological methods detect host anti-trypanosome antibodies (Nantulya, 1990).

There are two types of serological assays; a) the primary and b) the secondary binding assays (Tizard, 1991).

a) The primary binding assays measure the immune complexes resulting from the direct binding of the trypanosome antigen to the host immunoglobulins (Tizard, 1991).

The Indirect Fluorescence Antibody Test (IFAT) is one example of primary binding assays but this assay has a problem of standardisation of the antigen which has since been reduced by improvement in the antigen preparation (Katende *et al*, 1987). IFAT can, to some extent, differentiate trypanosomes at species level (Nantulya, 1990). However, IFAT is limited by its requirement of sophisticated and expensive equipment which precludes it from use in the routine diagnosis in the field (Luckins, 1977).

The microplate Antibody detecting Enzyme Linked Immuno Sorbent Assay (Ab ELISA), is another example. It is relatively simple and straightforward and it is as sensitive as IFAT(Masake and Nantulya, 1990). ELISA is ideal for large scale screening of cattle because a lot of samples can be run on one plate (Luckins, 1977; Nantulya, 1990).

Ab-ELISA has limitations in that the antigen used is a crude trypanosome lysate which is not well defined and, therefore, makes it difficult to standardise its sensitivity and specificity (Nantulya, 1990). The improvement in the antigen preparation (Ijagbone *et al*, 1989) has improved the specificity of the assay.

b) The secondary binding assays measure the secondary reaction consequent upon the initial binding of the trypanosome antigen to the host anti trypanosome immunogloblin such as the activation of the classical complement path way in the Complement Fixation Test (Tizard, 1991). The antigen used in these tests is not well defined and, therefore, makes it difficult to standardise the test with regard to the specificity and sensitivity. Furthermore, such assays can not differentiate the infecting trypanosome species (Nantulya, 1990).

Since antibody based serological assays measure the antibody response to trypanosome infection, whose levels persist even after treatment, they can not differentiate active infections from the cured ones, giving more of a presumptive diagnosis than a confirmatory one. Also they have a poor ability to differentiate the trypanosome at species level. However, serological assays have not shown cross reactions with other genera of haemoparasites, e.g., *Theileria, Anaplasma, Babesia* etc.(Luckins, 1977; Nantulya, 1990).

C) ANTIGEN DETECTION

The demonstration of the trypanosome antigen in the host blood is synonymous with demonstrating the parasite itself (Voller and De Savigny, 1981). The modification of ELISA to an antigen capturing assay (Nantulya and Lindqvist, 1989) and the development of species/sub species specific monoclonal antibodies (MoAbs) (Nantulya, 1981; Nantulya

et al, 1984; 1986; 1987; Richardson et al, 1986) brought another advancement in the diagnosis of African animal trypanosomiasis. The antigen detecting ELISA(Ag-ELISA) offers a better prospect for confirming trypanosomiasis diagnosis in animals at species level (Nantulya, 1990).

Ag-ELISA, makes use of species/subspecies specific monoclonal antibodies (MoAbs) which are raised against conserved, invariant trypanosome surface coat antigens released upon disintegration of the parasites (Nantulya et al, 1987; Nantulya and Lindqvist, 1989). These MoAbs are coated on micro ELISA plates for them to recognise and capture the specific trypanosome antigen present in sera, (Rae and Luckins, 1984; Nantulya, 1990). Since the trapped antigen has several binding sites, a second monoclonal antibody labelled with an enzyme, when added, binds to the antigen on the free binding sites. This reaction is revealed by a colour change when a substrate and chromogen are added (Nantulya and Lindqvist, 1989; Nantulya, 1990). The antigens recognised by Ag-ELISA are water soluble and they occur in circulation either as free antigens or as immune complexes (Nantulya and Lindqvist, 1989; Nantulya et al, 1989). Since the test detects dead trypanosomes, the appearance of antigenaemia coincides with the first peak of parasitaemia and the antigenaemia continues to rise even when the parasitaemia can not be detected by the buffy coat technique (Nantulya and Lindqvist, 1989; Masake and Nantulya, 1990; Nantulya, 1990). The Ag-ELISA has a high sensitivity of about 96% which is about four (4) times that of the haematocrit method. This assay can reveal infections in parasitologically negative animals and no false positives have been suspected since all sera from trypanosomiasis-free areas test negative under this method(Masake and Nantulya, 1990).

The assay also has high specificity that it can reveal mixed infections of *Trypanosome species* (Nantulya, 1990). Few false negative results (~5%) are normally encountered in acute phase infections when the antigen levels are still very low, such as before the remission of the first parasitaemic peak (Nantulya and Lindqvist, 1989; Nantulya, 1989).

Ag ELISA can easily be adapted for large scale screening of animals in the epidemiological surveys of trypanosomiasis with the added advantage of its ability to identify *Trypanosome species*. Another advantage of Ag-ELISA is that a single assay can be applied to various animal species since unlike the Ab ELISA, the host species specific anti-immunoglobulin enzyme conjugates are not required (Nantulya et al, 1989; Masake and Nantulya, 1990). The need for electrophotometer for optic density determination could be a major limiting factor

D) THE POLYMERASE CHAIN REACTION (PCR)

PCR works on the principle of DNA biosynthesis (i.e. replication) *in vivo*. Replication may be defined as the process by which each new strand of the parental DNA dublex is copied precisely by base pairing with complementary nucleotides to form two dublex helices identical to the parent dublex (Conn and Stumpf, 1976). Important components of replication include 1) the template, which is the DNA chain that provides precise information for the synthesis of a complementary strand of the DNA, 2) the primer, which is the initial terminus upon which additional units are added to form the final product (Conn and Stumpf, 1976). In this case , the oligonucleotides serve as the primer upon which the

four deoxy-ribonucleotides (dNTPs) are added for the synthesis of daughter strands(the final product).

Conn and Stumpf (1976), describe in detail the process of replication. Briefly, the process may be devided into 3 phases; initiation, elongation and termination. Initiation occurs in a 5'-3' direction. For initiation to start, the double helix must be separated to allow polymerases to function on the strands. Unique proteins of low molecular weight(the primers) bind specifically to one of the two strands. This binding appears to relate to those regions of the dublex rich in Adenine-Thymine (A-T) base pairing. This reaction occurs in the presence of an RNA polymerase to form small lengths of hybrid dublexes.

Elongation starts in the presence of a co-polymers and ATP to form an active complex with the primer template. When elongation begins, deoxy-ribonucleotides are properly positioned for a nucleophilic attack by the 3'OH terminus of the growing RNA-primer chain. Further elongation is catalysed by a DNA coplymerase in a 5'-3' direction until 500-1000 deoxyribonucleotide residues will have been added. During termination, three events must occur; the excision of the RNA-primer fragment; filling of the remaining gaps with deoxy-ribonucleotide residues; fusion of the DNA fragments by a diester bond to form a continuous DNA daughter helix.

The sensitivity and specificity of PCR is based on two factors; 1) hybridisation, the measure of complementarity between different nucleic acids (Conn and Stumpf, 1976) and 2) Stringency, measure of the number of mismatched base pairs when two nucleic acid molecules form a DNA dublex (Ternover and Unger, 1993). During hybridisation, when

sample DNA molecules are carefully heated, their strands separate and when carefully cooled, there is reformation of double strands with primers where ever complementarity occurs (Ternover and Unger, 1993). Since most of the nucleotide sequences in different regions of the DNA from one organism are unique, the reformed helices should have their two strands representing the same (and unique) regions of the original DNA.(Conn and Stumpf, 1976). Single stranded primers are, therefore, used to prevent self annealing.

The running protocol of PCR involves repeated cycles of 3 steps; 1) high heat denaturation of the target DNA, which allows for the melting apart of target strands and , therefore, making them accessible to hybridisation by specific oligonucleotide primers; 2) annealing step which cools the mixtures to allow the primers fuse (anneal) to their complementary sequences and; 3) the extension of the annealed primers on the DNA template by a DNA Polymerase done at an intermediate temperature (Saiki 1990, Persing 1993a). These steps are linked in what is called a thermal cycle from which there is a theoretical doubling of the target sequence (Persing, 1993a). The reaction is terminated by lowering the temperature to below 7°C. The efficiency of PCR amplification is increased by use of primers with two priming sites which must be in close proximity to each other. This physical linkage of two priming sites in DNA synthesis, which in itself increases stringency of primer - target hybridisation, results in a primer directed DNA synthesis of new priming sites which gives PCR much of its sensitivity and specificity (Persing, 1993b).

Certain innovations have further enhanced the high specificity and sensitivity of PCR. These include, 1) The inclusion of a thermal stable enzyme, Taq Polymerase, which is isolated

from thermophilic bacteria, *Thermus aquaticus,* obtained from hot springs. This enzyme has a half life of 40 min. at 95°C and, therefore, it is able to withstand repeated thermal cycling. Conducting the annealing and extension reactions at elevated temperatures reduces non specific amplification and, 2) The development of an automated thermocyler, has eliminated the tedious task of transferring reaction tubes between water baths or heating blocks (Persing, 1993b).

The primers used for trypanosome PCR are prepared from species/ subspecies specific nuclear DNA. No cross reactions with non-targeted trypanosome DNA or huge excess of host DNA have been reported (Kukla *et al*, 1987; Gibson *et al*, 1988; Mullis and Faloona, 1987).

Chapter Three

3.0 MATERIALS AND METHODS

3.1 Research design

Area

This study was conducted in Petauke district (143 ° S, 314 ° E) in the Eastern Province of Zambia (Appendix.1). It has a Savannah type of vegetation with the major part covered by *Mubombo* woods. Ten (10) crush pens within a radius of 60 Km were identified in a high trypanosomiasis and tsetse fly challenge area located within the common tsetse fly belt of Zambia, Malawi, Mozambique and Zimbabwe.

Animals

Animals used in the study were locally bred, mostly of the indigenous Angoni breed of cattle with naturally acquired trypanosomes. Ten (10) trypanosomiasis positive animals were selected by screening between twenty (20) and thirty (30) cattle from each crush pen using the haematocrit method. Screening was continued until the 10^{th} positive animal per crush pen to obtain a total sample size of one hundred (100) cattle. Blood samples from the selected animals were collected for further examination using the stained dry thin blood smear (TBS), Ag- ELISA and the PCR techniques. For each method, the *Trypanosome species* were determined using the respective procedures as indicated in the methodologies. To establish the actual infection in each animal, the results from the respective diagnostic tests were pooled and termed net infection.

Recording of results.

I) Coding system: The coding system of No. '1' for positive and '0' for negative result was adopted and identified against each trypanosome spp observed which were designated as T.c, T.v, T.b. for *T. Congolense, T. vivax & T. b. brucei* respectively.

ii) Statistical analysis: The net infection results were then analysed statistically by Analysis ofVariance (ANOVA) using the Completely Randomised Block Design (CRBD) to test if there was any significant difference in the statistical means of infection rates for the three trypanosome species (used as treatments in the design) as well as to test if there was any influence on the results from the crush pens (blocks).

Specimen collection

Blood was collected from cattle at two sites

1) The ear vein:

Blood was collected in two capillary tubes. One of the capillary tubes was used for the haematocrit examination. The other one was used to make a thin blood smear for parasitological examination and to blot a blood spot on filter paper for the PCR examinations. The blood spots were stored at -20°C until needed.

2) The jugular vein;

Blood collected in plain vacutainer tubes was stored overnight at 4°C before sera was separated and preserved at -20°C for Ag-ELISA.

3.2 Parasitological examination

1) Haematocrit Examination

Blood collected in capillary tubes was centrifuged for five minutes at 15,000 RPM to obtain the buffy coat. The tube was then cut just below the buffy coat region and a plasma drop with buffycoat contents was placed on a glass slide and covered with a cover slip. These preparations were examined under a light microscope (Olympus, Mg x40). Trypanosome search was done in up to 100 microscopic fields. The *Trypanosome species* were determined by observing the movement patterns of the organisms and the relative size according to Stephen, (1986).

2. Thin blood Smear Examination

A drop of whole blood collected in the second capillary tube was placed on microscopic glass slide and a thin blood smear was made. The thin smears were air dried and fixed in alcohol (methanol) for five minutes and air dried before they were stained for 20 minutes in 10% Giemsa (diluted in PBS pH 7.2 at room temperature) and later washed in tap water and air dried. The thin smears were examined under the oil immersion objective lens (Mg x100) of a light microscope (Olympus).

3.3 PCR examination

Template DNA isolation:

Trypanosome sample DNA was isolated from dried blood spots by Chelex-100, an anionic resin, using modifications of the methods described by Walsh and Higushi (1991) and Kain and Lamer(1991)

About 5mm^2 in area of dried blood spot on filter paper (Whatman no. 41) was cut and placed in a 1.5 ml microcentrifuge tube to which 1 ml of ice cold 0.15% Saponin (Sigma) diluted in PBS pH 7.2 was added and the mixture incubated in ice for 10 minutes whilst occasionally mixing by gently inverting the tubes to destroy the erythrocytes. The mixture was then centrifuged at 10,000 RPM for 1 minute and washed with 1 ml ice cold PBS pH 7.2. The supernatant was then discarded.

To collect the DNA, 200 µl of 5% Chelex-100 Resin (Bio-Rad) in distilled water was added, taking care that the Chelex beads were evenly distributed by gently mixing the Chelex solution whilst pipetting. The mixture was vortexed for 10 seconds and incubated at 56°C for 15 minutes in a water bath before vortexing at high speed for 10 seconds and then incubated in boiling water for 8 minutes. After vortexing for 10 seconds, the mixture was centrifuged at 10,000 RPM for 1 minute and 100 µl of the supernatant was transferred into a fresh tube and saved as the sample template DNA.

PCR Cycling:

For each template DNA sample, three separate 0.5 ml vials were prepared for *T. congolense, T. vivax* and *T. brucei* amplification.

The standard PCR amplification were carried out in 10 µl reaction mixtures. The reaction mixtures were prepared by adding; 1) 1 µl of Taq DNA buffer containing 10m M Tris-HCl, pH 9.3, 50m M KCl, 1.5 mM $MgCl_2$; 2) 1 µl of the four deoxy-nucleoside triphosphates (dNTPs) - containing 125 mM of each of the dNTPs; 3) 1µl of the respective Primers at 0.25 mM; 4) 2 µl of chelex-100 isolated template DNA solution; 5) 0.1 µl Taq polymerase

(pharmacia) containing 0.5 U and, 6) 4.9 µl distilled water. The reaction mixtures were overlaid with 20 µl paraffin oil and cycled in a programmable heating blocks as follows: Samples were incubated at 94°C for 3 minutes in an initial denaturing step and subjected to 40 cycles involving denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. Samples were then incubated at 72°C for 7 minutes and then cooled to 4°C to stop the reaction.

To the PCR product, 2 µl of DNA loading buffer (Bioventures inc. USA) was added and 10 µl was placed on to a well of 2% agarose gel in 1 x TAE buffer (40 mM Tris acetate, 1 mM EDTA). In the first well, only 5 µl Biomaker (Bioventures inc.) was added. The product was then separated electrophoretically in 1 x TAE buffer at 100V until the dye had covered half way the gel, approximately 40 minutes.

The gel was then stained in 0.5 mg/ml Ethidium bromide for 7 minutes on a shaker and then washed 3 times for 5 minutes per washing. The reaction was then observed under UV light and photographed.

Primer sets used were referred to as reported sequences (Kirchoff and Donelson, 1993): TBR1 (CGAATGAATATTAAACAATGCGCAG) and (AGAACCATTTATTAGCTTTGTTGC) for *T. brucei brucei* and TCN1 (TCGAGCGAGAACGGGCACTTTGCGA) and (ATTAGGGACAAACAAATCCCGCACA) for *T. congolense,* Savannah, TVW1

(CTGAGTGCTCCATGTGCCAC) and TVW2 (CCACCAGAACACCAACCTGA) for *T. vivax* (Courtesy of Prof. Ken Katakura, Japan).

3.4 ANTIGEN-ELISA EXAMINATION.

This test was done using the kit (TRP9506029) supplied by joint FAO/IAEA programme (1995) and is based on a standard direct sandwich ELISA technique to determine the presence of *Trypanosoma brucei, T. congolense* and *T. vivax* as described by Nantulya *et al.* (1987), using the protocol TRAP 1.2 provided by FAO/IAEA (1995).

The procedure involved the following steps; 1) Coating of microplates with trapping antibodies, 2) addition of test and control sera, 3) addition of antibody conjugate, 4) addition of chromogen/substrate, 5) addition of stopping solution and 6) reading of results.

1) COATING

Three microplates (Nunc-Maxisorp-cat. # 4-39454) were prepared for each set of 40 test sera samples to test for *T. brucei, T. congolense* and *T. vivax* respectively. Trapping species specific monoclonal antibodies (MoAbs); 1) Mouse anti -*T. brucei* (clone T.b.r.7, IgM isotype), 2) Mouse anti -*T. congolense* (clone T.c.37 IgM isotype) and 3) Mouse anti -*T. vivax*(clone T.v.27, IgG1 isotype) were separately diluted 1:300 in coating buffer (0.05M Carbonate/Bicarbonate, pH 9.6). 100 µl of the diluted MoAbs were dispensed into all the 96 wells species microplates. The plates were sealed and stored over night at + 4°C.

2) ADDITION OF SERA.

The following day, 100µl of 40 neat test sera samples were respectively dispensed into rows A3-A12, C3-C12, E3-E12 and G3-G12 of a microplate (Appendix 2) (Flow labs.). 40 µl of each test sera were added to 760 µl of Serum diluent buffer (0.01M PBS, pH 7.4 plus

0.05% (V/V) Tween 20 and 0.5% (V/V) Normal mouse serum in corresponding micronic tubes to attain a dilution of 1:20. On another tube holder, 4 micronic tubes for each species were filled with 760 µl of the serum dilution buffer for controls (Cc, C+ +, C+ and C-). 40 µl of control sera for each species; C+ +(High infection), C+ (low infection) and C-(un infected bovine sera), were added in the corresponding tubes to attain a dilution of 1:20.

The coated plates were now washed once in washing buffer containing 0.01M PBS, pH 7.4 plus 0.5% (V/V) Tween 20. Using a multichanel pippet with 10 tips, 100 µl of the diluted test sera samples from wells in A3 - A12 were transferred to corresponding wells on row A and B of the coated microplates. Using a separate set of tips each time, the same was done to transfer test sera from C3 - C12 to rows C and D, from E3 - E12 to rows E and F and from G3 - G12 to rows G and H. Using a set of 4 tips, 100 µl of the controls were added to the corresponding species coated microplates as follows; Cc(serum dilution buffer) to AB1-2, C++ to CD 1-2, C+ to EF1-2 and C- to GH1-2. The microplates were incubated at 37°C in an Orbital shaker for 15 minutes and washed once.

3) ADDITION OF CONJUGATE

Detecting antibody conjugates: Horse radish peroxidase (HRP)-Mouse anti -*T. brucei* (clone T.b.r.7), HRP-Mouse anti-*T. congolense* (clone T.c.39) and HRP -mouse anti- *T. vivax* (clone T.v.27) were diluted 1:1,000 respectively in conjugate diluent buffer containing 1% (W/V)Bovine serum albumin, 0.01M PBS, pH 7.4 plus 0.5% (V/V)Tween 20. 100 µl of the detecting antibody conjugates were dispensed into all wells of the corresponding plates and incubated at 37°C for 15 minutes, washed and soaked for 10 minutes (repeated 3 times).

4) ADDITION OF CHROMOGEN/SUBSTRATE

The chromogen TMB peroxidase containing 0.4 g/l 3, 3',5,5'-tetra methyl benzidine in organic base, was mixed 1:2 with peroxidase substrate buffer (0.002% H_2O_2 in citric acid buffer). 100 µl were dispensed in all wells of column 1 of the blanking microplate and into all the wells of the test microplates. The plates were incubated at 37°C for 20 minutes with the counting down starting immediately after addition into the blanking plate.

5) ADDITION OF THE STOPPING SOLUTION

100 µl of stopping solution (1M H3P04) was added into the blanking column and all wells of the test microplates.

6) READING OF RESULTS

Reading of results was based on the measurement of the substrate development. This was done on a computer controlled Multiskan reader (Labysystem) at 450 nm absorbance, which gave results as Percentage Positivity values calculated by dividing Optical Density (OD) values of the sample by the OD values of the controls (FAO/IAEA, 1995).

Chapter four

4.0 RESULTS

4.1 Haematocrit (HCT)

Two hundred and forty (240) cattle were screened by the HCT method (HCT-1)from the ten (10) crush pens to select the experimental group of 100 trypanosomiasis positive cattle, which showed an overall trypanosomiasis prevalence of 41.7% (Table 1). From this experimental group, species identification (HCT-2) indicated that *Trypanosoma congolense* was the most predominant species with 89 (89%) infection rate followed by *T. vivax* 9 (9 %) and *T. brucei brucei* 3 (3%) giving an infection ratio of 29:3:1 respectively (Table 1). There was only 1 (1%) mixed infection detected between *T. congolense* and *T. vivax* and no mixed infections were recorded on *T. b. brucei* positive samples (Table 2). Comparison of the HCT-2 results to the Net infection figures, indicate that HCT-2 detected 89 (94%) of the net *T. congolense* infections whilst it detected much less of the *T. vivax* 9 (11%) and *T. b. brucei* 3 (4%) net infections (Table 3).

4.2 Thin Blood Smear (TBS)

Morphological characteristics of *Trypanosoma congolense, T. vivax* and *T. b. brucei* are shown in Fig. 1. Of the one hundred(100) cattle found positive by HCT-1 method, only 81 (81%) were confirmed positive by the thin blood smear examination (TBS) method. Of these, 62 (76.5%) were *T. congolense,* 23 (28.4%) were *T. vivax* and 14 (17.3%) were *T. b. brucei,* giving a species infection ratio of about 4:2:1 (Table 1). Distribution at species level showed higher single species infection rates for *T. congolense*, 49 (61%), and only 9 (11%) for *T. vivax* and 7 (9%) for *T. b. brucei* (Table 2). A few mixed infections were also

detected as 9 (11%) for *T. congolense and T. vivax*, 2 (3%) for *T. congolense* and *T. b. brucei*, 3 (4%) for *T. vivax* and *T. b. brucei* and 2 (3%) for a combination of all the three species (Table 2). Comparison of the TBS results to the Net infection figures, indicate that TBS detected 62 (65%) of the actual *T. congolense* positives, whilst it detected much less, 23(28%) of the actual *T. vivax* and 14 (18%) of the *T. b. brucei* infections (Table 3).

4.3 Antigen ELISA (Ag-ELISA) results

In this assay, the percentage positivity values of above 10% were taken to be positive. The Ag-ELISA confirmed 87 (87%) of the cattle in the experimental group as being positive for trypanosomiasis (Table 2). *T. congolense* was again the most predominant species with 82(94%)(Table 1). There was an increase in the detection of *T. vivax* with 51 (62.2%) and *T. brucei* with 58 (71%), which gives a lower species infection ratio of about 2:1:1 (Table 1). There was a marked reduction in the detection of single species infections, i.e. 11 (13%) for *T congolense*, 2 (2%) for *T. vivax* and non (0%) for *T. b. brucei* (Table 2). On the other hand, there was an increase in the detection of mixed infection for. *T. congolense* and *T. vivax* 16(18%), for *T. congolense* and *T. b. bruce* 24 (28%), for *T. vivax* and *T. b. brucei* 3(4%), and a combination of all species 31 (36%) (Table 2). On comparison to the net infection results (Table 3), Ag-ELISA detected, 82 (86%) of the actual *T. congolense* infections, 51 (63%) of the *T. vivax* and 58 (76%) of the *T. b. brucei.* The Ag-ELISA results were further compared to those of the TBS method (Table 4). It was observed that Ag-ELISA had a higher sensitivity for *T. congolense* as it detected 55 (88%) of the samples confirmed *T. congolense* positive by the TBS method and 23 (71%) of those missed by the TBS method. The next sensitivity was for *T. b. brucei* where it detected 10 (1%) of *T. b.*

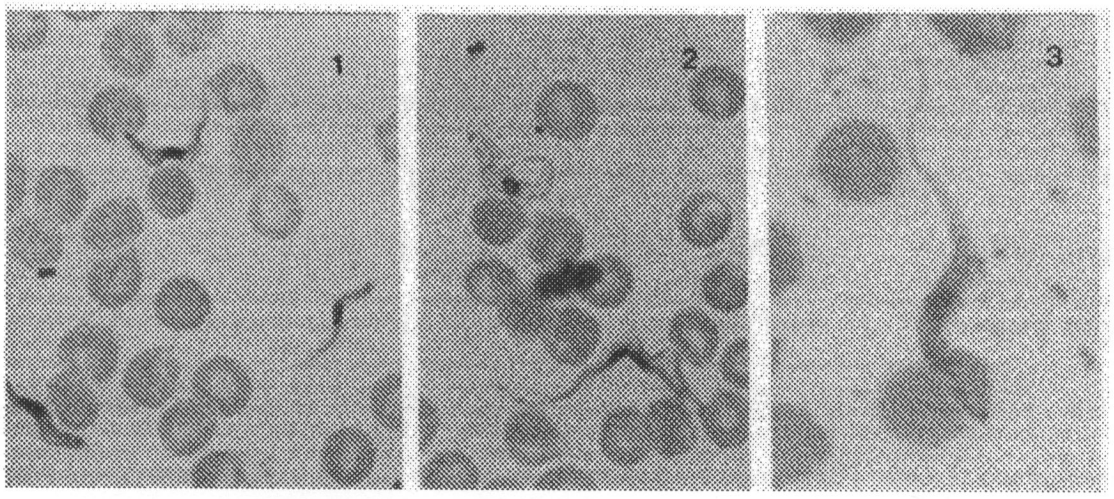
brucei confirmed by the TBS method and 48 (83%) of those negative by the TBS method. The least sensitivity was for *T. vivax,* where it detected 13 (57%) of those confirmed positive and 27 53%) of those which were negative for *T. vivax* under the TBS examination.

4.4 PCR

The DNA bands for *T. congolense, T. brucei* and *T. vivax* positive samples were seen on the Agarose gels at 370, 150 and 125 base pairs respectively as shown in Fig. 2.

The PCR results confirmed 86 (86%) of the experimental animals as positive for trypanosomiasis (Table 2). Of these, 73 (85%) were *T. congolense*, 37(43%) were *T. vivax* and 38 (44%) were *T. brucei* which gives an infection ratio of about 2:1:1 (Table 1). The species combinations as detected by PCR showed 37 (43%) *T. congolense*, 4 5%) *T. vivax* and *T. b. brucei* 2 (2.%) single species infections. The following mixed infections were detected; 7 (8%) *T. congolense* and *T. vivax*, 10 12%) *T. congolense* and *T. b. brucei*, 7 (8%) *T. vivax* and *T. b. brucei* and 19 (22%) with all the three species (Table 2).

When compared to the net infection results, PCR detected 73 (76%) of the *T. congolense*, 37 (45%) of the *T. vivax* and 38 (50%) of the *T. b . brucei* actual infections(Table 3). On comparison to the TBS results, PCR detected 50 (81%) of the *T. congolense* infections confirmed by TBS and another 23 (71%) of those negative by the TBS. PCR only detected 10 (43%) of the *T. vivax* positive infections confirmed by TBS and 27 (35%) of those negative by the TBS. For *T. brucei*, PCR detected only 5 (36%) of those confirmed positive by TBS and 33 (38%) of those negative on the TBS, confirming the lowered sensitivity for *T. vivax* and *T. b. brucei* (Table 5)



T.congolense (x1000) *T. brucei.* (x1000) *T.vivax*(x1500)

Fig 1 Morphological characteristics of *T. congolense, T. brucei* and *T. vivax* as seen on thin blood smears under a light microscope

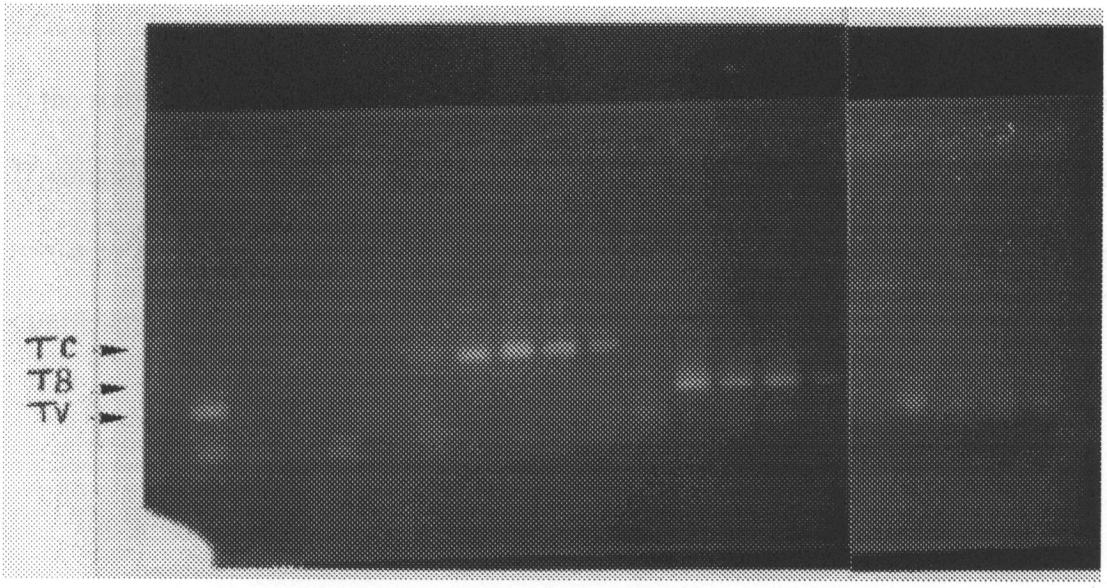


Fig 2 Photographs showing PCR postive DNA bands for *T. congolense* at 369 base pairs(BPs*), T. b. brucei*, at 150 Bps and *T. vivax,* at 125 BPs.

4.5 Net infection

The pooled results for *Trypanosoma congolense, T. vivax* and *T. b. brucei* infection from the respective diagnostic tests performed on each sample showed 95(95%) to be infected *with T. congolense*, 81 (81%) with *T. vivax* and 76 (76%) with *T. b. brucei*, giving an overall infection ratio of 1.3:1.1:1(~1:1;1) respectively (Table 1). There were very few single species infections detected; 7 (7%) for *T. congolense,* 1 (1%) for *T. vivax* and none for *T. b. brucei*(Table 2). Most of them were mixed infections; 16 (16%) *T. congolense* and *T. vivax*, 12 (12%) *T. congolense* and *T. b. brucei*, 4 (4%) *T. vivax* and *T. b. brucei*, and 60(60%) had *T. congolense, T. vivax* and *T. b. brucei* mixed infections (Table 2).

Testing of the pooled results by analysis of variance using the Complete Block Design at 95% confidence interval (Appendix 3), it was found that there was no significant difference in the statistical means of infection for the three species, suggesting an equal distribution of *T. congolense*, *T. vivax* and *T. b. brucei* infection in the cattle sampled. This situation seems to apply to all areas since on testing the block (crushpen) effect, there were no significant differences noted amongst them.

Table 1 Summary of the overall crush pen results for *T. congolense*(Tc), *T. vivax*(Tv) and *T. b.* brucei(Tb) infections identified by using different diagnostic methods to obtain the Pooled(Net) infection rates and ratios.

Crush pen(Cp)	HCT-1		HCT-2			TBS			Ag-ELISA			PCR			**NET INFECTION**		
	# sampled	# ve	Tc	Tv	Tb	Tc	Tv	Tb	Tc	Tv	Tb	Tc	Tv	Tb	**Tc**	**Tv**	**Tb**
Cp 1	30	10	7	3	1	6	2	1	8	3	4	4	5	1	**9**	**7**	**4**
Cp 2	28	10	8	1	1	4	0	0	7	1	3	7	8	7	**8**	**8**	**9**
Cp 3	27	10	9	1	0	5	1	1	8	7	7	6	5	6	**9**	**9**	**9**
Cp 4	24	10	10	0	0	6	2	4	9	7	8	10	0	0	**10**	**7**	**8**
Cp 5	29	10	10	0	0	7	2	2	10	5	9	8	0	3	**10**	**7**	**9**
Cp 6	26	10	9	1	0	4	2	0	9	2	7	5	3	0	**10**	**7**	**7**
Cp 7	23	10	8	2	0	9	1	0	10	10	3	7	0	0	**10**	**10**	**3**
Cp 8	20	10	8	1	1	5	5	4	8	7	7	8	0	5	**9**	**10**	**9**
Cp 9	22	10	10	0	0	9	4	1	9	9	6	10	10	10	**10**	**6**	**8**
Cp 10	21	10	10	0	0	7	4	1	4	0	4	8	6	6	**10**	**6**	**8**
TOTAL	**240**	**100**	**89**	**9**	**3**	**62**	**23**	**14**	**82**	**51**	**58**	**73**	**37**	**38**	**95**	**81**	**76**
SPP.INF .RATE		**41.7 ***	**89**	**9**	**3**	**76 .5**	**28 .4**	**17 .3**	**94 .2**	**58 .2**	**66 .7**	**85**	**43**	**44**	**95**	**81**	**76**
SPP.INF .RATIO			**29**	**3**	**1**	**4**	**2**	**1**	**2**	**1**	**1**	**2**	**1**	**1**	**1**	**1**	**1**

HCT-1----------Initial screening of cattle by the Haematocrit method

HCT-2----------Species identification by the HCT method.

TBS----------Thin Blood Smear

PCR----------Polymerase Chain Reaction

Ag-ELISA----------Antigen detecting Immuno Sorbent Assay.

NET Infection--------Actual infection established by pooling results of all the diagnostic tests for each animal.

* Overall prevalence of trypanosomiasis from the cattle screened at Kalinda(Cp.1), Nyampande (Cp.2), Filipo(Cp.3), Chitabe(Cp.4), Chilabila(Cp.5), Mtunda(Cp.6), Kasero-1(Cp.7), Kasero-2(Cp.8), Seya(Cp.9) and Kavundula(Cp.10).

Table 2 The different combinations of *T. congolense, T. vivax* and *T. brucei* detected by HCT, TBS, Ag-ELISA and PCR diagnostic methods.

		Tyrpanosome spp combination													
	Total +ve	+ve Tc		+ve Tv		+ve Tb		+ve Tc/Tv		+ ve Tc/Tb		+ve Tv/Tb		+ve Tc/Tv/Tb	
METHOD	Total #+ve (%)	#	%	#	%	#	%	#	%	#	%	#	%	#	%
HCT-2	100 (100)	88	88	8	8	3	3	1	1	0	0	0	0	0	0
TBS	81 (81)	49	61	9	11	7	9	9	11	2	3	3	4	2	3
Ag-ELISA	87 (87)	11	13	2	2	0	0	16	18	24	28	3	4	31	36
PCR	86 (86)	37	43	4	5	2	2	7	8	10	12	7	8	19	22
Net infection	100 (100)	7	7	1	1	0	0	16	16	12	12	4	4	60	60

Table 3. Evaluation of the diagnostic methods in detection of *T. congolense, T. vivax* and *T. b. brucei* in comparison to the net infection.

SPECIES	NET	METHOD							
	infection(#)	HCT-2		TBS		Ag-ELISA		PCR	
		#	%	#	%	#	%	#	%
T. congolense	95	89	94	62	65.	82	86	73	76
T. vivax	81	9	11	23	28	51	63	37	45
T. b. brucei	76	3	4	14	18.4	58	76	38	50

Table 4: Evaluation of Ag- ELISA results for the detection of *T. congolense, T. vivax* and *T. b. brucei* in comparison to the Thin Blood Smear results

SPECIES	# + VE(TBS)	#+VE ELISA	+VE TBS & ELISA		+VE TBS -VE ELISA		-VE TBS & +VE ELISA	
			#	%	#	%	#	%
T. congolense	62	82	55	88	7	12	27	71
T. vivax	23	51	13	56	10	43	27	53
T. b. brucei	14	58	10	71	4	29	48	83

Table 5. Evaluation of PCR results for the detection of *T. congolense, T. vivax* and *T. b. brucei* in comparison to the Thin Blood Smear results

SPECIES	# + VE (TBS)	#+VE PCR	+VE TBS & PCR		+VE TBS -VE PCR		-VE TBS & +VE PCR	
			#	%	#	%	#	%
T. congolense	62	73	50	81	12	19	23	71
T. vivax	23	37	10	43	13	57	27	73
T. b. brucei	14	38	5	36	9	64	33	38

Chapter five

5.0 Discussion

Although all the diagnostic methods used show more *Trypanosoma congolense* than *T. vivax* and *T. b. brucei* infections, the net infection results show a higher rate of mixed infections with as much as 60% of the experimental group having mixed infections of *T. congolense, T. vivax* and *T. b. brucei* and a very low rate of single species infection. Statistical analysis shows that there are no significant differences in the species statistical means of infection. This finding suggests a high rate of co-existence of the three trypanosome species in cattle. These results also indicate some inadequacies in the diagnostic methods routinely used to identify trypanosome species.

The HCT method, whose sensitivity is increased by concentrating the trypanosomes at the buffycoat (Nantulya, 1990), was more effective in the detection of *T. congolense*, a strictly plasma trypanosome. The low level of detection for *T. vivax* indicates that *T. vivax* attains low parasitaemic levels. One reason for this could be that *T. vivax* also tends to be more of a humoral group trypanosome like *T. b. brucei* as suggested by Stephen (1986). Since the detection of *T. vivax* and *T. b. brucei* infections under HCT were mostly obtained where *T. congolense* was absent, this could indicate that *T. congolense* due to its superior parasitaemia overshadows the detection of the movements of the other two species which attain low parasitaemic levels. Also, since *T. vivax* moves so rapidly across the microscopic field, and especially that it appears to be characterised by a low parasitaemia, the chances of noting the trypanosome is further reduced.

The TBS method provides a more definitive identification of the trypanosome species by actually noting the typical morphological features characteristic of each species (Hoare, 1970 b; Luckins, 1992). The problem with the TBS is its low sensitivity, which is dependent on the parasitaemia and often exacerbated by the tissue tropism phenomenon of the trypanosomes (Masake and Nantulya, 1990). *Trypanosoma congolense* was again, more readily detected using this method probably, because of being a strictly plasma (haematic) trypanosome (Losos and Ikede, 1972; Losos, 1978). Although *T. vivax* has been described as strictly a plasma trypanosome (Losos and Ikede, 1972), it was observed that in the *T. vivax* positive smears, normally only one trypanosome would be detected in about 100 microscopic fields which suggests the high chances of missing *T. vivax* in the TBS method. The poor rate of *T. vivax* detection by the TBS method suggests that *T. vivax* tends to be more of a humoral trypanosome than a haematic one or it could be that *T. vivax* infections are normally chronic contrally to West and East Africa where it is reported to be hyperacute (Willet, 1970). *Trypanosoma brucei brucei* was also poorly detected by the TBS method. This is as expected since *T. b. brucei* is mainly a humoral trypanosome (Losos and Ikede, 1972). However, when detected, several trypanosomes, mainly the dividing forms (long and slender and the intermediate forms) were seen. This seems to agree with the observations that during successive parasitaemias, *T. b. brucei* trypanosomes transform from long dividing forms towards the short and stumpy non dividing forms through the intermediate forms (Robertson, 1912; Vickerman and Tetley, 1978).

The Ag-ELISA results also revealed more *T. congolense* infections. However co-existence of trypanosome species was indicated by the increased detection of *T. vivax* and *T. b. brucei* which resulted in more mixed infections being detected, with infection ratio of about 2:1:1 for *T. congolense* to *T. vivax* to *T. b. brucei* respectively. Ag-ELISA is said to have a very high sensitivity (95%) and specificity (99%) (Nantulya, 1990). The lower prevalence rates associated with *T. vivax* and *T. brucei* detection in the study could be an indication of reduced sensitivity of Ag-ELISA for *T. vivax* and *T. b. brucei* than for *T. congolense.*

Considering that Antigen ELISA detects the species specific invariant antigens which are conserved in the trypanosome surface coat and only available for detection when they are released into circulation following the destruction of the trypanosome (Nantulya at al, 1987), it is possible that the false negative *T. vivax* and *T. brucei* could have been due to failure in detecting them in their first peak build up phase and before the remission (Nantulya, 1990). The level of antigenaemia could be another likely source of the false negative results especially for *T. vivax* which showed very low parasitaemia in the parasitological examinations. Further still, since the antigen used to prepare the Mouse anti-species monoclonal antibodies were not from the Zambian strains, it is also possible that there could be some regional variations in the invariant antigens, (especially for *T. vivax*, which showed the lowest sensitivity) such that the currently used monoclonal antibodies may not adequately recognise the local strains.

The Antigen ELISA is being validated for the second time in Zambia. In the first evaluation trials by Sinyangwe et. al. (1993), in the Gwembe Valley, there were more single species

infections recorded than mixed ones, which led to the withdrawal of the kit for further improvement. Further evaluation of the kit is still required and probably the native trypanosomes should be used to prepare the monoclonal antibodies in order to improve the Ag- ELISA sensitivity and specificity.

Results from the PCR also manifested higher *T. congolense* infection rates with *T. vivax* and *T. brucei* being at the same rate. The prevalence ratio of 2:1:1 for *T. congolense* to *T. vivax* to *T. b. brucei* was maintained as in the Ag-ELISA results, although the actual percentage of the trypanosomes detected by PCR were lower than those obtained by the Ag ELISA. Detection of African trypanosomes by PCR, focuses on the highly repetitive nuclear DNA sequences as targets for PCR amplification since the minicircle KDNA is highly heterogeneous. Foreign origin primer sets for the three *T. congolense* subspecies(Savannah, Kenyan and Forestry), *T. vivax* and for *T. brucei* are available (Kirchoff and Donelson, 1993). The Zambian *T. congolense* appears to have similar DNA to the Savannah type of *T. congolense* in Kenya as was shown in the preliminary tests (Katakura *et al*, in press).

Since PCR detection of trypanosomes from the blood is based on recognition of the DNA of the trypanosome in blood circulation, the results can be influenced by the level of parasitaemia. When the parasitaemia is very low, the chances of picking at least one trypanosome in the blood sample are greatly reduced and, therefore, further reducing the chances of isolating at least one trypanosome DNA for amplification. This appears to be

particularly true for *T. b. brucei* which is a humoral group trypanosome (Losos and Ikede, 1972). If the low parasitaemia/ aparasitaemia is the cause of the *T. vivax* low detection by PCR, then *T. vivax* should be considered more of a humoral trypanosome than a haematic one. However, this does not completely explain the low detection of *T. vivax* and *T. brucei* infections confirmed by the TBS examination. What was expected was a very high detection of these species since PCR only needs at least one DNA to detect the presence of the trypanosomes (Persing, 1993a). The probable reason, therefore, could be that some variations exist in the highly repetitive nuclear DNA sequences of the Zambian strains in comparison to those of the trypanosomes which were used to prepare the primer sets.

Since this has been the first time that trypanosome detection by PCR has been conducted in Zambia, further studies to evaluate PCR are required. It is particularly important to try and characterise the indigenous trypanosomes and, if possible,to prepare the primer sets from them.

Chapter six

CONCLUSION.

The three trypanosomes (*T. congolense, T. vivax* and *T. b. brucei)* are widely distributed in Zambia. These trypanosomes were found to statistically co-exist in cattle at a ratio of approximately 1:1:1. However, all diagnostic techniques showed higher sensitivity for *Trypanosoma congolense.* This was more pronounced in the parasitological methods than in the Ag-ELISA and PCR techniques, which revealed more *T. vivax* and *T. b. brucei* infections. The poor sensitivity for *T. vivax* and *T. b. brucei* observed in the parasitological methods, which are in routine use, has probably led to the apparent *T. congolense* predominance being reported in cattle. The species identificaton in the diagnosis of trypanosomes is a very important factor in epidemiological surveys. Because of the variations in drug susceptibility by the trypanosome species, knowledge of the type of trypanosome species present in animals is important in chemotherapeutic control programmes especially with respect to drug resistance development by trypanosomes. For instance, *T. brucei* requires higher Berenil dosage (7mg/Kg) than *T. congolense* and *T. vivax* whose recommende dose is 3 mg/Kg body weight. The advent of the highly sensitive and specific *in vitro* antigen detecting techniques (Antigen ELISA and PCR) seem to be the likely solution to this problem of diagnosis. However, further evaluations of these techniques is recommended and efforts should be made to use the local strains of trypanosomes in the preparations of monoclonal antibodies and the primer sets so that the problem of detection and identification of the trypanosome species could be resolved.

Meanwhile since no single test is capable of detecting all infecting trypanosomes in cattle, these methods where practicable, should be combined with the parasitological methods to yield more reliable results.

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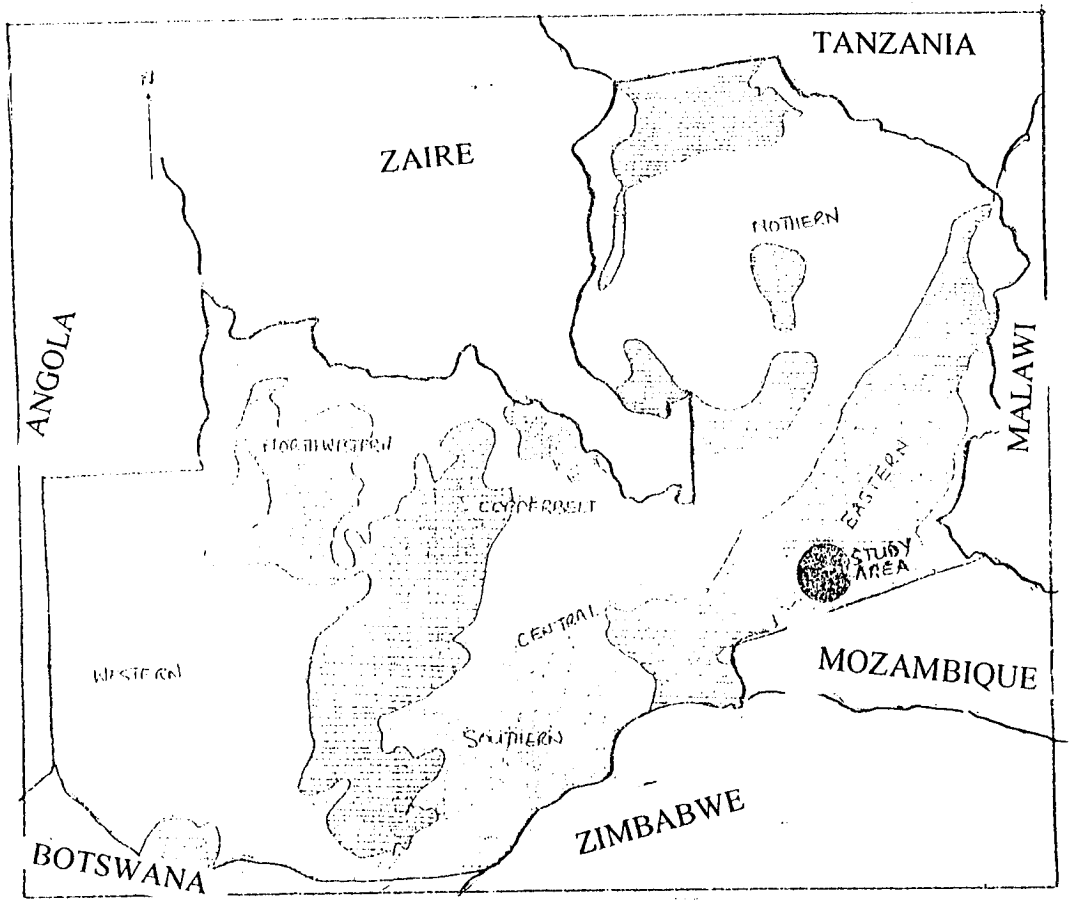
Appendix 1



Map of Zambia showing the Tsetse fly ditribution and the location of Petauke(study area).

Tsetse infested areas

Appendix 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cc	CC										
B	Cc	Cc										
C	C++	C++										
D	C++	C++										
E	C+	C+										
F	C+	C+										
G	C-	C-										
H	C-	C-										

Lay out of Ag-ELISA microplate

APPENDIX 3

ANOVA TABLE- Statistical analysis of the Net infection results

Source of variation	Degrees of freedom	Sum of squares	mean squares	variance ratio	critical F value (95% CI)
Treatments (SPECIES)	(K-1) = 2	SSTr=19	MSTr=sstr/df =9.5	MSTr/MSE =3.39	$F_{(2,18)}=3.5$
Block (Crush pen)	(B-1) = 9	SSBl=23	MSBl=msbl/df =2.5	MSBl/MSE =0.89	$F_{(9,18)}=2.5$
ERROR	(K-1)(B-1) = 18	SSE=51	MSE=sse/df =2.8		
TOTAL	29	SST= 93			

Hypothesis testing;

1)Treatment(species) effect
Hypothesis;
Ho: All means are equal (Utc=Utv =Utb)
Ha: not all means are equal
Where Utc= the mean of *T. congolense* infection
Utv=the mean for *T. vivax* infection
Utb= the mean for *T. b. brucei* infection
Decision rule; Reject Ho if V.R equal to or greater than critical value of F at 95% confidence interval.
Decision; since V.R (3.39) is less than critical F value(3.5) we fail reject the Ho . Therefore there seems to be no significant difference between means of infection amongst the three species.

2) Block (Crush pen) effect

Ho; All block means are equal
Ha; not all block means are equal

Decision rule ; Reject Ho if the V.R is equal to or greater than the F critical value
Decision; Since VR(0.89) is less than the F critical value at 95% C I, we fail to reject the Ho. Therefore, the site of sampling does not seem to affect the results.