DECLARATION

I, Kizito Mweene Bukowa, hereby declare that this thesis, submitted by me to the University of Zambia for the degree of Master of Science in Veterinary Parasitology has not been submitted at any other University.

Signature……

Date……

CERTIFICATE OF APPROVAL

This dissertation of **Kizito Mweene Bukowa** has been approved as fulfilling the requirements for the award of Master of Science in Veterinary Parasitology by the University of Zambia.

Supervisor …….. Sign……... Date……...

Examiner …….. Sign……... Date…….

Examiner …….. Sign……... Date…….

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ABSTRACT

A cross sectional study was carried out by microscopy and Loop-mediated isothermal amplification (LAMP), a novel molecular technique that amplifies target DNA under isothermal conditions, to determine the epidemiology of the re-emerging trypanosomosis in the Choma-Kalomo block, an important agricultural zone of the Southern Province of Zambia which was aerial-sprayed in 1987 in an attempt to eradicate tsetse flies and trypanosome infections. A total of 460 heads of cattle comprising 205 (44.6%) cows, 152 (33.0%) oxen, 56 (12.2%) female calves, 33 (7.2%) male calves and 14 (3.0%) bulls, were sampled from 13 crush pens. Blood collected from the superficial ear vein was used for microscopy and as a source of DNA in the trypanosome-specific LAMP reactions.

The overall prevalence of bovine trypanosomosis recorded by microscopy in the Choma-Kalomo block was 4.8%. Out of the 13 crush pens, bovine trypanosomosis was only detected in Nakalombwe (27.9%), Kakuse (18.6%) and Mbila A (8%). Further analysis of the same samples by LAMP revealed (i) an increase in the overall prevalence of bovine trypanosomosis to 12.8%, (ii) an increase in the sampling sites with trypanosome positive cattle from 3 by microscopy to 6, including Nakalombwe (53.5%), Kakuse (51.2%), Mbila A (36%), Fwentele (14.3%), Bungashiya (7.1%) and Maila Male (4.9%), (iii) detection of 6.8% mixed-infection cases of *T. congolense* and *T. b. brucei* while no mixed-infection case was detected by microscopy. Overall, LAMP was at least 2.5 times more sensitive than microscopy. Importantly, LAMP method was able to detect all parasitologically positive cases, in addition to the new cases it revealed, suggesting the reliability of LAMP, in addition to its superior sensitivity to microscopy.

Trypanosoma congolense accounted for 74.6% of total trypanosome infections recorded by LAMP in the Choma-Kalomo block. In agreement with previous reports, several cattle infected with *T. congolense* in this study were debilitated and anaemic. Adult cows seemed more likely to be infected with trypanosomes than any other category of cattle, possibly due to their being stressed through milking and at the same time being used for traction power, hence making them more susceptible to tsetse bite (less active) and trypanosomosis. Furthermore, trypanosome infections were mainly detected in sampling sites in close proximity to the Kafue National Park [Nakalombwe (53.5%) and Kakuse (51.2%)] where the tsetse vectors and wildlife reservoirs are in abundance.

Taken together, these results indicate a re-surgence of bovine trypanosomosis in the Choma-Kalomo block. Furthermore, data from this study suggest that LAMP is a much more sensitive and reliable technique for detection of bovine trypanosomes from field samples. Loop-mediated isothermal amplification is cheaper, quicker, simple and may easily be performed in the field. Thus, LAMP has the potential as an alternative molecular technique for detecting trypanosome infections from field specimens.

DEDICATION

This work is dedicated to my wife Carolyn, for her patience, encouragement and support she rendered during my studies, and to my parents, Mr and Mrs Bukowa for supporting my early childhood education that made me reach the level of tertiary education.

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LIST OF ABBREVIATIONS AND ACRONYMS

AAT	Animal African trypanosomosis
DNA	Deoxyribonucleic acid
DVLD	Department of Veterinary and Livestock Development
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation of the United Nations
HAT	Human African trypanosomosis
IFAT	Indirect fluorescent antibody test
ISCTRC	International Scientific Council for Trypanosomosis Research and Control
LAMP	Loop-mediated isothermal amplification
NALEIC	National Livestock Epidemiology and Information Centre
PCR	Polymerase chain reaction
PCV	Packed cell volume
RIME	Repetitive insertion mobile element
RPM	Revolutions per minute
RTTCP	Regional Tsetse and Trypanosomosis Control Programme
SRA	Serum resistant associated genes
SSA	Sub-Saharan Africa
VSG	Variant Surface glycoprotein
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

1.1 Background

African trypanosomes are extracellular protozoan haemoparasites causing sleeping sickness in man or *nagana* in livestock in sub-Saharan Africa (SSA). Human and animal trypanosome infections have serious socio-economic implications such that they remain a major obstacle to overall development in the region (Okomo-Assoumou *et al.*, 1995; Taylor, 1998; Njiru *et al.,* 2004). Whereas *Trypanosoma brucei rhodesiense* (*T. b. rhodesiense*) causes acute human African trypanosomiasis (HAT) in East and Southern Africa, *T. b. gambiense* causes chronic HAT in West and Central Africa (MacLean *et al*., 2007). About 60 million people are at risk of contracting HAT in the 37 sub-Sahara African countries (Chappuis *et al*., 2005).

Tsetse-transmitted animal infective trypanosomes include the highly virulent *T. vivax* and *T. congolense* and the lower virulent *T. b. brucei* that cause *nagana* in cattle and other domestic animals as well as *T. simiae* causing an acute fatal disease mainly in pigs in sub-Saharan Africa (Moloo *et al*., 2008). *Trypanosoma evansi* is mechanically transmitted by haematophagous arthropods, causing surra mainly in camels and horses in Asia, Middle East, North Africa, Central and South America (Brun *et al*., 1998). Recently a case of human infection of *T. evansi* has been reported in India making it a potential human pathogen (Joshi *et al*., 2005). The third animal trypanosome syndrome is Dourine, a sexually-transmitted infection of horses caused by *T. equiperdum* in Asia, Africa, South-Eastern Europe and South America (Brun *et al*., 1998; Gilbert, 1998).

The annual direct production losses due to *nagana* in cattle alone are between US$6,000 and US$12,000 million in the 37 tsetse-infected sub-Sahara African countries (Hursey and Slingenbergh, 1995). However, this is an under-estimation considering the enormous indirect losses due to reduction in livestock numbers, use of cattle manure and draught power, as well as increased veterinary expenses. Moreover, at least 10 million km^2 of potential grazing land is unsuitable for livestock production, putting over 46 million cattle, along with millions of sheep and goats, at risk of contracting animal African trypanosomosis (AAT) (Hursey and Slingenbergh, 1995).

Current control methods of AAT include (i) vector control using insecticide-impregnated targets and traps, (ii) reducing proximity of livestock to the vector and reservoir hosts, (iii) use of trypanocides and (iv) keeping trypanotolerant livestock (Taylor, 1998; Aksoy *et al.*, 2003). However, none of these methods or their combination gives absolute control. This is mainly due to (i) rapid evolution of drug-resistant trypanosomes, (ii) limited effectiveness of vector control methods, (iii) lack of an effective anti-trypanosome vaccine as a result of antigenic variation associated with trypanosomes (Deitsch *et al*., 1997; Taylor, 1998; Marcello *et al.,* 2007).

Along with Malawi, Mozambique and Zimbabwe, Zambia lies within the 'common fly belt' in which *Glossina morsitans morsitans* (*G. m. morsitans)* and *G. pallidipes* are the most abundant tsetse fly species (Fig.1). The Zambian part of the common fly belt is mainly centered on the Luangwa river valley and further extends to the Zambezi valley and their adjacent areas, which has a large population of wildlife in game parks, together with some domestic animals (Symeonakis *et al.,* 2007). The Choma-Kalomo block is an area of approximately 4,500 km^2 within the common tsetse fly-belt closer to the Kafue National Park (Lovemore, 1989). It

incorporates fairly large parts of Choma, Kalomo and small sections of Namwala and Itezhi tezhi districts and is an important agriculture area. The main activities in Choma-Kalomo block are crop farming and livestock rearing. However, trypanosomosis and other vector-borne diseases including theileriosis/East Cost Fever, anaplasmosis, babesiosis and heartwater, negatively affect livestock production.

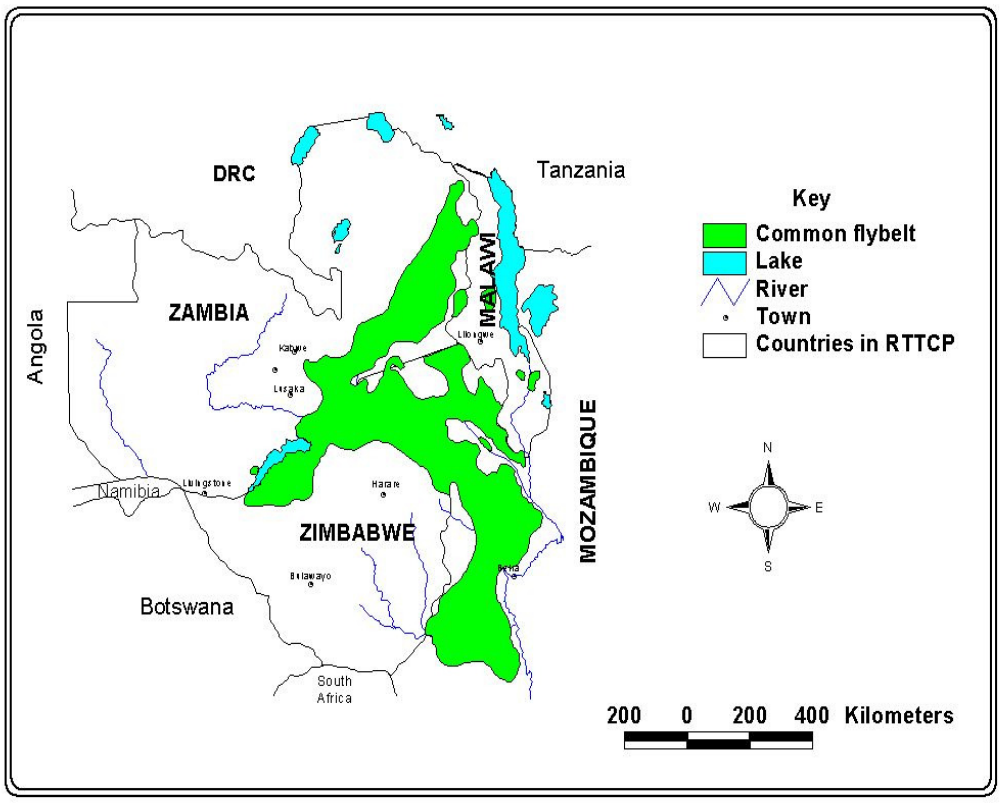

Figure 1: The common tsetse fly belt of approximately 322,000 km^2, covering Malawi, Mozambique, Zambia and Zimbabwe (Courtesy of RTTCP Zimbabwe, 1995)

The overall objective of the Regional Tsetse and Trypanosomosis Control Programme (RTTCP) of Malawi, Mozambique, Zambia and Zimbabwe was to eradicate the tsetse flies from a discrete common fly-belt of approximately 322,000 km^2 within the Zambezi, Rio Púngoè, Rio Buzi and Rio Save drainages and hence control trypanosomosis (Connor, 1989). As such, about 4,500 km^2 of the Choma-Kalomo block was aerial-sprayed in 1987. Surveillance results indicated that tsetse flies were successfully eradicated and trypanosomosis survey further revealed absence of trypanosomes from this important agricultural area (Connor, 1989). Furthermore, enhanced agricultural activities were also reported in the area including reduced livestock mortality rates, increased calving rates, increased livestock population and crop production (DVLD, 2003).

However, recent field reports from Choma-Kalomo block indicate a re-surgence of trypanosomosis (personal communication, Silavwe, Southern Province Principal Biologist and Soko, Choma district Veterinary Officer). This study thus aimed to investigate the prevalence and risk factors of bovine trypanosomosis in the Choma-Kalomo block.

1.2 Study justification

About 4,500 km^2 of the Choma-Kalomo block was aerial-sprayed in 1987 in order to eradicate the tsetse flies and trypanosomosis. This successfully eradicated the tsetse fly vectors in the area and led to improved cattle productivity and associated agricultural activities (Connor, 1989; DVLD, 2003). Unfortunately, recent field reports from Choma-Kalomo block indicate a re-surgence of trypanosomosis (Personal communication, Silawve, Southern Province Principle Biologist; Soko, Choma district Veterinary Officer). As such, there was need to conduct systematic studies to determine the prevalence of re-emerging trypanosomosis in that important agriculture region of Zambia.

1.3 Objective of the study

1.3.1 General objective

The main aim of the study was to determine the epidemiology of the re-emerging trypanosomosis in the Choma-Kalomo block of Southern Province of Zambia.

1.3.2 Specific objectives

1.3.2.1 To compare the sensitivity of microscopy and LAMP in the detection of trypanosomes in bovine blood samples from the Choma- Kalomo block.

1.3.2.2 To identify the trypanosome species found in Choma-Kalomo block.

1.3.2.3 To investigate the influence of cattle age and sex on the prevalence of trypanosomosis in Choma-Kalomo block.

1.3.2.4 To identify the species of tsetse flies present in Choma-Kalomo block.

CHAPTER 2: LITERATURE REVIEW

The prevalence of trypanosomosis relies on four interacting organisms: (i) the pathogenic parasite, (ii) the insect vector, (iii) the wild animal reservoir and (iv) the vertebrate host. While complex, this dependence on multiple players provides several opportunities for interventions since the interruption of any of these interactions can potentially reduce disease transmission (Butler, 2003; Aksoy *et al.,* 2003).

2.1 The Parasite

The causative agent of trypanosomosis is a protozoan parasite which belongs to the order Kinetoplastida, family Trypanosomatidae and genus *Trypanosoma*. The taxonomic classification of trypanosomes of interest in the present study is summarized as follows:

Kingdom:	Protista
Subkingdom:	Protozoa
Phylum:	Sarcomastigophora
Class:	Zoomastogophora
Order:	Kinetoplastida
Suborder:	Trypanosomatina
Family:	Trypanosomatidae
Genus:	*Trypanosoma*
Section:	Salivaria

On the basis of the site of development of the organism in the insect vector that transmits them, the genus *Trypanosoma* is divided into two sections: stercoraria and salivaria. Trypanosomes in

the stecoraria section such as *T. cruzi* which causes Chagas' disease in humans in Latin America, develop in the hindgut of the vector and are thus transmitted to the mammalian host via faeces (Engman and Leon, 2002; Benvenuti and Gutierrez, 2007). In contrast, Salivarian trypanosomes, encompassing most of the African trypanosomes such as *T. congolense, T. b. brucei, T. simiae* and *T. vivax*, develop in the anterior portion of the fly's digestive tract and are transmitted to the mammalian host via the saliva when an infected vector takes a blood meal (Barret *et al*., 2003). However, *T. vivax* may also be mechanically transmitted on the mouthparts of biting diptera and other arthropods (Yaman *et al.,* 2003; Desquesnes and Dia, 2004).

Salivarian trypanosomes are further divided into four major subgenera: *Dutonella, Nannomonas, Trypanozoon* and *Pycnomonas* (Majiwa *et al*., 1999). *Trypanosoma vivax* (subgenus *Dutonella*), *T. congolense* (subgenus *Nannomonas*) and the *T. brucei* complex (subgenus *Trypanozoon*) are the most economically important and widely spread species affecting domestic animals (Nonga and Kambarange, 2009). *Trypanosoma congolense* is considered to be the most pathogenic species in domestic ruminants in Southern and East Africa (Marcotty *et al.,* 2007). In Eastern Zambia for instance, about 34% of cattle have trypanosomosis, mainly caused by *T. congolense* (Marcotty *et al.,* 2007; Simukoko *et al.,* 2007). On the other hand, *T. vivax* is the most frequent cause of trypanosomosis among cattle in West Africa (Yaman *et al.,* 2003).

African trypanosomes are unusual among protozoan parasites in that they never enter the host's cells and yet persist for extended periods of time in mammalian blood and other tissues. They are protected from hostile immunological environment of the host by a thick protein coat called the variant surface glycoprotein (VSG) (Marcello *et al.,* 2007). Moreover, they constantly modify

their VSG by a process of antigenic variation (Pays *et al.,* 1994; Taylor, 1998; Lythgoe *et al.,* 2007). The host's antibodies facilitate the neutralization and killing of approximately 99% of the original protozoan population. However, during this time a few of the trypanosomes would have shed their coat, switched original VSG, and covered themselves with a new antigenically distinct VSG coat, which helps to give rise to a new population expressing the new VSG coat. The immune system again responds to this proliferated population by producing a new set of antibodies that successfully kill most of the trypanosome population. However, VSG switching among a small portion of trypanosomes renders them undetectable, and they successfully evade the host immune response. This cycle continues indefinitely, eventually causing the demise of the host (Deitsch *et al.,* 1997; Namangala, 2011).

2.2 The Tsetse Fly Vector

African trypanosomes are mainly transmitted by tsetse flies which belong to the genus *Glossina*, order diptera of different species only found in Africa. The genus *Glossina* consists of 23 species within which three groups are recognized on the basis of their preference for habitant. These groups are: (i) the riverine (*palpalis*) group, (ii) the forest (*fusca*) group and (iii) the savannah (*morsitans*) group (Geiger *et al.,* 2005).

The *palpalis* group consists of *Glossina fusca fuscipes* (*G. f. fuscipes*), *G. f. martini* and *G .f. quanzaenesis*. *Glossina palpalis* occupies the Congo basin, the basin of the upper Nile and other rivers of West Africa (Hargrove, 2003a; Geiger *et al.,* 2005). The *fusca* group may be divided into three sub-groups (Weitz and Glasgow, 2009). The first sub-group includes: *G. tabaniformis*, *G. haningatoni* and *G. nashi.* These are confined to the Congo basin and the forest of Gabon,

Southern Cameroon and the Guinea coast as far as Liberia. The second sub-group is distributed peripherally around the fly-belts of the forest of Gabon and West African Guinea coast and includes *G. fusca*, *G. medicorum*, *G. fusciepleuris* and *G. schwetzi*. The third sub-group includes *G. longipenis* and *G. brevipalpis.* These tsetse species are confined to Sudan (south east comer), Ethiopia (southern border), Somalia, Kenya and Tanzania (north eastern quarter) including South Africa in the south. The *morsitans* group includes *G. m. morsitans* and *G. m. submorsitans*. They are found in the deciduous woodlands and savannah vegetations forming a band around the main forest of the Congo basin and Guinea coast in West Africa (Van den Bossche and De Deken, 2002; Gieger *et al*., 2005). Tsetse flies belonging to the morsitans group are more effective vector of trypanosomes than the *palpalis* group (Laveissiere *et al.,* 1990).

The groupings simply reflect the fact that all tsetse flies have a need for a humid environment and protective shade from sunlight, which the leafy vegetation provides. Tsetse flies are found in Africa in a belt that stretches south of the Sahara and north of Kalahari desert between 14º North and 29º South of the equator (Aksoy and Rio, 2005). They feed exclusively on blood and transmit the parasites from one host to another during feeding (Aksoy *et al*., 2003).

Tsetse distribution has a spatially discrete pattern (Barret *et al*., 2003). In southern Africa, the distribution of tsetse flies occurs in (i) areas where livestock are absent, (ii) zones where livestock have been introduced in game areas with game still abundant and representing a major source of food for tsetse, (iii) areas where, due to human interference, the density of game animals is low with livestock as a main source of food for tsetse flies and finally (iv) areas where

livestock occupy the edge of tsetse-infested wildlife zones (game/livestock interface) (Van den Bossche and Vale, 2000).

2.3 The Trypanosome Hosts

Trypanosomes are found throughout the world and parasitise mammals, birds, reptiles, amphibians and fish (Hamilton *et al.,* 2004). Table 1 shows the vertebrate hosts of salivarian trypanosomes and their mode of transmission.

Table 1: Distribution of vertebrate hosts and mode of transmission of salivarian trypanosomes

Subgenus	Species	Geographical Distribution	Vertebrate host	Cyclic transmission	Non- Cyclic Transmission
Duttonella	*T.vavix*	West, Central and East Africa	Wild and domestic ungulates	*Glossina*	Biting Diptera
		West indies, South America		None	
Nannomonas	*T.congolense*	West, Central and East Africa	Ungulates+ carnivores	Glossina	Biting Diptera
	T.simiae	West, Central and East Africa	mainly siuds	Glossina	
Pycnomonas	*T. sius*	Central Africa	Suids	Glossina	Not known
Trypanozoon	*T.brucei*	West, Central and East Africa	carnivores + humans	Glossina	Biting Diptera
	T.evansi	North Africa, Asia, Central and South America	ungultes+carnivore	none	Biting Diptera
	T.equiperdum	Africa, Asia, South America	Equines	none	Coitus

Source: Hamilton *et al.,* 2004

In wild animals, such as warthogs, bushbucks, kudus or buffalos, trypanosomes become established but do not produce disease. This is because these animals and the parasites have evolved for many years such that a balanced relationship exists (Taylor, 1998; Aksoy *et al*.,

2003; Namangala, 2011). In domestic animals, the host/parasite relationship has not fully developed, therefore development of the disease occurs (d'Ieteren *et al.,* 1998; Namangala, 2011). Among the domesticated animals, humpless cattle such as the N'dama of West Africa (*Bos indicus*) were the first to be introduced into northern and western Africa from about 4,500 BC, hence they are adapted to tsetse-transmitted trypanosomes and are thus trypanotolerant (d'Ieteren *et al.,* 1998). The humped Zebu-type Boran (*Bos taurus*) which are more trypanosusceptible, were introduced into Africa about 3,000 years later than the *Bos indicus.* Goats and Sheep were introduced at almost the same time about 2,500 years later hence the host/parasite relationship has not fully developed and these species develop clinical disease when infected with the parasites (Taylor, 1998; d'Ieteren *et al.,* 1998).

Mammalian hosts may possess natural resistance to particular species of trypanosomes. For instance, cattle are not likely to be infected with *T. simiae* (Moloo *et al*., 2008). The human-infective and highly pathogenic *T. b. rhodesiense* is maintained in wild and domestic animals (Fevre *et al*., 2001; Acha and Szyfres, 2003; Ochan, 2004) where it does not cause disease. Furthermore, *T. b. brucei* is distinguished from the other two closely related species (*T. b. rhodesiense* and *T. b. gambiense*) in that it does not cause infection in man (Njiru *et al*., 2004).

2.4 Diagnosis of Trypanosomosis

The field diagnosis of AAT is difficult mainly because clinical and post-mortem signs of the disease are not pathognomonic. Therefore diagnosis must rely on laboratory techniques that confirm the presence in blood of trypanosomes or the presence of anti-trypanosomal antibodies.

2.4.1 Diagnosis of trypanosomosis by clinical signs and post-mortem findings

The course of trypanosomosis in cattle is variable depending on the factors associated with the host and the parasite. Generally, *nagana* in cattle and other domestic animals is characterized by the intermittent presence of parasites in the blood, intermittent fever, wasting, lymphadenopathy, lacrimation and abortion in pregnant animals. Adult animals are considered to be more susceptible to trypanosomosis than young animals (Taylor and Authie, 2004). According to Torr *et al*. (2006) and Torr and Mangwiro (2000), tsetse flies are attracted significantly more by odour of large animals (i.e. oxen, bulls, cows) and least by calves. The infected animal lags behind the herd (Pentreath, 1995; Masamu *et al.,* 2006). The course of the infection may be acute (few weeks), subacute or chronic (several months to one year or longer) and animals may die if untreated. Anaemia is thought to be the most pathogenic consequences of infection with African trypanosomes (Sigauque *et al.,* 2000; Van den Bossche and Rowlands, 2001).

Post-mortem examination of an animal that died of AAT reveal generalized carcass emaciation, enlarged lymph nodes, enlarged liver and petechial haemorrhages of the serosal membranes, especially in the peritoneal cavity (Taylor and Authie, 2004). Although these findings could help veterinarians suspect AAT in a herd, they are not fully pathognomonic as several other disease conditions including malnutrition, tuberculosis and intestinal helminthes may exhibit similar clinical signs and post-mortem findings (Taylor and Authie, 2004) (Fig. 2).

Figure 2. Cow suffering from animal African trypanosomosis

2.4.2 Parasitological diagnosis

The detection of circulating trypanosomes in host blood, cerebral spinal fluid and lymph node biopsy by light microscopy is a specific and definitive diagnosis of trypanosomosis (Chappuis *et al*., 2005; WHO, 2006). Body fluids such as blood can be directly examined as wet smears for trypanosome presence by light microscopy. The trypanosomes are detected by their movement among the blood cells (Hopkins, 1995; Chappuis *et al*., 2005). However, the disadvantage of this technique is its low sensitivity, often less than 50% (Yadvendra *et al*., 1998). To increase the sensitivity of this method, animal blood in a heparinised capillary tube may be centrifuged and the resultant buffy coat examined in an intact capillary tube or as wet smear on a blood slide (Woo and Rogers, 1974; Hopkins, 1995; Vreysen *et al*., 2004). The other advantage of

centrifuged heparinised capillary tube is that packed cell volume (PCV) can be determined at the same time (Vreysen *et al*., 2004).

In addition, parasites may be examined on Giemsa-stained thin or thick smears. Thin blood films preserve the morphology of trypanosomes and hence are useful in morphological differentiation of species (Hopkins, 1995; WHO, 2006; Buscher *et al*., 2009). The trypanosomes on stained Giemsa preparation reveal characteristic features including the size, shape, flagellum, undulating membrane, nucleus, kinetoplast and basal body (Table 2), all of which can be used to identify animal or human infective trypanosome species. Thick smears are useful for the detection of scanty trypanosomes when parasitaemia is low (Hopkins, 1995).

Table 2: Morphological structure of animal infective trypanosome

Species	**Size**	**Flagellum**	**Undulating Membrane**	**Shape of Posterior end**	**Size of kinetoplast**	**Position of Kinetoplast**
T.congolense	8-24µ	no free flagellum	not developed	Round	Medium	Marginal and sub terminal
T.vivax	18-31µ	Free flagellum	Slightly developed	Large and round	Large	Terminal
T.brucei	11-39µ	In slender form free flagellum	Developed	Bluntly pointed	Small	Marginal and sub terminal

Source: Hopkins, 1995

2.4.3 Xenodiagnosis

Xenodiagnosis is the inoculation of trypanosome-infected blood into rodents such as mice or rats. This technique can be used to detect some *T. congolense* and *T. brucei* complex (but not the non-rodent-adapted *T. vivax*) infections (Gathuo *et al*., 2007). The rodent inoculation technique is useful when the parasitaemia is scanty. However, its disadvantage is that not all trypanosome species, including some strains of *T. congolense* and *T. brucei* complex become established in rodents (Duleu *et al.,* 2004).

2.4.4 Serological diagnosis

The development of anti-trypanosomal antibody detection technique has been a major improvement in the serodiagnosis of AAT. Commonly used techniques include the indirect fluorescent antibody test (IFAT) (Luckins and Mehlitz, 1978) and the enzyme-linked immunosorbent assay (ELISA) (Luckins and Mehlitz, 1978; Greiner *et al.,* 1997; Hopkins *et al.,* 1998; Monzon *et al*., 2003). However, the disadvantages of serodiagnosis are (i) antibody ELISAs are not species-specific because of strong cross reactions between the pathogenic *trypanosoma* species (Desquesnes *et al.,* 2001) and (ii) no distinction can be made between the past and present infection restricting their usefulness to measuring challenge.

2.4.5 Molecular diagnosis

Molecular techniques such as polymerase chain reaction (PCR) have significantly improved the sensitivity and accuracy of trypanosome diagnosis compared to the traditional parasitological methods (Mugittu *et al.,* 2001). Molecular tests differentiate between trypanosome species and subspecies using specific primers (Desquesnes and Davila, 2002; Geysen *et al.,* 2003). Loop-mediated isothermal amplification (LAMP) is a new DNA amplification method and has been

applied in the detection of African trypanosomes (Thekisoe *et al*., 2007). Loop-mediated isothermal amplification uses four primers in the initial steps (F3, B3, FIP and BIP) and two primers in the subsequent steps (FIP and BIP) in order to ensure high specificity for target amplification (Iwasaki *et al*., 2003; Thekisoe *et al*., 2007; Njiru *et al*., 2008). Unlike PCR, LAMP does not require sophisticated equipment (all it requires is a water bath or a heating block) and is thus cheaper (only cost about $1 USD per test) and simpler to use. Furthermore, amplification of DNA during LAMP reactions occurs at constant temperature (60-65°C) within about 30 minutes, producing large amounts of DNA that can be visualized with naked eyes (Notomi *et al*., 2000). Moreover, LAMP has been recently developed for use in the diagnosis of both AAT and HAT and may be a better alternative molecular diagnostic method in resource-poor countries such as Zambia (Picozzi *et al*., 2002; 2008).

2.5 Treatment and control of animal African trypanosomosis

2.5.1 Use of trypanocidal drugs

There are a number of drugs available for the treatment of AAT (Van den Bossche *et al*., 2006). Early diagnosis of the disease is important for effective treatment. The choice of drug, dosage and route of administration vary depending on the animal species affected, local preference and presence or absence of trypanosome drug resistance (McDermott *et al*., 2003; Tuntasuvan *et al*., 2003). Diminazene aceturate (Berenil®) offers numerous advantages for use in AAT-infected cattle and other domestic animals including (i) its high activity against *T. congolense* and *T. vivax,* particularly on those strains resistant to other trypanocides, (ii) its low toxic effects in cattle and (iii) its easy utilization (Taylor, 1998). Furthermore, cattle infected with *T. congolense*, T. *vivax* or *T. b. brucei* may be treated curatively with Diminazene aceturate (Berenil®) or

prophylactically with Isometamidium (Samorin®). Berenil, as well as Suramin and Quinapyramine, are effective in the treatment of surra (Tuntasuvan *et al*., 2003).

The appearance of resistant strains of trypanosomes has been associated with the extensive and prolonged usage of the few anti-trypanosomal drugs (Diminazene aceturate, Homidium, Isometamidium, Quinapyramine and Suramin) available on the market. Evidence of drug resistance in several areas in East and Southern Africa exist (Chitambo and Arakawa, 1992; Anne *et al*., 2001; Geerts *et al.,* 2001; Sinyangwe *et al*., 2004). Currently, single or multiple trypanocidal drug resistant has been reported in 17 African countries where the problem is increasing and spreading at a fast rate (Delespaux *et al*., 2008).

2.5.2 Vector control

Biological control of insect vectors (tsetse flies) involves clearing of vegetation, game elimination (Allsopp, 2001) and use of sterile insect technique (Vreysen *et al*., 2004). Vegetation clearance results in denial of a suitable tsetse habitat while destruction (shooting) of wild animals deprives the flies of blood meal sources and may lead to their starvation and death (Allsopp, 2001; Aksoy *et al.*, 2003). This latter method proved to be successful in Zimbabwe in the 1930s as large areas of the country were freed from tsetse flies following shooting of large wild animals in the Zambezi basin (Allsopp, 2001). However, game and vegetation destruction are unpopular among conservationists and unsustainable due to their implications on the environment (Allsopp, 2001; Aksoy *et al.*, 2003). Furthermore, the sterile insect technique requires a substantial reduction in the fly population via other control methods to levels at which sterile males can successfully compete with the wild non-sterile males (Mkanyi and Feldmann, 2000; Vreysen *et al.,* 2004).

Tsetse flies may also be controlled through use of traps and insecticide-impregnated targets (Barret *et al*., 2003; Hargrove, 2003a). However, the cost of maintaining traps and targets and their destruction by local communities limit their success.

The use of insecticide (e.g. Pyrethroid) treated livestock in tsetse control appears to be cost effective (Eisler *et al*., 2003). In this technique, tsetse flies ingest a lethal dose of insecticide during a blood meal taken from an animal which has been hand-sprayed, dipped or had pour-on application (Vale *et al.,* 1999; Eisler *et al*., 2003). Although this has been done with a lot of success (Chizyuka and Luguru, 1986; Thomson and Wilson, 1992; Bauer *et al*., 1995), the effective application of insecticides onto the animal requires special skills (Hargrove *et al.,* 2003b).

Sequential aerial spraying with organo-chloride compounds is very expensive but may lead to vector and AAT eradication (Aksoy *et al*., 2003). Aerial spraying has recently been successfully done in Botswana, Namibia, Angola and the Kwando-Zambezi region of Zambia in an attempt to eradicate tsetse flies in the region. However, it has been shown that re-infestation of the vector in the previously cleared areas occurs resulting in establishment of AAT as evidenced in Nigeria (Omotainse *et al.,* 2004), Zambia - Siavonga tsetse belt, (DLVD, 2003) and Choma-Kalomo block (Personal communication, Silawve, Southern Province Principal Biologist; Soko, Choma district Veterinary Officer).

CHAPTER 3: METHODOLOGY

3.1 Study area

The study was carried out in Choma-Kalomo block (Fig. 3) of the Southern province of Zambia in December 2009 during the rainy season. The Choma-Kalomo block, approximately 4,500 km^2 in size, is an agriculture zone which incorporates fairly large parts of Choma, Kalomo and a small section of Namwala and Itezhi tezhi districts.

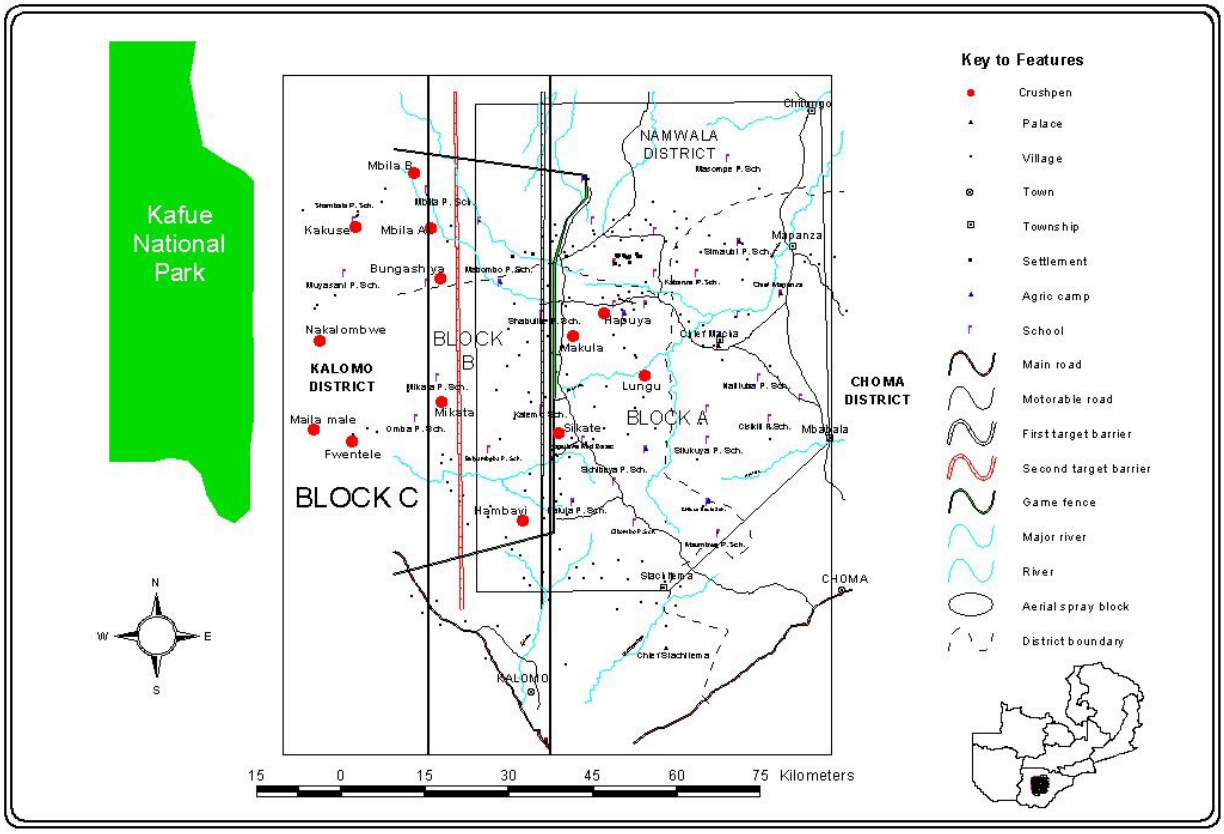

Figure 3: Map showing the study area and the sampling sites

(Source: RTTCP, Zambia, 2002)

3.2 Sampling sites and sample size calculation

A cross sectional study was carried out. As shown in figure 3 above, a total of 13 sampling sites were identified. Geo-spatial references of the selected crush pens are shown in table 3.

Table 3: Location and geo-spatial references of selected crush pens as sampling site

District	Veterinary camp	Crush pen name	Geo-spatial reference	
			Latitude	**Longitude**
Choma	Lungunya A	Lungu	S: 16.45846	E: 026.23309
		Hapuya	S: 16.27259	E: 026.31499
		Sikaate	S: 16.38766	E: 026.48587
		Makula	S: 16.38764	E: 026.43072
Itezhi tezhi	Basanga	Mbila A	S: 16.17338	E; 026.31499
		Mbila B	S: 16.22934	E: 026.33706
		Kakuse	S: 16.38676	E: 026.49305
Kalomo	Lungunya B	Hambayi	S: 16.43545	E: 026.38589
		Bungashiya	S: 16.36614	E: 026.14082
	Nkanda zovu	Mikata	S: 16.46427	E: 026.30394
		Fwentele	S: 16.55185	E: 026.13389
		Nakalombwe	S: 16.38044	E: 026.15285
		Maila Male	S: 16.49340	E: 026.15802

The sample size was determined as described by Cannon and Roe (1982), with an estimated trypanosomosis prevalence of 1%, confidence level of 95% and total cattle population of 75,000:

$$n = \left(1-(1-P)^{1/d}\right) * \left(N - \frac{(d-1)}{2}\right)$$

Where;

n = Sample size.

P = is the prevalence = 1%.

d = number of (detectable) cases in the population

N = is the population size of cattle estimated at 75 000 (DVLD, 2003).

Thus, we had to sample a minimum of 299 cattle. Due to overwhelming response from the farmers, a total of 460 heads of cattle were actually sampled in this study.

A proportional simple random sampling method was applied to select animals at each crush pen. During sampling of cattle, the following parameters were recorded (i) PCV, (ii) animal age and (iii) sex. Cattle of 12 to 48 months were considered calves, while those above 48 months of age (cows, bulls and oxen) were considered adults. Calves were kept at homestead for the purpose of milking and were not sampled.

3.3 Blood collection and diagnosis

Blood was collected from the superficial ear vein into three heparanised microhaemetocrit capillary tubes from each animal. The capillary tubes were sealed with "cristaseal" (Hawksley, UK) and centrifuged immediately in a haematocrit centrifuge for 5 minutes at 2,500 rpm and PCV values were determined and recorded. The buffy coat was examined as a wet preparation for motile trypanosomes under the light microscope (Chappius *et al.,* 2005). Thin blood smears

were further made from trypanosome positive samples and stained with Giemsa for species identification by light microscope (WHO, 2006; Buscher *et al*., 2009). The trypanosome species were distinguished with reasonable accuracy by microscopy using their size, position of the kinetoplast, presence of undulating membranes and length of the free flagella in the laboratory (Picozzi *et al.,* 2002). Furthermore, trypanosome-infected cattle were treated with anti-trypanosomal drugs - Diminazene aceturate (Berenil®) or Isometamidium (Samorin®) during sampling. All the above procedures were done in the field at the sampling site (Fig. 4).

Figure 4: Examination of buffy coat preparation at the sampling site

The buffy coat and uppermost layer of red blood cells of remaining two centrifuged heparanised microhaemetocrit capillary tubes of each specimen were extruded onto a labelled filter paper (Whatman no. 1, Whatman®) as buffy coat spot (Fig. 5) for extraction of trypanosomal DNA. The buffy coat spots were kept in zip lock plastic bags containing silica gel at -20°C until DNA extraction.

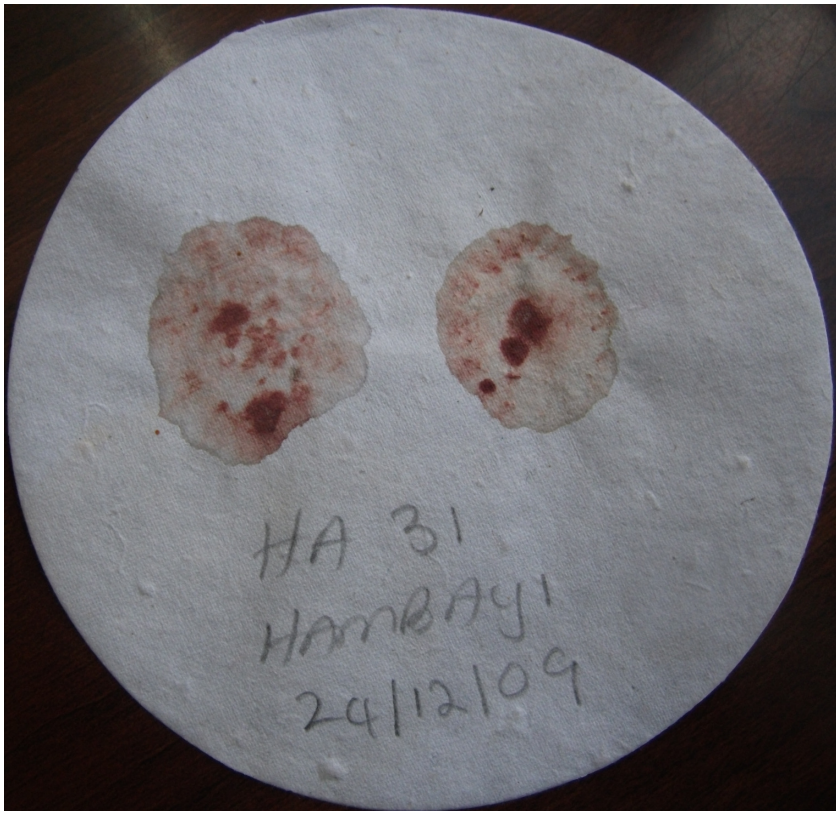

Figure 5: Buffy coat spots on filter paper

3.3.1 DNA extraction

In order to extract trypanosome DNA from filter paper spots, a modified methanol fixation method as described by Johanson *et al.* (2009) was adopted. The buffy coat on the filter paper was first fixed with 100µl of 100% methanol and then left to dry at room temperature for 20 minutes. After drying, 3 sample disks of at least 3mm diameter were punched (using a manual one-hole punch) from the center of the buffy coat spot and further fixed with 100µl of 100% methanol three times (first time left to dry at room temperature for 20 minutes and twice incubated at +37°C for 40 minutes after each fixation process). The dried disks were placed in labelled 0.5ml sterile reaction tubes containing 40µl of elusion buffer (10µl of 10X PCR buffer + 90µl of double distilled water), and later heated at 95°C in a heating block for 30 minutes. After pulse vortexing the tubes, the filter paper disks were removed using sterile tip and discarded while the eluted DNA was stored at -20°C until use.

3.3.2 Loop-mediated isothermal amplification

A LAMP reaction of 25 µl was performed using a Loopamp DNA Amplification Kit (Eiken Chemical, Tochigi, Japan) and the parasite DNA as template, according to the manufacturer's instructions, with minor modifications. Briefly, 2 µl of template DNA was added to a 23 µl master mix containing 12.5 µl of reaction buffer (40 mM Trish-HCl (pH 8.8), 20 mM KCl, 16 mM $MgSO_4$, 20 mM $(NH_4)_2SO_4$, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM of each dNTP), 1 µl of *Bst* DNA polymerase enzyme (provided in the Loopamp DNA Amplification kit), 1 µl Loopamp fluorescent detection reagent (Eiken Chemical, Tochigi, Japan), 2 µl primer mix and 6.5 µl distilled water. Table 4 shows a summary of the LAMP primers used in this study for the amplification of *T. b. brucei* (Njiru *et al*., 2008), *T. b. rhodesiense* (Radwanska *et al*., 2002) and *T. congolense* (Thekisoe *et al.,* 2007). The primer mixes were made as follows: (i) *T. congolense*

amplification targeting the CON2 18s rRNA gene (BIP and FIP at 40 pmol each, F3 and B3 at 5 pmol each); (ii) *T. b. brucei* amplification targeting the repetitive insertion mobile element (RIME) gene (FIP and BIP at 40 pmol each, Loop F and Loop B at 20 pmol each, F3 and B3 at 5 pmol each); (iii) Furthermore, all RIME-LAMP positive samples were tested for *T. b. rhodesiense* targeting the human serum resistant associated genes (SRA), (FIP and BIP at 40 pmol each, Loop F and Loop B at 20 pmol each, F3 and B3 at 5 pmol each). The reaction mixture was incubated at 64^0C for 30 minutes in a heat block (Dry Thermounit DTU 1B, TAIEC Co., Saitama, Japan) and then at 95^0C for 2 minutes to terminate the reaction. The LAMP products were visualized using a transilluminator (WD, H19, Good design award Co., Japan).

Table 4: LAMP primers used for the amplification of *T. b. brucei, T. b. rhodesiense* and *T. congolense*

Target gene	Primer	Sequence	Specificity	Reference
RIME	FIP	5'-GGAATACAGCAGATGGGGCGAGGCCAATTGGCATCTTTGGGA-3'	*Trypanozoon*	Njiru *et al*., 2008
	BIP	5'-AAGGGAGACTCTGCCACAGTCGTCAGCCATCACCGTAGAGC-3'		
	F3	5'-CTGTCCGGTGATGTGGAAC-3'		
	B3	5'-CGTGCCTTCGTGAGAGTTTC-3'		
	LF	5'-GCCTCCCACCCTGGACTC-3'		
	LB	5'-AGACCGATAGCATCTCAG-3'		
SRA	FIP	5'-GGACTGCGTTGAGTACGCATCCGCAAGCACAGACCACAG-3'	*T. b. rhodesiense*	Radwanska *et al*., 2002
	BIP	5'-CGCTCTTACAAGTCTTGCGCCCTTCTGAGATGTGCCCACT-3'		
	F3	5'-GCGGAAGCAAGAATGACC-3'		
	B3	5'-TCTTACCTTGTGACGCCTG-3'		
	LF	5'-CGCGGCATAAAGCGCTGAG-3'		
	LB	5'-GCAGCGACCAACGGAGCC-3'		
CON2 18s rRNA	FIP	5'-GCGCATGCGTCGGTGTTATTTTCGCGTGTGTGTTCATGTCA-3'	*T. congolense*	Thekisoe *et al*., 2007
	BIP	5'-ACTCTCCCCCCAAAATGGTTGTCCAAGCACGCAAATTCACAT-3'		
	F3	5'-TGTGTGTTTGTCGTGGAAGC-3'		
	B3	5'-ATTCGTGACCGCGTCAAA-3'		

3.4 Tsetse fly capture and identification

Epsilon traps (Fig. 6) were set at each sampling site in different locations where cattle graze for trapping tsetse flies as previously described by Barret *et al*. (2003). The species and sex of the caught tsetse flies were recorded and identified according to the method described by Krinsky (2009).

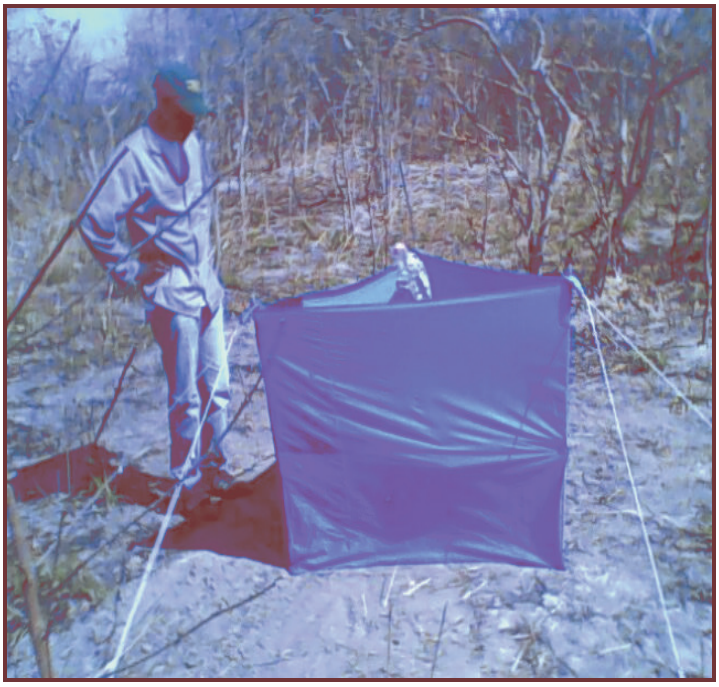

Figure 6: Epsilon trap used to capture tsetse flies

3.5 Data analysis

Data was stored in basic excel format for easy handling and storage. The data was transferred to Stata / SE 8.0 (©1994 – 2003. Stata Corporation) for statistical analysis. Independent sample t-test was used to determine the association between continuous variables, while fisher's exact test was used to determine the association between categorical variables. Odds ratio was used to measure the effect of age and sex on trypanosomosis. P values < 0.05 were considered statistically significant.

CHAPTER 4: RESULTS

4.1 Comparison of trypanosome detection in cattle blood samples from the Choma-Kalomo Block by Microscopy and LAMP

A total of 460 heads of cattle comprising 205 (44.6%) cows, 152 (33.0%) oxen, 56 (12.2%) female calves, 33 (7.2%) male calves and 14 (3.0%) bulls, were sampled from 13 crush pens within the Choma-Kalomo block. According to the results obtained by microscopic examination of the Giemsa-stained cattle blood smears, 22 (4.8%; 95% CI: 2.8 – 6.7%) of the animals were infected with trypanosomes. Of those infections, 21 *(*4.6%; 95% CI: -4.2 – 13.4%) were caused by *T. congolense* (Fig.7A) while only 1 (0.2%; 95% CI:-1.7 – 2.1%) animal was infected with *T. brucei* (Fig.7B). No animal was detected with *T. vivax* infection by microscopy.

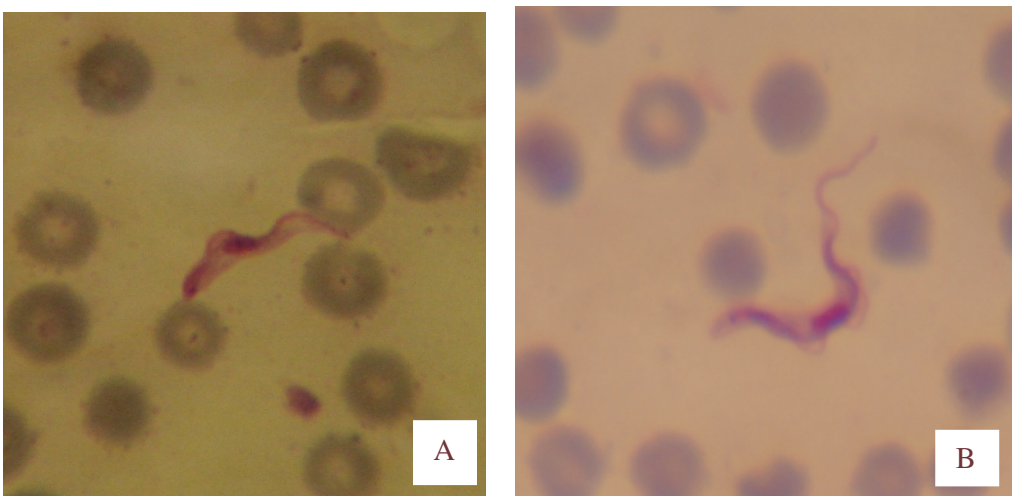

Figure 7: Giemsa-stained thin blood smears from cattle showing *T. congolense* (A) and *T. brucei* (B) parasites

The 460 cattle blood samples were also examined for the presence of trypanosomes by LAMP using the extracted DNA from cattle blood as a template. The LAMP reactions used in this study employed primers targeting the RIME and CON2 18rRNA genes to specifically amplify the sub-genus *Trypanozoon* and *T. congolense*, respectively. All RIME-LAMP positive samples were subjected to further screening by the SRA-LAMP which specifically amplifies the human infective *T. b. rhodesiense*. The RIME-LAMP positive but SRA-LAMP negative samples were assumed to be *T. b. brucei*. In this study, *T. vivax* infection was not determined in cattle by LAMP because of the current non-availability of *T. vivax*-specific LAMP primers.

LAMP positive samples could clearly be distinguished from negative ones through colour change (Fig. 8). All parasitologically positive samples were also positive on LAMP. The LAMP method of trypanosome detection further increased the number of animals infected with trypanosomes from 4.8% (95% CI: 2.8 – 6.7%) by microscopy to 12.8% (95% CI: 9.7 – 15.8%), with infections being mainly caused by *T. congolense* 44 (9.5%; 95% CI: 2.0 – 17.0%) while 11 (2.4%; 95% CI: -1.5 – 6.3%) animals were infected with *T. b. brucei.* In addition, LAMP detected 4 (0.9%; 95% CI: -1.5 – 13.3%) animals that had mixed-infections of *T. congolense* and *T. b. brucei*. No *T. b. rhodesiense* was detected.

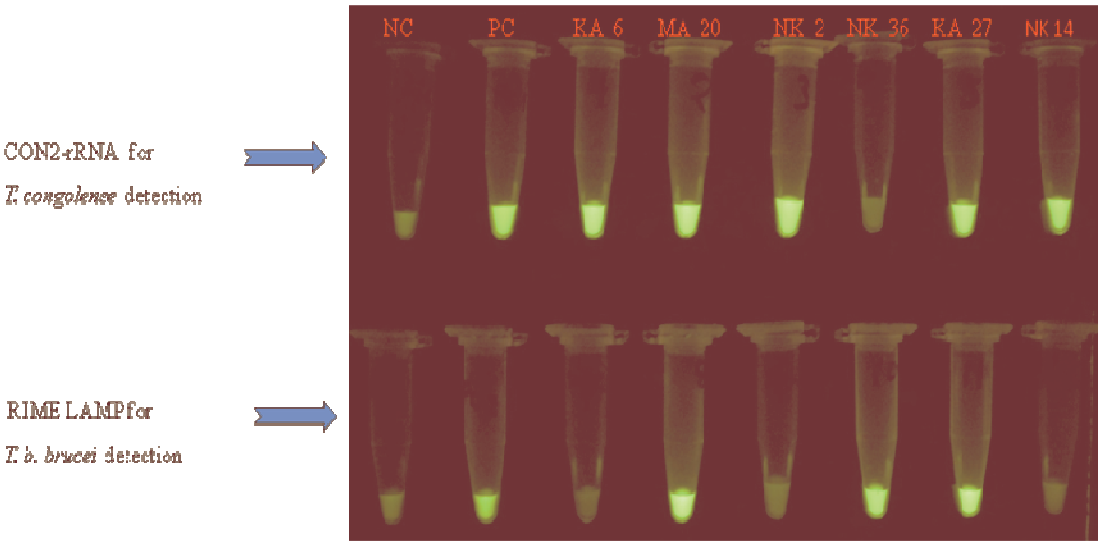

Figure 8: Representative LAMP results for *T. congolense* and *T. b. brucei* detection in cattle blood.

NC - Negative control; PC - Positive control; KA - Kakuse; NK - Nakalombwe; MA - Mbila A; numbers 2, 6, 14, 20, 36, 27 signify sample identity.

Overall, whereas the majority of the infections (over 95%) detected by microscopy were those caused by *T. congolense*, LAMP detected more animals infected with *T. b. brucei* and cattle mixed-infected with *T. congolense* and *T. b. brucei* than microscopy, hence the monolytic *T. congolense* infected cattle detected by LAMP reduced to about 74% (Fig. 9).

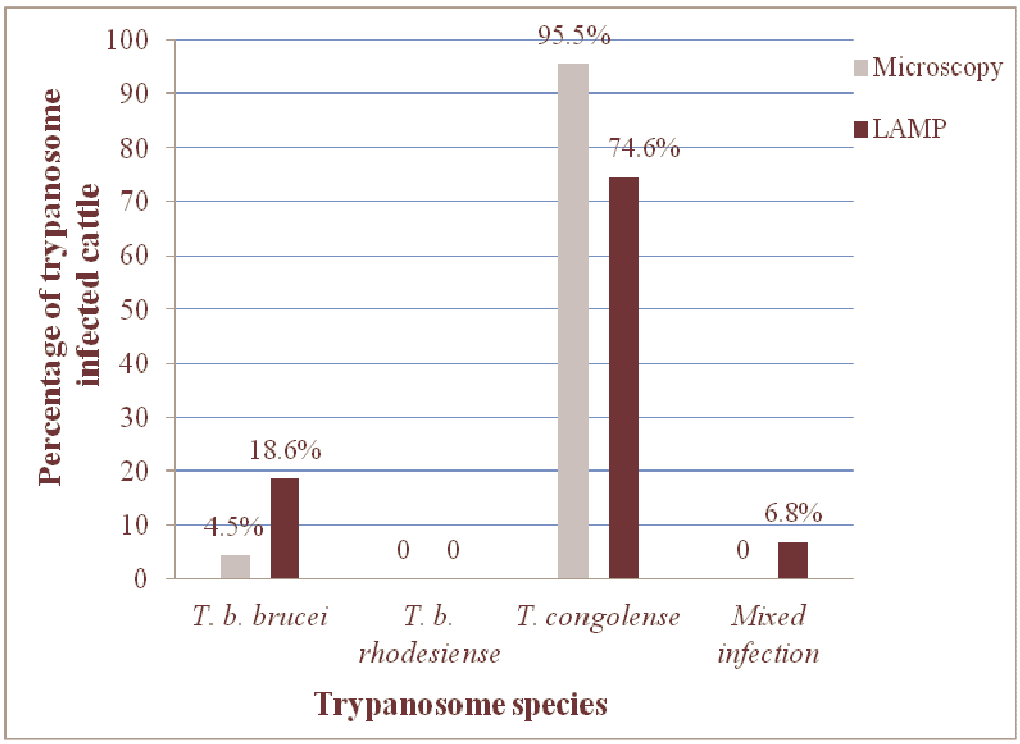

Figure 9: Determination of trypanosome species affecting cattle by microscopy and LAMP Mixed infection = infection of *T. b. brucei* and *T. congolense*

As an indicator of anaemia, cattle PCV values were determined next. As shown in table 5, the average PCV values of animals detected to have trypanosomosis tended to be lower compared to that of uninfected animals. However, table 5 further shows that there was no significant difference in the average PCV of cattle detected to be infected with trypanosomes by microscopy or LAMP and those that were not infected ($P > 0.05$).

Table 5: Average Packed cell volume of non-infected and cattle detected to be infected with trypanosomes by microscopy or LAMP

Category	**Microscopy**		**LAMP**	
	Average (%) PCV (95% CI)	Total	Average (%) PCV (95% CI)	Total
Infected cattle	24.2 (19.2 – 29.3)	22	28.6 (22.6 – 34.7)	59
Non-infected cattle	33.2 (28.0 – 38.5)	438	33.4 (28.1 – 38.7)	401

It was also observed that the prevalence of trypanosomosis in specific sites or crush pens that recorded positive cases within the Choma-Kalomo block differed significantly. As shown in figure 10, only 3 crush pens recorded positive cases of trypanosomosis by microscopy, with Nakalombwe having the highest prevalence of 27.9% (95% CI: 14.9 – 41.3%), followed by Kakuse 18.6% (95% CI: 7.0 – 30.2%) and Mbila A recorded the lowest 8% (95% CI: -2.6 – 18.6%). The use of LAMP for trypanosome detection led to an increase in the number of crush pens that recorded positive cases from 3 by microscopy to 6 by LAMP as follows: Nakalombwe 53.5% (95% CI: 39.0 – 68.4%), Kakuse 51.2% (95% CI: 36.3 – 66.1%), Mbila A 36.0% (95% CI: 17.2 – 54.9%), Fwentele 14.3% (95% CI: -11.6 – 40.2 %), Bungashiya 7.1% (95% CI: -2.4 – 16.6 %) and Maila Male 4.9% (95% CI: -1.7 – 11.5 %) (Fig. 10).

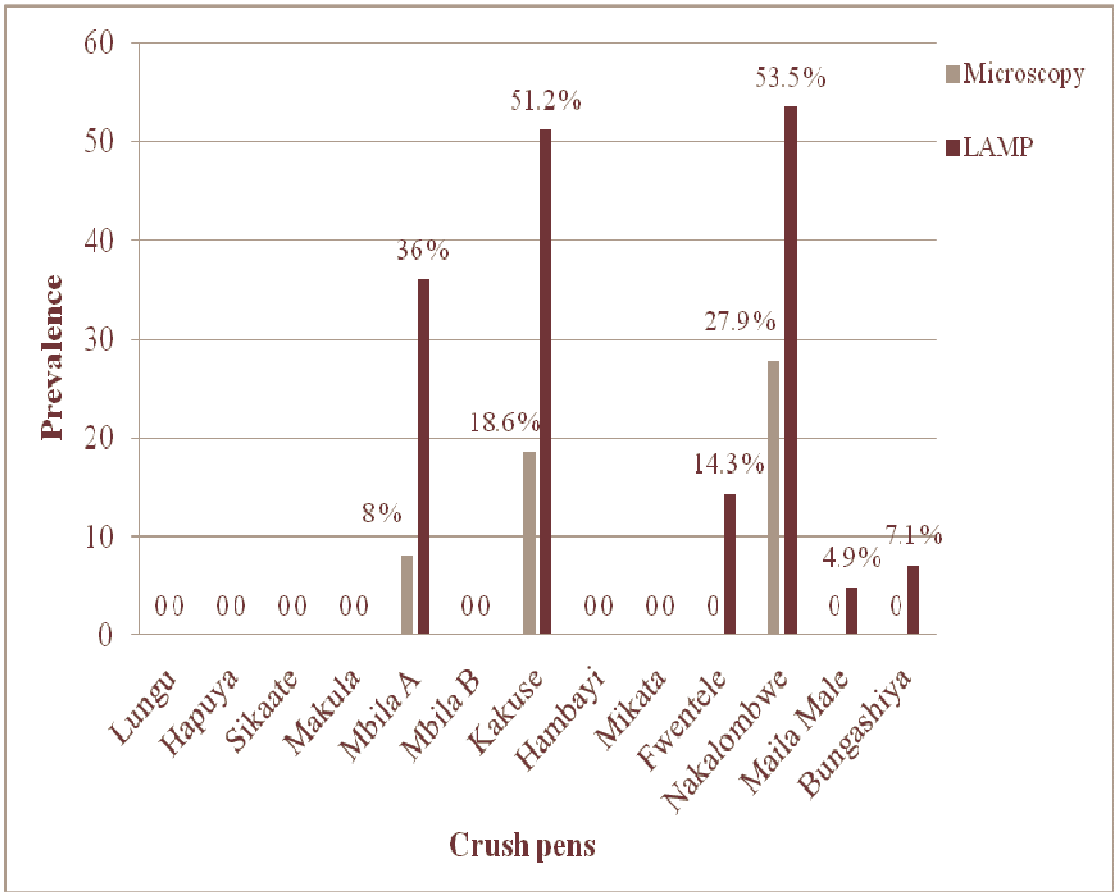

Figure 10: Determination of the distribution of trypanosome infected cattle across crush pens by microscopy and LAMP

The influence of age and sex on the prevalence of bovine trypanosomosis in sampling sites that recorded positive cases of trypanosome infections within the Choma-Kalomo block was determined. Table 6 shows trypanosome infections in different categories of cattle, detected by microscopy and LAMP. As shown in table 6, both microscopy and LAMP tended to detect more trypanosome infections in cows than any other category. On the other hand, neither microscopy nor LAMP detected trypanosome infection in female calves.

The odds ratio was next determined for all categories except for female calves which were all negative for trypanosomosis, using cows as reference data at 95% confidence intervals (CI). As shown in table 7, although there was a tendency of detection of more trypanosome infections in cows than any other category, there was no significant likelihood of cows being infected with trypanosomes as compared to most of the other categories by either microscopy or LAMP. However, a significant ($P < 0.05$) odds ratio of 4.46 (95% CI: 1.4 – 14.6) was observed when the likelihood of cows being infected with trypanosomes was compared to that of male calves by microscopy.

Table 6: Detection of trypanosome infection in different categories of cattle by microscopy and LAMP

Category	*T. b. brucei*		*T. congolense*		*T. b. brucei / T. congolense*		Total number of positives		Total sampled
	Microscopy	LAMP	Microscopy	LAMP	Microscopy	LAMP	Microscopy	LAMP	
Male calves	0	3	5	6	0	0	5	9	33
Female calves	0	0	0	0	0	0	0	0	56
Cows	1	6	7	21	0	3	8	30	205
Bulls	0	0	1	3	0	0	1	3	14
oxen	0	2	8	14	0	1	8	17	152
Total	**1**	**11**	**21**	**44**	**0**	**4**	**22**	**59**	**460**

Table 7: Odds ratio to determine the influence of age and sex on bovine trypanosomosis

Category	Odds ratio		Standard error		P-value		95% CI	
	Microscopy	LAMP	Microscopy	LAMP	Microscopy	LAMP	Microscopy	LAMP
Cows vs. Male calves	4.46	2.06	2.70	0.89	0.01	0.09	1.4 – 14.6	0.9 – 4.8
Cows vs. Bulls	1.96	1.5	2.11	1.01	0.55	0.55	0.2 – 16.5	0.4 – 5.7
Cows vs. Oxen	1.41	0.75	0.73	0.24	0.50	0.38	0.5 – 3.9	0.4 – 1.4

4.2 Capture and identification of tsetse flies from the study site

During sampling, a total of 105 tsetse flies were caught from Kakuse and Nakalombwe crush pens, the two sampling sites with higher trypanosome infection rates of cattle. Using the criteria described by Krinsky (2009), all the caught flies were identified to be *G. m. morsitans* (Fig. 11A) of which 59.0% (95% CI; 49.6 – 68.4%) were females (Fig. 11B) and 41.0% (95% CI; 31.6 – 50.4) were males (Fig. 11C). Figure 12 summarizes the proportion of male and female flies caught in the two crush pens.

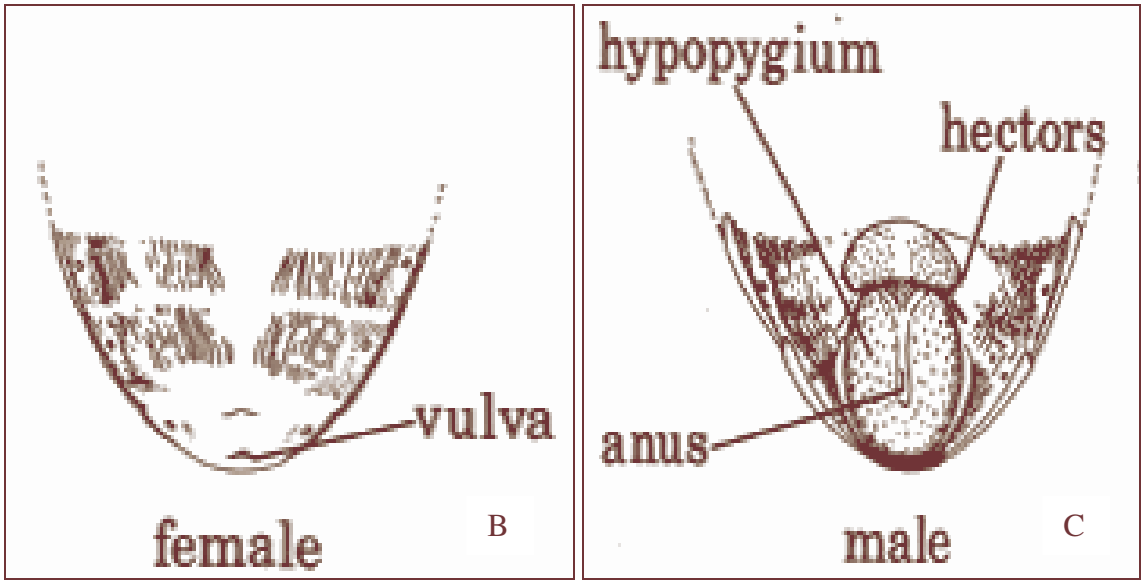

Figure 11: *Glossina morsitans morsitans* at rest after a blood meal (A), female *G. m. morsitans* showing vulva (B) and male *G. m. morsitans* showing hypopygium (C) (Krinsky, 2009)

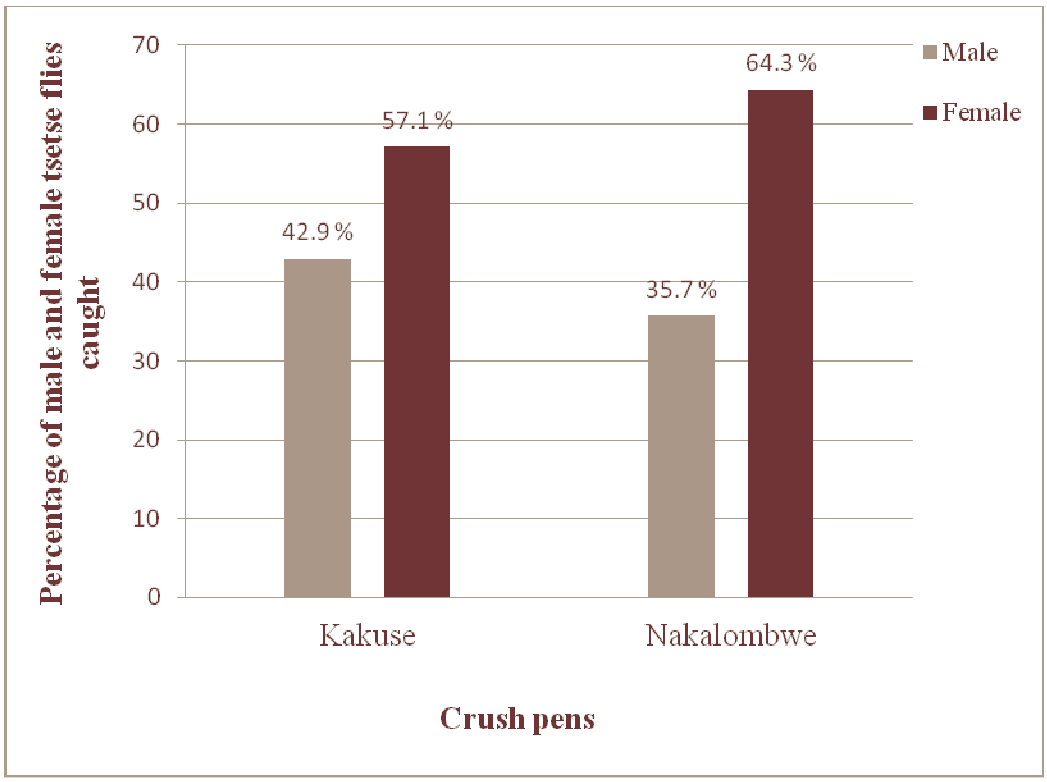

Figure12: Percentage of *G. m. morsitans* caught at each sampling site (crush pen) according to sex

CHAPTER 5: DISCUSSION

Bovine trypanosomosis is continuously being reported in different parts of the 37 tsetse infested countries in SSA (Shereni, 1990; Anne *et al.*, 2001) including Zambia (Machila *et al.,* 2001; Sinyangwe *et al.,* 2004), in areas where various control measures were previously instituted. The discontinuation of implementation of tsetse and/or trypanosomosis control methods by African governments due to financial difficulties has led to delayed detection of the vector and/or trypanosomes, resulting into re-surgence of bovine trypanosomosis (Shereni, 1990; DLVD, 2003; Omotainse *et al.,* 2004). Parasitological results from the present study indicated bovine trypanosomosis prevalence of 4.8% while molecular diagnosis using LAMP technique revealed a prevalence of 12.8% in the Choma-Kalomo block in which tsetse flies and trypanosomosis had previously been eradicated (Connor, 1989). It was thus noted that the ability of the diagnostic tests (microscopy and LAMP) used to determine the prevalence of the re-emerging trypanosomosis differed significantly in sampling sites that recorded positives cases. In Nakalombwe, the prevalence of bovine trypanosomosis by LAMP was 53.5% as compared to 27.9% by microscopy, while Kakuse recorded a prevalence of 51.2% by LAMP as compared to 18.6% by microscopy. Similarly, Mbila A recorded a prevalence of bovine trypanosomosis of 36% by LAMP as compared to 8% by microscopy. Thus these sampling sites recorded much higher prevalence of bovine trypanosomosis than the overall prevalence in the entire Choma-Kalomo block, whether parasite detection was by microscopy or LAMP. This is due to the fact that most of the sampling sites did not record any positive cases of bovine trypanosomosis and hence diluted the overall prevalence in the region. The higher prevalence in crush pens such as Nakalombwe and Kakuse where more than half the sampled animals were detected to have trypanosomosis by LAMP is worrying and calls for quick interventions. Overall, these data are in

agreement with previous reports that suggest that LAMP is at least 2.5 times more sensitive than microscopy in the detection of trypanosomes (Thekisoe *et al.*, 2005; 2007; 2008; Alhassan *et al*., 2007). Although the buffy coat method improves the sensitivity of trypanosome detection by microscopic examination through parasite concentration, this method, as shown in this study, still suffers from relatively poor sensitivity as compared to modern molecular techniques such as LAMP (Picozzi *et al.,* 2002; Thekisoe *et al*., 2005; Simukoko *et al.,* 2007). Indeed, even though the buffy coat method concentrates the parasites, this technique failed to detect some chronically infected animals in a previous study (Picozzi *et al.,* 2002). The previous surveillance reports from Choma- Kalomo block, which used microscopic examination of buffy coat (DVLD, 2003), could have underestimated the problem of bovine trypanosomosis. This has serious implications as it influences the ultimate decisions of policy makers including the type of control strategies put in place.

Loop-mediated isothermal amplification is a new DNA amplification method performed under isothermal conditions. It relies on auto-cycling strand displacement DNA synthesis performed by *Bst* DNA polymerase. Furthermore, the advantage of *Bst* DNA polymerase used is that, unlike the conventional PCR polymerase, it is not inhibited by impurities (hemoglobin and/or myoglobin) found in blood or tissues (Kuboki *et al*., 2003). As such, it is very suitable for reactions involving DNA extracted from filter papers or FTA cards as was the case in the present study. Loop-mediated isothermal amplification is as specific as PCR but tends to be more sensitive than PCR and indeed much more sensitive than microscopy (Thekisoe *et al*., 2005).

However, although molecular techniques are more sensitive and specific, the cost of equipment and reagents is usually prohibitive in most resource-poor countries. As such, detection of trypanosomes by microscopy, which is usually improved through parasite concentration methods such as buffy coat, is currently one of the most useful and reliable methods of parasite diagnosis in most resource-poor countries including Zambia.

In the present study, in addition to being over 2.5 times more sensitive than microscopy, LAMP was capable of detecting cases of trypanosome infections in areas where no case was detected by microscopy. Although microscopy only detected monolytic infections of *T. congolense* and *T. b. brucei*, LAMP revealed 6.8% of cattle that had mixed-infection with *T. congolense* and *T. b. brucei*. The fact that LAMP detected all parasitologically positive cases (in addition to the new cases), suggests that this technique is reliable. Loop-mediated isothermal amplification has previously been used in the detection of various pathogens such as African trypanosomes (Kuboki *et al*., 2003; Thekisoe *et al.,* 2005; 2007; 2008), *Babesia gibson* (Ikadai *et al*., 2004), *Theileria parva* (Thekisoe *et al*., 2010), *Plasmodium* species (Poon *et al*., 2006), *Mycobacteriun* species (Iwamoto *et al*., 2004), *Edwardsiella tarda* in fish (Savan *et al*., 2004) and simplex virus (Enomoto *et al*., 2005). Taken together, these observations suggest that LAMP could prove to be a useful molecular diagnostic tool for trypanosome detection in field samples in resource-restricted countries like Zambia as it can even be performed under field conditions.

About 75% of the recorded trypanosome infections in cattle in this study were caused by *T. congolense*. *Trypanosoma congolense* and *T. vivax* (though not detected in this study) are highly virulent parasites that cause debilitation, loss of productivity and death in cattle and other

domestic livestock (Moloo *et al*., 2008). Our data is in conformity with previous reports in which *T. congolense* was documented to be the most common trypanosome species affecting cattle in southern Africa (Kheir *et al.,* 1995; Van den Bossche and Vale, 2000). Furthermore, Simukoko *et al.* (2007) also reported that *T. congolense* accounted for about 34% of bovine trypanosomosis in Eastern province of Zambia. *Trypanosoma congolense* infection in cattle is usually characterized by emaciation, loss of condition, anaemia, fever and reduced productivity. Anaemia, characterized by low PCV values (Van den Bossche and Rowlands, 2001), is documented to be the most important pathogenic consequences of *T. congolense* infection in cattle (Taylor, 1998; Sigauque *et al.,* 2000; Vreysen *et al*., 2004). Interestingly, the average PCV values of *T. congolense* infected cattle in this study tended to be lower compared to those of uninfected cattle, suggesting the occurrence of anaemia in the infected cattle. It should however be born in mind that several other infections including worms (Bath *et al.,* 2001) and tick-borne diseases (Fandamu *et al.,* 2007) may cause anaemia in cattle. On the other hand, the apparently lower average PCV values mainly observed in trypanosome infected cattle and not in the uninfected cattle suggests that the anaemia could have been induced by trypanosome parasites, in particular *T. congolense*.

Trypanosoma vivax infections are predominant as distance from tsetse fly zones increases due to its mechanical transmission. This could probably explain why *T. vivax* was not detected in our study, although it may also be due to lower trypanosome species sensitivity and specificity of microscopy. Unfortunately, this study was unable to determine the presence of *T. vivax* by LAMP due to the current non-availability of *T. vivax*-specific LAMP primers. According to studies by Kindanemariam *et al.* (2002) in southern Ethiopia and Abdalla *et al.* (2005) in Sudan,

T. vivax infections occur in tsetse-free areas. Furthermore, *T. vivax* infections seem to be more prevalent during certain times of the year when the tsetse densities and activities are relatively lower, favoring the development of shorter life cycle of this parasite species (Simokoko *et al.,* 2007).

According to the LAMP results, 18.6% of the recorded trypanosome infections in the study were caused by *T. b. brucei* species. Unlike the highly virulent *T. congolense* and *T. vivax*, *T. b. brucei* and *T. b. rhodesiense* are thought to be low pathogenic to domestic livestock such as cattle (Ochan, 2004; Njiru *et al*., 2004). On the other hand, *T. b. rhodesiense* is highly virulent in humans, causing HAT. Cattle and other domestic animals have previously been documented to play a role as reservoir hosts by habouring the human infective *T. b. rhodesiense* (Acha and Szyfres, 2003; Njiru *et al*., 2004). Consequently, in Uganda, screening of livestock for *T. b. rhodesiense* has been advocated to prevent the spread of HAT due to cattle movement (Fevre *et al.,* 2001; Njiru *et al.,* 2004). Because *T. b. rhodesiense* uniquely expresses the SRA gene which enables it to establish infection in humans and which distinguishes it from its closely related *T. b. brucei*, the SRA gene has been exploited in the specific diagnosis of *T. b. rhodesiense* (Radwanska *et al*., 2002). However, no *T. b. rhodesiense* was detected in the Choma-Kalomo block in this study. This may not be surprising as there are no cases of sleeping sickness being reported from that region. Even though there was no *T. b. rhodesiense* detected in the Choma-Kalomo block during the study, it is necessary to carry out routine sampling of the cattle due to the interaction between cattle and wildlife, especially in those sampling sites closest to the park.

The prevalence of bovine trypanosomosis differed substantially between sampling sites within the Choma-Kalomo block. More importantly, trypanosome infections were generally only detected in sampling sites that were within close proximity to the Kafue National Park. To that effect, the highest prevalence of bovine trypanosomosis recorded by LAMP was at Nakalombwe (53.5%) and Kakuse (51.2%) respectively, which were closest to the Kafue National Park. As the distance from Kafue National Park increase, the prevalence of the disease tended to decrease (36% in Mbila A; 14.3% in Fwentele; 7.1% in Bungashiya and 4.9% in Maila Male). In sampling sites that were much further away from the park, no infections were recorded. Considering that trypanosomes are tsetse-transmitted parasites, this observation may not be surprising as the tsetse density is expected to be higher within and in close proximity to the Kafue National Park which also habour large populations of wildlife reservoirs (Symeonakis *et al.,* 2007). It is also documented by Van den Bossche and Vale, (2000) that tsetse flies are more abundant in areas where livestock occupy the edge of tsetse-infested wildlife zones such as the Kafue National park.

Data from this study suggest differences in trypanosome infections among age and sex categories of cattle. Most of the trypanosome infected cattle were cows. Adult cows were at least twice more likely to be infected with trypanosomes than any other category. The higher prevalence of bovine trypanosomosis in cows could be due to their being stressed through milking. Besides being milked, as a result of massive loss of cattle (mainly oxen) mainly due to theileriosis, cows in Southern province of Zambia are now being used for traction power i.e. as draught animals, which previously was restricted to oxen. This is further evidenced by the relatively lower numbers of oxen in the sampling sites as compared to cows. Such stressed cows tend to be weak

and may fail to actively use their tails, ears or limbs to chase tsetse flies that wish to take a blood meal from them. This could further explain the discrepancy between our findings and those of Simukoko *et al*. (2007) in which oxen exhibited higher likelihood of being infected with trypanosomes than any other category. According to Torr *et al.* (2006) and Rowlands *et al.* (2001), tsetse flies are attracted significantly more by the odour of large animals (such as bulls, oxen and cows) and animals that showed less defensive behavior such as stressed cows, and least in calves (especially when calves are kept as part of the herd). However, calves are kept at homestead in most cases for the purpose of milking. In agreement with Simukoko *et al.* (2007), such differences in the attractiveness to tsetse flies may also translate into differences in prevalence of bovine trypanosomosis.

This study reveals that tsetse flies were caught in the Choma-Kalomo block during the sample collection from cattle. Using the criteria described by Krinsky (2009), the flies were identified to be *G. m. morsitans*. Indeed, *G. m. morsitans* is the commonest species of tsetse flies found in the savannah regions of southern Africa. Although the study did not investigate the presence of trypanosomes in the caught tsetse flies, *G. m. morsitans* is documented to be responsible for cyclic transmission of *T. b. brucei* and *T. congolense* to cattle during a blood meal (Kubi *et al.,* 2007). The infection rate in tsetse population undergoes variation and the tsetse density and activity determines the rate of infection in livestock (Kubi *et al.,* 2007). To that effect, the high density of tsetse flies and the increased frequency of blood meals taken by the flies at the beginning of the rainy season is thought to be responsible for the high rate of trypanosome infections in cattle at this time of the year (Van den Bossche and De Deken, 2002; Kubi *et al.,* 2007). This study was conducted during the month of December, which is the beginning of the

rainy season in Zambia and could thus explain the relatively high prevalence of bovine trypanosomosis recorded in the Choma-Kalomo block by LAMP.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

To the best of our knowledge, this is the first time molecular diagnosis (LAMP) has been used to estimate the prevalence of bovine trypanosomosis in the Choma-Kalomo block. Our data confirms the re-surgence of tsetse infestation and trypanosomosis in the previously aerial sprayed Choma-Kalomo block. Highest prevalence of bovine trypanosomosis cases were diagnosed by LAMP, which was at least 2.5 times more sensitive than microscopy. Of note, LAMP detected all parasitological cases (in addition to several new cases not detected by microscopy), suggesting that this technique is reliable. Loop-mediated isothermal amplification is a novel molecular technique that is easier and simpler to use, quicker, cheaper as compared to other molecular techniques such as PCR and yet as specific and sensitive as PCR, and LAMP products may be visualized by naked eyes. All these attributes make LAMP more user friendly for detection of such infections as trypanosomosis in field samples in developing countries with limited resources, such as Zambia.

Successful tsetse eradication in aerial-sprayed areas requires continuous monitoring and surveillance. Lack of surveillance programs lead to delayed detection of the vector and/or the trypanosomes resulting into re-surgence of tsetse fly and hence trypanosomosis as is the case in the Choma-Kalomo block. The proximity to the tsetse-infested areas such as the Kafue National Park appears to influence the prevalence of bovine trypanosomosis. Nakalombwe and Kakuse sampling sites, which were closer to the park, had the highest prevalence, with more than half the sampled animals being infected with trypanosomes, while sampling sites further away from the park did not record any trypanosome infection. *Trypanosoma congolense* seems to be the major

cause of bovine trypanosomosis in the Choma-Kalomo block and cows, probably due to being stressed from milking and being used for traction power exhibited higher likelihood of being infected with trypanosomes than all categories used in the present study. Finally, although *T. b. brucei* was detected in the infected cattle, this study did not report any case of *T. b. rhodesiense* infection in cattle. This is in conformity with the fact that although cattle are known to habour the human-infective *T. b. rhodesiense*, there are currently no cases of sleeping sickness being reported in the Choma-Kalomo block.

In order to successfully control trypanosomosis in the previously tsetse-eradicated areas, the study suggests the following recommendations:

I. Disease prevention

1. Important agricultural regions of Zambia like the Choma-Kalomo block should be regularly aerial sprayed at specified intervals so as to reduce the tsetse burden and in turn control trypanosomosis.

2. It is further important to conduct regular screening of bovine trypanosomosis and treating positive animals with trypanocides before spraying is effected.

3. The use of more sensitive trypanosome detecting techniques such PCR and/or LAMP should be encouraged instead of sorely relying on the relatively lower sensitivity parasitological methods.

4. Settlements within or close to game management areas should be discouraged. This requires farmer education/sensitization based on scientific evidence such as the data obtained from this study.

5. Continuous community-based tsetse monitoring and more trypanosomosis surveillance programs should be instituted in tsetse infested regions such as the Choma-Kalomo block.

6. The presence of other pathogenic animal infective trypanosomes such as *T. vivax* should be investigated using PCR since the *T. vivax*-specific LAMP primers are currently unavailable.

II. Future research areas

    1. Large scale study should be conducted to determine the effect of seasonality on the prevalence of bovine trypanosomosis in the Choma-Kalomo block.

    2. The rate of trypanosome infections in male and female tsetse vectors in the Choma-Kalomo block should be determined.

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