EPIDEMIOLOGICAL STUDIES ON *TRYPANOSOMA BRUCEI GAMBIENSE* IN BENUE AND TARABA STATES OF NIGERIA

BY

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EPIDEMIOLOGICAL STUDIES ON *TRYPANOSOMA BRUCEI GAMBIENSE* INFECTIONS IN BENUE AND TARABA STATES OF NIGERIA

BY

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AUGUST, 2014

DECLARATION

I declare that the work in this dissertation entitled: "Epidemiological studies on *Trypanosoma brucei gambiense* infections in Benue and Taraba States of Nigeria" has been carried out by me in the Department of Veterinary Public Health and Preventive Medicine under the supervision of Prof. I. Ajogi, Prof. Idris A. Lawal, and Dr. G. Mohammed. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

SOLOMON NGUTOR KARSHIMA		
Name of Student	**Signature**	**Date**

CERTIFICATION

This dissertation entitled "EPIDEMIOLOGICAL STUDIES ON *TRYPANOSOMA BRUCEI GAMBIENSE* INFECTIONS IN BENUE AND TARABA STATES OF NIGERIA" by Solomon Ngutor KARSHIMA meets the regulations governing the award of the degree of Doctor of Philosophy of the Ahmadu Bello University and is approved for its contribution to knowledge and literary presentation.

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DEDICATON

This work is dedicated to the Almighty God and to my dear wife Mrs. Juliana Torkwase Karshima.

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To Him who sits in heaven, the maker of heaven and earth is all glory, honour, praise, worship and adoration for giving me life, sustaining it and making this dream a reality. From the depth of my heart, I sincerely appreciate my wonderful team of supervisors in persons of Professors I. Ajogi, Idris A. Lawal, and Dr. G. Mohammed for their immeasurable advice and patience during the course of this study. No amount of words can express my gratitude to you all because any credit that this work deserves is attributable to you. I earnestly pray that the Almighty God bless you all. I also thank Dr. O.O. Okubanjo for all his support during the course of this work. I wish to appreciate the Departmental Heads and all the Academic and technical staff of the Departments of Veterinary Public Health and Preventive Medicine and Veterinary Parasitology and Entomology for their immense support which contributed to the success of this work. I wish to sincerely acknowledge my dear wife Mrs. Juliana Karshima who is truly the reason for my success during this study. I am so delighted to have you as part of me; you are truly the best encouragement I need. To my sweet daughters Miss Iveren Karshima and Miss Iyuana Karshima, I salute your courage and also thank you for your patience. I love you so sincerely. To my sweet mummy Mrs. Esther Karshima, my dear daddy Mr. Emmanuel Karshima, my beloved sister Mrs. Hembafan Zeku and all my other siblings, I sincerely thank you for your moral supports during the course of this study. These friends were indispensable, Doctors Peter Ehizibolo, Shalangwa B. Ishaku, Emmanuel Vandi and Blessed Madaki to mention but a few. It is my earnest prayers that the good Lord blesses everyone mentioned in this acknowledgement and those not even mentioned for lack of space.

ABSTRACT

The aim of this study was to determine the epidemiology of *Trypanosoma brucei gambiense* infections in Benue and Taraba States of Nigeria using participatory epidemiology, serology, as well as parasitological, entomological and molecular techniques. Of the 1,200 blood samples of humans examined using the card agglutination test for *T. b. gambiense* (CATT), 21 were positive giving an overall human sero-prevalence of 1.75%. State based sero-prevalence rates were 1.3% (8/600) for Benue and 2.2% (13/600) for Taraba States. Local Government Area based sero-prevalence rates in Benue were 0.8% (5/600), 0.5% (3/600) and 0% (0/600) for Gboko, Vandekya and Ukum respectively, while, Gashaka, Ibi and Karim Lamido in Taraba recorded sero-prevalence rates of 1.2% (7/600), 0.2% (1/600) and 0.8% (5/600) respectively. Gender based sero-prevalence rates in Benue were 1.0% (6/600) for males and 0.3% (2/600) for females while in Taraba, 1.33% (8/600) and 0.83% (5/600) were recorded in males and females respectively. There was no significant association between the sex based prevalence rates (p>0.05). Age based sero-prevalence rates in Benue was 0.67% (4/600) for both children and adults, while, children and adults in Taraba recorded sero-prevalence rates of 1.2% (7/600) and 1.0% (6/600) respectively. There was no significant association between the age based prevalence rates (p>0.05). Out of the 1,200 blood samples of animals examined using the CATT, 107 were positive, giving an overall animal sero-prevalence of 8.9%. Of this, pigs and cattle recorded sero-prevalence rates of 10.7% (64/600) and 7.2% (43/600) respectively. Highest sero-prevalence rates in Benue and Taraba were observed in Gboko 5.7% (34/600) and Gashaka 3.7% (22/600) Local Government Areas respectively. There was significant association between the LGA based prevalence rates (p<0.05). Management based sero-prevalence rates were 8.7% (52/600) for extensively managed and 2.0% (12/600) for intensively managed pigs. Breed based

sero-prevalence rates in cattle were 5.33% (32/600), 1.5% (9/600) and 0.33% (2/600) for White Fulani, Bokoloji and Muturu respectively. There was significant association between the breed based prevalence rates (p<0.05). Sex based sero-prevalence rates were 4.7% (28/600) in females and 6.0% (36/600) in males for pigs as well as 2.83% (14/600) in males and 4.83% (29/600) in females for cattle. The highest tsetse catch in Benue and Taraba were in Gboko 78/531 (14.7%) and Gashaka 226/531 (42.6%) respectively. Two species of tsetse flies identified during the study were *Glossina palpalis* 87.2% (463/531) and *Glossina tachinoides* 12.8% (58/531) with infection rates of 3.4% and 1.2% respectively. A total of 11 *Trypanosoma* isolates of which 6 (54.5%) originated from humans, 3 (27.3%) from pigs and 2 (18.2%) from cattle were characterized by the identification of internal transcribed spacer-1 (ITS-1) rDNA, serum resistance antigen (SRA) and *Trypanosoma gambiense* specific glycoprotein (TgsGP) genes. Kinetoplastids (KIN) primer sets identified all the 11 purified *Trypanosoma* isolates from mice blood giving 100% sensitivity. The KIN primer sets identified all human isolates as belonging to the *T. brucei* genome, while in animal samples both *T. brucei* and *T. congolense* were identified. ITS-1 primer sets identified *T. brucei, T. congolense* forest and *T. congolense* savannah sub-species from isolates of cattle and pig origin. PCR targeting serum resistance antigen gene excluded the presence of human infective *T. b. rhodesiense* in all the 6 human and 5 animal isolates. A strain-specific TgsGP primer set characterized all the 6 human, 1 cattle and 2 swine isolates as type 1 *T. b. gambiense*. Of the 30 tsetse blood-meals analyzed for the identification of their sources of blood-meals, 10 (33.3%), 9 (30.0%), 7 (23.3%) and 4 (13.3%) fed on cattle, pigs, dogs and humans respectively. The differences between the feeding frequencies of tsetse on the individual vertebrates showed no significant association (p>0.05). There were mixed feedings involving human-cattle-pig-dog among 2 (6.7%) and cattle-dog-pig among 5 (16.7%) of the

analyzed tsetse flies. Dual feeding patterns involving human-dog 2 (6.7%), human-cattle 4 (13.3%) and human-pig 3 (10.0%) were also observed. There were no significant associations between tsetse feeding frequencies on both multiple and dual feeding patterns (p>0.05). The respondents in the participatory epidemiology rated HAT among seven other endemic diseases to have the highest mortality of 85.0% (34/40) and economic impact of 70% (28/40). Wildlife species, domesticated animals and humans were identified by participants as reservoirs of *T. b. gambiense* and their proportionate ratings in disease transmission were 34/40 (85.0%), 28/40 (70.0%) and 7/40 (17.5%) respectively. Game reserves and homestead areas were assigned the highest and lowest sources of infection with proportions of 29/40 (72.5%) and 10/40 (25.0%) respectively. Farming was identified by respondents as the major risk occupation while shepherding was considered the least risk occupation with proportions of 30/40 (75.0%) and 7/40 (17.5%) respectively. The age brackets 16-30 and >75 years were considered as having the highest 33/40 (82.5%) and lowest 3/40 (7.5%) HAT impacts in the studied communities. The findings of this study have established the presence of *T. b. gambiense* type 1, forest and Savannah sub-species of *T. congolense*, the roles of animal reservoirs, species-specific, dual and mixed feeding patterns of tsetse flies in the epidemiology of HAT in the study areas.

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LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
bp	Base pair
CATT	Card Agglutination Test for Trypanosomosis
CDC	Centres for Disease Control and Prevention
CI	Confidence Interval
CIATT	Card Indirect Agglutination Test for Trypanosomosis
CNS	Central Nervous System
CSF	Cerobrospinal Fluid
CVL	Cervical Lymph Node
DALYs	Daily Adjusted Life Years
DDT	Dichloro-diphenyl-tetrachloroethane
DEAE	Di-ethyl-amino-ethyl
DFMO	Difluoromethylornithine
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside Ttiphosphate
DRC	Democratic Republic of Congo
EDTA	Ethylene Diamine Tetra-acetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
ES	Expression Site
ESAG	Expression Site Associated Gene
FAO	Food and Agriculture Organization
GR	Game Reserve
HAT	Human African Trypanosomosis
HIV	Human Immunodeficiency Virus
IFA	Immuno-fluorescent Assay
IgG	Immunoglobulin G

IgM	Immunoglobulin M
ITS	Internal Transcribed Spacer
KIN	Kinetopastids
KIVI	Kit for In-Vitro Isolation
LFD	Lateral Flow Dipstick
LGA	Local Government Area
mAECT	Mini Anion Exchange Centrifugation Technique
mHCT	Micro-haematocrit Centrifugation Technique
MTOC	Micro-tubule Organising Centre
ODC	Ornithine Decarboxylase
PCR	Polymerase Chain Reaction
PSG	Phosphate Saline Glucose
QBC	Quantitative Buffy Coat
RC	Republic of Cameroon
rDNA	Ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SRA	Serum Resistant Antigen
SS	Sleeping Sickness
TgsGP	*Trypanosoma gambiense* specific glycoprotein
UV	Ultra-Violet
VAT	Variable Antigen Type
VSG	Variable Surface Glycoprotein
WBC	White Blood Cell
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 General Introduction

Human African Trypanosomosis (HAT) or Sleeping Sickness is a vector-borne zoonosis caused by protozoan parasites of the genus *Trypanosoma* (Musere, 1990). The disease causes high mortality of up to 100% in untreated cases (Murray, 1994; Odiit *et al.,* 1997). The socio-economic impact of HAT ranks third after malaria and schistosomiasis in affected parts of sub-Saharan Africa (Cattand *et al.,* 2001). HAT is the prototype of a neglected disease, affecting the poorest people of the poorest continent. The development of new diagnostic tests and drugs has been severely affected by this neglect (Trouiller *et al.,* 2002; Stich *et al*., 2003).

Sixty million people are continuously exposed to the risk of infection in 36 African countries where active transmission is reported to be taking place. However, only 3-4 million of the population at risk is covered by surveillance (WHO, 1998; 2000), and 0.3-0.5 million people were said to be infected, with 0.1 million people dying of the disease yearly (Cattand *et al.*, 2001).

Human African trypanosomosis is caused by the human infective forms of *Trypanosoma brucei*. *Trypanosoma brucei rhodesiense* causes acute disease referred to as the Rhodesian sleeping sickness in East and Southern Africa and *T. brucei gambiense* causes a chronic disease referred to as the Gambian sleeping sickness in West and Central Africa (Hide and Tait, 1991; Tait, 2000; Welburn *et al.,* 2001a). *Trypanosoma brucei brucei* is an animal trypanosome which is similar to *T. brucei gambiense* and *T. brucei rhodesiense,* but the presence of human serum resistance

antigen (SRA) gene distinguishes *T. b. rhodesiense* from the others (Gibson *et al*., 2002). The human SRA gene allows *T. brucei rhodesiense* to survive exposure to normal human serum (Xong *et al.,* 1998). Two types of *T. brucei gambiense* have been identified. Type 1 is reported as the cause of the chronic disease in West and Central Africa (Tait *et al.,* 1984; Bacchi and Yarlett, 1993; Mathieu-Daude *et al.*, 1995), while type 2 is said to be associated with an acute like disease in Nigeria (Hide *et al.,* 1990).

In natural conditions, transmission of the disease from human to human, human to animal and animal to human is cyclical through bites of infected tsetse flies including *Glossina palpalis, G. tachinoides* and *G. fuscipes* for Gambian sleeping sickness. These vectors are especially common at watering places like rivers or lakes where people frequently visit to collect water and do their washings, and animals visit to drink water (Service, 1980). *Glossina morsitans, G. swynnertoni* and *G. pallidipes* are important vectors of Rhodesian sleeping sickness and occur in the savanna areas of East Africa (Service, 1980). It is postulated that domestic cattle play a central role in the epidemiology of Rhodesian sleeping sickness (Fevre *et al.,* 2001).

Two clinical forms of HAT are recognized. The first or haemolymphatic stage is associated with the presence of parasites in the lymphatics, blood, and other body systems, excluding the central nervous system (CNS). The second stage or stage of CNS involvement is associated with parasite damage to the CNS and characterized by increased white blood cell (WBC) counts, protein levels, presence of parasites in the cerebrospinal fluid (Bisser *et al.*, 2000), and manifested by sleep disturbances (Chappuis *et al*., 2005).

The involvement of wild and domesticated animals as reservoirs of Gambian sleeping sickness has been reported (Njiokou *et al*., 2006; Simo *et al*., 2006; Mbaya *et al*., 2009; Karshima *et al*., 2012). Welburn *et al*. (2001b) reported 18% prevalence of *Trypanosoma brucei rhodesiense* in cattle from Seroti, Uganda by means of serum-resistance-antigen (SRA) gene identification, while a 34.3% prevalence of *T. brucei gambiense* was reported in pigs from the Republic of Cameroon using polymerase chain reaction (Simo *et al*., 2006).

Diagnosis of the disease in humans and animals is important to the patients as well as the community. This is because undiagnosed, and of course, untreated patients will continue to spread infection through activities of teneral tsetse flies (Cattand *et al*., 2001). As long as vaccination or prophylaxis against the infection and effective vector control are lacking, ability for early diagnosis and treatment remain important tools in the control of the disease (WHO, 1998).

1.2 Statement of Research Problem

Human African trypanosomosis caused by *T. b. gambiense* is a debilitating, complex, widespread and fatal parasitic zoonosis transmitted by tsetse flies. The disease has re-emerged in the 1990s as a serious public health problem in sub-Saharan Africa including Nigeria (Stich *et al.,* 2003; Waiswa *et al.,* 2003), and poses a major health risk to tourists visiting tropical Africa (Conway-Klassen *et al.,* 2002; Jelinek *et al.,* 2002). The disease affects economically active adults with about 25% of cases occurring in the 20-29 age groups, and just over 60% in those aged from 10-39 years (Swallow, 2000). The involvement of the central nervous system (CNS) in HAT is

manifested by sleep disturbances making the diseased population economically inactive (Atouguia and Kennedy, 2000).

In Nigeria, human infection with *T. b. gambiense* in the Gboko endemic area of Benue State was first reported in 1974, and since then no successful attempt has been made to control the tsetse vector in the affected area (Aiyedun and Amodu, 1974). Airauhi *et al.* (2006) and Osue *et al*. (2008) reported an active transmission of the Gambian type of sleeping sickness in Abraka, Delta State by the use of the card agglutination test for trypanosomosis (CATT). Karshima *et al*. (2012) also reported the presence of *T. b. gambiense* Li Tat 1.3 surface antibodies in humans and cattle in some Local Government Areas (LGAs) of Taraba State using the CATT.

Previous workers in Nigeria did not characterize *T. b. gambiense* and also failed to establish the sources of blood-meals of tsetse vectors using molecular studies. However, the reports of Wastling *et al*. (2011) which showed the occurrence of latent epidemic of *T. b. gambiense* in children and adults in Uganda necessitated this study, since this kind of epidemic may be possible in any other sleeping sickness focus.

1.3 Justification

More recently in Nigeria, Karshima *et al.* (2012) reported a prevalence of 1.25% (5/400) and 7.25% (29/400) for humans and cattle respectively in some LGAs of Taraba State using the CATT. Airauhi *et al*. (2006) and Osue *et al.* (2008) also reported an active transmission of Gambian type of sleeping sickness in Abraka, Delta State. The outbreak in Gboko, Benue State

was reported about four decades ago (Aiyedun and Amodu, 1974) indicating the existence of the risk to the human population in Nigeria.

Sixty million people are continuously exposed to the risk of infection in 36 African countries where active transmission is reported to be taking place. However, only 3-4 million of the population at risk is covered by surveillance (WHO, 1998). It is stipulated that once sleeping sickness is established in an area, the disease is unlikely to disappear unless drastic control measures are put in place (Chappuis *et al*., 2005).

Studies on wild animals revealed the involvement of numerous animals such as primates, reptiles, antelopes and wild bovids (Herder *et al*., 2002; Njiokou *et al*., 2006; Mbaya *et al*., 2009), as well as domesticated animals such as pigs, goats, sheep and cattle (Welburn *et al*., 2001b; Nkinin *et al*., 2002; Simo *et al*., 2006) as suitable reservoirs for *T. brucei gambiense* and consequently their roles in the transmission of the disease.

HAT is not routinely diagnosed in Nigerian hospitals, even in endemic foci. HAT patients are therefore left undiagnosed for long periods allowing the disease to develop to the second stage which is very fatal and difficult to treat. This study will identify undiagnosed HAT patients thereby protecting them against the life threatening syndromes associated with the parasites' invasion of the CNS and toxicity of second stage chemotherapeutic agents like melarsoprol and eflornithine.

There is paucity of information on human African trypanosomosis in Taraba and Benue States, despite the presence of predisposing factors that can facilitate the occurrence of the disease such as the presence of Gashaka-Gumti game reserve which habours many wildlife species which can serve as reservoirs for the infection. Other factors are the established endemic focus in Benue State (Aiyedun and Amodu, 1974), as well as the relationship between the two States and the Fontem sleeping sickness focus of the Republic of Cameroon (Simo *et al*., 2006). Diagnosis of the disease in humans and animals in the two States, vector control and of course, treatment of infected population remain the major tools in the control of sleeping sickness (Cattand *et al*., 2001).

1.4 Aim of the Study

The aim of this study was to conduct epidemiological studies on *Trypanosoma brucei gambiense* infections in Benue and Taraba States of Nigeria.

1.4.1 Objectives of the study

The specific objectives of this study were to:

i. determine the sero-prevalence of *T. b. gambiense* in humans cattle and pigs using the CATT.
ii. characterize *Trypanosoma brucei* isolates from *T. b. gambiense* sero-positive humans cattle and pigs using polymerase chain reaction.
iii. identify possible vectors of *Trypanosoma* species in the study areas.
iv. determine the infection rates of tsetse population with trypanosomes.

v. identify sources of blood-meals among tsetse flies in the study areas.

vi. gain information on the sources of HAT infection, HAT risk occupations and the distribution of tsetse flies in the study areas.

1.5 Research Questions

i. Are the humans, cattle or pig populations of the study areas infected with *T. b. gambiense*?

ii. Is there any genetic relationship between *T. brucei* isolates from *T. b. gambiense* sero-positive humans, cattle and pigs?

iii. Are there vectors of *T. b. gambiense* in the study areas?

iv. Are tsetse flies in the study areas infected with trypanosomes?

v. Do tsetse flies in the study areas feed on man, cattle, and pigs?

vi. Are the people of Benue and Taraba States aware of the sources of HAT infection, HAT risk occupations, distribution of tsetse flies and the impacts of the disease in their communities?

CHAPTER TWO

LITERATURE REVIEW

2.1 History of Human African Trypanosomosis

The symptoms of African sleeping sickness were documented by Atkins in 1742, but the association of the clinical syndrome with its etiological agent, the trypanosome, was not documented until 1902 by Forde (Despommier *et al*., 1995). In the Journal of Tropical Medicine, Forde chronicled his treatment of a 42 year-old European male colonialist who was presented to his clinic in the Gambia Colony in May 1901. The patient complained of fever and malaise, leading Forde to make a preliminary diagnosis of malaria. He initiated anti-malarial quinine treatment, but there was no improvement several days after, which led him to the preparation of a slide from the patient's blood. This examination ruled-out malaria due to lack of malarial parasites found in the blood. Only later, Dutton, a second physician from the Liverpool School of Tropical Medicine, made the identification of *Trypanasoma brucei* in the patient's blood (Forde, 1902). Due to the probable location of the patient, the case could be attributed to the species *T. b. gambiense*.

The identification of *T. b. rhodesiense* as another species of trypanosome that causes African sleeping sickness was not documented until 1910. Stephens and Fantham described a strain of trypanosome observed in a blood smear of a patient who presented with symptoms of African trypanosomosis. The patient had no history of travel within a region known to be endemic with *T. b. brucei*, yet his blood smear clearly indicated trypanosomal infection. The novel morphology was believed to be a new species of *T. brucei*. Because the patient was believed to

have been infected in Rhodesia (present day Zimbabwe), the new parasite was thus named *T. b. rhodesiense* (Stephens and Fantham, 1910).

The reports of Kinghorn and Yorke in 1912 proved that *T. b. rhodesiense* could be transmitted from humans to animals by the tsetse fly. They also concluded through their research that many game animals in East Africa, including waterbuck, impala, and warhog, served as reservoirs for *T. b. rhodesiense* in that region of the continent (Kinghorn and Yorke, 1912).

2.2 Antigenic Variation in *Trypanosoma brucei* Genome

Antigenic variation is a survival strategy utilized by trypanosomes to evade the immune system of its hosts. *Trypanosoma brucei* is continuously exposed to antibody challenge from its host as a result of its extracellular lifestyle. To evade this challenge, the parasite uses antigenic variation which is a surface protein called the variable surface glycoprotein (VSG). It has been shown that over 10^7 molecules of these VSG are expressed on the cell surface of each *T. brucei* parasite, creating a dense coat that prevents adaptive immunity from detecting or accessing invariant antigens (Borsch, 2002; Stockdale *et al*., 2008). This variant surface glycoprotein (VSG) determines the variable antigen type (VAT) of the parasites. The density of VSG molecules is sufficiently high for the VSG layer to be visible by electron microscopy as a 12 nm thick surface coat (Vickerman, 1969) that occupies a surface area of 180 mm^2 approximately (Jackson *et al*., 1985). The surface coat acts as a physical barrier, preventing access to the underlying plasma membrane by components of nonspecific immune responses (Ferrante and Allison, 1983; Barry and McCulloch, 2001).

However, antibodies against each of the expressed VSG are generated at each moment by the host immune system, and parasite survival is based on periodic switches to an immunologically distinct VSG coat. Such switches are pre-emptive of the immune response and contribute to the pattern of trypanosome growth seen in an infected host. This is seen in the increased parasitaemia which subsequently drops as VSG-specific antibodies are raised by the host (Barry *et al*., 2005). Cells that have switched to another VSG coat survive this killing and initiate another phase of parasitaemia which is again decimated by anti-VSG immune killing. As a survival strategy, antigenic variation succeeds by prolonging the time that the parasite resides in the host producing the chronic nature of trypanosomosis and enhancing transmission to a new host via bites of the tsetse vector. The second goal of antigenic variation is to allow trypanosomes to infect new hosts that already have immunity to some VSGs as a result of previous infections (Mehlert *et al*., 2002; Barry *et al*., 2005).

2.2.1 Requirements for VSG switching in *Trypanosoma brucei*

The strategy of immune evasion by *T. brucei* gemone is governed by three basic requirements. First, a family of genes encoding antigenically distinct surface antigens is needed in which the *T. brucei* genome contains over 1,000 of such VSG genes (Marcello and Barry, 2007). Second, one variant antigen gene must be expressed by a single trypanosome at a time to avoid exhausting the surface antigen repertoire so that the infection can survive over a long period, and finally, a mechanism is needed to switch the single expressed antigen gene. Two strategies have evolved to meet these requirements. One involves antigen switching and occurs by purely transcriptional mechanisms that are not associated with DNA rearrangements (Marcello and Barry, 2007). In this strategy, a single antigen gene is expressed, but periodically that expression is silenced and a

second gene activated. The other route for switching is based on recombination where a singular expression relies on the presence of a site for antigen gene expression, and switching to new variants is achieved by recombination into the expression site. In fact, most such recombination appears to be driven by gene conversion (Brayton *et al*., 2002; Palmer and Brayton, 2007), where a silent antigen gene is copied and duplicated into the expression site, deleting the resident gene. Both strategies are utilized by *T. brucei* genome making antigenic variation in these parasites very unique.

2.2.2 Mechanisms of VSG switching during antigenic variation in *T. brucei*

The subtelomeric gene arrays in the eleven diploid megabase chromosomes host the majority of the VSG of *T. brucei.* This parasite also contains 1-10 and 100 intermediate and mini chromosomes respectively which harbour further VSGs at the telomeres. Most VSG pseudogenes found in the subtelomeric arrays also contribute to antigenic variation in *T. brucei* (Vanhamme *et al*., 2001a; b; Marcello and Barry, 2007). Individual VSGs activation is achieved by recombination into the VSG expression sites (ESs), which are located on the telomeres of some megabase and intermediate chromosomes. The exact number of ESs in the *T. brucei* genome is still being determined, however it is estimated to be as high as 20 (Becker *et al*., 2004). The normal composition of an ES include; a single VSG, a number of expression site–associated genes (ESAGs) and an array of 70-bp repeats upstream of the VSG. Unusually for protein-coding genes, transcription of the ESs is driven by RNA polymerase I (Navarro and Gull, 2001; Pays *et al*., 2001; Gunzl *et al*., 2003), which is normally reserved for expression of rRNA genes. In addition, ES expression is directed by a single promoter upstream of the ESAGs. This

means that a multigene primary transcript is synthesised from which individual mature mRNAs are generated by trans-splicing and polyadenylation (Gerrits *et al*., 2002; Gunzl *et al*., 2003).

Variant surface glycoprotein genes are activated in an order of hierarchy. First to be activated are the telomeric VSGs, followed by array VSGs and finally pseudogene VSGs (Stockdale *et al*., 2008). Switching of VSG proteins occurs at a rate substantially higher than the background mutation rate of other genes in the parasite suggesting that it is a regulated process. The VSG switching process is partially dependent on homologous recombination of DNA, which is mediated in part by the interaction of the *T. brucei* BRCA2 gene with RAD51 (Hartley and McCulloch, 2008). In addition to homologous recombination, transcriptional regulation also plays an important role in antigen switching. This is in contrast to other pathogens, where antigenic variation is typically mediated by DNA rearrangements or transcriptional regulation. The process by which VSG switching occurs has not been fully understood, but it is known that activation of VSGs requires recombination of the VSG genes into a VSG expression site (Hartley and McCulloch, 2008).

2.3 Aetiology of Human African Trypanosomosis

HAT is caused by the human infective subspecies of *Trypanosoma brucei. T. brucei* has been classically defined into three subspecies. *Trypanosoma brucei brucei* is infective to animals and is found throughout the tsetse belt of Africa (Hide *et al.*, 1990). *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are both infective to humans. *T. b. gambiense* is associated with chronic disease in West and Central Africa while *T. b. rhodesiense* causes acute form of the disease in Eastern and Southern Africa. The three subspecies are morphologically

indistinguishable (Losos, 1986), except that the human serum-resistance-antigen (SRA) gene found in *Trypanosoma brucei rhodesiense* is lacking in the other two (Gibson *et al.*, 2002).

2.3.1 The biology and classification of *Trypanosoma brucei*

Trypanosoma brucei belongs to the genus *Trypanosoma*, family Trypanosomatidae, a large group of unicellular protozoan parasites, and the order kinetoplastida. It is a spindle-shaped cell measuring 20-30 by 1.5-3.5 μm with a single flagellum (Chappuis *et al.*, 2005). This flagellum emerges from the posterior end, runs along the cell membrane, and extends beyond the anterior part of the cell. The base of the flagellum is associated with the kinetoplast, a large particle containing the DNA of the single mitochondrion (Chappius *et al.*, 2005). The trypamastigote is the only form observed in the mammalian host whereas the epimastigote is the form that occurs within the testse fly. During the entire life cycle, *T. brucei* cells multiply by binary fission and are exclusively extracellular (Chappuis *et al.*, 2005).

The bloodstream forms of *Trypanosoma brucei* are typically polymorphic, measuring between 11 to 30 μm in length (Chappuis *et al*., 2005). This polymorphism is represented by three forms namely: (i) the long slender forms measuring 23 to 30 μm in length, and possess a long free flagellum, a well-developed undulating membrane, a sub-terminal kinetoplast and a narrow drawn-out posterior end, (ii) short stumpy forms that measure between 12 to 22 μm, are stout, lack free flagellum, possess a well-developed undulating membrane and have the kinetoplast near the broadly rounded posterior end, and (iii) intermediate forms that measure between 20-25 μm with a shorter flagellum and a blunter posterior end than the slender forms (Chappuis *et al*., 2005).

The entire cell surface of the bloodstream and metacyclic forms and the flagellum, are covered with a coat that provides general protection against non-specific host resistance mechanisms (Barry and McCulloch, 2001; Borsch, 2002). The surface coat is a prominent immunogen and elicits the production of high titres of antibodies by the host immune system that is lytic to the parasite. Through antigenic variation, a few trypanosomes change their surface coats and survive the host immune system to multiply and produce a new wave of parasitaemia. Each variant is termed a distinct variable antigen type (Barry and McCulloch, 2001; Borsch, 2002). The genome of *T. brucei* contains up to 1,000 different genes encoding the variant surface glyco-proteins (Borsch, 2002; Vanhamme *et al*., 2001a). This phenomenon explains the fluctuating number of circulating trypanosomes in the patient's blood, which contributes to the limited sensitivity of parasite detection methods in clinical practice.

There are two forms of HAT caused by different subspecies of *T. brucei*. The Gambian Sleeping Sickness is caused by *T. b. gambiense* in West and Central Africa while the Rhodesian Sleeping Sickness is caused by *T. b. rhodesiense* in Eastern and Southern Africa. (Hide and Tait, 1991; Tait, 2000; Welburn *et al.*, 2001a). Two types of *T. b. gambiense* have been identified. The type 1 or classical *T. b. gambiense* was classified based on its association with chronic disease in Western and Central Africa. It is distinctly identified by other molecular markers such as the absence of the Antat 1.1 antigen gene and restriction fragment length polymorphisms (RFLPs) in the Antat 1.8 antigen gene (Gibson *et al.*, 1980; Tait *et al.*, 1984; Mathieu-Daude *et al.*, 1994, 1995). It also shows differences in susceptibility to the trypanocidal drug difluoromethylornithime (Bacchi and Yarlett, 1993). The type 2 was grouped together with cattle

stocks of *T. brucei* from Nigeria using the RFLP analysis and was found to be associated with acute-like disease in Nigeria (Hide *et al.*, 1990).

2.4 Genomics of *Trypanosoma brucei*

The two genomes of African trypanosomes are located within the kinetoplast and the nucleus. There are clear evidence to show that nuclear genome is diploid, however, haploid genomes have also been reported especially among the variant surface glycoprotein genes, their expression sites and mini-chromosomes.

2.4.1 Chromosome structure

The *Trypanosoma brucei* genome is made of 11 diploid pairs of megabase chromosomes, as well as one to five intermediate chromosomes and approximately 100 minichromosomes of unknown ploidy (El-Sayed *et al*., 2000; Tait *et al*., 2002). The ends of these linear chromosomes contain typical telomere repeats with the sequence TTAGGG. Essentially, all the housekeeping genes are encoded in the megabase chromosomes and are transcribed as long polycistronic units with few exceptions (Clayton, 2002; Daniels *et al*., 2010; Ersfeld, 2011). The 26 megabase chromosome genome contains approximately 9000 genes, including 1000 non-expressed variant surface glycoprotein (VSG) genes which of most of them are said to be pseudogenes (Simarro *et al*., 2012).

Trypanosoma brucei lives extracellularly in the mammalian hosts and evades the immune response by means of antigenic variation (Vickerman, 1978). These parasites express a single surface coat protein which is called variant surface glycoprotein (VSG) from one of

approximately 15 expression sites (ESs), which are located proximal to the telomeres of megabase or intermediate chromosomes. Notably, expression of the VSGs is driven by RNA polymerase I from a special nuclear site, called the expression site body (Navarro and Gull, 2001). Although VSGs are highly immunogenic, *T. brucei* manages to escape the immune response by switching the expression of VSG up to once per 100 cell divisions (Turner and Barry, 1989; Morrison *et al*., 2005; Lythgoe *et al*., 2007). This VSG switching often involves gene conversion of VSG cassettes into the active ES, creating and expressing a novel VSG gene that has not previously been seen by the immune system (Robinson *et al*., 1999 Boothroyd *et al*., 2009). This intricate monoallelic expression and periodical switching of VSGs enable the parasites to evade the host immune response, making the development of effective vaccine difficult. The parasite possesses approximately 100 minichromosomes that harbour additional VSG genes that serve as templates for recombination into one of the ESs (Sloof *et al*., 1983; Van-Deursen *et al*., 2001). Consistent with the concept that these small chromosomes are important for antigenic variation, they are segregated faithfully during cell division (Zomerdijk *et al*., 1992; Wickstead *et al*., 2003). Minichromosomes are mostly composed of the 177 bp repeats of unknown function (Melville *et al*., 2000; Wickstead *et al*., 2004). In addition to these linear chromosomes, circular DNA of up to 400 kb, called Nuclear Repeat (NR) elements, are found in many strains, although their function remains unknown (Alsford *et al*., 2003).

2.4.2 Centromere structure

The centromere is the chromosomal locus where kinetochores assemble to mediate the interaction with microtubules. Despite its fundamental importance, centromere structures are highly divergent and three different types are found: the regional centromere, point centromere

and holocentric types (Henikoff *et al*., 2001; Berriman *et al*., 2002). It is thought that regional centromeres represent the ancestral form, whereas point centromeres and holocentricity are derived features (Malik and Henikoff, 2009; Melters *et al*., 2012). Determining the position of a centromere can be done in several ways; (i) by mapping the region of a given chromosome that confers mitotic and/or meiotic stability (Clarke and Carbon, 1980; Copenhaver, 1999), (ii) by determining the DNA sequence that associates with kinetochore/ centromere proteins and (iii) by mapping the position of primary constrictions using fluorescence in situ hybridization approach (Maloney *et al*., 2012). The trypanosomatid centromere was first mapped in *T. cruzi* (Obado *et al*., 2005). This was achieved through a functional mapping (telomere-associated chromosome fragmentation) and delineation of the 11–16 kb GC-rich regions that confer mitotic stability. The same regions also exhibit an increased topoisomerase II activity (Obado *et al*., 2007), strongly suggesting that these GC-rich regions represent the centromeres in *T. cruzi*. However, this is quite unusual because centromeric DNA is comparatively AT-rich in essentially all studied eukaryotes (Talbert *et al*., 2009). This unusual feature might be related to the absence of CENP-A. The *T. brucei* centromere was subsequently mapped based on topoisomerase II activity. Unlike *T. cruzi*, its centromere contains AT-rich repetitive arrays (20–120 kb), which are restricted to a single site on each megabase chromosome (Obado *et al*., 2007; Echeverry *et al*., 2012). The unit repeat size/sequence varies among chromosomes, although some chromosomes such as chromosomes 4, 5, 8, 9, 10 and 11 share similar sequences. These chromosomes possess the almost identical sequence of 147 bp referred to as the CIR147 repeat, whereas chromosome 3 has a unique 120 bp sequence (Obado *et al*., 2007). However, it remains to be shown that the AT-rich repeat region confers mitotic stability and centromere activity. Transposable elements are found at the centromere of trypanosomes as in many other eukaryotes (Wong and Choo,

2004). In addition, rRNA gene arrays are present adjacent to the AT-rich repeats of chromosomes 1, 2, 3, 6 and 7, although their significance is currently unknown (Obado *et al*., 2007).

Repetitive sequences found at the centromere of many species are thought to promote the formation of heterochromatin using endogenous RNAi pathways (Folco *et al*., 2008; Lejeune *et al*., 2010). Components of the RNAi machinery are present in *T. brucei*, and small RNAs are detected from the CIR147 repeats (Patrick *et al.*, 2009; Tschudi *et al*., 2012), which are present on the centromeric region of chromosomes 4, 5, 8, 9,10 and 11 (Obado *et al*., 2007). On the other hand, small RNAs are not found from the AT-rich repeats of the centromeres of chromosomes 1, 2, 3, 6 and 7 (Tschudi *et al*., 2012). However, these centromeres contain rRNA gene arrays which may substitute the role of CIR147 repeats. Although it remains unknown whether conventional heterochromatin is formed at the centromere, knockdown of argonaute leads to chromosome segregation defects in *T. brucei* (Durand-Dubief *et al*., 2007). Notably, repetitive sequences are not found at the centromeric region in *T. cruzi* (Obado *et al*., 2007), an organism that does not possess a functional RNAi pathway (Tschudi *et al*., 2012).

2.4.3 Cell cycle

Similar to other eukaryotes, the cell cycle of trypanosomes consists of distinct G1, S, G2 and M phases (Hammarton, 2007; Li, 2012). Trypanosomes possess two DNA-containing organelles namely; kinetoplast and nucleus, both of which must be segregated faithfully. During the cell cycle of procyclic form *T. brucei*, there are distinct replication and segregation periods for kinetoplast DNA and nuclear DNA (Woodward and Gull, 1990; Siegel *et al*., 2008; Gluenz *et*

al., 2011). The kinetoplast finishes its DNA replication first and the kinetoplast elongation/division occurs during the S phase of nuclear division. The nuclear division then occurs, followed by cytokinesis that results in the formation of two daughter cells that contain one kinetoplast and one nucleus. It remains unknown how these temporal orders are established and regulated.

Trypanosomes, like many other protists and fungi (Pickett-Heap, 1974; Heath, 1980), do not disassemble their nuclear envelope during mitosis; a process referred to as closed mitosis (Vickerman and Preston, 1970), and a mitotic spindle forms within the nucleus. Many eukaryotes rely on two microtubule organizing centres (MTOCs) to nucleate a bipolar spindle (Tanenbaum and Medema, 2010; Nigg and Stearns, 2011). Although no distinct structure such as a centrosome or a spindle pole body is detected in *T. brucei*, electron microscopy has visualized ring-like structures, inside the nucleus and close to the nuclear membrane, that appear to nucleate spindle microtubules during mitosis (Ogbadoyi *et al*., 2000). It appears probable that this is a truly intranuclear MTOC specific to the spindle because *T. brucei* is one of the organisms that lack a Brr6 domain protein that appears critical to the process of nuclear envelope fenestration in spindle morphogenesis (Tamm *et al*., 2011). This provides yet more evidence for a set of distinct and dispersed cytoplasmic and nuclear MTOCs in *T. brucei* whose differential activation will require regulation at specific points of the cell cycle (Gull *et al*., 2004). A rhomboid-shaped bipolar spindle is initially assembled and converges into two poles at opposite ends of the nucleus. Later during mitosis, this focal organization is lost and the spindle becomes bifurcated at both ends (Ogbadoyi *et al*., 2000). Spindle pole-specific components have not been identified thus far, and the mechanism of bipolar spindle assembly remains largely unknown in trypanosomes.

2.3.4 Conserved mitotic players

Despite the long evolutionary distance, trypanosomes do possess a reasonable proportion of the basic mitotic machinery discovered in conventional model eukaryotes. This includes the CDK/Cyclin system (Hammarton *et al*., 2003; Tu and Wang, 2004), cohesin complex (Gluenz *et al*., 2008; Bessat and Ersfeld, 2009), separase (Bessat and Ersfeld, 2009), condensing complex (Gluenz *et al*., 2008), Aurora B (Tu *et al*., 2006; Li *et al*., 2009), APC/C (Kumar and Wang, 2005) and proteasome (Mutomba *et al*., 1997; La-Count *et al*., 2001; Bringaud *et al*., 2001). Therefore, the most basic cell cycle machinery appears to be conserved in these distant eukaryotes. Similar to all other eukaryotes, tubulins are highly conserved in trypanosomes and are essential for the segregation of both large and small chromosomes (Ersfeld and Gull, 1997; Bringaud *et al*., 2002a, b). Homologues of microtubule-associated proteins are also present, including XMAP215, EB1 and CLASP, although their relevance to mitotic events remains to be investigated. Similar to other eukaryotes, Kinesin-13 (Wordeman and Mitchison, 1995) plays important roles in faithful chromosome segregation (Chan *et al*., 2010; Wickstead *et al*., 2010). Polo-like kinase is also present, but it does not appear to play critical roles in chromosome segregation (Kumar and Wang, 2006; Hammarton *et al*., 2007; De-Graffenried *et al*., 2008). Some components of the nuclear pore complex have been detected at kinetochores in metazoans (Mishra *et al*., 2010; Wozniak *et al*., 2010), and while nuclear pore components have also been identified in *T. brucei* (De-Grasse *et al*., 2009), none have been detected at trypanosomatid kinetochores thus far.

2.5 Epidemiology of HAT

The first documented reports of Gambian Sleeping Sickness were in 1902 and 1903 in Gambia (Forde, 1902) and Uganda (Bruce, 1903) respectively. This was followed by the discovery of Rhodesian Sleeping Sickness in Rhodesia seven years after (Stephens and Fantham, 1910). The disease was thought to have been largely conquered by the 1960s, until its re-emergence as a serious public health problem in sub-Saharan Africa in the 1990s (Cattand *et al.,* 2001). It is estimated that sixty million people are continuously exposed to the risk of HAT in nearly 200 separate active foci from 36 sub-Saharan African countries where active transmission is reported to be taking place. However, only 5 to 6.7 percent of the population at risk is covered by surveillance (WHO, 1998; Cattand *et al.,* 2001). Despite the absence of reliable epidemiological figures, Cattand *et al*. (2001) estimated that 0.3 to 0.5 million people are currently infected and 0.1 million deaths are caused each year by the disease.

Gambian sleeping sickness is restricted to Western and Central Africa. The most severely affected countries are the Democratic Republic of Congo, the Republic of Congo , Angola, Central African Republic and Southern Sudan, where HAT has re-emerged during the last decades mainly due to long-standing geo-political instability and subsequent erosion or collapse of control programmes (Moore and Richer, 2001; Stanghellini and Jesenando, 2001; Van-Nieuwenhove *et al.,* 2001).

T. b. gambiense is transmitted by the bite of infected tsetse flies of the palpalis group. Human to vector contacts occurs mostly in forested rivers and shores where people frequently visit to collect water and do their washings, but is also pre-domestic when huts are built in or near

plantations (Burri and Brun, 2003). *T. b. gambiense* is morphologically indistinguishable from the human infective *T. b. rhodesiense* and the animal infective *T. brucei brucei,* which has until recently greatly inhibited the understanding of the epidemiology of Sleeping Sickness (Chappuis *et al*., 2005). Gambian trypanosomosis is a chronic disease and lasts for several years (WHO, 1998), but untreated cases usually end fatally. Undiagnosed individuals may continue to spread infection through the activities of teneral tsetse flies (Cattand *et al*., 2001).

Trypanosoma brucei rhodesiense is present in Eastern and Southern Africa. Wild animals are usually affected, but epidemics occasionally occur in domestic animals and humans. The parasite is transmitted by the bite of tsetse flies of the *Glossina morsitans* or *G. fuscipes* groups, and human-vector contact typically occurs in savanna Woodland but can be peridomestic during epidemics (Burri and Brun, 2003). The incidence of Rhodesian Sleeping Sickness is currently much lower than that of the Gambian disease, but large epidemics were observed in the past. Welburn *et al.* (2001b) reported a prevalence of 18% of *T. b. rhodesiense* in cattle from Seroti, Uganda, using the serum-resistant-antigen (SRA) gene identification. Similarly, a 34.3% prevalence of *T. b. gambiense* was reported in pigs from the Republic of Cameroon by means of polymerase chain reaction (Simo *et al.,* 2006).

It has been shown that 50 or less fewer of HAT are diagnosed yearly outside Africa (Lejon *et al*., 2003a, b). Visitors to some game parks in Eastern Africa are said to be at risk of *T. b. rhodesiense* infection (Sinha *et al*., 1999; Jelinek *et al*., 2002). Migrants from countries where *T. b. gambiense* is highly endemic can have HAT that remains unrecognized for years thereby spreading the disease among uninfected population (Sahlas *et al*., 2002).

Studies conducted in Western and Central Africa have failed to find an increased risk of HAT among human immunodeficiency virus (HIV) infected individuals, however, no definite conclusion can be drawn from the available data (Louis *et al*., 1991; Pepin *et al*., 1992; Meda *et al*., 1995). Moreover, there have been no studies describing the clinical presentation and the validity of diagnostic tests in co-infected patients.

In Nigeria, Airauhi *et al*. (2006) reported a prevalence of 17.1% for *T. b. gambiense* in human samples examined from Abraka, Delta State by the use of the card agglutination test. Two years later Osue *et al.* (2008) reported a prevalence of 9.8% in 491 human samples examined from the same area using the same technique, indicating a decline in the prevalence of the disease in the area over two years. Karshima *et al*. (2012) reported prevalence rates of 1.25% and 7.25% in humans and cattle in some LGAs of Taraba State using the card agglutination test for *T. b. gambiense*.

The distribution of animal trypanosomosis follows the pattern of tsetse spread in Nigeria covering about 80% of the total land mass between latitude 4 ^{O}N to 13 ^{O}N including the highlands of Jos, Mambilla, and the Obudu plateau which were said to be free of tsetse and animal trypanosomosis (Omotainse *et al.,* 2004). Onyiah, (1997) reported a prevalence of 10.0% in cattle, 8.6% in sheep and 8.1% in goats in some Northern States of Nigeria between 1993 and 1996. Similar studies showed a prevalence of 22.2% in ruminants from Lere LGA of Kaduna State. In some LGAs of Kano State, prevalence rates were reported in cattle, sheep, and goats to be 5.3%, 12.0%, and 0.7% respectively (Kalu and Lawani, 1996). Omotainse *et al.* (2000)

reported in Konshisha LGA of Benue State prevalence rates of 57.1%, 33.9%, and 36.8% in sheep, goats, and pigs respectively. Studies in Gombe State showed prevalence rates of 7.4% in sheep and 5.0% in goats from Alkaleri LGA (Daniel *et al.,* 1993), while in Yamaltu-Daba LGA prevalence rates were shown to be 10.4% and 8.85% in cattle and sheep respectively (Omotainse *et al*., 2001).

2.6 The Burden of Human African Trypanosomosis

The available estimates for HAT indicate that 60 million people are at risk of both the Gambian and Rhodesian forms of Sleeping Sickness in sub-Saharan Africa in approximately 200 distinct foci (WHO, 1998). The greatest burden of reported cases is due to *Trypanosoma brucei gambiense* representing over 90% of total HAT cases (Cordon-Obras *et al*., 2010; Wombou *et al*., 2011). A total of 23,832, 19,901, 17,036, 15,651, and 11,382 of *T. b. gambiense* and 655, 514, 580, 727, and 486 *T. b. rhodesiense* cases were reported in 2002, 2003, 2004, 2005, and 2006, respectively (WHO, 2006; Simarro *et al*., 2008). Approximately two-thirds of reported *T. b. gambiense* cases occur in the Democratic Republic of Congo (DRC) (Simarro *et al*., 2008). These data, especially for *T. b. gambiense*, illustrate an encouraging trend for countries such as Angola, Democratic Republic of Congo and Sudan where concerted efforts have been mounted to control HAT. The decrease in disease incidence in recent years is due in large part to enormous efforts involving active case detection. These efforts need to be maintained despite the increasing cost per patient of detecting additional cases in successful control programmes (Shaw and Cattand, 2001). There is much still to do before elimination of HAT can be considered a real option in the near future. It has been estimated that up to 70,000 cases actually occur annually (WHO, 2006) against the previous estimate of 300,000 (WHO, 1998). However, most of these cases go unnoticed especially in Nigeria where control programmes are not sustained for reasons

related to lack of funds. The disparities in these estimates illustrate the need for formal methods to quantify the substantial hidden burden of HAT.

The combined burdens of *T. b. gambiense* and *T. b. rhodesiense*, was considered in the first global burden of disease assessments (Fevre *et al*., 2008b), with an estimated 1.78 million daily adjusted life years (DALYs) lost across Africa, using a standard West 26 life table (life tables describe age-specific mortality in a population and determine, for example, the number of years of life lost following death at a given age), and an annual incidence of 32,000 reported cases, including 24,000 deaths (Murray and Lopez, 1996b)**.** Subsequent assessment gave an estimated 1.34 million DALYs lost to HAT in 2000 (WHO, 2008) and 1.54 million DALYs lost in 2002 (WHO, 2003; Mathers *et al*., 2007) and 48,511 deaths. Issues in assessing the burden of HAT and quantifying disease impact is necessary for providing information required for effective decision making in relation to planning of control and intervention strategies (Murray and Lopez, 1996a). Burden can be measured in terms of impact at a range of scales, the individual, family groups and society at large. For decision-making at the societal level (e.g., government policy, national or regional budgetary allocation, etc.), a societal, or population-based approach, is most appropriate. For this, a range of tools are available (Barry *et al*., 2005; Sassi, 2006); and the DALY is a useful and now well-established measure (Murray *et al*., 2004; Mathers and Loncar, 2006; Stein *et al*., 2007). Proper quantification matters greatly to the neglected diseases, because a primary reason for their neglect is that their true impact on society is not known. For focal diseases such as HAT, it is necessary to choose an appropriate scale at which the assessment of burden is carried out in many sub-Saharan African countries.

Also, HAT not only occurs focally, but occurs in both endemic and epidemic situations. While relative disease burdens during periods of endemic and epidemic periods matter in themselves, it would be a mistake to calculate a DALY for an epidemic and assume that this was then the more general baseline level of burden for longer term planning. Rather, when determined for epidemics or outbreaks this should be explicit (Fevre *et al*., 2008a). Routine data collection during periods of endemic transmission may be lacking in many settings, while epidemics or outbreaks focus attention on a disease, resulting in greater availability of data (Fevre *et al*., 2008b). Epidemic situations do however, present a particular set of conditions for the assessment of the cost-effectiveness of control as well as long-term investment (Fevre *et al*., 2008b), and DALYs, as an outcome measure of such analyses, can add significantly to the valuation of alternate investment options. The time units chosen for burden assessments also matter, particularly if outputs influence resource allocation. Recent decreases in the number of reported HAT cases (Simarro *et al*., 2008) could, for example, lead to a reduction in effort in detecting future cases, a situation which has previously led to disease resurgence (Van- Nieuwenhove *et al*., 2001; Lutumba *et al*., 2005). It becomes important that temporal trends in incidence are accounted for in the choice of time period for assessing burden, though the sporadic nature of data availability may make this difficult in practice.

2.7 Economics of Human African Trypanosomosis

2.7.1 Economics of case detection

In Gambian trypanosomosis, surveillance and active case detection paly a central role in disease control by ensuring that diseased individuals are diagnosed and treated, thereby reducing the size of the human reservoirs. Over the years, case detection depended on passive detection where

patients with marked clinical signs are referred from local health clinics to HAT special centres for definitive diagnosis and treatment. However, the most yieldful case detection method is the active and exhaustive case-finding using mobile teams and screening large sections of the population at risk. The appropriate intensity of case-finding should be a function of the local prevalence of the disease (WHO, 1998). In order to support and direct active case-finding to control the disease, a systematic approach for surveillance has been developed based on mapping foci of the disease (Cattand *et al*, 2001). Villages and hamlets are categorized according to their infection status, endemic villages are grouped into foci, and decisions are taken as to where to direct control activities and where to initiate specific surveillance activities based on serologically testing a proportion of the population.

Several studies analyzed the costs of case-finding and treatment in Uganda (WHO, 1998), Cote d'Ivoire (Trowbridge *et al*., 2001) and Central African Republic (Perry and Randolph, 1999). According to Walker and Fox-Rushby (2000), the naira equivalence of the approximate cost of screening one person by mobile teams in the Central African Republic 14 years ago was estimated at 425 naira (2.5 US dollar). However, the use of this screening test is still limited in Nigeria due to the unavailability of the screening kits.

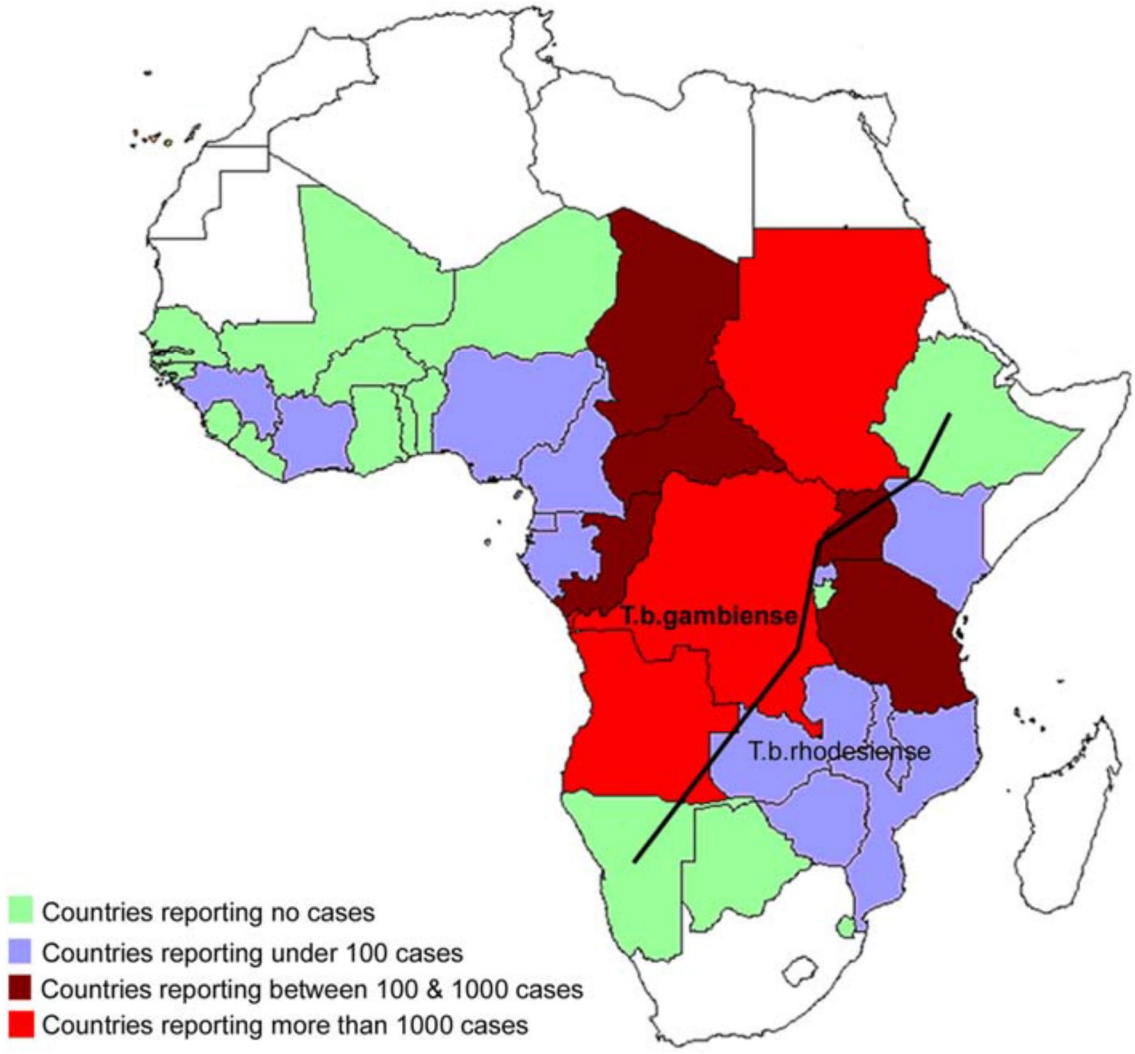


Fig 2.1: Distribution of Sleeping Sickness in relation to number of cases reported annually (Simarro *et al*., 2008).

2.7.2 Economics of treatment

Taking cognizance of the timing of drug administration and duration of hospitalization, different treatment regimes have been in use for several decades with considerable variations from country to country (WHO, 1998). Some of the costs of treatment are borne by family members, who may provide food, pay some costs or accompany the patient (WHO, 1998). An estimate of treatment costs as presented by the WHO (1998) gives first-stage treatment cost at US$ 107 (#18,190) for treatment using pentamidine at a subsidized price, at against US$ 227 (#38,590) at full market cost and US$ 114 (#19,380) for suarmin. For second-stage treatment, the estimates were US$ 257 (#43,690) for treatment using melarsoprol and US$ 675(#114,750) using eflornithine. Since then, WHO's public/private partnerships have assured a cost-free supply of drugs until 2006. Second-stage treatment regimes using melarsoprol are usually based on 20-30 days hospitalization, however, new 10 day schedule for treating second-stage Gambian sleeping sickness is being tested. This would improve the cost-effectiveness of treatment and, to a certain extent, relieve the financial burden on families.

2.7.3 Economics of vector control

Several vector control activities at different timings and localities targeted HAT control. Field costs for vector control from some of these schemes have been documented. Tsetse control activities using traps and screens and involving local communities have been undertaken for Gambian Sleeping Sickness in Cote d'Ivoire and Congo and for Rhodesian Sleeping Sickness in Uganda. Trowbridge *et al*. (2001) summarized the work of many years in Cote d'Ivoire, citing costs for traps, screens and vehicle running, which came to US$ 39/Km^2 (#6,630//Km^2) protected

and US$ 2.3/person (#391/person) protected. This study was also accompanied by active case-finding.

2.8 The Testse Vector of Human African Trypanosomosis

Tsetse flies include all flies of the genus *Glossina*. They are large biting flies inhabiting much of mid-continental Africa between the Sahara and the Kalahari Deserts (Rogers *et al.*, 1996). The flies are narrow-bodied, yellowish to dark brown and 6 to 15 mm long. At rest, the wings are held over the back, overlapping almost completely. The body of tsetse usually has dark and lighter patches, making the insect difficult to see when it is settled on bark of trees, rock and soil (FAO, 2009). Tsetse flies belong to the kingdom Animalia, phylum Arthropoda, class Insect, order Diptera, subsection Calyptratae, superfamily Hippoboscoidea, family Glossinidae and genus *Glossina* (FAO, 2009).

2.8.1 Morphology and biology of tsetse fly

The knowledge of tsetse morphology and biology is important in the institution of tsetse prevention and control strategies. Like all other insects, the tsetse fly has a tough outer covering or cuticle. This cuticle is elastic so that it can stretch and allow for large bloodmeal (FAO, 2009). Tsetse flies are readily distinguished from all other biting flies and similar sized non-biting flies by the combination of a rigid and forwardly projecting proboscis (Urguhart *et al*., 2003), and a characteristic wing venation. In between vein four and five of the wing is a closed cell which looks like an upside down hatchet and is referred to as “hatchet cell” (FAO, 2009). At rest, tsetse flies normally appear quite slim because the wings are placed one over the other on the back as a

pair of a closed scissors (Services, 1980). Immediately after bloodmeal, the abdomen becomes larger, rounded, and reddened.

The proboscis (haustellum) is made of three parts: the labium, the labrum, and the hypopharynx. The labrum forms the tube (food canal) through which blood is sucked from the host while, the hypopharynx is a narrow tube through which saliva, and of course saliva contaminated with infective metacyclic forms in infected tsetse is injected into the host animal during a blood-meal (FAO, 2009). The male genital organ (hypopygium) is seen at the posterior end of the abdomen. Just in front of the hypopygium is a plate bearing dark hairs called hectors. The female genital organ contains a small opening (vulva) through which the larva emerges. This opening may have a few small plates around it. The number and arrangement of these plates can be used for species identification (FAO, 2009).

2.8.2 Life cycle of the tsetse fly

After mating, the female tsetse takes bloodmeals which support the development of a single egg in one of the two ovaries. The egg completes its maturation, passes down the common oviduct into the uterus where it is fertilized by the release of spermatozoa from the spermathecae (Service, 1980). The adult female deposits the third stage larvae within approximately 10 days. At this stage, the larva is creamy-white, segmented, and has a pair of prominent ear-shaped protuberances on the posterior end which are referred to as polyneustic lobes, which is respiratory in function. After deposition, the larva wriggles into loose soil to a depth of few centimeters and form a rigid dark-brown, barrel-shaped puparium (Urquhart *et al*., 2003). The pupal period may last for about 4 to 5 weeks depending on environmental temperature. On

emergence, the female fly requires several bloodmeals over a period of 16 to 20 days in order to produce its first larva (Urguhart *et al*., 2003).

In natural conditions, the most important vectors of *T. b. gambiense* are *Glossina palpalis* and *Glossina tachinoides* in West Africa and *G. fuscipes* in Central Africa. These vectors are especially common at watering places like rivers or lakes where people frequently visit to collect water and do their washings (Service, 1980). *Glossina morsitans*, *G. swynnertoni* and *G. pallidipes* are important vectors of *T. b. rhodesiense* and occur in the Savanna areas of East Africa (Service, 1980).

2.9 Reservoir Hosts of Human African Trypanosomosis

The zoonotic nature of *T. b. rhodesiense* was confirmed since 1958 when Heisch *et al*. (1958) tested human 'volunteers' with parasites collected from an infected bushbuck and the infection resulted in Sleeping Sickness (Chappuis *et al*., 2005). This observation led to an over-emphasis being placed on the wild animal reservoir, and on the bushbuck in particular, in relation to the epidemiology of the disease (Chappuis *et al*., 2005). Subsequently, Onyango *et al.* (1966) carried out a similar study in human 'volunteers' using parasites collected from infected cattle. This study confirmed the involvement of domestic animals as reservoirs of Sleeping Sickness. There is no definitive human 'volunteer' experiment on the zoonotic nature of *T. b. gambiense,* but indirect evidence based on isoenzymes and DNA showed that *T. b. gambiense* also has animal reservoirs (Mehlitz *et al.*, 1981, 1982; Paindavoine *et al.*, 1986).

Subsequent studies reported the involvement of various species of wild and domesticated animals as reservoirs of the causative agents of Sleeping Sickness. Welburn *et al.* (2001b) reported 18% prevalence of *T. b. rhodesiense* in cattle from Seroti, Uganda using the serum resistance antigen gene as a marker. A recent study by Karshima *et al*. (2012) in some parts of Taraba State reported a prevalence of *T. b. gambiense* in cattle indicating the involvement of livestock in the transmission cycle of the disease in the State. In addition, a 34.4% prevalence of *T. b. gambense* was reported in pigs from the Fontem sleeping sickness focus of the Republic of Cameroon using polymerase chain reaction (Simo *et al.*, 2006). Mbaya *et al*. (2009) reported the effect of DL-α-Difluoromethylornithine on biochemical changes in Baboons (*Papio anubis*) experimentally infected with *T. b. gambiense* and noticed an acute-like disease that resembled experimental *Trypanosoma brucei brucei* infection in animals. The existence of animal reservoirs of Sleeping Sickness has consequences for the origin of the disease foci, the spread of the disease, as well as in effective control despite the effective diagnosis and treatment approach used (Chappuis *et al*., 2005).

2.10 Life Cycle and Transmission of *T. brucei*

The infection of a mammalian host starts with the bite of an infected tsetse fly (*Glossina* species), which injects the metacyclic trypomastigote forms of the parasite present in its saliva via its hypopharynx during the course of taking a blood-meal (Chappuis *et al.*, 2005). The trypanosomes multiply locally at the site of the bite and form a local skin reaction known as chancre 4 to 14 days post-infection. The chancre resolves in about 2 to 3 weeks (Chappuis *et al*., 2005). Few days post-infection the trypanosomes enter the bloodstream and transform into bloodstream trypomastigotes, which are carried to other tissues (CDC, 2009). The primary

reason for this transformation is adaptation to the energy source (glucose) and for proliferation (Katelyn and Mathews, 2007). The trypomastigotes multiply by binary fission in various body fluids such as the blood, 1ymph, and spinal fluid. These parasites can cross the blood-brain barrier and cause the final and fatal stage of Sleeping Sickness (CDC, 2009).

The bloodstream trypomastigote form of *T. brucei* enters the tsetse fly vector during the cause of ingestion of the bloodmeal from an infected mammalian host (CDC, 2009). Using flagellum for propulsion, it travels to the midgut of the fly. In the midgut, the bloodstream trypomastigotes transform into procyclic trypomastigotes which multiply by binary fission (CDC, 2009). The primary reason for this transformation is to facilitate proliferation, because mitosis requires a great deal of energy, the parasite must develop a means of utilizing the available nutrients in its environment. In the midgut of the fly, the nutrient available is proline while in the blood the main source is glucose. The parasite transforms such that it can utilize the glucose for mitosis (Katelyn and Mathews, 2007).

The procyclic trypomastigotes leave the midgut and transform into epimastigotes. These epimastigotes multiply in the salivary gland and transform into infective metacyclic trypomastigotes. Following the bite of an infected tsetse fly, the infective metacyclic trypomastigotes are injected under the skin at the site of bite and the cycle is repeated as shown in figure 2.1 (Chappuis *et al.,* 2005; CDC, 2009).

2.11 Pathogenesis and Pathology of HAT

The pathogenesis of HAT is traditionally divided into two stages namely the first or haemolymphatic stage and the second or central nervous system (CNS) stage. The first or early phase is associated with the presence of parasites in the lymphatics, blood and other body systems without any CNS involvement (Pentreath, 1995; Kennedy, 1999). It is referred to as the phase of haemolymphatic involvement in which there are no obvious signs of central nervous system (CNS) pathology, nor alterations in the cellular composition. The protein contents are not seriously affected and absence of the parasites in the cerebrospinal fluid (Pentreath, 1995; Kennedy, 1999). The second phase is seen during the late stage when the parasite damages the CNS and the condition may be diagnosed by marked increases in the white blood cell (WBC) counts, increase in protein levels, and the presence of parasites in the cerebrospinal fluid (Molyneux *et al.*, 1996).

The CNS involvement may occur within a few weeks to several months, or it may take years after the initial infection. However, this usually develops gradually, with a range of neurological signs and symptoms, and mental disturbances that may occur long before diagnostically detectable changes in the CSF could occur. The pathology occurs in different body systems of the untreated patient and progresses inexorably, with no single cause of death (Chappuis *et al*., 2005).

2.11.1 Initial response and pathologies in the non-nervous body systems

2.11.1.1 *Skin reactions in HAT patients*

At the sites of bite by infected tsetse flies, where trypanosome parasites are deposited in the subcutaneous tissue, a hard, painful and red nodule usually 2 to 3 cm in diameter may develop. It arises 4 to 14 days following an infected tsetse bite and resolves in about 2 to 3 weeks thereafter. Morphologically, the chancre is an inflammatory process, with oedema, infiltration of macrophages and lymphocytes, and multiplying parasites (Chappuis *et al*., 2005).

Skin rashes are observed most commonly on the shoulder, trunk, and thighs, appearing as oval, erythematous areas with clear centres. Skin rashes are common in fair-skinned patients and are considered cardinal diagnostic symptoms of infected Europeans. The rashes are accentuated by heat, sweating or cold, do not itch, and is not always correlated with other disease features such as fever episodes. Pruritis occurs in about 50% of all cases of HAT (Chappuis *et al*., 2005). It is important to know that pruritis is common to a wide range of infections and disorders where foreign substances or altered levels of endogenous mediator chemicals may affect sensory nerve endings.

2.11.1.2 *Lymph nodes*

Enlargement of lymph nodes follow parasites multiplication and spread from the chancre. Cervical lymph node enlargement is marked in the chronic Gambian disease and is an important diagnostic feature referred to as Winterbottom's sign (Chappuis *et al*., 2005). There is also expansion of the germinal centres of the lymphocyte-plasma cell series, macrophages and

general oedema. It is important to note that except during new variant development where parasites exist predominantly, the most preferred site for these parasites is the lymph tissue. After the development of these new variants, the trypanosomes again escape into the extracellular spaces in the tissue. The chronic nature of this disease allows for these waves of parasitaemia making parasites undetectable in peripheral blood. However, during these periods, swollen lymph nodes are rich sources for isolating trypanosomes (Pentreath *et al*., 1997).

2.11.1.3 *Blood*

The nutritional requirements of the rapidly multiplying variants of trypanosomes are obtained from the patients' blood resulting in serious changes in the glucose and a range of other substrates and metabolites (Apted, 1970; Jenkins and Facer, 1985). There is a fluctuation in the waves of parasitaemia which occurs between 1 and 7 days and separated by intervals of several days or even a month during which parasites are undetectable in the blood (Apted, 1970; Jenkins and Facer, 1985). The blood composition becomes affected as a result of the physiological mechanisms associated with the pathological changes in most body systems, together with the attempted repair processes. This results in a decline in both erythrocyte and leucocyte counts (Chappuis *et al*., 2005). As a strategy of defence by the host, the determinant B cells are activated via both T cell-dependent and independent pathways during the development of new variant surface glycoprotein (VSG). Parasite elimination results from immune lysis and their destruction in the Kupffer cells of the liver following an elevated level of immunoglobulins especially IgM. Following the elimination of particular population of variant trypanosomes, the variant antigenic type (VAT) specific IgG and IgM levels decline to low concentrations but the levels of the invariant trypanosome antigens remain high. Polyclonal immunoglobulins directed

against a large range of host autoantigens, including components of the nervous system such as myelin also increase. Although the responsiveness of B and T cells is generally increased, there is paradoxical progressive immunosuppression due to impairment of T-cell responses (e.g. helper, suppressor and cytotoxic functions) to the persistent trypanosome antigens (Vincendeau *et al*., 1999).

The anaemia that occurs in HAT is as a result of the destruction of erythrocytes in trypanosome-infected individuals. *T. brucei* expresses cysteine protease enzymes that have been shown to digest the surface of erythrocytes. Subsequently, the deformed erythrocytes are not recognized by the host immunological mechanism and are therefore phagocytosed (Mbawa *et al.,* 1992; Chagas *et al.,* 1997). In dyserythropoiesis, erythrocyte synthesis is not sufficient to compensate for the destroyed cells (Chappuis *et al*., 2005). Trypanosome specific antibodies are produced which combine with corresponding trypanosome antigens attached to the surface of the erythrocyte. This antigen-antibody reaction results in the formation of anti-trypanosomal antibody-antigen complex. The antigen-antibody complex binds to erythrocyte surface and subsequent lysis occurs once complement becomes involved (Chappuis *et al*., 2005). The nature of the anaemia observed in HAT is usually macrocytic normochromic or microcytic hypochromic (Kagira *et al.,* 2006).

2.11.1.4 *Spleen*

Following infection, splenomegaly may occur. The Sinusoid becomes packed with activated macrophages (Chappuis *et al*., 2005).

2.11.1.5 *Heart and blood vessels*

The heart is the main organ at risk, especially in the acute Rhodesian Sleeping Sickness. In many patients, damage to the heart is manifested by tachycardia or bradycardia, cardiac murmurs, myocarditis with congestive heart failure, pericarditis, pericardial effusions, and pancarditis (Chappuis *et al*., 2005). Another characteristic of the disease is increased capillary fragility and permeability with extensive petechiations. This is associated with generalized oedema, especially of the extremities and face (Chappuis *et al*., 2005). The cause of the oedema is not known but obvious possibilities are the increased cellular trafficking across the capillary endothelium, substances released (e.g proteases) or a range of substances released from tissues by the parasite, including vasoactive amines and peptides, cytokines and nitric oxide (Boreham, 1985: Pentreath, 1999). There is also pathological loss of a number of blood substances due to damage to the glomerular filter of the kidney.

2.11.1.6 *Hormonal and endocrine changes*

Dysfunctions of the endocrine system are very common and occur in majority of Sleeping Sickness patients. In females, endocrine changes may include; menstrual disorders, sterility, abortion, premature birth, still-birth, and perinatal deaths. These changes are associated with uterine hypolasia, and atrophy of the sexual organs. In males, alterations commonly include impotence and testicular atrophy (Chappuis *et al*., 2005). In the late satge, a state of gynaecomastia develops, where the fat tissue adopts a feminine distribution, and this together with the generalized oedema may give the superficial appearance of a well-nourished person (Apted, 1970).

Endocrine rhythms and the sleep-wake cycle are associated with several types of interaction. For instance, hormones such as prolactin, have sleep-dependent secretory patterns, even when sleep patterns are altered in disease states (Radomski and Brandenberger, 1999). Substances such as growth hormone and plasma rennin are tightly coupled to the internal sleep structure, whereas other hormones such as cortisol are relatively independent of the sleep-wake rhythm generator. Measurement of the substances in Sleeping Sickness patients has shown that, despite the disorganization of the sleep pattern such as day time somnolence, the relationship between the hormonal pulses and the sleep phases was preserved. Thus the slow wave sleep (SWS) episodes remained associated with increases in cortisol and growth hormone, and rapid eye movement (REM) sleep is associated with the pineal hormone, which can modify the circadian timing system (Radomski and Brandenberger, 1999).

2.11.2 Nervous system and neuropathology

The neurological disturbances associated with human African trypanosomosis include a wide range of mental, motor, sensory, and circadian patterns (e.g. sleep) of disturbances. These signs are usually associated with late stage disease but may occur even early in more acute form as seen in type 2 *T. b. gambiense* and *T. b. rhodeisense* (Atouguia and Kennedy, 2000). Other signs and symptoms such as irritability, loss of ability to concentrate, lassitude, anxiety, violent mood swings, uncontrolled sexual impulse, delirium, hallucinations, suicidal tendencies, and manic episodes are characteristic of the nervous stage of the disease (Atouguia and Kennedy, 2000).

The presentation of signs such as tremors of the finger and tongue, fasciculations involving the muscles of the face, neck, arms and upper trunk as well as the paralysis of the skeletal muscles

and cerebellar ataxia which usually lead to difficulty in walking and speech are all manifestations of motor involvement. Moreso, hyperaesthesia, paraesthesia, anaesthesia, and intensive generalized pruritis are indicators of sensory changes. Sleep disturbance which is the final stage of the disease is more prominent in Gambian disease than Rhodesian disease (Atouguia and Kennedy, 2000).

2.12 Clinical Features of Human African Trypanosomosis

The clinical presentations of the Gambian and Rhodesian trypanosomosis are remarkably different. While the former causes a chronic illness that lasts for years, the later usually presents as an acute febrile illness that is fatal within weeks or months if left untreated. Exceptions to this rule occur, since fulminant illness has been described with *T. b. gambiense* infections and chronic disease caused by *T. b. rhodesiense* has also been reported in Uganda (Buyst, 1975; Boatin *et al.*, 1986; Burri and Brun, 2003). The clinical features can be indicative of HAT but are not sufficiently specific to be used to detect all infected individuals, confirm the diagnosis, or stage the disease (Jannin *et al.*, 1993; Bisser *et al.*, 1997; Lejon *et al.*, 2001). Diseases such as malaria, enteric fever, tuberculosis, meningitis and HIV infection can mimic or even coexist with HAT. Therefore, clinical suspicion must be confirmed by laboratory tests.

At the site of the infecting bite, parasites proliferate, and after 5 to 15 days, the site produces a nodule or ulcer called a chancre which resolves within a few weeks. In African patients, chancres are generally absent at the time of diagnosis in both forms of HAT (Buyst, 1975; Boa *et al.*, 1988). This contrasts with the high prevalence of chancres observed in European patients, particularly those infected with *T. b. rhodesiense* (Duggan *et al.*, 1966; Jelinek *et al.*, 2002). In the largest published review of Europeans diagnosed with HAT, chancres were more frequently

observed in patients returning from Eastern Africa (46%) than in patients returning from Western or Central Africa (23%) (Duggan *et al.*, 1966).

2.12.1 The Gambian trypanosomosis

After a long but variable initial asymptomatic period, patients with Gambian trypanosomosis can present with intermittent nonspecific symptoms such as fever, fatigue, headache, arthralgia, and pruritis. The skin rash, often observed in white skin patients is usually not visible on dark skin (Duggan *et al.*, 1966). Transient limb or face oedema can occur. Enlarged, painless, rubbery cervical lymph nodes in the posterior cervical triangle were recognized as an alert sign for HAT long ago by Sir Thomas Masterman Winterbottom, who noted that slave traders in the late 18th century used neck swelling as an indicator of the sleepiness or abnormal behavior that made particular slaves undesirable (Barrett *et al.*, 2003). Splenomegaly is another nonspecific sign and is more commonly found than hepatomegaly (Boa *et al.*, 1988). This highly variable clinical picture grossly corresponds to the first or hemolymphatic stage of the disease. Later in the disease, neuropsychiatric symptoms and signs due to the CNS invasion by trypanosomes and the resulting immune response (Greenwood and Whittle, 1980), gradually become more prominent.

The interval between the start of the infection and the second, or neurological stage is in the order of months or years (Burri and Brun, 2003). The clinical features can be grouped into categories such as psychiatric, motor, sensory, and sleep abnormalities. The mental disturbances may include irritability, lassitude, headache, personality change, and overt psychiatric presentations such as psychosis. Pyramidal (e.g. focal paralysis), extrapyramidal (e.g. rigidity and tremor), and cerebellar (e.g. dysarthria and ataxia) disorders are common. Delayed and

increased sensation to pain (Kerandel's sign) can also be noticed (Kennedy, 2004). Reversal of the normal sleep-wake cycle, with daytime somnolence alternating with nocturnal insomnia is typical. Weight loss and endocrine abnormalities such as amenorrhea and impotence are also frequent complains. If left untreated, patients ultimately die from the consequences of severe wasting, dysfunction of the immune system, deep coma, and seizures, often due to pneumonia or meningitis (Kennedy, 2004).

2.12.2 The Rhodesian trypanosomosis

HAT caused by *T. b. rhodesiense* infection presents as an acute febrile illness starting 1 to 3 weeks after the infective bite. It cannot be distinguished clinically from other tropical fevers such as malaria, enteric fever, and bacterial meningitis. Compared to *T. b. gambiense* illness, febrile episodes are more pronounced and frequent and lymphadenopathy is usually generalized (Burri and Brun, 2003). Keratitis and conjunctivitis have been observed. There is less demarcation between first and second-stage illness, and CNS involvement can be clinically limited to drowsiness and tremor (Greenwood and Whittle, 1980; Burri and Brun, 2003). Pancarditis with congestive heart failure, arrhythmia, and pericardial effusion can kill the patient before pronounced CNS involvement becomes apparent (Stich *et al.*, 2002). Most deaths (80%) occur within 6 months of onset of illness (Odiit *et al.*, 1997).

2.13 Diagnosis of Human African Trypanosomosis

Diagnosis of HAT involves the demonstration either in the blood, CSF or other body fluids of the parasite causing Gambian and Rhodesian Sleeping Sickness. In HAT diagnosis, clinical, biological, serological and molecular features are all considered as indirect evidence of disease

while the demonstration of parasites still remains the only direct evidence of the disease. For reasons related to costs and risks associated with treatment, the demonstration of the parasite is mandatory for primary diagnosis (Chappuis *et al*., 2005). The diagnosis of the disease involves three major steps which include screening, diagnostic confirmation, and staging. The majority of control programmes rely on active case detection through mass population screening. Screening tools therefore need to be sensitive, practical, quick, and cheap (Chappius *et al.*, 2005). For that purpose, the card agglutination test for trypanosomosis (CATT/*T. b. gambiense*), currently used in most areas of endemic infection, is a more efficient screening method than the cervical lymph node (CLN) palpation and puncture (Robays *et al*., 2004). Diagnostic confirmation then relies on the finding of trypanosomes in the blood, lymph nodes, or cerebrospinal fluid (CSF). Unfortunately, it is estimated that 20 to 30% of patients are missed by the standard parasitological techniques (Robays *et al*., 2004). Staging of the disease is a key step that allows classification of the patient into the first (haemolymphatic) or second (meningoencephalitic) stage of the disease. In the absence of reliable blood tests able to detect CNS invasion by the parasite, HAT staging relies on the CSF examination. Some of the techniques used in diagnosis of HAT include:

2.13.1 Biological blood parameters

Biological blood parameters such as increased sedimentation rate and low haematocrit reflect the systemic chronic inflammation present in Sleeping Sickness patients and are non-specific. There is mild or absence of thrombocytopenia, and features of disseminated intravascular coagulopathy are not found (Chappius *et al.*, 2005). Total plasma proteins show decreased albumin and increased immunoglobulins, especially of immunoglobulin G (Bisser *et al.*, 1997). Clinical

features such as chancre formation, enlargement of cervical lymph nodes, cardiovascular disturbances, and disturbances of consciousness and sleep can be considered as indirect evidence of the disease (Chappuis *et al*., 2005).

2.13.2 Serological diagnosis

2.13.2.1 *The card agglutination test for trypanosomosis (CATT)*.

This is a fast and simple agglutination assay developed for detection of *T. b. gambiense* specific antibodies in the blood, plasma, or serum of Sleeping Sickness patients (Magnus *et al.*, 1978). Since the introduction of the CATT/*T. b. gambiense*, it has proven to be useful for mass population screening in the diagnosis of Gambian Sleeping Sickness, limiting the number of parasitological examination to patients found with a positive serology (Chappuis *et al.*, 2005).

The antigen consists of lyophilized bloodstream forms of *T. b. gambiense* variable surface antigen type Li Tat 1.3. Antigen production is a fastidious process based on the extraction of trypanosomes from infected rat blood. The trypanosomes are then fixed, stained with Coomassie blue, and freeze-dried (Chappuis *et al.*, 2005). The reagent is produced under standard quality control procedure, and is currently made only at the Institute of Tropical Medicine in Antwerp, Belgium. The field kit containing the essential reagents, control sera, and a 12/220-V card rotator are commercially available (Chappuis *et al.*, 2005).

To perform the test, one drop of the reagent is mixed with one drop of test blood on the card area and shaken for 5 minutes on the rotator, and the development of agglutination visible to the

naked eyes can be read (Chappuis *et al.*, 2005). The sensitivity of the CATT on undiluted whole blood (CATT-wb) varies from 87 to 98% (Penchenier *et al.,* 2003; Simarro *et al.*, 2003; Robays *et al.,* 2004). When the CATT is performed on undiluted blood or serum with low dilution (less than 1:4), the agglutination can be inhibited, a phenomenon referred to as prozone. This is caused by complement factors, and affects the sensitivity of the test. This problem can be overcome by the addition of ethylene diamine tetra-acetic acid (EDTA) to the dilution buffer (Pansaerts *et al.*, 1998). Despite a reported specificity of around 95%, the positive predictive value of the CATT-wb remains limited because the test is used for mass screening in populations where the prevalence of HAT is usually below 5% (Truc *et al.*, 2002; Penchenier *et al.*, 2003; Robays *et al.*, 2004). False-positive results can occur in patients with malaria and other parasitic diseases such as transient infection by non-human trypanosomes (Magnus *et al.*, 1978).

2.13.2.2 *The latex agglutination test for T. b. gambiense*

The latex agglutination test for *T. b. gambiense* has been developed as a field alternative to the CATT (Buscher *et al.,* 1999). The test is based on the combination of three purified variable surface antigens, Li Tat 1.3, 1.5, and 1.6, coupled with suspended latex particles. The test procedure is similar to the CATT, including the use of similar rotator (Chappuis *et al.,* 2005). In recent field studies in Western and Central African countries, the latex/*T. b. gambiense* showed a higher specificity (96 to 99%), but a lower or similar sensitivity (71 to 100%) as compared to the CATT (Jamonneau *et al.*, 2000; Truc *et al.*, 2002; Penchenier *et al.*, 2003).

2.13.2.3 *Immunofluorescence assay (IFA)*

The use of immunofluorescence assays have been reported with high sensitivity and specificity for the control of HAT in Equatorial Guinea, Gabon, and the Republic of Congo (Noireau *et al.*, 1988). The availability of standardized antigen at low cost has greatly improved the reliability of the test (Magnus *et al.*, 1978). Further evaluations are needed before it can be recommended for routine field use.

2.13.2.4 *Enzyme-linked immunosorbent assay (ELISA)*

This method can be performed with serum, filter paper eluates, and cerebrospinal fluid (CSF) with strict standardization and quantification (Lejon *et al.*, 1998). Using ELISA, trypanosome antibodies have been detected from a group of 23 confirmed Sleeping Sickness patients (Lejon *et al.*, 2003a; b). Antibody levels in the saliva were found to be 250-fold lower than in the serum and could not be detected by the CATT or the LATEX/*T. b. gambiense* in the vast majority of these patients (Lejon *et al.*, 2003a; b). The use of IFA and ELISA is limited to a few laboratories because of the sophisticated equipment required for these techniques.

2.13.2.5 *Antigen detection tests*

Antigen detection is an attractive concept that would allow, unlike methods detecting antibodies, a distinction between active and cured HAT. By detecting antigens released by non circulating trypanosomes sequested in the liver, spleen, lymph nodes, or CNS, antigen detection has the potential to improve the sensitivity of current parasitological methods. Following promising results of specific antigen detection by ELISA (Nantulya *et al.*, 1992), the card indirect

agglutination test for trypanosomiasis (CIATT) was developed by Brentec Diagnostics, Nairobi, Kenya for field use. A preliminary evaluation of the TrypTectCIATT showed a high sensitivity compared to other parasitological techniques (Nantulya, 1997), but the results of subsequent studies raised strong doubts about its specificity (Asonganyi *et al*., 1998).

2.13.3 Management of serologically suspect individuals

Parasitological methods have a limited sensitivity and therefore do not allow all HAT patients with an initial positive screening to be confirmed to have the disease and receive treatment (WHO, 1998). These undiagnosed patients will return home and will either be diagnosed at a later stage of the disease or simply die. Moreover, they contribute to the reservoir of parasites and thus to transmission of the disease. One option is to examine these serologically suspect individuals at regular intervals (e.g. every 3 months) for 1 to 2 years (WHO, 1998). However, compliance with follow-up visits is usually low, and the efficiency of this strategy is poor (Chappuis *et al*., 2004). A much more promising option is to determine a subgroup of serologically suspected individuals at high risk of being infected with *T. b. gambiense* and to treat them. Of 86 serologically suspect individuals (CATT serum 1:4) being monitored in Angola, 52% of individuals with a CATT plasma end titer of 1:16 were diagnosed with HAT during follow-up but none of the individuals with lower CATT end titers were diagnosed with the disease (Simarro *et al*., 1999). Similar results were obtained from a group of 749 serologically suspect individuals (CATT, 1:4) who were examined at least once during a 12-month follow-up period in Kajo-Keji county, Southern Sudan (Chappuis *et al*., 2004). Individuals with a CATT plasma end-dilution titer of 1:16 had a 50% person-year risk of being confirmed with HAT during follow-up, compared with a 10 and 14% person-year risk for 1:4

and 1:8 end-dilution titers, respectively. The authors of these studies recommend treatment for all serologically suspect individuals with a CATT end titer of 1:16, when the prevalence of HAT in the investigated population is sufficiently high. Which prevalence threshold to choose remains an open question, but it should probably be not less than 1%. In a study conducted in a low-prevalence area in Cote 'd'Ivoire, only one patient was diagnosed with HAT during a 2-year follow-up of 77 serologically suspect patients (Garcia *et al*., 2000). However, the CATT end-dilution titer was limited to 1:4 in that study. Other factors, such as poor access to care and absence or limited availability of parasitological diagnosis, should positively influence the decision to perform titration of serum and treat individuals with a CATT end titer of 1:16.

2.13.4 Trypanosome detection

Parasitological diagnosis involves the examination of lymph node aspirates, blood, or CSF under a light microscope for the presence of trypanosomes. It provides a direct evidence for trypanosome infection and is considered a definite diagnosis. Failure to demonstrate the parasite does not exclude the occurrence of infection (Chappuis *et al.*, 2005). The main parasite detection methods available under fieldwork conditions include:

2.13.4.1 *Chancre aspirate examination*

Trypanosomes can be detected in the chancre a few days earlier than in the blood. This involves puncturing the chancre, collecting the fluid which is then microscopically examined as a fresh or Giemsa-stained preparation (Chappuis *et al.*, 2005). The method is rarely used because most HAT infections are detected after the chancre has resolved.

2.13.4.2 *Lymph node aspiration*

Systematic palpation of cervical lymph nodes (CLN) should be done in patients positive for CATT. Enlarged CLN should be punctured and the fresh aspirate placed onto a clean glass slide, covered with a coverslip, and examined under x 40 magnification using a light microscope in order to detect the presence of motile trypanosomes (Chappuis *et al*., 2005). The technique is simple and cheap. The sensitivity of this technique depends on the parasite strain, the stage of the disease (being highest during the first stage), and the presence of other diseases causing lymphanopathy. The sensitivity varies between 40 and 80% (Simarro *et al.*, 2003).

2.13.4.3 *Wet blood film*

In wet blood film, about 5 to 10µl of finger prick blood is applied on a clean glass slide and a coverslip placed on it after which it is examined microscopically. Samples that are positive for trypanosomes will reveal parasites moving between erythrocytes (Chappuis *et al*., 2005). The sensitivity of this technique is low, detecting limits being 10,000 trypanosomes/ml of blood (Chappuis *et al*., 2005). The technique is still in use because of its low cost, its simplicity, and immediate results obtained.

2.13.4.4 *Thick blood film*

Examination of 20 µl of the finger prick blood in a stained thick blood film slightly improves the sensitivity, with a detection threshold of about 5,000 trypanosomes/ml of blood. It is the technique of choice in the absence of a centrifuge (Henry *et al.*, 1981). This technique is time consuming, requiring 10 to 20 minutes per slide, and requires also expertise to recognize the

parasites, which are frequently deformed in this preparation. Other parasites such as microfilaria and *Plasmodium* can also be detected using this technique (Chappuis *et al.*, 2005).

2.13.4.5 *Microhaematocrit centrifugation technique (mHCT)*

This technique also referred to as the Woo test was developed by Woo in 1971 and is still in use in many HAT control programmes. In mHCT, capillary tubes containing anticoagulants are three-quarters filled with a sample of the finger-prick blood. The dry end is sealed using plasticine and centrifuged at high-speed in a haematocrit centrifuge. This allows for the concentration of trypanosomes at the level of the white blood cells (WBC), between the plasma and the erythrocytes (Woo, 1971). The capillary tubes are then mounted in a special holder and examined under low power magnification for mobile parasites (Woo, 1971). This technique can detect as low as 500 trypanosomes/ml of blood.

2.13.4.6 *Quantitative buffy coat technique (QBC)*

The QBC has been used with success in the diagnosis of Sleeping Sickness (Bailey and Smith, 1992). QBC has the advantages of having the parasites concentrated by centrifugation, and morphologically identifying the stained nucleus and kinetoplast of trypanosomes with acridine orange, thus allowing a better discrimination from WBC. To carry out the test, blood is centrifuged at high-speed as in mHCT using special capillary tubes containing EDTA and acridine orange and provided with a floating cylinder. Motile trypanosomes can be identified by their fluorenscent kinetoplast and nucleus between the WBC in the expanded buffy coat (Chappuis *et al.*, 2005). Ultraviolet light is generated by a ‘cold light source’ connected by a

glass fibre to a special objective containing the appropriate filter. The technique is highly sensitive (Chappuis *et al*., 2005).

2.13.4.7 *Mini anion exchange centrifugation techniques (mAECT)*

The mAECT was introduced by Lumsden *et al.* (1979). The technique separates trypanosomes from venous blood by anion chromatography and concentrates them at the bottom of a sealed glass tube by low-speed centrifugation (3000 rpm). The larger blood volume (300µl) enables detection of fewer than 100 trypanosomes per milliliter of blood (Lumsden *et al.*, 1979).

2.13.4.8 *In-vitro culture and animal inoculation.*

When inoculated into a suitable medium, viable trypanosomes multiply but are not detected for days or weeks. A ready-for-use kit for *in vitro* isolation (KIVI) has been developed (Aerts *et al*., 1992). After inoculation of 5 to 10 ml of blood, the bottles are kept at ambient temperature. Bloodstream-form trypanosomes transform into proliferating large procyclic forms that can be detected within 3 to 4 weeks. The recorded sensitivity of the KIVI is variable (Truc *et al*., 1992; Truc *et al*., 1994). The yield of isolation of *T. b. gambiense* in rodents is low unless neonates or immunosuppressed rodents are used (WHO, 1998). The high cost and the long delay before obtaining the result are definite obstacles to routine field use of these techniques.

2.13.4.9 *Proteomic signature analysis*

Proteomic signature analysis is a promising technology that has been recently used with sera from patients with HAT and other diseases (Papadopoulos *et al*., 2004). The accuracy of this

experimental method was high with 100% sensitivity and 98.6% specificity. However, this technique needs to be confirmed in prospective studies. This method is impracticable in the field but could identify discriminating biomarkers that could lead to the development of more conventional and simpler tests (Papadopoulos *et al*., 2004).

2.13.4.10 *Imaging techniques*

Neuroimaging techniques such as computed tomography and magnetic resonance imaging are very effective and accurate in the diagnosis of CNS stage of Sleeping Sickness. However these techniques are not available in areas of endemic infection, except in few referral centers. They reveal nonspecific features that are occasionally useful to stage the disease. Brain magnetic resonance imaging may show diffuse white matter abnormalities, ventricular enlargement, hyperintensities in the basal ganglia, and signs of meningitis (Gill *et al*., 2003).

2.13.5 Stage determination: cerebrospinal fluid (CSF) examination

Accurate disease phase (stage) determination is essential to select an adapted treatment with minimal risk for the patient. In the absence of sufficiently specific clinical signs and blood tests indicating the evolution from first to second stage HAT, staging of patients still relies on the examination of CSF obtained by lumbar puncture. This is a vital step in the diagnosis process (Chappuis *et al*., 2005). According to WHO recommendations, second-stage HAT is defined by the presence in the CSF of raised while blood cell count (greater than 5 cells/µl of CSF), increased protein content (greater than 370 mg/liter, as measured by the dye-binding protein assay), and trypanosomes (WHO, 1998).

2.13.5.1 *White blood cell count*

The CSF white blood cell count is the most widely used technique for stage determination. After collecting the CSF sample, the cell count should be determined immediately to prevent cell lysis. If fewer than 20 cells /µl of CSF are counted, it is recommended to repeat the counting procedure and calculate the mean count value. The CSF pleocytosis is of lymphocytic origin, consisting mainly of B-cells (Greenwood and Whittle, 1976).

2.13.5.2 *Protein concentration*

In normal healthy individuals, protein in the CSF consists mainly of albumin (70%) and IgG (30%), both originating from the serum (Chappuis *et al.*, 2005). Protein concentrations in the CSF are elevated in HAT patients and range from 100 to 2,000 mg/liter (Bisser *et al.*, 2002; Lejon *et al.,* 2003a*)*. Recent evidence suggest that the protein concentration threshold set by WHO (370 mg/liter) is too low and should be raised to 750 mg/liter to reflect blood-brain impairment, astrocyte activation, and neurodegeneration (Legon *et al.*, 2001; 2003a).

2.13.5.3 *Trypanosome detection*

The detection of trypanosomes in CSF allows immediate classification of a patient as being in the second stage of illness. Trypanosomes in the CSF start to lyse within 10 minutes after sample collection, it is therefore very important to examine the CSF immediately after lumbar puncture (Chappuis *et al.*, 2005). Trypanosomes can simply be detected during cell counting of CSF. This technique is simple and cheap, but suffers insufficient sensitivity. Sensitivity can be increased by

centrifugation of the CSF sample, especially when a double centrifugation method is used (Cattand *et al.*, 1988).

2.13.5.4 *Antibody detection*

High levels of immunoglobulins, especially IgM have been reported several years ago in the CSF of second stage HAT patients (Lambert *et al.*, 1981; Bisser *et al.*, 1997). An increased CSF IgM concentration is thus considered as a strong potential marker of second-stage HAT (Greenwood and Whittle, 1980). Techniques such as ELISA and IFA (Lejon *et al.*, 1998) as well as CATT (Magnus *et al*., 1978) and LATEX (Buscher *et al.*, 1999) have been used to detect the presence of trypanosome specific antibodies. The presence of anti-galactocerebroside antibodies in CSF, assessed by ELISA seems to be a promising marker for CNS involvement (Bisser *et al.*, 2000).

2.13.6 Molecular diagnosis

Molecular techniques can be applied on various sample types to provide evidence of infection. Molecular diagnostic techniques are complex and their present application is limited to research purposes. Although these techniques are very sensitive, test samples should be large enough and well stabilized to contain the required minimum amount of template DNA or RNA (Chappuis *et al*., 2005).

The polymerase chain reaction (PCR) is a molecular technique which theoretically is very sensitive and specific. Its diagnostic potential and use in Sleeping Sickness have been reported (Schares and Mehlitz, 1996; Kabiri *et al.*, 1999). PCR tests that are able to distinguish *T. b.*

gambiense and *T. b. rhodesiense* from the other members of the subgenus *Trypanozoon* have now been developed (Welburn *et al.*, 2001b; Radwanska *et al.*, 2002a). The PCR technique used for DNA detection involves the mixing of Taq polymerase, primers, and a template in a test tube which is then sealed and placed in a thermal cycler for amplification. Cycling involves denaturation between 94 and 95 ^{0}C for 1 minute, primer annealing usually between 55 and 72 ^{0}C for 1 minute and finally primer extension at 72 ^{0}C for 4 minutes and then cooled to 5 ^{0}C (Radwanska *et al.*, 2002b).

In the last decade, a DNA amplification platform called loop-mediated isothermal amplification (LAMP) was developed. This technique has been used to develop LAMP tests specific for the subgenus *Trypanozoon* (Kuboki *et al.*, 2003; Thekisoe *et al.,* 2007; Njiru *et al*., 2008a) as well as *T. b. gambiense* and *Trypanosoma brucei rhodesiense* (Njiru *et al*., 2008b). The major advantages of LAMP include (i) rapidity and the use of six to eight primers providing high specificity; (ii) ability of the technique to amplify target DNA from partially processed template; (iii) requirement for only a simple heating device, such as a water bath; and (iv) results that can be inspected visually through the use of varied detection formats, such as turbidity (Mori *et al.,* 2001), fluorescent dye (Boehme, 2007), probes (Mori *et al.,* 2006), lateral-flow dipstick (LFD) format and microfluidic chips (Fang *et al.,* 2010). LAMP has attracted much interest as an easily applicable yet highly sensitive molecular tool with great potentials for diagnosis in resource-poor rural settings, where HAT typically occurs.

The molecular characterization of human-infective trypanosomes indicates that the majority of Gambian HAT cases are caused by a genetically homogeneous group 1 *T. b. gambiense*

(Kanmogne *et al.,* 1996), with group 2 *T. b. gambiense* accounting for an insignificant percentage (Mehlitz *et al.,* 1982). In addition, studies have identified a *T. b. gambiense*-specific glycoprotein (TgsGP) gene (Berberof *et al.,* 2001) that is specific to group 1 *T. b. gambiense* and absent in isolates from group 2 (Gibson *et al.,* 2010). The 3′ end of the TgsGP gene has been used to develop the only reliable PCR tests for *T. b. gambiense* (Radwanska *et al.,* 2002a).

2.13.6.1 *DNA-based techniques*

The diagnosis of trypanosomes has been improved since the 1980s, by DNA-based techniques. DNA diagnosis is either based on hydridization profiles of parasite DNA with DNA probes or polymerase chain reaction (PCR) technology. A DNA-probe is a known DNA sequence which can be obtained by cloning or by PCR with labelled nucleotides (enzymes or isotopes). DNA probing entails exposing a denatured DNA sample fixed on nitro-cellulose to a labelled DNA-probe under specific salt and temperature conditions. If the complementary DNA sequence is present in the sample, the probes will bind to it and remain on the nitro-cellulose where they can be visualised (Desquesnes and Dàvila, 2002). Probes have been developed for the main pathogenic trypanosomes (Masiga and Gibson, 1990), but the sensitivity of this technique is limited to 100 parasites (Masiga *et al*., 1992), which is not sufficient for trypanosome detection in mouthparts of the vectors or in host blood when the parasitaemia is low.

The polymerase chain reaction (PCR) is an *in vitro* technique which allows the amplification of a specific deoxyribonucleic acid (DNA) region that lies between two regions of a known DNA sequence. The technique exploits a thermostable enzyme, *Taq* polymerase, which synthesises a new strand of DNA by copying an original DNA template (Mullis, 1986). PCR was originally

developed to amplify sequences of interest and to increase the sensitivity of detection using DNA-probes (Saiki *et al*., 1986). However, since the DNA-probe technique is laborious and time consuming (Desquesnes and Dàvila, 2002), in recent years the development of PCR alone has been mainly devoted to diagnosis without the need of probes. PCR amplification of DNA is achieved by using oligonucleotide primers. These are short, single-stranded DNA molecules which are complementary to the ends of a defined sequence of DNA template. The primers are extended on a single-stranded denatured DNA template by a DNA polymerase, in the presence of deoxynucleoside triphosphates (dNTPs) under suitable reaction conditions. This results in the synthesis of a new strand complementary to the template strands. Following 30-40 cycles, the DNA will have been amplified several million times, and the resulting PCR product can be visualised on an agarose or polyacrylamide gel, after staining with ethidium bromide and exposing it under ultra-violet light. The specific size of the PCR product is evaluated by simultaneous migration of molecular size markers and a positive control. A negative control is run together to evidence any DNA contamination. The actual sequence amplified can be analysed by sequencing. Generally, once the specificity of the primers has been established, the size of the PCR product is sufficiently characteristic for diagnostic purposes (Newton and Graham, 1997; Desquesnes and Dàvila, 2002). The first developments of PCR for trypanosome identification were published in Moser *et al*. (1989).

2.13.6.2 *Diagnostic targets of PCR*

In most cases, PCR diagnosis aims to identify the parasite at the species level, which can be done using various targets. The preferred targets are those which are present in a high copy number in the genome of trypanosomatids and the more copies of the target, the greater the chances of

amplifying it by PCR. Single copy genes are more difficult to amplify (Mcleod *et al*., 1997) and are rarely targeted since low parasitaemia is a characteristic of trypanosome infection and the sensitivity would be too low (Desquesnes and Dàvila, 2002). Mini-chromosomes of the nuclear DNA contain satellite DNA which has been the most favoured target in the development of species-specific primers able to detect very small amounts of parasite DNA. Such primers were developed for the main pathogenic trypanosomes: *Trypanozoon* (Moser *et al*., 1989; Masiga *et al*., 1992), *T. vivax* and *T. evansi* (Masiga *et al*., 1992). Other repetitive sequences have also been investigated, for example, in the detection of *T. vivax* DNA (Clausen *et al*., 1998, Morlais *et al*., 2001).

Because of the diversity of *Trypanosoma* species potentially present in a single host, PCR diagnosis carried out on host material requires several PCR reactions; for example, in cattle, up to five reactions per sample may be required. Research is now utilizing diagnosis based on the amplification of the internal transcribed spacer-1 (ITS-1) of ribosomal DNA which presents the advantages of being a multi-copy locus (100–200), having a small size (300–800 bp), which varies from one taxon to another but is conserved in size in a given taxon. This may lead to the development of a multi-species-specific diagnostic protocol using a single PCR (Desquesnes and Dàvila, 2002). The limitation of this technique is that the sensitivity of the primers is lower than that of satellite DNA primers, due to partial homology of the sequences and their limited repetitiveness. ITS-1 sequences are only repeated 100-200 times in comparison with 10, 000-20,000 times for satellite DNA. Consequently, the design of new primers is required to reach 100% homology with all pathogenic trypanosomes. Geysen *et al*. (2003) demonstrated that a single polymerase chain reaction (PCR) restriction fragment length polymorphism (RFLP) assay can be used to characterize all important pathogenic trypanosome species. This was the first

report of a sensitive pan-trypanosome PCR assay amplifying all species including *T. vivax* to a comparable extent, using a single primer pair. Restriction enzyme analysis using *Msp*1 and *Eco*571 gave a clear destinction between *T. congolense*, *T. brucei*, *T. vivax* and *T. theileri*. Several subgroups within the *T. congolense* group could be distinguished using this method, although no difference could be detected between the species belonging to the subgenus *Trypanozoon*.

Delespaux *et al*. (2003) further evaluated this method using mixed infections with *T. b. brucei, T. theileri*, *T. congolense* and *T. vivax*. The results showed that all mixed infections gave clear profiles that could be easily differentiated except in the case of *T. theileri* and *T. congolense* mixed infections where the *T. theileri* band was concealed by the *T. congolense* profile. By reducing the cost of the PCR diagnosis, this technique would allow a greater number of field samples to be tested in epidemiological studies and/or would increase the variety of *Trypanosoma* species that could be detected. Further investigations are required to develop and optimise multi-species-specific diagnostic tools for trypanosomes, which could also serve as a model for such tools in other pathogens.

2.13.6.3 *Specificity and sensitivity of PCR diagnosis and typing*

Several levels of specificity have been achieved from sub-genus to species, sub-species and even types. Another work based on microsatellite sequences has provided markers for population genetic studies. Similarly, PCR has allowed an increase in the specificity of diagnosis in vectors such as tsetse flies. Direct determination of the trypanosome species-type in the vector or host is

likely to provide a more reliable estimate of parasite prevalence since no selection is exerted on the ability of the parasite to grow either in culture or in vertebrate hosts (Majiwa *et al*., 1993).

Many natural trypanosome infections of either tsetse or livestock are due to more than one trypanosome species (Nyeko *et al*., 1990). Precise evaluation of such infections by PCR requires that the oligonucleotide primers for PCR retain their specificities under standard assay conditions. Species-specific DNA probes have been shown to detect simultaneous infection of cattle with *T. vivax*, *T. b. brucei*, and *T. congolense* when conventional methods revealed only single infections (Nyeko *et al*., 1990). Clausen *et al*. (1998) indicated that PCR coupled with DNA probe hybridisation could prove to be a highly sensitive tool for the diagnosis and assessment of the therapeutic efficacy and disease progress especially in chronic trypanosomosis.

Individual hosts often harbour more than one parasite clone. It was previously thought that certain *Nannomonas* species were limited to particular ecological niches but it has been proved that this is not the case, as revealed when these probes are used in epidemiological studies (McNamara *et al*., 1995a; b). *T. congolense* comprises morphologically identical but genotypically heterogeneous trypanosomes that express different phenotypes in terms of vector and host specificities, and disease symptoms (Young and Godfrey, 1983; Majiwa *et al*., 1985). Four genotypic groups of *T. congolense* have been recognised. They are designated Savannah-type *T. congolense*, West African forest/riverine-type *T. congolense*, Kilifi-type *T. congolense* and the Tsavo-type *T. congolense* (Young and Godfrey, 1983; Majiwa *et al*., 1985; Majiwa *et al*., 1993).

Repetitive, randomly arranged DNA sequences and oligonucleotide primers, specific for each of these types, for use in polymerase chain reaction (PCR) amplification, have been described (Majiwa *et al*., 1985; Dickin and Gibson, 1989; Majiwa *et al*., 1993). The availability of such reagents for rapid, specific and highly sensitive detection and identification of the different types of trypanosomes will ultimately enable a comprehensive evaluation of their role in the epidemiology of the disease they cause (Hide and Tait, 1991). Applied to the routine detection of *Trypanosoma* infections in animals, PCR provides a highly specific diagnosis. So far, the most used primer sets for trypanosomes have never failed in terms of their specificity (Desquesnes and Dàvila, 2002). However, species-specific primers have not yet been described for some trypanosome species such as *T. equiperdum*. Moreover, although it is possible to distinguish *T. b. rhodesiense* from *T. b. brucei* using three minisatelite markers (Mcleod *et al*., 2000) or using serum-resistance-antigen gene (Welburn *et al*., 2001a), couples of sub-species-specific primers are not yet available (Desquesnes and Dàvila, 2002).

With regards to the sensitivity, PCR allows the detection of a single specific sequence of DNA; consequently, a single parasite (0.1 pg of DNA) can be detected when using satellite DNA (Desquesnes and Dàvila, 2002). This very sensitivity also brings a very high risk of false positive results since a very small amount of other biological materials can contaminate the sample. The sensitivity threshold of the trypanosome detection by PCR generally ranges from 1 to 20 parasites/ml of blood, depending on the technique used. Below this level of parasitaemia, PCR cannot detect the infection. It is not a problem of sensitivity; it is only due to the fact that there is no DNA in the sample investigated when the parasitaemia is so low (Desquesnes and Dàvila, 2002). To increase the sensitivity, it should then be necessary to increase the volume of the

samples processed and the concentration through the parasitological techniques described. In all cases, false negative results (in samples where substantial amount of the specific DNA is present) can occur due to the presence of inhibiting factors in a sample (haemoglobin, EDTA, etc.), or DNAses introduced into the sample, which provoke destruction of the DNA, and then the specific sequence under investigation (Desquesnes and Dàvila, 2002). There is still a need to design primers based on repetitive sequences for a sensitive detection and specific identification of *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense* for epidemiological studies in vectors, domestic and wild reservoirs of human Sleeping Sickness.

2.13.6.4 *RNA Detection*

Reverse transcriptase PCR (RT-PCR): This is a technique to detect ribonucleic acid (RNA), which is far less stable than DNA and which is indicative of active transcription of DNA sequence. This technique may allow differentiation between an active infection and persisting DNA derived from dead trypanosomes (Chappuis *et al*., 2005).

Hybridization: Fluorescent molecular probes that hybridize with ribosomal RNA have recently been developed for facilitated detection of the parasite in thick blood films or blood smears (Radwanska *et al.*, 2002b). DNA from *T. b. gambiense* Li Tat 1.3 surface antigen is extracted and immobilized on nitrocellulose paper. The DNA in solution is separated according to size by electrophoresis through agarose gel. This is followed by denaturation of the DNA fragments by soaking in alkali (0.5M NaOH or 1.5M NaCl). The single stranded DNA from *T. b. gambiense* fixed on the solid support can then hybridize with the single stranded labeled DNA probe indicating that the DNA is complimentary with the labelled DNA probe (Radwanska *et al.*,

2002b). The nitrocellulose paper containing the two DNAs is then washed so that areas of hybridization can be detected using autoradiography by placing nitrocellulose paper in contact with a photographic film (Radwanska *et al.*, 2002b).

2.14 Prevention and Control of HAT

The prevention and control of HAT can be achieved through the combination of chemotherapy and vector control. As chemotherapy takes care of the Sleeping Sickness agents in the human host, tsetse control takes care of the transmission of the disease agents from infected individuals and animal reservoirs to uninfected individuals.

2.14.1 Chemotherapy of human African trypanosomosis

Generally, four drugs have been registered for the treatment of this disease in humans. Pentamidine and suramin are trypanocides both registered for the treatment of the first stage of the disease while melarsoprol and eflornithine are used against the second stage (Chappuis *et al*., 2005).

2.14.1.1 *Pentamidine*

This drug is a water soluble synthetic aromatic diamidine introduced since 1930s, and has been registered for the treatment of the first stage of Gambian trypanosomosis (Sands *et al.*, 1985). It is a first choice for the first-stage Gambian Sleeping Sickness with cure rates of up to 98% (WHO, 1986). Some cures of second-stage infections have also been reported, but cerebrospinal

fluid (CSF) drug levels are usually insufficient to guarantee a trypanocidal effect in the central nervous system (WHO, 1986; Brunner *et al.*, 1991).

African trypanosomes have a nucleoside (adenine/adenosine: P2) transporter that takes up pentamidine, resulting in the concentration of the agent at levels many times than in plasma (Fairlamb *et al*., 1992; Carter *et al*., 1995). Recent studies have found two other transporters, besides P2, that also transport pentamidine and may be responsible for 50% of its uptake (de-Koning, 2001). Many studies have focused on the mechanism of pentamidine action; however, none appears to conclusively define the target. It is known to bind to the minor groove of kinetoplast (mitochondrial) DNA, and to promote the cleavage of kinetoplast minicircle DNA, eventually leading to the development of dyskinetoplastic cells. Despite its effects on kinetoplast DNA, pentamidine has no effect on nuclear DNA, and dyskinetoplastic forms can persist in the bloodstream of mammals (Wang, 1997; Barrett *et al*., 2007). Pentamidine was also found to be a reversible inhibitor of S-adenosylmethionine (AdoMet) decarboxylase, an enzyme in the polyamine biosynthetic pathway, but this is unlikely as the primary mechanism of action (Barrett *et al*., 2007). It is now clear that the concentration of the drug in trypanosomes is achievable via uptake through the P2 nucleoside–pentamidine transporter, and two other recently discovered transporters HAPT1 and LAPT1 (de-Koning, 2001; Delespaux and de-Koning, 2007). Other targets studied previously in trypanosomes include the inhibition of glycolysis and lipid metabolism, as well as effects on amino acid transport and ion exchange.

Pentamidine is generally well tolerated if given as an intramuscular injection at a dose rate of 4 mg/kg body weight for 7-10 days (Sands *et al.*, 1985). However, minor adverse, but reversible

reactions including hypotension, dizziness, collapse and shock may occur. Simultaneous application of plasma expanders and a prolonged infusion time may alleviate this reaction, but the pulse rate and blood pressure of the patients must be monitored closely during treatment with pentamidine (Brunner *et al.*, 1995). Nausea and/or vomiting have also been reported.

Local reactions at the site of injection may include pain, development of sterile abscesses, necrosis, and systemic reactions (acotaemia due to a nephrotoxic effect, leucopenia, hypo and hyperglycaemia) may occur. There is no evidence of mutagenicity or fetotoxicity (Brunner *et al.*, 1991). The advantages of the use of pentamidine over Suramin are brevity of the treatment course and ease of application.

2.14.1.2 *Suramin*

Suramin is a sulfonated naphthylamine, which has been in use since 1922. Suramin does not penetrate the blood-brain barrier and is not used for CNS-stage disease. The drug was developed from the closely related azo dyes, trypan red, and trypan blue (Barrett *et al.*, 2007). It is registered for the treatment of first stage of both the Gambian and Rhodesian forms of the disease. However, it is favoured in the Rhodesian form (Kuyu *et al.*, 1999). Combination of Suramin and Pentamidine may reduce the adverse drug reactions of both components. Suramin has also been administered prior to Melarsoprol treatment in second-stage Rhodesian Sleeping Sickness (WHO, 1986). The most commonly used dosage regimen consist of a test dose of 4-5 mg/kg body weight on the first day, followed by five injections of 20 mg/kg body weight on days 3, 5, 12, 19 and 26. The maximum dose per injection is 1g (WHO, 1986).

Suramin has an extremely long half-life in humans, 44-54 days. Suramin binds to many plasma proteins including low density lipoproteins (LDL), which trypanosomes avidly bind and endocytose as a result of specific membrane receptors. LDL is a prime source of sterols for bloodstream trypanosomes (Vanterkenburg *et al*., 1993; Barrett *et al*., 2007). Suramin has been shown to inhibit all of the glycolytic enzymes in *T. b. brucei* and also other enzymes, including those of the pentose phosphate pathway (Barrett *et al*., 2007). This specificity for trypanosomal enzymes was attributed to higher isoelectric points for the parasite enzyme than the mammalian enzymes, allowing the negatively charged suramin to bind preferentially to the parasite enzymes. In practice, because most trypanosome glycolytic enzymes are contained in a membrane-bound cytosolic organelle, the glycosome, it is not likely that rapid massive binding occurs (Wang, 1997). This would rapidly induce lysis in bloodstream forms that depend on glycolysis as the sole energy-generating source. Rather, animals that are heavily infected with trypanosomes and given suramin show a slow decrease in parasite numbers, indicating that enzyme inhibition occurs slowly. Suramin may be affecting newly synthesized enzyme molecules in the cytosol before they are imported into the glycosome. Suramin has also been found to affect thymidine kinase and dihydrofolate reductase. It is likely that suramin's action may be attributable to the inhibition of several of these enzymes (Wang, 1997).

Adverse drug reactions of Suramin are dependent on nutritional status, concomitant illness (especially Onchocerciasis) and the general clinical condition of the patient (Chappuis *et al*., 2005). These adverse reactions include pyrexia, mild and reversible nephrotoxicity manifested by early albuminuria and haematuria. Regular urine checks during the course of treatment are therefore mandatory (Chappuis *et al*., 2005). Other reactions include nausea, circulatory collapse

and urticaria, exfoliative dermatitis, haemolytic anaemia, peripheral neuropathy, and bone marrow toxicity with agranulocytosis, thrombocytopenia and reactive encephalopathy (Chappuis *et al*., 2005).

2.14.1.3 *Melarsoprol*

This is an organo-arsenical drug developed since 1949 and traded as Mel B® and Arsobal®. The drug is registered for use against the second stage of Sleeping Sickness. The drug is available in vials of 5 ml as a 3.6% solution in propylene glycol (WHO, 1998). The drug is associated with severe and life threatening side effects, the most important being encephalopathic syndromes. These syndromes occur in 5-10% of all treated cases, with 10-70% ending fatally (Pepin and Milord, 1994; WHO, 1998; Blum *et al.,* 2001). Clinically, the symptoms are manifested as convulsions, progressive coma, psychotic reactions or abnormal behaviour. Simultaneous administration of corticosteroids (Prednisolone at 1 mg/kg body weight) has been shown to reduce the incidence of the encephalopathic syndrome (Pepin *et al*, 1989). Other adverse reactions that may occur include pyrexia, headache, and general malaise. Skin reactions may include pruritis, thrombophlebitis and exfoliative dermatitis (WHO, 1998). Peripheral neuropathy, cardiac failure, renal dysfunction as well as hepatotoxicity have also been reported (Van-Nieuwenhove, 1999).

Although the mechanism of melarsoprol action has been extensively studied, it still remains unclear. Parasites exposed to low (1–10 M) levels rapidly lyse. Because the bloodstream forms are intensely glycolytic, any interruption of glycolysis or interference with redox metabolism should produce this effect. Melarsoprol has also been reported to cause inhibition of

trypanosome enzymes like pyruvate kinase, phosphofructokinase and fructose-2,6-bisphosphatase. It is likely that the rapid inhibition of fructose 2, 6, bis-phosphate production is a key factor in halting glycolysis through down-regulation of pyruvate kinase (Wang, 1997). Other studies indicated that melarsoprol and melarsen oxide formed adducts with trypanothione (N1, N8-bisglutathionyl spermidine), a metabolite unique to trypanosomes and believed to be responsible for the redox balance of the cell and detoxification of peroxides (Fairlamb *et al*., 1992). The melarsen-trypanothionine adduct (Mel T) inhibits trypanothione reductase, which has been attributed to the mode of action. However, melarsoprol and related arsenicals may also bind to other sulfhydryl-containing agents in the cell, including dihydrolipoate and the closely adjacent cysteine residues of many proteins. Similar to pentamidine and diminazene, melarsoprol uptake into African trypanosomes has been attributed to the P2 purine nucleoside transporter; thus, significant levels can be concentrated in the cell from a low plasma concentration (Fairlamb *et al*., 1992; Carter *et al*., 1995). Although most laboratory-generated melarsoprol-resistant strains have lost or modified the P2 transporter, clinical isolates appear to have retained uptake capacity (de-Koning, 2001).

2.14.1.4 *Eflornithine*

This drug with the chemical name α-difluoromethylornithine (DFMO) was approved in 1990 by the United States Food and Drug Administration Commission for the treatment of second stage Gambian Sleeping Sickness (Iten *et al*., 1997). DFMO is an enzyme-activated irreversible inhibitor of ornithine decarboxylase (ODC), and is the most recently developed agent for late-stage Gambian Sleeping Sickness. The drug is administered orally at a dose rate of 100 mg/kg

and 150 mg/kg body weight for adults and children respectively, at 6 hour intervals for 14 days (Pepin *et al.*, 2000).

DFMO rapidly and irreversibly binds to the catalytic site (cysteine 360) in mouse ODC, inactivating it. In culture, it blocks division of bloodstream trypanosomes, but it is not trypanocidal. In laboratory infections, DFMO cures when administered continuously in drinking water as a 2% solution. Within 48 hours of administration, DFMO reduces putrescine levels to zero, and reduces spermidine levels by 75% (Bacchi *et al*., 1990). Trypanothione levels are also significantly reduced (Bacchi *et al*., 1990). As noted, DFMO is not trypanocidal and depends on a functional immune system to rid the host of non-dividing forms (Bacchi *et al*., 1990).

Morphologically, trypanosomes exposed to DFMO have multiple kinetoplasts and nuclei as well as forms resembling ‘‘stumpy’’ blood forms (Bacchi *et al*., 1990). DFMO is curative for laboratory infections of *T. b. brucei* and *T. b. gambiense*, but not to all strains of *T. b. rhodesiense* (Bacchi *et al.,* 1990). The reason for this selectivity is not completely evident, although it is not due to uptake of DFMO, because it enters by passive diffusion, not transport. Iten *et al*. (1997) found that *T. b. rhodesiense* isolates have an ODC with a shorter half-life than *T. b. gambiense*, which could result in lowered susceptibility to DFMO. Also levels of AdoMet are highly elevated in DFMO-treated susceptible *T. b. rhodesiense* strains, but less so in refractory isolates (Bacchi and Yarlett, 1995). This elevation is the result of the block in putrescine synthesis and the resulting inability to make spermidine. The elevated level of AdoMet is the result of an AdoMet synthase insensitive to its product. Trypanosome ODC is missing in the C-terminal PEST sequence in both procyclic and bloodstream trypanosomes, and

this appears to be the major reason for the stability of the trypanosome enzyme. The remainder of the ODC molecule has ~60% sequence identity with the mammalian enzyme, including a cysteine 360 residue at the demonstrated DFMO-binding site for the mammalian enzyme (Ghoda *et al*., 1990). Beyond this, trypanosomes lack a polyamine oxidase, which in mammalian cells converts spermine to the biologically active spermidine. Trypanosomes are also limited in their ability to transport putrescine and spermidine (Bacchi and Yarlett, 1995).

Adverse reactions associated with the use of eflornithine may include bone marrow toxicity manifested as anaemia, leucopenia, and thrombocytopenia. Gastrointestinal symptoms such as nausea, vomiting, and diarrhoea have also been reported. Alopecia and convulsion may be observed (Chappuis *et al.,* 2005).

2.14.1.5 *Nifurtimox*

Nifurtimox is traded as Lampit®. It is an alternative drug used against HAT. The drug was introduced in the late 1960s for use against Chagas disease. It is not registered for Sleeping Sickness and therefore its use is restricted to compassionate treatment (Van-Nieuwenhove, 1988). The drug is administered orally at a dose of 8-10 mg/kg body weight per day for adults and 10-15 mg/kg body weight per day for children in divided doses given every 8 hours for 60-90 days (Van-Neiuwenhove, 1988).

2.14.1.6 *Diminazene aceturate*

This drug was developed by Hoechst and marketed as Berenil® for use against animal trypanosomes and is not registered for Sleeping Sickness therapy. However, its administration via the intramuscular route against first-stage of both Gambian and Rhodesian infections has been reported (Peregrine and Mamman, 1993). The drug is administered as an intramuscular injection at doses of 2-5 mg/kg body weight for 7-10 days (Peregrine and Mamman, 1993). Like pentamidine, berenil has also been linked to kinetoplast DNA binding at the minor groove and cleavage of minicircle DNA. As with pentamidine, berenil may also interfere with RNA trans-splicing (Wang, 1997). Berenil is also a more effective and noncompetitive inhibitor of AdoMet decarboxylase in trypanosomes, resulting in the reduction of spermidine content and elevating putrescine in the parasite. Berenil uptake, as with pentamidine, occurs via the P2 nucleoside transporter, which allows significant accumulation from the external environment.

The other pentamidine transporters, HAPT1 and LAPT1 appear to play only a minor role in berenil uptake (de-Koning, 2001). Although berenil has been used for many years on thousands of Sleeping Sickness patients, there are little studies published on its toxicity (Barrett *et al*., 2007; Delespaux and de-Koning, 2007). This may in part be due to physicians who are unwilling to document human studies with an agent licensed for veterinary use. However, personal accounts of those using berenil in humans indicate it is well tolerated (Peregrine and Mamman, 1993; de-Koning, 2001).

2.14.1.7 Post-treatment follow-up

An 18-month to two-year post-treatment follow-up is necessary to declare a patient cured. Therefore, patients must have a lumbar puncture during each of the biannual examinations. A recent report stated that stage 2 follow-up could be shortened to a maximum duration of 12 months, based on CSF WBC counts (Mumba *et al*., 2010). However, in recent practice in the Democratic Republic of Congo, several relapses were observed 18 months after proper treatment (Mumba *et al*., 2010). Improvement in patient condition and resolution of the clinical and biological abnormalities indicate a good response to treatment. However, negative CATT findings are not indicative of a cure; therefore, CATT should not be used to monitor treatment outcome (Lejon *et al*., 2010). Although PCR has been used along with parasite detection techniques for diagnosis and staging of *T. b. gambiense* HAT, it cannot be used during post-treatment follow-up, because of false positives after successful treatment, due to the persistence of DNA material from living or dead parasites (Deborggraeve *et al*., 2011). Relapse is marked by a new increase in the CSF WBC count, and patients should be retreated. When patients are treated early, complete recovery usually occurs; however, treatment in advanced stage of the disease is associated with the relapse.

2.14.2 Tsetse control

2.14.2.1 *Insecticidal control of tsetse*

The biology of the tsetse fly (*Glossina* spp.) is unique in many ways and only chemicals with specific properties have proved suitable in the control of the fly (Allsopp, 1990). In the choice of insecticides, toxicity to the insect is very important but has not been a major determinant. Low

toxicity to the operators has been a key factor in their choice (WHO, 1990; Nagel, 1995; Grant, 2001).

In the control of tsetse flies, only two chemical groups have enjoyed wide success. These are the organochlorines such as dichlorodiphenyltetrachloroethane (DDT), dieldrin, and endosulfan as well as the pyrethroids which include permethrin, cypermethrin, cyfuthrin, lambda-cyhalothrin and deltamethrin (WHO, 1990; WWF, 1998). Various methods have been developed for treating tsetse habitats with residual deposits of insecticides. These include the use of spraying machines attached to vehicles, the use of helicopters and pressurized knapsacks sprayers for hand spraying of insecticides (Hursey and Allsopp, 1983). Insect growth regulators have been identified with a view to their use as biological insecticides which would have no undesirable side-effects on humans or their environment.

2.14.2.2 *The sterile insect technique (SIT)*

The SIT involves the production and systematic release of reproductively sterile male insects to mate and inseminate females. Such inseminated females become effectively infertile for the remainder of their lifespans (Bloem and Bloem, 2000; Hendrichs, 2000; Feldmann and Hendrichs, 2001). The males are sterilized by radiation at the appropriate developmental stage and then taken to the identified target area and released. Aerial release facilitates homogeneous and area-wide dispersal of the released sterile insects (Klassen, 2000; Allsopp and Philemon-Motsu, 2000; Vreysen *et al.*, 2000). For increased effectiveness, the released sterile males should significantly out-number the fertile, native male flies (Chandler and Faust, 1998; Lee *et al.*, 2000).

The concept of 'sterile male release' is based on the fact that tsetse females copulate only once, and if the male of a copulating pair is sterile the female will not produce during her lifetime. Thus, a series of field trials were carried out to evaluate the impact of the release of irradiated male tsetse on tsetse populations. Trial work was carried out at Mkwaja Ranch in Tanzania and in Zimbabwe, Burkina Faso, Nigeria and Zambia. Although under field conditions it has been demonstrated that this approach can significantly reduce tsetse populations, the majority opinion is that sterile male release is not a practical proposition because it is too sophisticated and too expensive. It is also estimated that ten sterile males are required per female. In order to reduce the number of sterile males required it is necessary to carry out two to three insecticide sprays and then to release 12000 sterile males per km^2, even in areas where the tsetse density is low. The cost of this method is estimated to be US$ 3000 to US$ 4000 (#510,000-#680,000) per km^2. As the effect is tsetse-species specific, the cost increases with the number of species to be controlled.

2.14.2.3 *Application of bait*

Baits have been in use since the early days of tsetse control. In the Zulu land, traps were in use since 1932 and DDT- treated oxen were used effectively to combat tsetse in Tanzania since 1949 ((Lovemore, 1999). The use of bait in the control of tsetse is dependent on factors such as size and odour of the bait, distribution of the tsetse population in relation to position of the baits, and tsetse preference for the bait as compared to wildlife (Vale, 1993). Stationary baits were proved more effective in areas that lack cattle (Green, 1994) while movable baits such as insecticide-treated animals were proved more successful in areas highly populated by animals (Lovemore,

1999; Vale *et al.*, 1999). Research is also being carried out on the isolation of pheromones (sex hormones) and their characterisation for use as attractants in traps. Other methods used in tsetse control include the destruction of wild game reservoirs, destruction of tsetse breeding sites as well as breeding of predators such as ducks to feed on the larva of the flies.

2.15 Future Prospects for Vaccination

There is no evidence that immune responses against antigens common to trypanosomes contribute to host protection. Thus, the major constraint to the development of a commercially applicable vaccine against trypanosomosis is the phenomenon of antigenic variation, as it is now well established that host protective responses are affected by antibodies directed against surface coat antigens of the trypanosome (Murray and Urquhart, 1977). The repertoire of these antigens generated by bloodstream forms of the parasite is large, with the result that the development of a field vaccine has been considered impossible. However, there is now evidence to indicate that the repertoire of antigens produced by metacyclic parasites following transmission through the tsetse is much more limited. Thus, it has been possible to immunise cattle against tsetse-transmitted homologous (but not heterologous) strains by prior exposure to metacyclic parasites which have been propagated in tissue culture, or by prior infection via tsetse flies followed by trypanocidal drug treatment (Murray and Urquhart, 1977). The immunity produced has been shown to last for as long as 5 months. Nevertheless, the feasibility of production and the efficacy of a vaccine against metacyclic trypanosomes will depend on the relative stability of the metacyclic antigen repertoire for each species of trypanosome and on the number of strains which occur in the field. Current research is directed towards these objectives. It is thought, however, that the number of strains of *T. congolense*, *T. vivax* and *T. brucei* is likely to be

prohibitively large for the production of a 'cocktail' vaccine containing the appropriate metacyclic antigens. As a result, the development of a conventional vaccine against African trypanosomosis is unlikely in the foreseeable future. Enzymes in tsetse saliva and gut are also being identified with a view to using them as immunogens to vaccinate the host against tsetse (Murray and Urquhart, 1977).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study was carried out in Benue and Taraba States of Nigeria between January and December, 2013.

3.1.1 Benue State

Benue State is located in the North-central zone of Nigeria between longitudes 7^{o} 47$^{'}$ and 10^{o} 00$^{'}$ East and latitudes 60 25$^{'}$ and 8^{o} 8$^{'}$ North. It shares boundaries with five other States, namely: Nassarawa (north), Taraba (east), Cross River (south), Enugu (south-west) and Kogi (west), and with the Republic of Cameroon (south-east). The State covers an area of 34,089 km^2 representing 3.7% of the total land area of Nigeria. The State has a tropical sub-humid climate, with two distinct seasons which are wet and dry seasons. The wet season lasts for seven months (April-October) while the dry season occurs between November and March. The two major occupations of the people of the State are farming and fishing. The State has twenty three Local Government Areas (LGA) which are grouped into three Senatorial Districts (Fig: 3.1).

3.1.2 Taraba State

Taraba State is located in North-eastern Nigeria between longitude 8^{o}00$^{'}$ N and latitude 10^{o}30$^{'}$ E and shares borders with Gombe (north), Adamawa (north-east), Plateau (west), Bauchi (north-west), Benue (south-west) States, and with the Republic of Cameroon (south). The State covers an area of 54,473 km^2 representing 5.9% of the total land area of Nigeria. It has two seasons, the rainy and dry seasons. The rainy season starts in April and ends in October, while the dry season

begins in November and terminates in March. The State comprises of three types of vegetation, namely; the guinea savannah in the southern part, the sub-Sudan in the northern part, and the semi temperate in the Mambila Plateau (Chapman *et al*., 2004). The major occupation of the people of the state is agriculture (Fig: 3.1).

3.2 Sample Size

The sample sizes used in the study were determined using the method of Thrusfield (1997).

$$n = \frac{Z^2pq}{d^2}$$

Where

n = sample size

p = expected prevalence

q = 1 – p

d = desired absolute precision of 5% (0.05)

Note: The expected prevalence for humans was 17.1% (Airauhi *et al.,* 2006), cattle 9.25% (Karshima *et al*., 2012) and pigs 26.4% (Simo *et al.*, 2006).

$$for\ humans\ n = \frac{1.96^2 \times 0.171 \times (1 - 0.171)}{(0.05)^2} = 217.8$$

However, a total of 600 individuals were sampled from each State.

$$for\ cattle\ n = \frac{1.96^2 \times 0.0925 \times (1 - 0.0925)}{(0.05)^2} = 128.99$$

A total of 600 cattle were sampled in Taraba State.

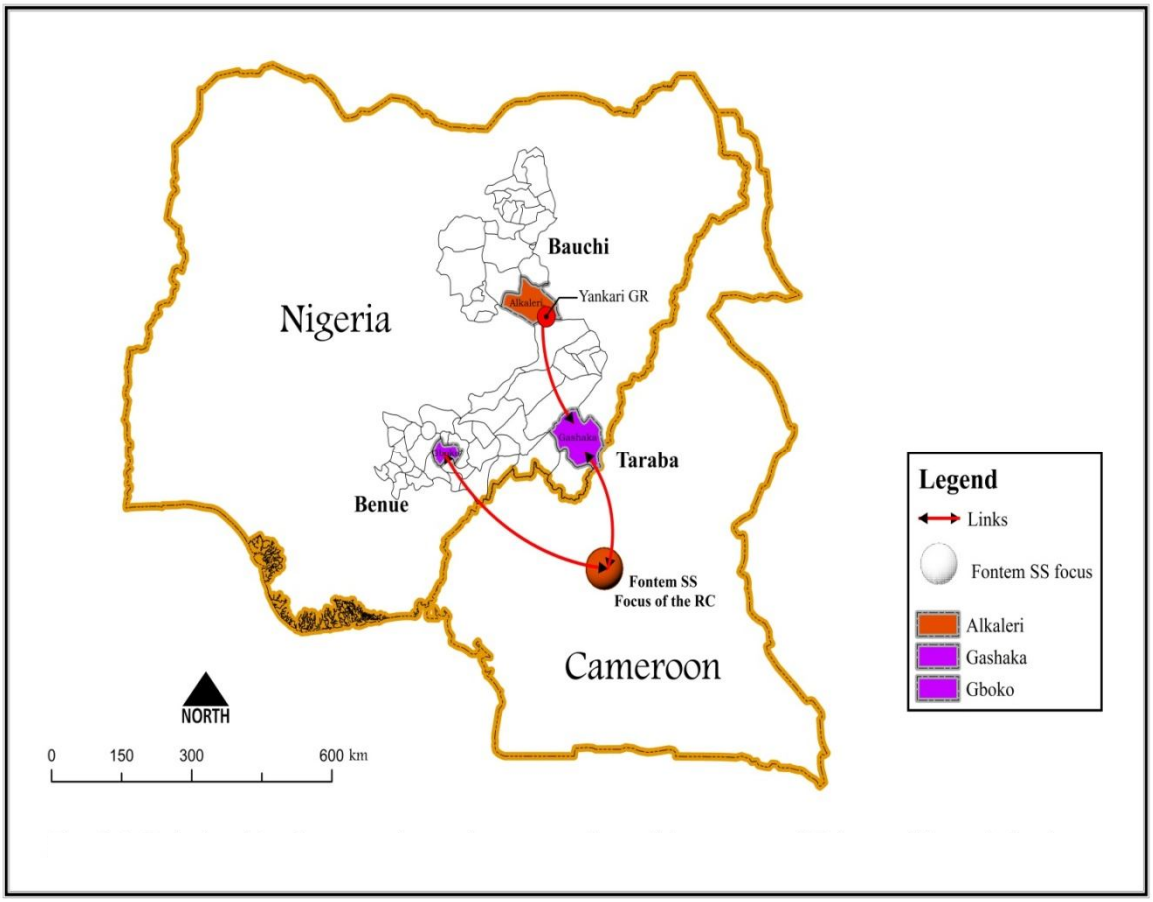


Fig 3.1: Relationship between the study areas and the Gashaka-Gumti game reserve, Yankari game reserve and the Fontem sleeping sickness focus of the Republic of Cameroon.

Game reserve (GR), Republic of Cameroon (RC) Sleeping Sickness (SS)

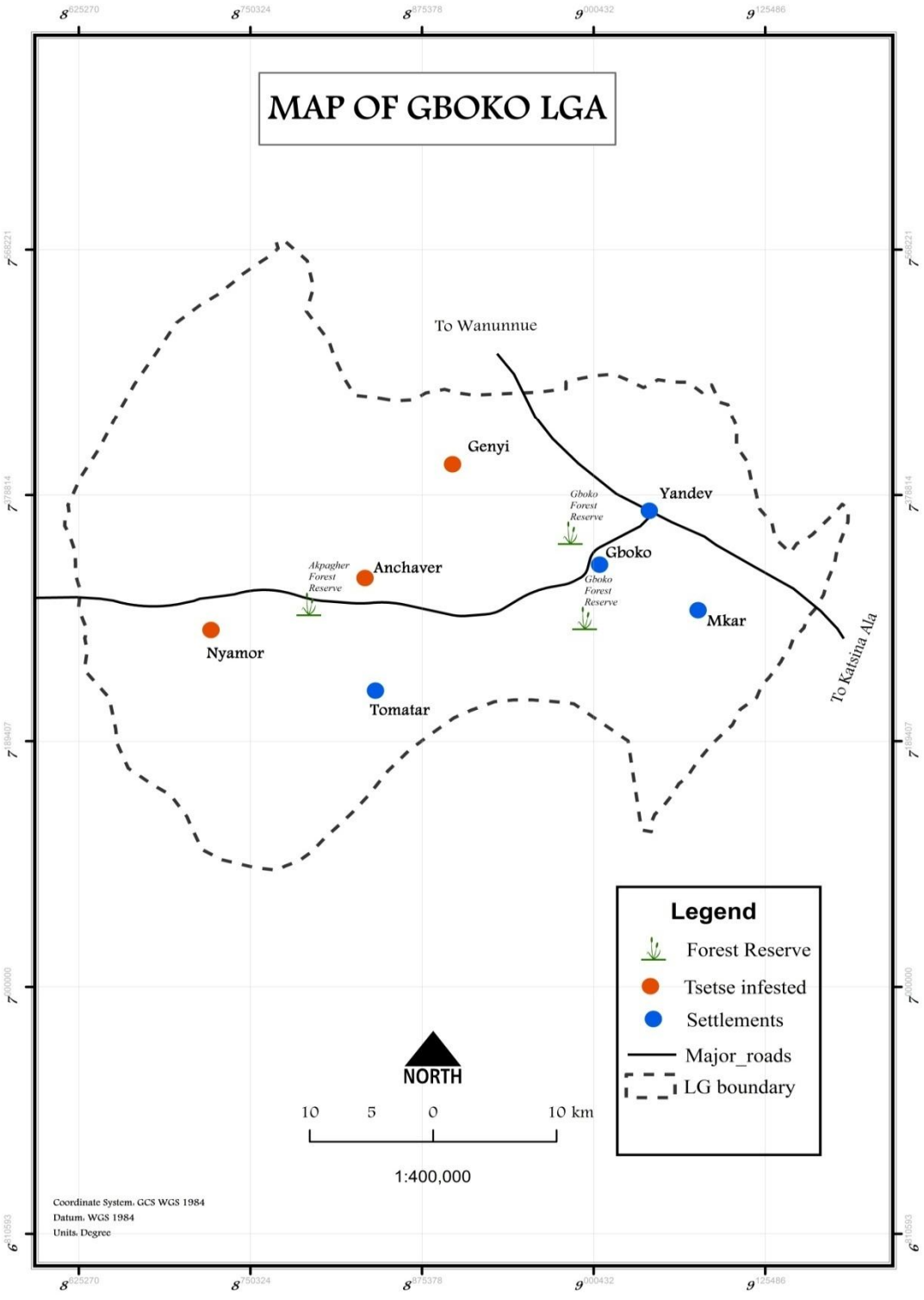


Fig 3.2: Map of Gboko Local Government Area showing tsetse infested areas as provided by respondents during the participatory epidemiology.

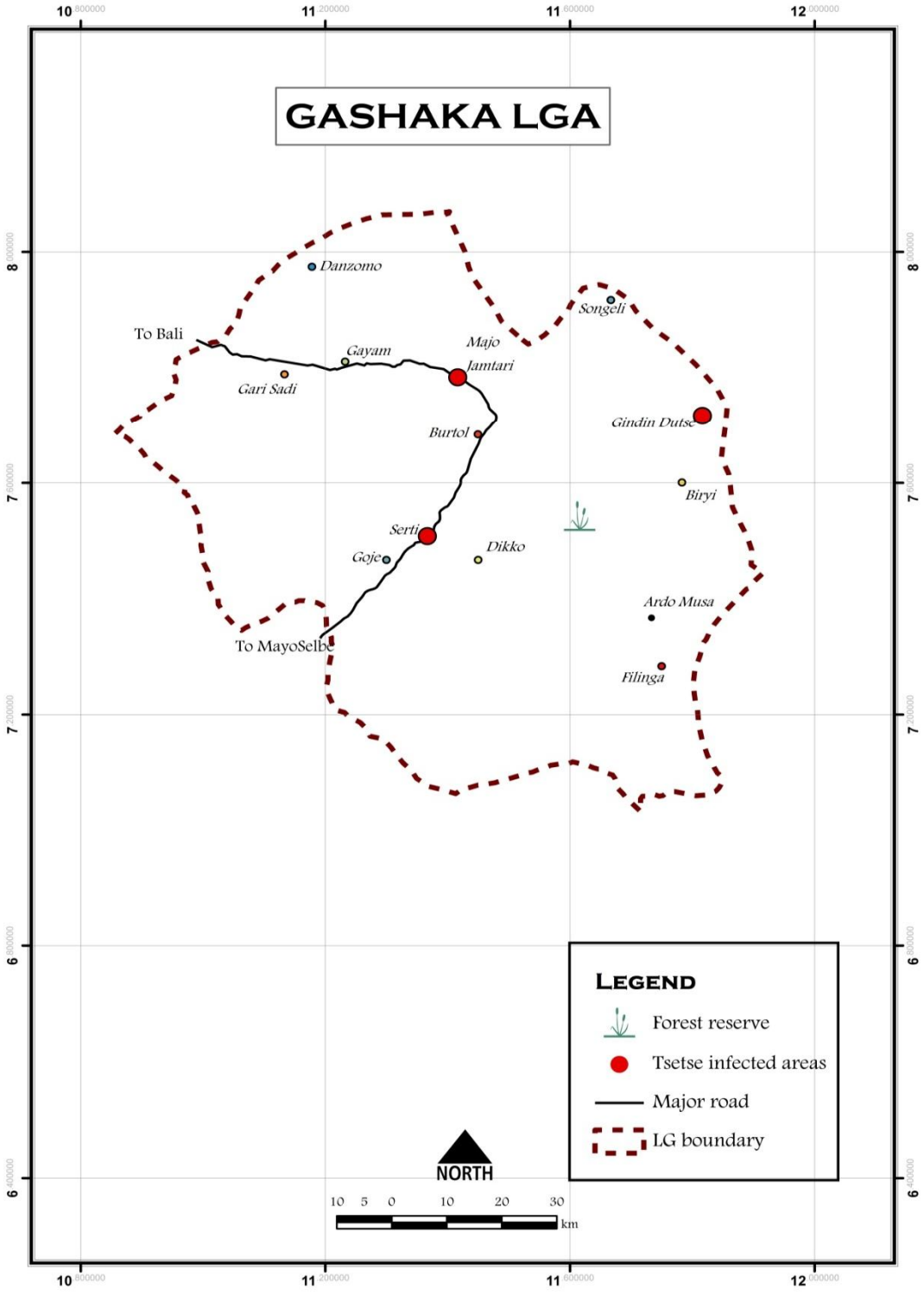


Fig 3.3: Map of Gashaka Local Government Area showing tsetse infested areas as provided by respondents during the participatory epidemiology.

$$for\ pigs\ n = \frac{1.96^2 \times 0.264 \times (1 - 0.264)}{(0.05)^2} = 298.6$$

A total of 600 pigs were sampled in Benue State.

A grand total of 2,400 samples were subjected to serological analysis.

3.3 Study Design

A cross sectional study was conducted in Benue and Taraba States of Nigeria. Three LGAs were randomly selected from each of the States using simple balloting; these were Gboko, Ukum and Vandikya in Benue State as well as Gashaka, Ibi and Karim Lamido in Taraba State. Within a LGA, three Villages were randomly selected using simple balloting. For human sampling, house holds were sampled systematically by selecting every 5^{th} house hold following the order to which houses were built, and individuals were selected using balloting by names.

Herds of cattle and swine were identified from the selected villages from which animals were selected using cluster sampling. From each of the randomly selected villages in each LGA, a total of fifteen herds were selected using simple balloting. From each selected herd, 30% or 50% of animals were sampled from herds with sizes of ≥50 or <50 animals respectively. The selected animals were stratified based on breed, sex and age groups for the purpose of this study (Cattle ≤ 1year as young, > 1 year as Adult; Pigs ≤ 6 months as Young, > 6 months as Adult).

3.4 Sample Collection

3.4.1 Ethical issues

Ethical clearance was obtained from the Taraba State Ministry of Health before the commencement of this study. Consents of participants were sought either verbally or in written before the commencement of sampling. Only individuals that agreed were sampled for this study. The study also observed all the guidelines governing the use of laboratory animals in research.

3.4.2 Participatory epidemiological study

This study utilized open communication, interviews and proportional pilling to obtain information on community HAT ranking, community beliefs on sources of infection, reservoirs of infection, disease impact and risk occupations practiced in the communities studied (Plates I and II). Before the commencement of this study, the participatory epidemiology team visited the village heads of the communities studied. A total of twelve respondents were nominated by each village head to participate in the study. Two villages from each of the two States studied were subjected to the participatory epidemiological study to validate all information in a process referred to as triangulation. Proportional pilling was employed at every stage of the discussions to obtain a proportion of every opinion submitted. The proportions from each of the four villages were averaged and expressed as percentages to represent each of the variable measured. Information on the distribution of tsetse flies and their densities were obtained and mapped as a guide for tsetse trapping during the study.

3.4.3 Blood sampling of human subjects

Two ml of blood was aseptically collected from each human subject via the median cubital vein by Medical Laboratory Scientist using a 5 ml syringe and 21 G needle and transferred immediately into clean labeled sample bottles containing ethylene diamine tetra-acetic acid (EDTA) at 1 mg/ ml and gently shaken until the blood was properly mixed with the anticoagulant. These samples were subjected to the card agglutination test for *T. b. gambiense* within one hour of collection as described by Magnus *et al*. (1978). All CATT positive samples were then inoculated into mice immuno-suppressed with cyclophosphamide to isolate trypanosomes.

3.4.4 Blood sampling of animals

Two ml of blood was aseptically collected from cattle via the jugular vein using 5 ml syringe and 18 G needles. The same volume of blood was also aseptically obtained from pigs via the ear vein using 5 ml syringe and 21 G needles. The blood samples were labelled appropriately and processed as described in section 3.4.3 over-leaf.

3.4.5 Sampling of vectors

Tsetse flies were trapped by setting biconical and Nitse traps around streams and animal grazing sites as described by Charllier and Laveissiere (1973). The traps were emptied every 24 hours and the flies were identified in the Parasitology Laboratory of the National Institute for Trypanosomosis Research, Vom, using the method of Murray *et al*. (1983). Tsetse flies collected were caged until needed for dissection within 48 hours (Plates XIV and XV).

3.5 Laboratory Analysis of Samples

3.5.1 The card agglutination test for *T. b. gambiense* (CATT)

The CATT reagent and kit were obtained from the Institute for Tropical Medicine, Antwerp, Belgium. Blood samples were analyzed using the CATT as described by Magnus *et al.* (1978). This test is based on the detection of *T. b. gambiense* specific Li Tat 1.3 antibodies using a purified *T. b. gambiense* variable surface antigen. To perform the test, one drop of test blood was put on a test card area and a drop of a reconstituted homogenized CATT antigen suspension was added. This was mixed and spread using a stirring rod. The test card was then rocked for 5 minutes as recommended by the manufacturer.

The results of the test were read to determine very strong agglutination, strong agglutination, moderate agglutination, weak agglutination and absence of agglutination (Plates V and VI). These were interpreted as strongly positive (+++), moderately positive (++), positive (+), weakly positive (±), and negative (−) results respectively using the manufacturers guide.

3.6 Preparation of PSG Buffer

Phosphate-saline glucose (PSG) buffer was reconstituted by adding 15.0 g of D-glucose, 6.8 g of disodium hydrogen phosphate dehydrate (Na_2HPO_4), 1.7 g of sodium chloride (NaCl) and 0.3 g of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) to some amount of distilled water, dissolved and made up to one litre at a pH of 8.0. The mixture was thoroughly mixed until the solutes were completely dissolved.

3.7 Mice Infection and Parasitaemia Estimation

A total of 60 mice were obtained from the Small Animal Experimental Unit of the National Veterinary Research Institute, Vom, Nigeria for the study. The mice were screened for ectoparasites and endoparasites by standard techniques (Schalm *et al.,* 1975), and acclimatized for two weeks before inoculation. The mice were divided into two groups of 30 animals each (A and B) representing the two states studied. Mice allocated for each state were further divided into two groups of 15 animals each which were designated as A_h B_h and A_a B_a for human and animal sample inoculation respectively. The mice were immunosuppressed using intraperitoneal administration of cyclophosphamide at 200 mg per kg body weight. Human blood samples were treated as described in section 3.4.3 and 0.5 ml of the sero-positive blood samples were enriched with 0.5 ml of PSG buffer and 0.3 ml of the PSG buffer enriched sero-positive blood was then inoculated intraperitoneally into immunosuppressed mice.

For animal blood samples, a total of four serologically positive samples were pooled together and treated as described in sections 3.4.3 and 3.4.4 above and the sero-positive blood samples obtained were enriched with 0.5 ml PSG buffer. Each enriched sample was then inoculated intraperitoneally into immunosuppressed mice. Tail-blood samples were collected from each mouse every 24 hours to estimate parasitaemia using the rapid matching technique as described by Herbert and Lumsden (1976).

3.8 Parasitological Evaluations

3.8.1 Laboratory detection of *Trypanosoma* species

Trypanosomes were detected in mice tail-blood using wet, thick and thin blood smears, and the microhaematocrit centrifugation techniques (Woo, 1971), from which the buffy coat (Bailey and Smith, 1992) was evaluated (Plates XVI and XVII).

3.8.2 Identification and dissection of *Glossina* species

Flies were identified using morphological characteristics as described by Murray *et al*. (1983). Tsetse flies were dissected to check for the different developmental stages of the parasites (Service, 1980), and to obtain DNA from blood-meals for PCR in order to identify blood-meal sources (Plate X).

3.9 Molecular Analysis

3.9.1 Isolation and purification of trypanosomes

The bloodstream form trypanosomes originating from animal and human samples and propagated in mice were separated from the infected mice blood using a DEAE 52 column (Whatman, Maidstone, Kent, UK). The column was packed with DEAE 52 and two ml of infected mice blood was poured into the packed column and the eluted parasites free from blood cells were collected as described by Lanham and Godfrey (1970). The eluted parasites were centrifuged at 2,612 g for 10 minutes and this was followed by washing three times in phosphate

saline glucose (PSG) buffer by re-suspension and centrifugation (Plate XVIII). These parasites were then stored at 4 ^{O}C until needed for DNA extraction.

3.9.2 Extraction of DNA from purified trypanosomes

Trypanosome DNA extraction was done using GeneJET genomic DNA extraction kit (Thermo Scientific, Germany). A total of 200 µl of purified trypanosomes was lysed by adding 400 µl of lysis solution and 20 µl of proteinase K and vortexing to obtain a homogenous suspension. This was incubated at 56 ^{O}C for 10 minutes and then 200 µl of absolute ethanol was added and mixed by votexing. The prepared lysate was transferred to a genomic DNA purification column inserted in a collection tube and centrifuged at 1,469 g for 3 minutes. The collection tube was discarded and the DNA purification column was transferred into another collection tube and centrifuged at 2,612 g for 3 minutes after adding 500 µl of Wash Buffer I. The flow-through was discarded and the purification column was placed back into the collection tube and centrifuged at 5,876 g for 5 minutes after adding 500 µl of Wash Buffer II. At this point, the collection tube was discarded and the DNA purification column was transferred into a 1.5 ml micro-centrifuge tube. To elute the DNA collected in the column, 200 µl of elusion buffer was added to the centre of the column and centrifuged at 1,469 g for 3 minutes. This step was repeated to maximize DNA yield, after which the DNA was incubated at room temperature for 2 minutes. The DNA purification column was then discarded and the DNA collected in the micro-centrifuge tube was stored at -20 ^{O}C until needed for Polymerase Chain Reaction (PCR) at the Biotchnology Laboratory of the Ahmadu Bello University, Zaria, Nigeria.

3.9.3 PCR detection of trypanosomes

PCR detection was based on two different primer sets to validate the presence of trypanosome DNA in the blood of infected mice. Both ITS-1 and KIN primer sets were used to identify the presence of DNA of trypanosomes species in the blood of infected mice.

All PCR were carried out in a final volume of 11 µl containing 5 µl phusion flash high fidelity PCR master mix (phusion flash II DNA polymerase, dNTPs, $MgCl_2$), 1 µl each of forward and reverse primers, 1 µl of extracted DNA and 3 µl of PCR grade water. PCR conditions for ITS-1 and KIN primers were as follows: an initial denaturation step for 10 seconds at 98 °C to activate the phusion flash II DNA polymerase, four cycles of amplification with 1 second denaturation at 98 °C, 5 seconds hybridization at 58 °C and 15 seconds elongation steps at 72 °C; eight cycles of amplification with 1 second denaturation at 98 °C, 5 seconds hybridization at 56 °C and 15 seconds elongation steps at 72 °C; 23 cycles of amplification with 1 second denaturation at 98 °C, 5 seconds hybridization at 54 °C and 15 seconds elongation steps at 72 °C; and a final extension step of 5 minutes at 72 °C as described by the manufacturer.

3.9.4 Detection of TgsGP and SRA genes

To rule out the presence of human infective *T. b. rhodesiense* among the isolates, the primer set SRA was used to amplify the human serum resistant antigen (SRA) gene which is unique to the sub-species *T. b. rhodesiense*. Finally, the primer set TgsGP was used to characterize the *T. brucei* isolates as *T. b. gambiense* type 1 by amplifying the TgsGP gene which is unique to this strain. Cycling conditions for both SRA and TgsGP were; denaturation at 98 °C for 10 seconds to activate the phusion flash II DNA polymerase, followed by 40 cycles at 98 °C for 1 second,

annealing at 63 °C for 30 second, 30 seconds elongation at 72 °C and a final extension at 72 °C for 5 minutes. All amplified products were analyzed by electrophoresis in a 2% agarose gel and UV illumination after ethidium bromide staining and documented by photographing under ultra-violet illumination.

3.10 Identification of Sources of Tsetse Blood-meals.

3.10.1 Extraction of DNA from tsetse blood-meals

Tsetse blood-meal DNA was extracted from tsetse abdomens by a modified salt procedure as described previously (Norris *et al*., 2001; Muturi *et al*., 2011). Fully engorged tsetse abdomens were detached from the thorax and head and homogenized in 100 µl of extraction buffer containing 0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris-HCl, 0.05 M EDTA, pH 9.1, and 0.5% sodium dodecyl sulfate (SDS) and incubated at 65°C for one hour. To precipitate the SDS, 15 µl of cold 8 M potassium acetate was added to each homogenate and samples were incubated on ice for 45 minutes. Samples were then centrifuged at 2,612 g for 10 minutes to remove cellular debris, and DNA was precipitated by adding 250 µl of 100% ethanol to the transferred supernatant. Following 5 minutes incubation at room temperature, DNA was pelleted by a 15 minutes centrifugation at 5,876 g. Finally, the supernatant was discarded and dried pellets were washed using wash buffers I and II. DNA was eluted by the addition of 200 µl of elution buffer and centrifugation at 1,469 g for 3 minutes. DNA collected was then stored at -20 ^{O}C until needed for polymerase chain reaction.

Table 3.1: Primer Pairs and Targeted Amplification Genes for the Characterization of Trypanosomes.

Organism	Target gene	Primer	Sequence (5^1-3^1)	Amplicon size (bp)	Tm (^OC)	Reference
Trypanosoma spp	ITS1 rDNA	ITS1 F	CCGGAAGTTCACCGATATTG	411-780	58	Njiru *et al*. (2005)
		ITS1 R	TTGCTGCGTTCTTCAACGAA			
Trypanosoma spp	ITS1 rDNA	KIN 1	GCGTTCAAAGATTGGGCAAT	510-750	56	McLaughlin *et al. (1996)*
		KIN 2	CGCCCGAAAGTTCACC			
T. b. rhodesiense	SRA	SRA-F	ATAGTGACAAGATGCGTACTCAACGC	287	68	Radwanska *et al.* (2002a)
		SRA-R	AATGTGTTCGAGTACTTCGGTCACGCT			
T. b. gambiense	TgsGP	TgsGP-F	GCTGCTGTGTTCGGAGAGC	308	63	Radwanska *et al.* (2002b)
		TgsGP-R	GCCATCGTGCTTGCCGCTC			

3.10.2 PCR amplification of the mitochondrial DNA cytochrome b gene

Primers with known sequences (Table 3.2) were used to amplify the mitochondrial DNA cytochrome b gene which is unique in each vertebrate animal (Boakye *et al.,* 1999; Oshaghi *et al.,* 2006a, b). These PCR amplifications were used to identify the sources of tsetse blood-meals. Cycling conditions were denaturation at 98 °C for 10 seconds to activate the phusion flash II DNA polymerase, followed by 36 cycles at 98 °C for 1 second, annealing for 50 seconds at temperatures of 58.5 °C, 59.5 °C, 62.0 °C and 62.5 °C for cattle, dog, pig and human primers, respectively. Extension was at 72 °C for 40 seconds and final extension at 72 °C for 5 minutes. All amplified products were analyzed by electrophoresis in a 2% agarose gel and UV illumination after ethidium bromide staining and documented by photographing under ultra-violet illumination. In all cases, 100 base pair molecular ladder were used.

3.11 Data Analysis

All data obtained during the study were analyzed using Graph-Pad Prism 4.0. The prevalence rates of HAT were calculated by dividing the number of infected individuals by the total number of individuals examined and expressed as percentages. This was done for different variables such as sex, age, breed, and management practices. The Chi square (X^2) and Fisher's exact tests as well as Odd ratio (Kuzma and Bohnenblust, 2001) were used where appropriate to compare the prevalence rates based on different variables such as sex, age and LGA for humans as well as sex, age, LGA, breed and management practices for cattle and swine. The X^2 test was also used to determine differences in the tsetse feeding frequencies for individual vertebrate species as well as multiple and dual feeding frequencies. Values of $p<0.05$ were considered significant.

Table 3.2: Forward Primers and a Universal Reverse Primer for the Amplification of Mitochondrial Cytochrome b Gene in Tsetse Blood-Meals.

mtDNA-Cyt b gene	Primer	Sequence 5^1-3^1	Tm	Amplicon size	Reference
Human and Dog	DupHD-F	GGCTTACTTAGTACTTGTACCCTTCCTC	66.08	486 (Humans) 680 (Dog)	Lord *et al*. (1998)
Cattle	Cow 121-F	CATCGGCACAAATTTAGTCG	58.35	561	Irwin *et al*. (1991)
Pig	Pig 573-F	TTAGTCGCCTCGCAGCCGTA	64.48	453	Irwin *et al*. (1991)
Universal Reverse	UNREV 1025	GGTTGTCCTCCAATTCATGTTA	58.95	-	Irwin *et al*. (1991)

CHAPTER FOUR

RESULTS

4.1 CATT Based Prevalence of *T. b. gambiense* Among Humans in Benue State.

The overall sero-prevalence of *T. b. gambiense* among humans in Benue State as revealed by CATT test was 1.3% (8/600). Gboko LGA had the highest prevalence of 0.8% (5/600), while Vandekya and Ukum LGAs had 0.5% and 0.0% prevalence rates respectively (Table 4.1). Prevalence rates among humans in relation to sex were 1.0% (6/600) and 0.3% (2/600) for males and females respectively, while adult and children had 0.67% (4/600) each. There was no significant association between the sex and age based prevalence rates ($p>0.05$) as shown in Table 4.2.

4.2 CATT Based Prevalence of *T. b. gambiense* Among Pigs in Benue State

This study revealed an overall *T. b. gambiense* prevalence of 10.7% (64/600) among pigs in Benue State. Of this, Gboko, Ukum and Vandekya LGAs had 5.7% (34/600), 1.5% (9/600) and 3.5% (21/600) respectively (Table 4.3). The sex based prevalence rates were 4.7% (28/600), and 6.0% (36/600) for males and females, respectively (Table 4.4). The prevalence for young pigs was 3.83% (23/600), while 6.83% (41/600) was observed for the adults (Table 4.4). The highest prevalence of 8.7% (52/600) was observed among extensively managed pigs as against the 2.0% (12/600) for intensively managed pigs. There were significant associations between the LGA, age and management based prevalence rates among pigs ($p<0.05$), however, sex based prevalence showed no signi ficant association ($p<0.05$) as shown in Table 4.4.

Table 4.1: Sero-Prevalence of *T. b. gambiense* Among Humans in Benue State in Relation to Local Government Area.

LGA	No. Examined	No. Positive	LGA Specific Prevalence (%)	Overall Prevalence (%)
Gboko	200	5.0	2.5	0.8
Vandekya	200	3.0	1.5	0.5
Ukum	200	0.0	0.0	0.0
Total	**600**	**8.0**	**-**	**1.3**

Table 4.2: Sex and Age Based Sero-Prevalence of *T. b. gambiense* Among Humans in Benue State.

SEX	**No. Examined**	**No. Positive**	**Specific Prevalence (%)**	**Overall Prevalence (%)**	**Odds ratio**
Male	300	6.0	2.0	1.0	
Female	300	2.0	0.6	0.3	
Total	**600**	**8.0**	**-**	**1.3**	3.041
AGE					
Adult (≥ 18 Yrs)	200	4.0	2.0	0.67	
Children (< 18 Yrs)	400	4.0	1.0	0.67	
Total	**600**	**8.0**	**-**	**1.3**	2.020

SEX: p= 0.2858, 95% CI = 0.6086, 15.1900, **AGE**: p= 0.4508, 95% CI = 0.4998, 8.167

Table 4.3: Sero-Prevalence of *T. b. gambiense* Among Pigs in Benue State in Relation to Local Government Areas.

LGA	No. Examined	No. Positive	LGA Specific Prevalence (%)	Overall Prevalence (%)
Gboko	200	34.0	17.0	5.7
Ukum	200	9.0	4.5	1.5
Vandekya	200	21.0	10.5	3.5
Total	**600**	**64.0**	**-**	**10.7**

$X^2 = 16.41$, $p < 0.0003$, df = 2

Table 4.4: Sero-Prevalence of *T. b. gambiense* Among Pigs in Benue State in Relation to Sex, Age and Management Practices.

SEX	No. Examined	No. Positive	Specific Prevalence (%)	Overall Prevalence (%)	Odds ratio
Male	300	28.0	9.3	4.7	
Female	300	36.0	12.0	6.0	0.7549
Total	**600**	**64.0**	**-**	**10.7**	
AGE					
Young (≤ 6 month)	300	23.0	7.7	3.83	
Adult (> 6 months)	300	41.0	13.7	6.83	0.5245
Total	**600**	**64.0**	**-**	**10.7**	
Management practice					
Intensive	213	12.0	5.6	2.0	
Extensive	387	52.0	13.4	8.7	0.3846
Total	**600**	**64.0**	**-**	**10.7**	

SEX: $X^2 = 1.119$, $p = 0.2900$, 95% CI = 0.4495, 1.273, **AGE**: $X^2 = 5.667$, $p = 0.0173$, 95% CI = 0.3062, 0.8984, **MANAGEMENT PRACTICE**: $X^2 = 8.778$, $p = 0.0030$, 95% CI = 0.2004, 0.7380

4.3 CATT Based Prevalence of *T. b. gambiense* Among Humans in Taraba State.

This study revealed an overall prevalence rate of 2.2% (13/600) for *T. b. gambiense* among humans in Taraba State. The LGAs of Gashaka, Ibi, and Karim Lamido recorded prevalence rates of 1.2% (7/600), 0.2% (1/600) and 0.8% (5/600) respectively (Table 4.5). Table 4.6 shows prevalence rates of *T. b. gambiense* among humans in Taraba State in relation to sex and age. Males represented 1.33% (8/600) and females 0.83% (5/600) of the overall prevalence. Prevalence rates of 1.0% (6/600) and 1.2% (7/600) were recorded for adults and children respectively. There was no significant association between the LGA, sex and age based prevalence rates in humans from Taraba State ($p>0.05$).

4.4 CATT Based Sero-Prevalence of *T. b. gambiense* Among Cattle in Taraba State

The overall prevalence recorded among cattle in Taraba State was 7.2% (43/600) as shown in Table 4.7. Of this overall prevalence rate, 3.7% (22/600), 1.3% (8/600), and 2.2% (13/600) were observed in Gashaka, Ibi, and Karim Lamido LGAs, respectively (Table 4.7). Breed based prevalence rates were 5.33% (32/600), 1.5% (9/600), and 0.33% (2/600) for White Fulani, Bokoloji, and Muturu breeds, respectively (Table 4.8). Male cattle recorded a prevalence rate of 2.33% (14/600), while 4.83% (29/600) was observed among the females. In relation to age, 1.7% (10/600) and 5.7% (33/600) represented the prevalence rates for calves and adult cattle, respectively (Table 4.9). There was significant association between the LGA and breed based prevalence rates among cattle from Taraba State ($p<0.05$), however, sex and age based prevalence rates showed no significant association ($p>0.05$)

Table 4.5: Sero-Prevalence of *T. b. gambiense* Among Humans in Taraba State According to Local Government Areas.

LGA	No. Examined	No. Positive	LGA Specific Prevalence (%)	Overall Prevalence (%)
Gashaka	200	7.0	3.5	1.2
Ibi	200	1.0	0.5	0.2
Karim Lamido	200	5.0	2.5	0.8
Total	**600**	**13.0**	**-**	**2.2**

$p < 0.1124$, df = 2

Table 4.6: Sex and Age Based Sero-Prevalence of *T. b. gambiense* Among Humans in Taraba State.

SEX	No. Examined	No. Positive	Specific Prevalence (%)	Overall Prevalence (%)	Odds ratio
Male	300	8.0	2.7	1.33	
Female	300	5.0	1.6	0.83	1.616
Total	**600**	**13.0**	**-**	**2.2**	
AGE					
Adult (≥ 18 Yrs)	250	6.0	2.4	1.0	
Children (< 18 Yrs)	350	7.0	2.0	1.2	1.205
Total	**600**	**13.0**	**-**	**2.2**	

SEX: $p = 0.5768$, 95% CI = 0.5225, 5.000, **AGE**: $p = 0.7811$, 95% CI = 0.3999, 3.631

Table 4.7: Sero-Prevalence of *T. b. gambiense* Among Cattle in Taraba State According to Local Government Areas.

LGA	No. Examined	No. Positive	LGA Specific Prevalence (%)	Overall Prevalence (%)
Gashaka	200	22.0	11.0	3.7
Ibi	200	8.0	4.0	1.3
Karim Lamido	200	13.0	6.5	2.2
Total	**600**	**43.0**	**-**	**7.2**

$X^2 = 7.565$, $p < 0.0228$, df = 2

Table 4.8: Breed Based Sero-Prevalence of *T. b. gambiense* in Cattle in Taraba State.

Breed	No. Examined	No. Positive	Breed Specific Prevalence (%)	Overall Prevalence (%)
White Fulani	300	32.0	10.7	5.33
Bokoloji	200	9.0	4.5	1.5
Muturu	100	2.0	2.0	0.33
Total	**600**	**43.0**	**-**	**7.2**

$p < 0.0001$, df = 2

Table 4.9: Sex and Age Based Sero-Prevalence of *T. b. gambiense* in Cattle in Taraba State.

SEX	No. Examined	No. Positive	Specific Prevalence (%)	Overall Prevalence (%)	Odds ratio
Male	232	14.0	6.0	2.33	
Female	368	29.0	7.9	4.83	0.7507
Total	**600**	**43.0**	**-**	**7.2**	
AGE					
Calf (≤ 1 Yr)	171	10.0	5.8	1.7	
Adult (> 1 Yr)	429	33.0	7.7	5.5	0.7453
Total	**600**	**43.0**	**-**	**7.2**	

SEX: $X^2 = 0.7288$, $p = 0.3933$, df = 1, 95% CI = 0.3879, 1.453, **AGE**: $X^2 = 0.6251$, $p = 0.4291$, df = 1, 95% CI = 0.3588, 1.548

4.5 Distribution of Tsetse Flies Trapped in the Study Areas

In this study, a total of 531 tsetse flies were trapped. Of this, 19.6% (104/531) were from Benue State and 80.4% (427/531) from Taraba State. A total of 78 (14.7%), 21 (3.9%), and 5 (0.9%) were trapped in Gboko, Vandekyaa, and Ukum LGAs of Benue State, respectively while 226 (42.6%), 115 (21.7%), and 86 (16.2%) were trapped in Gashaka, Karim Lamido, and Ibi LGAs of Taraba State, respectively (Figure 4.1). There was an overall infection rate of 4.6% (24/531) with 0.6% and 4.0% observed in Benue and Taraba States, respectively (Figure 4.1). The two species of tsetse flies identified were *Glossina palpalis* and *Glossina tachinoides* which represented 87.2% (463/531) and 12.8% (68/531) of the total catch. Tsetse based infection rates were 3.4% (18/531) and 1.2% (6/531) for *Glossina palpalis* and *Glossina tachinoides*, respectively (Table 4.10).

4.6 Molecular Characterization of Trypanosome Isolates

A total of 11 isolates originating from humans, 6 (54.5%), cattle, 2 (18.2%) and pigs, 3 (27.3%) were characterized by the identification of the ITS-1 rDNA, human serum antigen (SRA) and *T. gambiense* specific glycoprotein (TgsGP) genes. Using PCR, purified trypanosomes showed 100% (11/11) sensitivity (Plate 4.1). The KIN PCR characterized all the human trypanosome isolates as belonging to the *T. brucei* genome, while both the KIN and ITS-1 PCRs identified *T. brucei* and *T. congolense* from the trypanosome stocks obtained from cattle and pigs (Plates 4.1 and 4.2). PCR using strain-specific SRA primer excluded the presence of human infective *T. b. rhodesiense* in all the 6 human and 5 animal isolates. A strain-specific TgsGP primer characterized all the 6 human, 1 cattle and 2 swine isolates as type 1 *T. b. gambiense* (Plates 4.3 and 4.4).

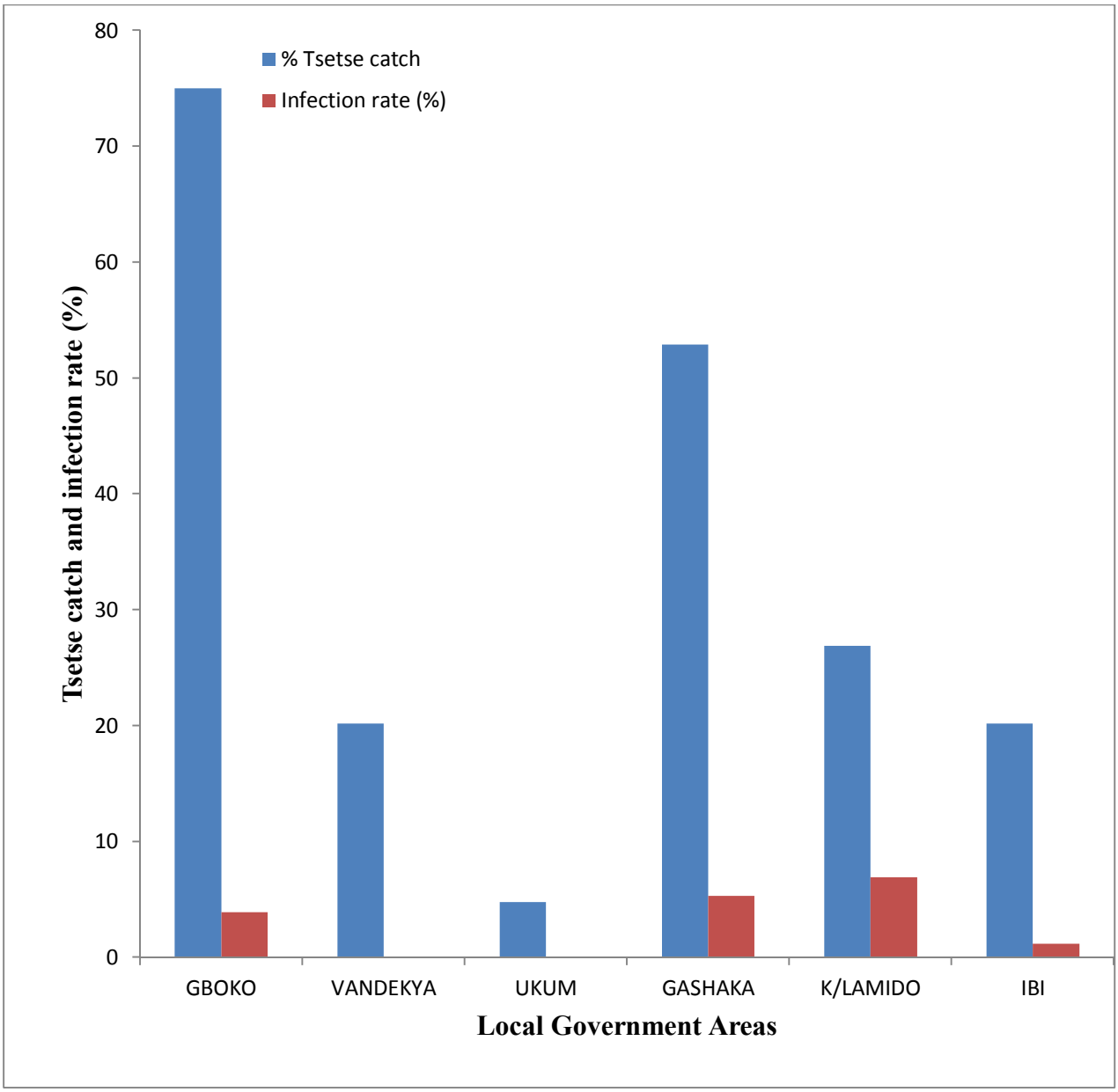


Fig. 4.1: Infection Rates of Tsetse Flies Trapped in Benue and Taraba States According to Local Government Areas.

Table 4.10: Infection Rates of Species of Tsetse Flies Trapped in Benue and Taraba States.

Species	No. trapped	No. positive for trypanosomes	Species specific incidence rate	Overall incidence rate
Glossina palpalis	463	18.0	3.9	3.4
Glossina tachinoides	68	6.0	8.8	1.2
Total	**531**	**24.0**	**-**	**4.6**

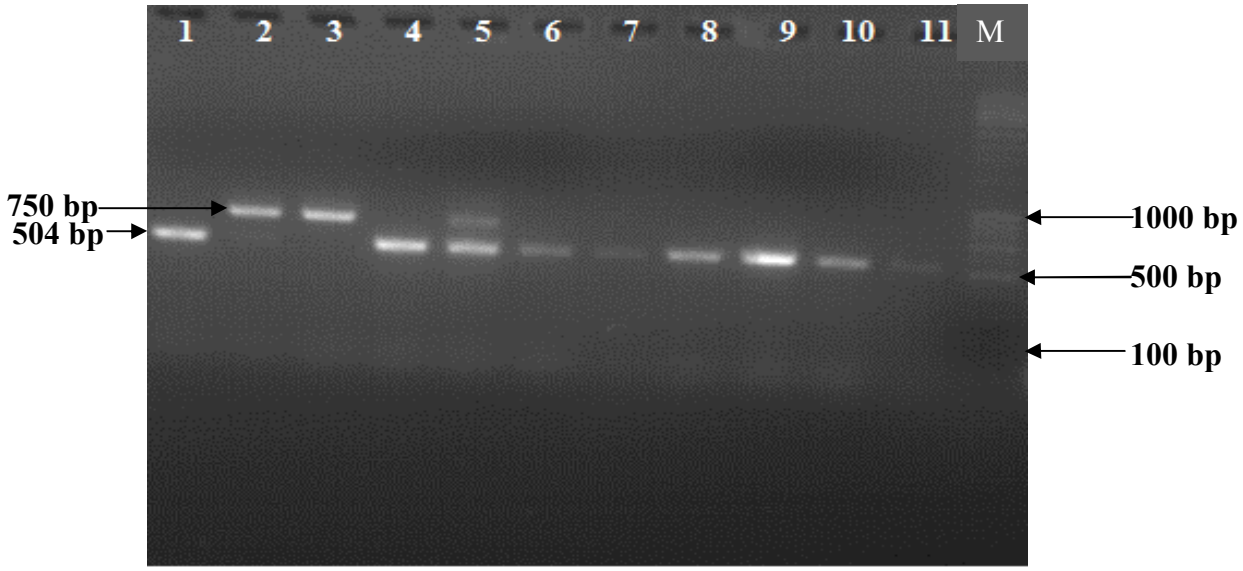


Plate 4.1: Ethidium Bromide Stained Agarose Gel Showing KIN Polymerase Chain Reaction Products Amplified from Field Trypanosome Isolates in lanes 1 to 11: lane 1 (TAC015), lane 2 (TAC401), lane 3 (BEP069), lane 4 (BEP227), lane 5 (BEP410), lane 6 (BEH008), lane 7 (BEH047), lane 8 (BEH118), lane 9 (TAH011), lane 10 (TAH312), lane 11 (TAH576) and lane M (100 bp molecular ladder).

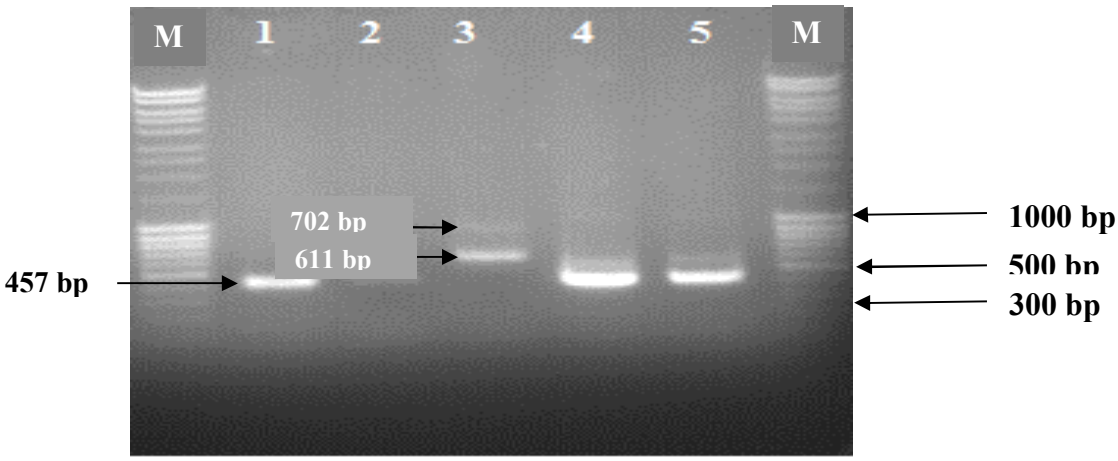


Plate 4.2: Ethidium Bromide Stained Agarose Gel Showing ITS-1 Polymerase Chain Reaction Products Amplified from Field Trypanosome Isolates in lanes 1 to 5: lane 1 (BEP069), lane 2 (BEP227), lane 3 (BEP410), lane 4 (TAC015), lane 5 (TAC401) and lanes M (100 bp molecular ladder).

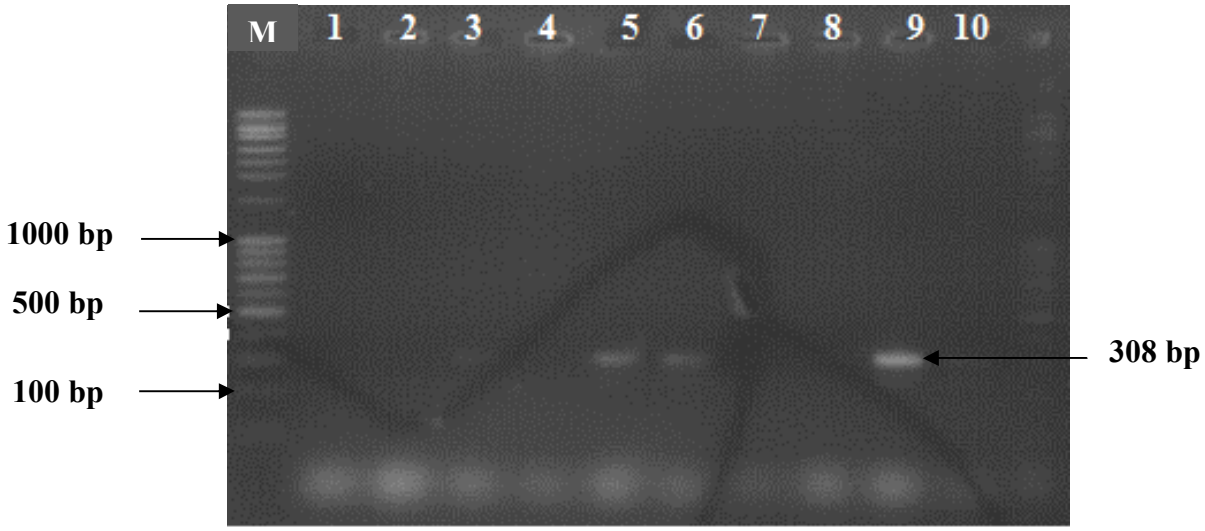


Plate 4.3: Ethidium Bromide Stained Agarose Gel Showing TgsGP Polymerase Chain Reaction Products Amplified from *T. brucei* Stocks Isolated from Humans in lanes 1 to 10: lane 3 (BEH008), lane 5 (BEH047), lane 7 (BEH118), lane 8 (TAH011), lane 9 (TAH312), lane 10 (TAH576) and lane M (100 bp molecular ladder).

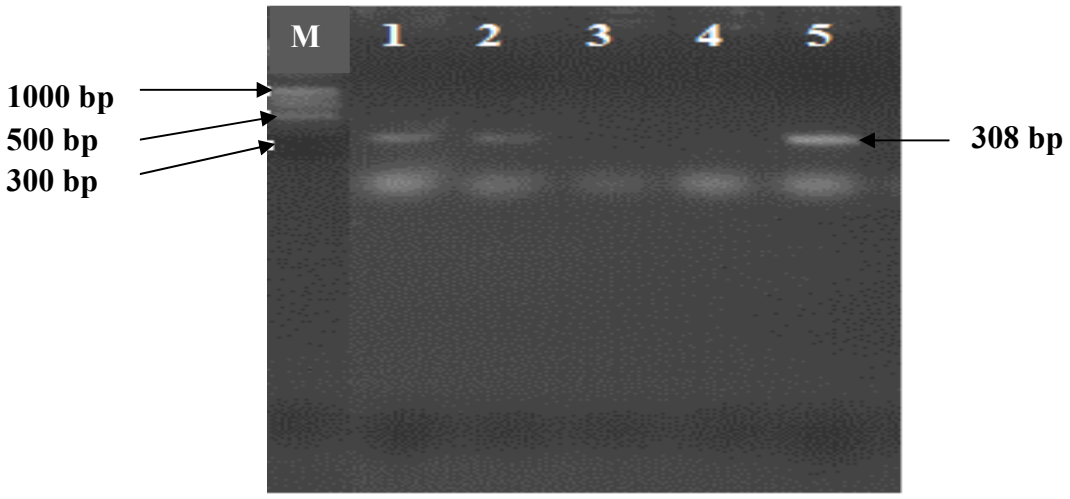


Plate 4.4: Ethidium Bromide Stained Agarose Gel Showing TgsGP Polymerase Chain Reaction Products Amplified from *T. brucei* Stocks Isolated from Cattle and Pigs in lanes 1 to 5: lane 1 (BEP069), lane 2 (BEP227), lane 5 (TAC401) and lane M (100 bp molecular ladder).

4.7 Molecular Identification of Sources of Tsetse Blood-Meals

Blood-meal sources from 30 selected engorged tsetse flies were identified by amplifying a species-specific sequence of the mitochondrial cytochrome b gene using PCR. Of the 30 tsetse flies analyzed, 10 (33.3%), 9 (30.0%), 7 (23.3%) and 4 (13.3%) were identified to have fed on cattle, pigs, dogs and humans respectively (Plates 4.5, 4.6 and 4.7). The study revealed multiple feeding patterns involving human-cattle-pig-dog among 2 (6.7%) and cattle-dog-pig among 5 (16.7%) of the analyzed tsetse flies. Dual feeding patterns involving human-dog 2 (6.7%), human-cattle 4 (13.3%) and human-pig 3 (10.0%) were also observed (Plates 4.5, 4.6 and 4.7).

4.8 Participatory Epidemiology

A total of eight diseases were listed by the respondents as major problems to their communities (Figures 4.2, 4.3 and 4.4). Of these diseases, HAT was rated highest in terms of mortality (86%) and economic impact (70%). Although the rating, in relation to economic impact was the same as that recorded for HIV/AIDS, mortality rating according to the respondents opinions was higher in HAT (86%) than HIV/AIDS (80%) as shown in Figures 4.2 and 4.3. Availability of drugs and control programmes for HAT were both rated 0% (Figure 4.4).

Considering reservoirs of *T. b. gambiense* in the study areas, the respondents assigned the highest proportion of 32/40 (80.0%) to wildlife, followed by 28/40 (70.0%) for domestic animals while humans were assigned the lowest proportion of 7/40 (17.5%) as shown in Table 4.3. As regards sources of infection, game reserves were assigned the highest proportion of 29/40 (72.5%), followed by 27/40 (67.5%) and 21/40 (52.5%) for watering points and farm lands, respectively. Homestead areas were assigned the lowest proportion of 10/40 (25.0%) as shown in Table 4.4.

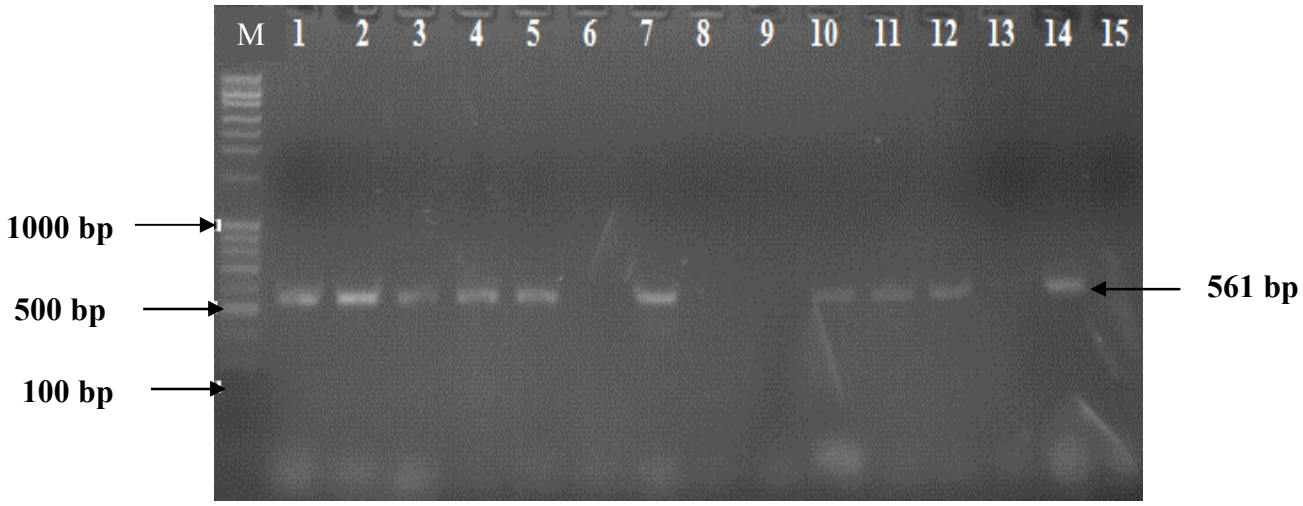


Plate 4.5: Ethidium Bromide Stained Agarose Gel Showing Cattle Specific Mitochondrial DNA Cytochrome b Polymerase Chain Reaction Products Amplified from Blood-Meal DNA Extracted from Field Captured Tsetse Flies in lanes 1 to 15: lane 1 (G01), lane 2 (G04), lane 3 (G07), lane 4 (G10), lane 5 (G17), lane 7 (G18), lane 10 (G22), lane 11 (G27), lane 12 (G29), lane 14 (G30) and lane M (100 bp molecular ladder).

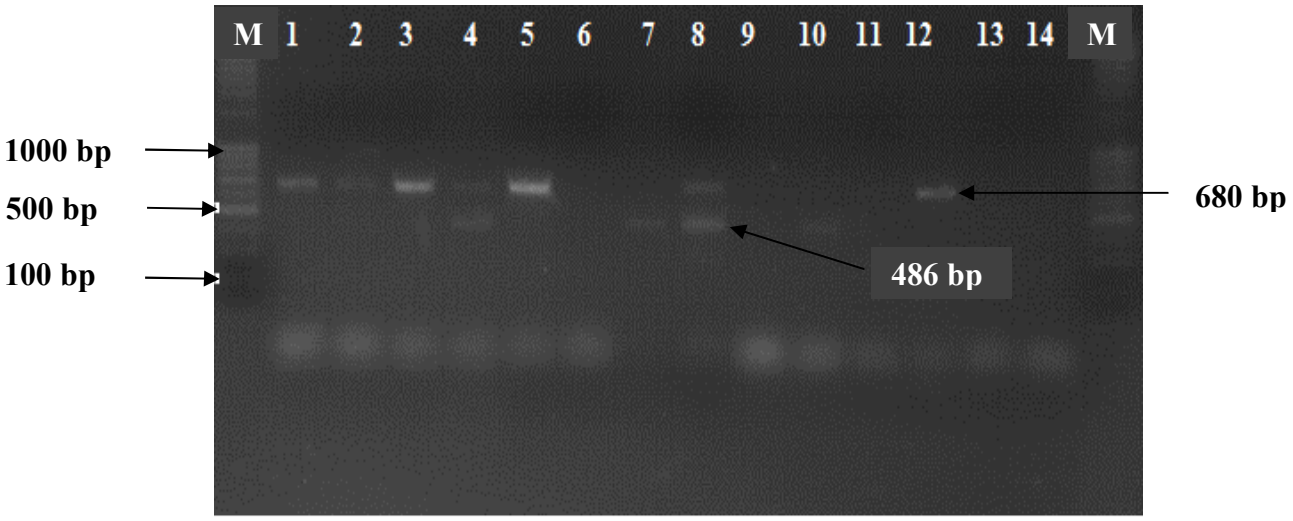


Plate 4.6: Ethidium Bromide Stained Agarose Gel Showing Human and Dog Specific Mitochondrial DNA Cytochrome b Polymerase Chain Reaction Products Amplified from Blood-Meal DNA Extracted from Field Captured Tsetse Flies in lanes 1 to 14: lane 1 (G01), lane 2 (G04), lane 3 (G07), lane 4 (G10), lane 5 (G17), lane 7 (G18), lane 8 (G22), lane 10 (G27), lane 12 (G29) and lane M (100 bp molecular ladder).

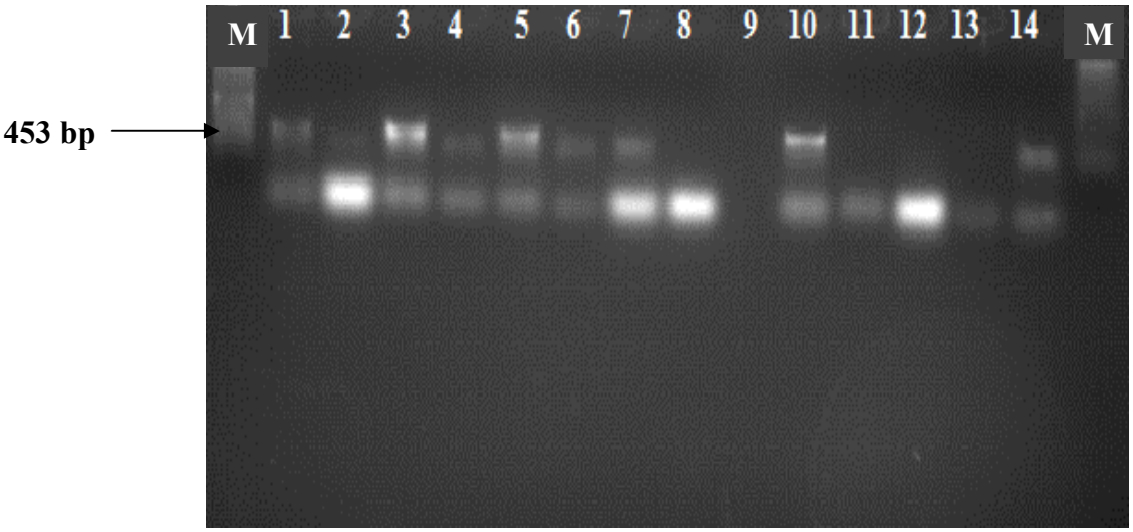


Plate 4.7: Ethidium Bromide Stained Agarose Gel Showing Pig Specific Mitochondrial DNA Cytochrome b Polymerase Chain Reaction Products Amplified from Blood-Meal DNA Extracted from Field Captured Tsetse Flies in lanes 1-14: lane 1 (G01), lane 2 (G04), lane 3 (G07), lane 4 (G10), lane 5 (G17), lane 6 (G18), lane 7 (G22), lane 10 (G27), lane 14 (G30) and lane M (100 bp molecular ladder).

Risk occupations associated with HAT as identified by inhabitants of the study areas were farming, hunting, fishing and shepherding. The proportions assigned to these risk occupations were 30/40 (75.0%), 28/40 (70.0%), 23/40 (57.5%) and 7/40 (17.5%), respectively (Table 4.13). Age based impact assessment of HAT showed that people within the age bracket of 16-30 years were said to be the most affected group with 33/40 (82.5%), followed by 32/40 (80.0%) for the age bracket of 31-45 years. The proportions of 18/40 (45.0%), 13/40 (32.5%), 11/40 (27.5%), and 3/40 (7.5%) were assigned to the age brackets of 46-60, 0-15, 61-75, and >75 years, respectively (Table 4.14). Respondents also identified Genyi, Anchaver, and Nyamor in Gboko, Benue State and Mayo Jamtari, Gindin Dutse, and Serti in Gashaka, Taraba State as tsetse infested areas (3.2 and 3.3).

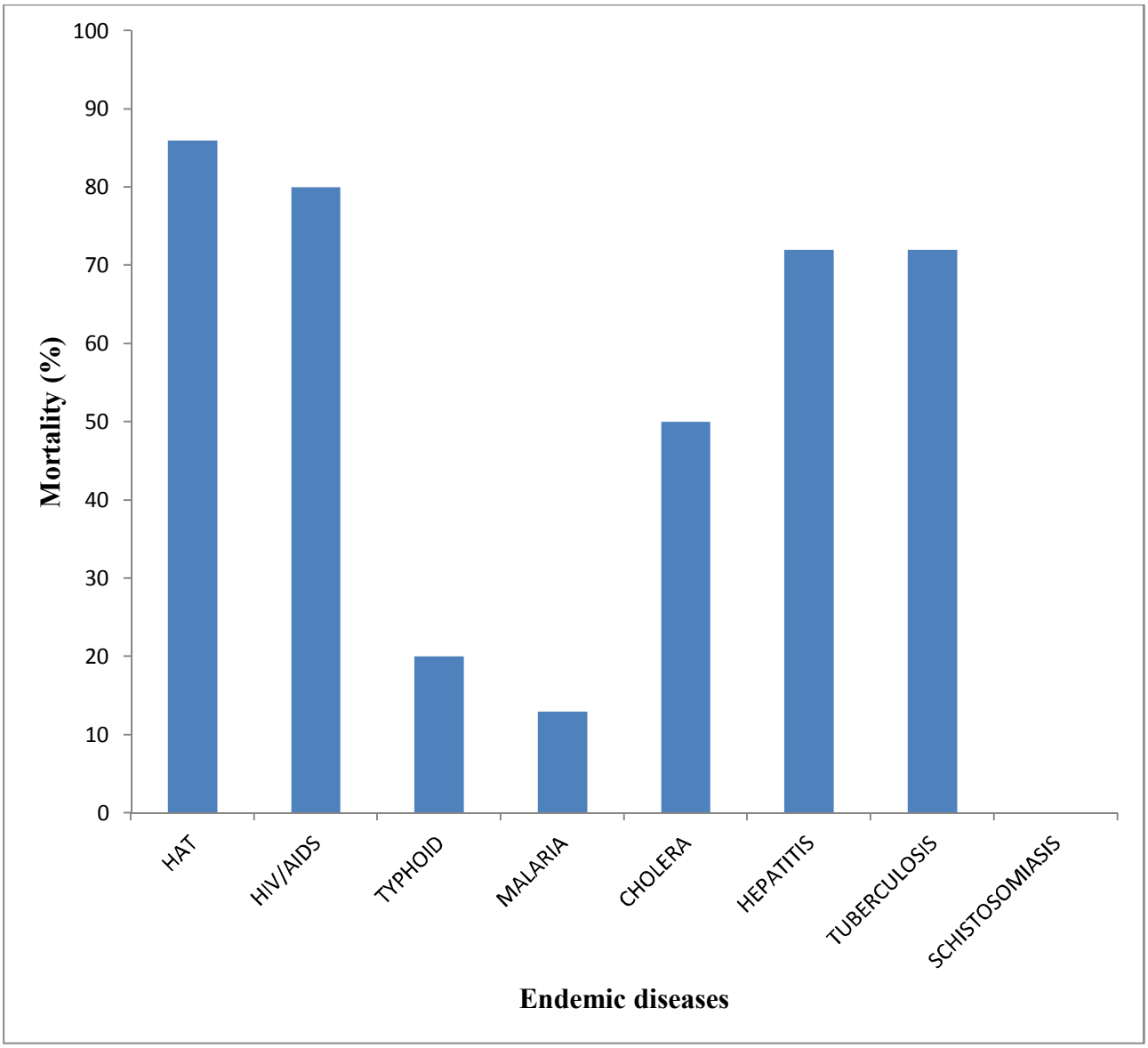


Fig. 4.2: Comparative Ranking of HAT with Other Listed Endemic Diseases by Respondents in the Study Areas Based on Perceived Mortality.

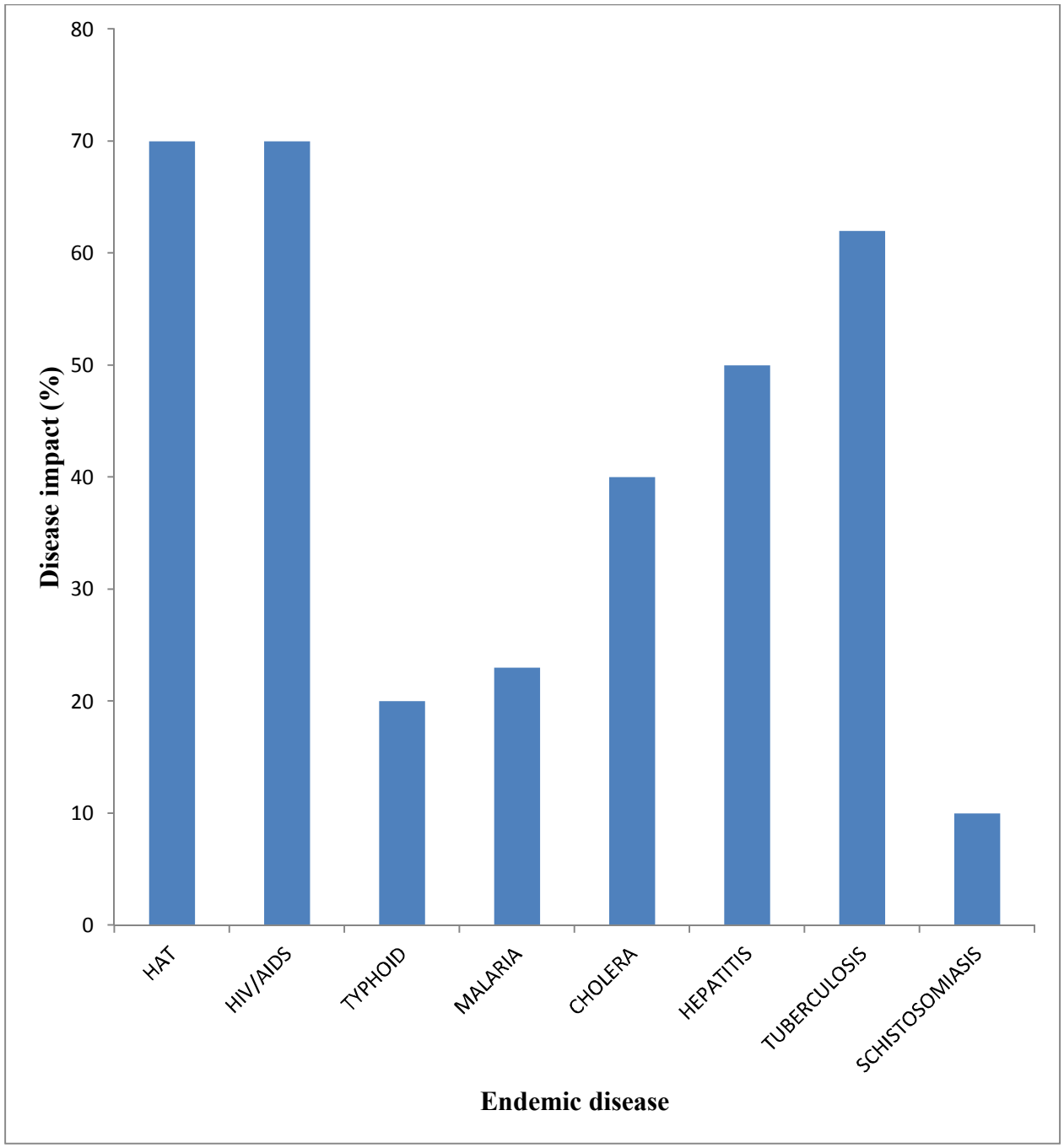


Fig. 4.3: Comparative Ranking of HAT with Other Listed Endemic Diseases by Respondents Based on Disease Impact.

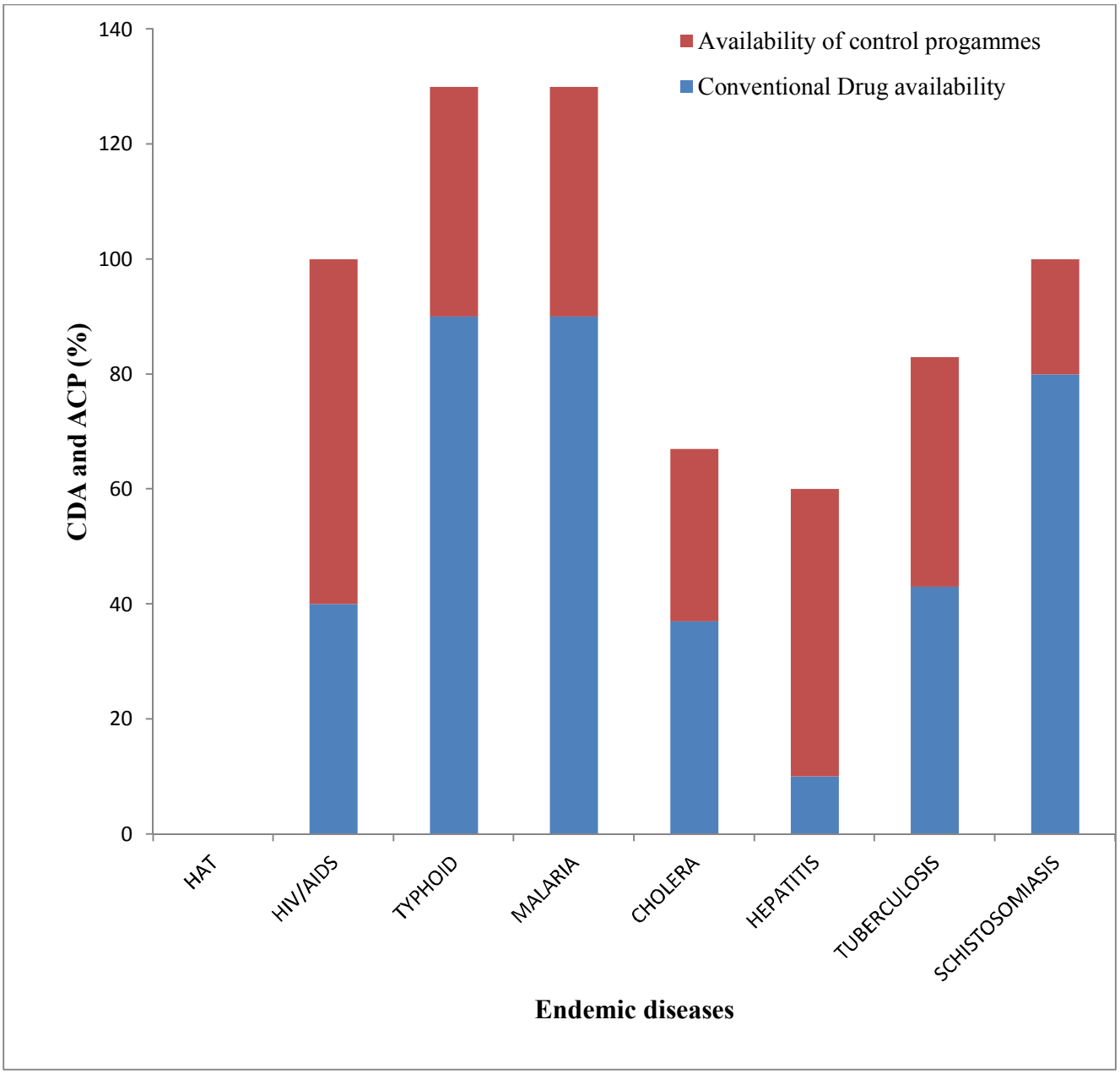


Fig. 4.4: Comparative Ranking of HAT with Other Endemic Diseases Based on Conventional Drug Availability (CDA) and Availability of Control Programmes (ACP).

Table 4.11: Possible Reservoirs of *Trypanosoma brucei gambiense* as Provided by Respondents.

	Proportional Pilling Ratings						
Possible reservoirs	**1st (x/10)**	**2nd (x/10)**	**3rd (x/10)**	**4th (x/10)**	**Total (x/40)**	**Ave (x/10)**	**(%)**
Humans	2.0	0.0	3.0	2.0	7.0	1.75	17.5
Domestic animals	5.0	7.0	8.0	8.0	28.0	7.0	70.0
Wildlife	8.0	8.0	9.0	7.0	32.0	8.0	80.0

$X^2 = 600$, $p < 0.0001$, df = 4

Table 4.12: Sources of Human Infections with *Trypanosoma brucei gambiense* as Provided by Respondents.

	Proportional Pilling Ratings						
Sources of infection	**1st (x/10)**	**2nd (x/10)**	**3rd (x/10)**	**4th (x/10)**	**Total (x/40)**	**Ave (x/10)**	**(%)**
Watering points	7.0	8.0	5.0	7.0	27.0	6.75	67.5
Farm lands	8.0	4.0	5.0	4.0	21.0	5.25	52.5
Homestead areas	2.0	3.0	2.0	3.0	10.0	2.5	25.0
Game reserves	6.0	9.0	6.0	8.0	29.0	7.25	72.5

$X^2 = 1200$, $p < 0.0001$, df = 9

Table 4.13: Common HAT Risk Associated Occupations Practiced by Inhabitants of the Study Areas as Sourced from Respondents.

	Proportional Pilling Ratings						
Occupations	**1st (x/10)**	**2nd (x/10)**	**3rd (x/10)**	**4th (x/10)**	**Total (x/40)**	**Ave (x/10)**	**(%)**
Farming	5.0	9.0	8.0	8.0	30.0	7.5	75.0
Fishing	6.0	4.0	7.0	6.0	23.0	5.75	57.5
Hunting	7.0	6.0	7.0	8.0	28.0	7.0	70.0
Shepherding	2.0	1.0	4.0	0.0	7.0	1.75	17.5

$X^2 = 1200$, $p < 0.0001$, df = 9

Table 4.14: Age Based Impact Assessment of HAT in the Study Areas as Provided by Respondents.

	Proportional Pilling Ratings						
Age group (Years)	**1st (x/10)**	**2nd (x/10)**	**3rd (x/10)**	**4th (x/10)**	**Total (x/40)**	**Ave (x/10)**	**(%)**
0-15	3.0	2.0	4.0	4.0	13.0	3.25	32.5
16-30	8.0	8.0	9.0	8.0	33.0	8.25	82.5
31-45	7.0	9.0	8.0	8.0	32.0	8.0	80.0
46-60	5.0	3.0	6.0	4.0	18.0	4.5	45.0
61-75	4.0	1.0	2.0	4.0	11.0	2.75	27.5
>75	1.0	1.0	1.0	0.0	3.0	0.75	7.5

$X^2 = 3000$, $p < 0.0001$, df = 25

CHAPTER FIVE

DISCUSSION

The results of this study have shown the serological evidence for the occurrence of HAT in the two states. Previous reports showed that Benue State was endemic for HAT (Aiyedun and Amodu, 1974). The findings of the present study indicate that the area may still be endemic for HAT and tsetse which may be due to lack or inadequate government efforts towards the control and eradication of the disease. Benue State shares border with the Fonten Sleeping Sickness focus of the Republic of Cameroon, thus making transboundry transmission a possibility. This may be an additional reason for the existence of the disease in the state. The riverine nature of the state makes it suitable for tsetse habitat (Service, 1980), and therefore a probable reason for the occurrence of the disease in the state. The major occupations in the state are farming, fishing and hunting (Chapman *et al*., 2004) which are all considered as risk occupations in the spread of HAT. This is because these occupations expose people to infected tsetse bites which may result in the transmission of infection. These could be additional reasons for the occurrence of the disease in the state.

Gboko LGA recorded a higher prevalence than Vandekyaa and Ukum LGAs. This difference may not be unconnected with the old HAT endemic focus in the area (Aiyedun and Amodu, 1974). The presence of the disease in Vandekya LGA might probably be as a result of easy movements of humans and animals between these two LGAs which share boundries and the presence of teneral tsetse flies that may be capable of transmiting the infection from HAT infected patients originating from the Gboko endemic focus. The prevalence recorded in Benue State was lower than that reported for Taraba State (Karshima *et al.*, 2012). However, Airauhi *et al*. (2006) reported prevalence 9.8% in Abraka, Delta State. This may not be unconnected with

the fact that the Abraka report was restricted to Abraka endemic focus only while this work covered a much larger area in an active surveillance. The mangrove thick and rain forest vegetations (Isaac *et al*., 2010) in Abraka might have promoted tsetse breeding thereby resulting in higher HAT transmission. This may be another possible explaination for the higher prevalence reported in that area.

The variations in the prevalence of *T. b. gambiense* in humans in relation to sex in both Benue and Taraba States is in agreement with the earlier report of Karshima *et al*. (2012), who also reported a higher prevalence in males than in females in some parts of Taraba State. This may be due to the greater overall involvement of males in occupations such as farming, fishing and hunting which put them at more risk than the females. This finding however contradicts the report of Mohammed *et al*. (2010) who reported higher prevalence in females in Southern Sudan probably due to differences in vegetation, tsetse density and vectoral capacities of tsetse flies in the Sudan. Although there was no significant difference between the prevalence in adult and children in Benue State, however, the prevalence in children may not be unconnected with their a practice of swimming around rivers and streams which may expose them to tsetse bites.

The zoonotic nature of *T. b. gambiense* had remained doubtful for over a century until recent reports of its detection from different animal hosts (Njiokou *et al*., 2010; Njitchouang *et al*., 2010; Karshima *et al*., 2012). This study revealed an overall prevalence of 10.7% among pigs in Benue State which is lower than earlier findings of 34.3% (Simo *et al*., 2006), 19.54% (Njiokou *et al*., 2010), and 65.3% (Njitchouang *et al*., 2010) from different HAT foci in the Republic of Cameroon. The possible explanations for these variations may include differences in the

sensitivity and specificity of the diagnostic tests used by the various studies, vector densities in the different study areas and the confinement of most of the other studies to established HAT endemic foci rather than the general survey conducted by this study. The epidemiological significance of this finding is that the animal populations of these study areas may serve as reservoirs of HAT, posing serious public health threat to the human population and playing a role in the maintenance and resurgence of HAT, and consequently difficulty in the control of the disease.

Pigs were earlier said to be capable of acting as reservoirs of *T. b. gambiense* in Benue State (Stephen, 1986). The present finding may therefore be a confirmation to this speculation. The prevalence observed in pigs in both Ukum and Vandekya LGAs may not be unconnected to the free movement of livestock within the State. Local and International trade (particularly with the neighbouring Cameroon Republic) of pigs which is a delicacy in the state may be an additional factor. The role of trade pigs in the spread of human serum resistant *Trypanosoma* species was also reported earlier (Onah and Ebenebe, 2003).

The prevalence of HAT in both pigs and cattle in relation to sex was higher in females than males which agree with the earlier reports of Karshima *et al*. (2012) in Taraba State and Shah *et al*. (2004) in the Republic of Cameroon. This may be attributed to stress associated with hormonal imbalances during pregnancy and lactation. Also because of the chronic nature of *T. b. gambiense* infection, the chances of detection in females might be higher because they are kept longer in herds for the purpose of breeding, thus allowing the chronic infection to persist.

Adult animals are known to release higher concentrations of insect attractants such as phenols in their urine (Okech and Hassanali, 1990; Madubunyi *et al*., 1996; Kremar *et al*., 2006) as well as acetone and octenol in their breath (Kremar *et al*., 2005; Torr *et al*., 2006; Kremar, 2007). These substances attract tsetse flies to the animals resulting in more frequent bites and subsequent HAT infection. Tsetse flies are also known to be more attracted to larger animals than smaller ones. These may be possible reasons for the higher prevalence observed among adult pigs and cattle than the younger ones. This finding corresponds with the earlier report of Karshima *et al*. (2012) in some selected LGAs of Taraba State. Extensively managed pigs usually visit swampy areas and streams to swim thereby being exposed to the risk of bites by riverine species of tsetse flies which were the actual species captured during the study. This may be a probable explanation for the higher prevalence observed among extensively managed pigs. However, teneral tsetse flies that breed and live around human settlements may be responsible for the low prevalence observed among intensively managed pigs.

The occurrence of HAT in Taraba State may be attributed to the boundaries the state shares with the established endemic areas of Benue State (Aiyedun and Amodu, 1974) and the Republic of Cameroon (Simo *et al*., 2006). Many factors which include availability of several wildlife species in the Gashaka-Gumti (Taraba State) and Yankari (Bauchi State) game reserves and the occupational hazards through farming and fishing might have contributed to the higher prevalence (Chapman *et al*., 2004). Furthermore, evidence of lack of sustained vector control measures due to lack of funds (Omotainse *et al*., 2004), the high cost and inability of the available animal trypanocides to be adequately used in the curative treatment against *T. b.*

gambiense, and the increasing trend of treatment failure **(**Mumba *et al*., 2010**)** might have also contributed to the occurrence of the disease in the state.

The overall *T. b. gambiense* sero-prevalence rate of 2.2% revealed by this study in humans in Taraba State is higher than the earlier reports by Karshima *et al*. (2012) and is suggestive of an active transmission of the disease in the state. However, this prevalence is lower than the 17.1% (Airauhi *et al*., 2006) and 9.8% (Osue *et al*., 2008) reported in Abraka, Delta State, Nigeria and the 11.1% (Mohammed *et al*., 2010) and 14.4% (Chappuis *et al*., 2010) reported in the Sudan and Kenya respectively. These variations may be associated with the sensitivity and specificity of diagnostic techniques that were used, the intensity of tsetse flies in the different study areas and the confinement of most of these studies to existing endemic foci. The higher prevalence observed in Gashaka LGA in both humans and cattle may be attributed to the presence of the Gashaka-Gumti National park which harbours several wildlife species that can serve as reservoirs of *T. b. gambiense*. The common grazing land shared by both wildlife species and the cattle population of the LGA may be an additional factor. The extension of the Yankari game reserve forest into the Jevjev and Binari areas of Karim Lamido LGA may explain also the high prevalence observed in the area. The riverine nature of this LGA might have contributed to the high prevalence observed as riverine areas are known to promote tsetse breeding (Abenga and Lawan, 2005).

The Bokoloji and Muturu cattle breeds are known to be trypanotolerant. This tolerance to *Trypanosoma* species may explain the lower prevalence recorded in these two breeds. The higher prevalence observed among the White Fulani may be attributable to their trypanosusceptibility

and perhaps due to their much high representation in the sampling. Of the three cattle breeds studied, the White Fulani breed that are raised using the nomadic system of management may also explain the higher prevalence recorded by this group.

The trypanosomes identified in Giemsa stained smears in cattle and pigs were *T. brucei* and *T. congolense,* which were also earlier reported by several other workers in Nigeria (Kalu and Lawani, 1996; Omotainse *et al*., 2000; Ogunsanmi *et al*., 2000; Mbaya *et al*., 2010; Karshima and Bobbo, 2011) and elsewhere (Shah *et al*., 2004; Mbahin *et al*., 2013). *Trypanosoma brucei* had the highest rate of occurrence. This observation contradicts the findings of Kalu and Lawani (1996) and Omotainse *et al*. (2000), who reported higher occurrence of *T. vivax* than *T. brucei* in Benue State and the neighbouring Plateau State. The predominance of *T. brucei* over *T. congolense* as revealed by this study may be due to the higher susceptibility of *T. congolense* to most of the trypanocidal drugs used in Nigeria when compared to *T. brucei* (Ogunsanmi *et al*., 2000). The fluctuating nature of parasitaemia associated with *T. congolense* infection may be an additional factor for the low detection rate of this parasite. The detection of *T. congolense* savannah and forest sub-species is suggestive that ITS-1 PCR is an ideal diagnostic test for trypanosomosis caused by *T. congolense.*

The recovery of tsetse flies in the study areas is suggestive of the existence of transmission of *T. b. gambiense* to humans and possible animal populations in the two states. The vector density as observed by this study may be associated with lack of vector control programmes in the areas. Invasion of the flies from the neighbouring endemic states and the neighbouring Republic of Cameroon may contribute to the fly density recorded in this study.

The overall infection rates recorded in this study involving both Benue (2.9%) and Taraba (4.9%) States were higher than the reports of earlier investigators; Ogunsanmi *et al*. (2000) in South-Western Nigeria and Karshima *et al*. (2011) in some LGAs of Taraba State. These differences may be associated with seasonal variations, availability of suitable breeding sites existing in game reserves for tsetse flies and un-sustained vector control programmes. The swampy nature of the study areas and the possibility of trans-boundary tsetse flies invading the study areas from neighbouring Republic of Cameroon may be additional factors.

The species of tsetse flies trapped in this study were reported among the eleven species of tsetse reported in Nigeria (Baldry, 1967) and were also earlier reported in some LGAs of Taraba State (Krashima *et al*. 2011). This observation evidently suggests the continuous existence of these vectors in the study areas probably as a result of collapse of previous tsetse control programmes or re-infestation from other tsetse endemic states such as Nasarawa, Enugu and Cross River States.

One of the major objectives of this study was to isolate and characterize *T. brucei* stocks from serologically positive humans, cattle and swine. This was necessary because of the roles of domestic and wild animals in the epidemiology of *T. b. gambiense* as earlier reported (Abenga and Lawan, 2005; Simo *et al*., 2006; Njiokou *et al*., 2010; Karshima *et al*., 2012), and atypical infection of humans with trypanosomes of animal origin (Joshi *et al*., 2005, 2006; Deborggraeve *et al*., 2008; Truc *et al*., 1998, 2007, 2013).

A total of 11 isolates that were subjected to KIN-specific primers detected *T. brucei* in 72.7% and *T. congolense* in 27.3% of the isolates. The predominance of *T. brucei* among human isolates was not supprising since all human infective trypanosomes belong to the *T. brucei* genome. However, a mixed infection of *T. brucei* and *T. congolense* was detected from swine. To validate our findings, the animal isolates were subjected to a second PCR using ITS-1 primer sets to comfirm the results obtained with the KIN primer sets. The results showed that both KIN and ITS-1 primers were able to detect *T. brucei* and *T. congolense*. However, ITS-1 was able to identify sub-species of *Trypanosoma congolense*.

In the current study, mixed infections involving different *Trypanosoma* species were observed in both cattle and pigs, which have also been previously reported (Mekata *et al*., 2008; Malele *et al*., 2011). The molecular epidemiology component of this study has confirmed the existence of mixed infections of forest and savannah sub-species of *T. congolense* in the field, which could not have been detected by the classical parasitological methods. The mixed infections could have occurred as a result of high chances of mixed *Trypanosoma* infections in tsetse flies or bites by different infected tsetse flies.

It has been established that the TgsGP gene is present in all group 1 *T. b. gambiense* isolates examined to date but not in *T. b. brucei*, *T. b. rhodesiense* or group 2 *T. b. gambiense* (Berberof *et al*., 2001; Gibson *et al*., 2010; Capewell *et al*., 2013). This gene is specific to group 1 *T. b. gambiense* and is shown to confer resistance to human serum. The *T. brucei* stocks identified using KIN PCR from humans were all confirmed as group 1 *T. b. gambiense* using TgsGP PCR which detected the presence of strain specific *T. gambiense* serum glycoprotein. The

epidemiological and clinical significance of detecting only one species of human infective trypanosomes in the study area is that, the risk of treatment failure associated with mixed infections of *T. b. rhodesiense*, types 1 and 2 *T. b. gambiense* which responds differently to HAT chemotherapy (Brun *et al*., 2001) will be overcome. It was also not supprising to have detected only type 1 *T. b. gambiense* among all human isolates since it has been reported to be associated with over 90% of cases of human African trypanisomosis (Cordon-Obras *et al*., 2010; Wombou *et al*., 2011).

From the results of this study, three out of the four animal *T. brucei* isolates characterized using the KIN and ITS-1 PCR were also confirmed as group 1 *T. b. gambiense* using TgsGP PCR. This suggests a possible existence of animal reservoirs of group 1 *T. b. gambiense* in the study areas. The epidemiological significance of this finding is that at least cattle and swine populations may play a role in the maintenance and possible resurgence of the disease in the study areas. Another implication is that, these reservoir animals might be traded for breeding purposes thereby initiating and spreading new HAT foci in non infected areas.

This study also determined the sources of blood-meals taken by tsetse flies captured within the two states (Clausen *et al*., 1998; Torr *et al*., 2001; Steuber *et al*., 2005; Mwandiringana *et al*., 2012). The current study revealed multiple host feeding on a range of vertebrates including human, cattle, pigs and dogs.

The results of this study have revealed that over 30% of the captured tsetse flies had fed on cattle indicating the significance of this host in the epidemiology of HAT in the study areas. This agrees with the findings of Torr *et al*. (2001). The nomadic system of cattle rearing which relies on grazing of cattle on free grazing reserves in the study areas make these animals readily available for feeding by flies and may provide additional reasons for cattle blood being a major source of tsetse blood-meal. Pigs represent a good percentage of the livestock population of the study areas. This may explain why pigs were the second largest group of animals fed upon by tsetse flies.

The results of this study showed that up to 13.3% of tsetse blood-meals involved humans which may indicate that human activities such as farming, hunting and fishing among others expose them to tsetse bites. Multiple feeding patterns involving humans, cattle, pigs and dogs were also observed and might have arisen from host preference by tsetse based on the availability of hosts as earlier reported (Chaves *et al*., 2010). The epidemiological implication of this is the risk of transmission of *T. b. gambiense* and other vector-borne diseases among human and animal populations in the study areas. The epidemiological consequences of this transmission to animals would seem to be the maintenance and possible resurgence of HAT over time. This finding also explains the occurrence of *T. b. gambiense* among humans, cattle and swine populations in the study areas, which could provide additional information in understanding the epidemiology of Gambian Sleeping Sickness.

The findings of the participatory epidemiological study assigned the highest mortality to HAT as compared to the seven other diseases identified by members of the community. This may not be

unconnected with the non-availabilty of conventional drugs like pentamidine and suramine used in the treatment of first stage HAT as well as melarsoprol and eflornithine used in the treatment of the CNS stage (Trouiller *et al*., 2002). Most African countries including Nigeria still depend on the World Health Organisation for the supply of these drugs leading to lack of treatment and death of patients (Cattand *et al*., 2001). This may also be the possible explanation for the 0% drug availability response assigned by these participants.

Victims of HAT are usually the economically active members of the populations in communities where the disease is endemic because members of these groups are the ones usually involved in the risk-associated occupations such as farming, fishing, hunting, and shepherding. This may explain the higher economic impact assigned to this disease by the respondents. The collapse of vector control programmes in HAT endemic foci in the country in the 1970s (Aiyedun and Amodu, 1974) resulting from lack of funds as reported by Omotainse *et al*. (2004) may explain the zero percent availability of control programmes revealed by this study.

The high proportion assigned to wildlife species as the possible source of *T. b. gambiense* infection by respondents may not be unconnected with the large numbers of these species present in the study areas (Chapman *et al*., 2004), especially within the Gashaka-Gumti Game Reserve and the extended forest of Yankari Game Reserve into Karim Lamido LGA of Taraba State. Domestic animals share the same grazing sites with wildlife species where they may acquire infections which are subsequently transmitted through tsetse to other animals and humans (Abenga and Lawan, 2005).

Game reserves, watering points, farm lands and homestead areas were all identified as sources of infection, because these areas are usually visited for hunting, bathing, washing, fishing and farming among many other activities. These activities expose humans to either the forest or riverine species of tsetse flies which transmit HAT (Service, 1980). The highest proportion assigned to farming as the main risk occupation in the study areas may not be unconnected to the fact that farming is the major occupation in the two states. The continuous search for "bushmeat" as delicacy in the study areas may explain the high proportion assigned to hunting by the inhabitants of the study areas.

The highest impact of HAT was assigned to the age groups 16-30 and 31-45 years probably because of the activeness of these age groups in farming, fishing, hunting and shepherding. This agrees with the report of Swallow (2000) who observed that HAT affects the economically active members of the population mainly rendering them inactive as a result of the sleep disturbances associated with the CNS stage of the disease.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

This study determined the epidemiology of *T. b. gambiense* in Benue and Taraba States of Nigeria using participatory epidemiology, serology, as well as parasitological, entomological and molecular techniques. A total of 2,400 blood samples were analyzed, of which 600 samples each were from humans from Benue and Taraba States, as well as 600 each from pigs and cattle from Benue and Taraba States. The overall sero-prevalence rates of HAT were 1.75% (21/1200) and 8.9% (107/1200) for humans and animals respectively. State based sero-prevalence rates in humans were 1.3% (8/600) and 2.2% (13/600), while those in pigs and cattle were 10.7% (64/600) and 7.2% (43/600) for Benue and Taraba States respectively. In addition, a total of 531 tsetse flies were captured during this study. Of these, 463 (87.2%) and 58 (12.8%) were *G. palpalis* and *G. tachinoides* respectively. The infection rates of tsetse flies were 3.4% and 1.2% for *G. palpalis* and *G. tachinoides* respectively. *Trypanosoma* species isolated from humans, cattle and pigs were identified based on the ITS-1 rDNA, human serum resistant antigen (SRA) and *T. b. gambiense* specific glycoprotein (TgsGP) genes. PCR identified all purified trypanosomes. The KIN PCR identified all the human trypanosome isolates as belonging to the *T. brucei* genome, while both the KIN and ITS-1 PCRs identified *T. brucei* and *T. congolense* from the *Trypanosoma* species obtained from cattle and pigs. PCR targeting serum resistance antigen gene excluded the presence of human infective *T. b. rhodesiense* in all the 6 human and 5 animal isolates. A strain-specific TgsGP primer pair identified all the 6 human, 1 cattle and 2 swine isolates as type 1 *T. b. gambiense*. Blood-meal sources from 30 randomly selected engorged tsetse flies were identified by amplifying a mammalian species-specific sequence of

the mitochondrial cytochrome b gene using PCR. Of these, 33.3% (10/30), 30.0% (9/10), 23.3% (7/30) and 13.3% (4/30) fed on cattle, pigs, dogs and humans respectively. The study revealed multiple feeding patterns involving human-cattle-pig-dog among 6.7% (2/30) and cattle-dog-pig among 16.7% (5/30) of the analyzed tsetse flies. Dual feeding patterns involving human-dog 6.7% (2/30), human-cattle 13.3% (4/30) and human-pig 10.0% (3/30) were also observed.

6.2 Conclusions

This study established the presence of type 1 *T. b. gambiense*, tsetse vectors of HAT, forest and savannah sub-species of *T. congolense* as well as the absence of *T. b. rhodesiense* and atypical human infections with animal trypanosomes in Benue and Taraba States of Nigeria. The roles of cattle and swine in the epidemiology of the disease in the two states were also established. In addition, species-specific, multiple and dual feeding patterns involving humans, cattle, pigs and dogs and the possible transmission cycle of the disease in the two states was understood. Risk occupations that exposed inhabitants of the study areas to HAT were also identified. This study also identified and mapped out tsetse infested areas through participatory epidemiology which will play a very important role in the institution of HAT control strategies in the study areas.

6.3 Recommendations

It is pertinent to:

i. follow-up on infected individuals and provide effective treatment where necessary.

ii. include animal intervention strategies in addition to the existing human and tsetse control in HAT control programmes so as to curtail disease transmission, maintenance and resurgence from animal reservoirs.

iii. conduct studies on other domesticated animals and wildlife species to ascertain their possible roles in the epidemiology of the disease in the study areas.

iv. carry out mass screening of individuals in the study areas for early disease detection so as to carry out stage one treatment and protect these individuals from the deadly consequences of second stage disease and its chemotherapy.

v. sequence *T. b. gambiense* isolates from humans and animals to establish the role of animals in HAT in the study areas.

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APPENDIX I

SAMPLE QUESTIONS USED FOR THE PARTICIPATORY EPIDEMIOLOGICAL STUDIES

1. Are you aware of the disease referred to as sleeping sickness?
2. Is it a problem in your community?
3. Have you ever been screened for sleeping sickness in this locality?
4. Would you like to know your sleeping sickness status?
5. Would you be willing to assist in the control of tsetse flies in your locality?
6. What are the common endemic diseases in your locality?
7. What rating can you assign these diseases in terms of mortality?
8. What rating can you assign these diseases in terms of economic impact?
9. What rating can you assign these diseases in terms of drug availability?
10. What rating can you assign these diseases in terms of control programmes availability?
11. What do you think are the possible reservoirs of sleeping sickness in your locality?
12. What rating can you assign these reservoirs in respect of their involvement in disease transmission?
13. What are the areas you think humans encounter tsetse flies in your locality?
14. Which of these areas do you think is the major source of human-tsetse contact?
15. What are the common occupations practiced in this locality that you think can expose humans to tsetse bites?
16. Which of these occupations do you think is the major source of human-tsetse contact?
17. Which age bracket do you think are mostly affected by sleeping sickness in your locality?
18. Why are people within these age bracket mostly affected?
19. Which areas of your locality do you think are mostly infested by tsetse flies?

APPENDIX II

INFORMED CONSENT FORM

I Dr. Karshima, Solomon Ngutor, a post-graduate student of the Department of Veterinary Public Health and Preventive Medicine, Ahmadu Bello University, Zaria wish to conduct a research work titled "Epidemiological and Molecular studies on *Trypanosoma brucei gambiense* in Benue and Taraba States of Nigeria". *Trypanosoma brucei gambiense* is a protozoan parasite that causes human African trypanosomosis (sleeping sickness) in humans. Two types of this parasite have been reported. The role of domesticated animals in the maintenance and resurgence of the disease has also been documented. There is paucity of information about the disease in Benue and Taraba States despite the availability of factors that can influence the existence of the disease in the state. This study will try to find out the number of people infected with this parasite, the types of parasites available in the study area and possible factors that influence the occurrence and distribution of the disease in the two states.

This study will involve asking questions through participatory epidemiology and taking 2 ml of blood sample from you.

Participation is voluntary. All information obtained from you during this study will be kept strictly confidential and your identity will not be disclosed.

If you accept to participate in the study, please kindly sign the space provided below.

I …… have asked questions regarding this research and have been satisfactorily answered. I therefore wish to participate in the research.

Thank you.

Signature of the Participant or Guardian…… Date ……

Signature of Witness …….. Date ……

Signature of investigator ……. Date …….

APPENDIX III

JOS UNIVERSITY TEACHING HOSPITAL
JOS, NIGERIA

Phone: 073-450226 - 9
e-mail: juth@infoweb.abs.net

Cables & Telegram: JUTH
P.M.B. 2076
Jos.

Ref: JUTH/DCS/ADM/127/XIX/5944

Date: 23rd May, 2014

Dr. Karshima, Solomon Ngutor
Department of Animal Health,
Federal College of Animal Health
and Production Technology,
P. M. B. 001 Vom,
Plateau State.

RE: ETHICAL CLEARANCE/APPROVAL

I am directed to refer to your application dated 28th March, 2014 on the research proposal titled:

"Epidemiological and Molecular Studies on *Trypanosome Brucei Gambiense* in Benue and Taraba States of Nigeria" and your appearance before the ethical committee on 2nd April, 2014.

Following recommendation from the Institutional Health Research Ethical Committee, I am to inform you that management has given approval for you to proceed on your research topic as indicated.

You are however required to obtain a separate approval for use of patients and facilities from the department(s) you intend to use for your research.

The Principal Investigator is required to send a progress report to the Ethical Committee at the expiration of three (3) months after ethical clearance to enable the Committee carry out its oversight function.

Submission of final research work should be made to the Institutional Health Research Ethical Committee through the **secretary in room 2, administration department**, please.

On behalf of the Management of this Hospital, I wish you a successful research outing.

Hajia R. Danfillo
For: Chairman, MAC.

INSTITUTIONAL HEALTH RESEARCH
ETHICAL COMMITTEE
DESPATCHED
23 MAY 2014
JOS UNIVERSITY TEACHING
HOSPITAL, P.M.B 2076 JOS



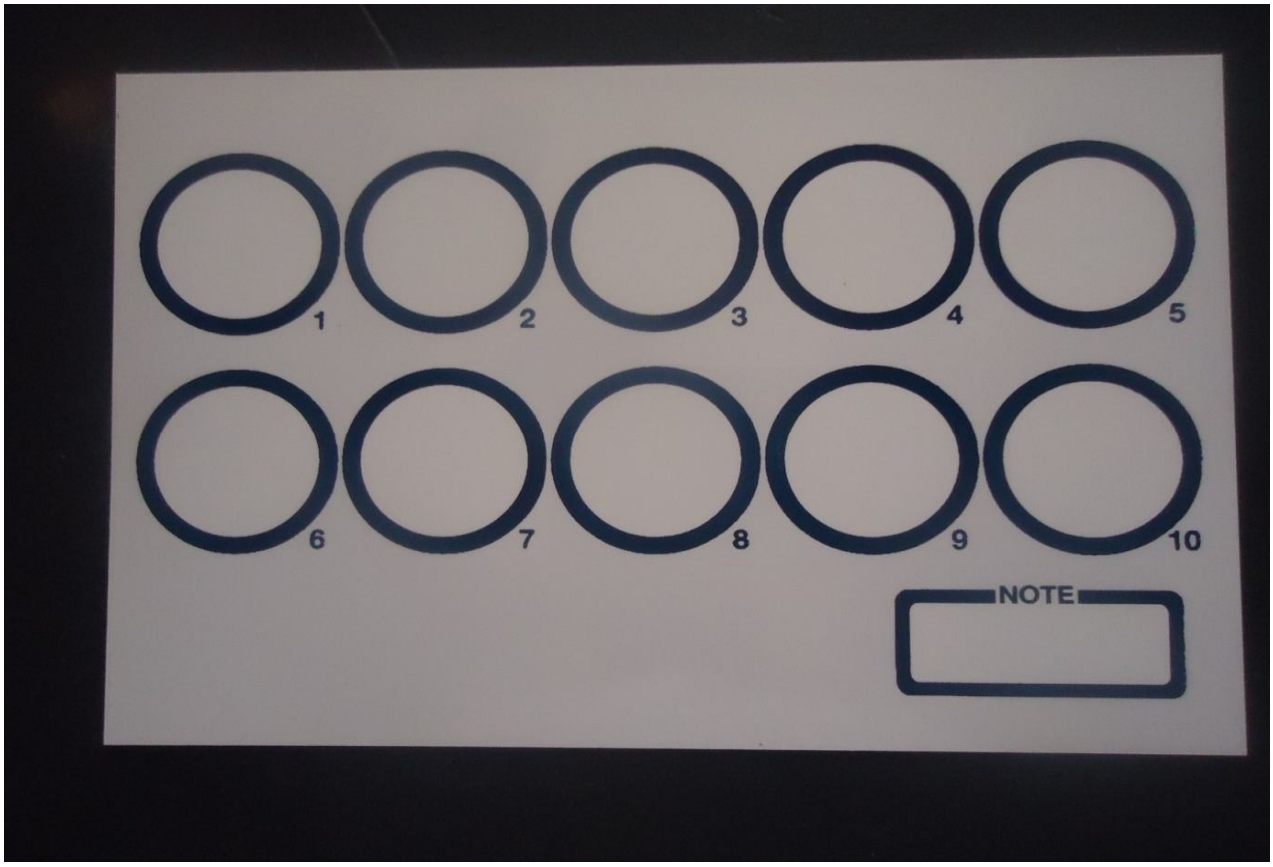
APPENDIX IV: A participant comparing HAT with seven other diseases by the use of proportional pilling during a session of participatory epidemiology.



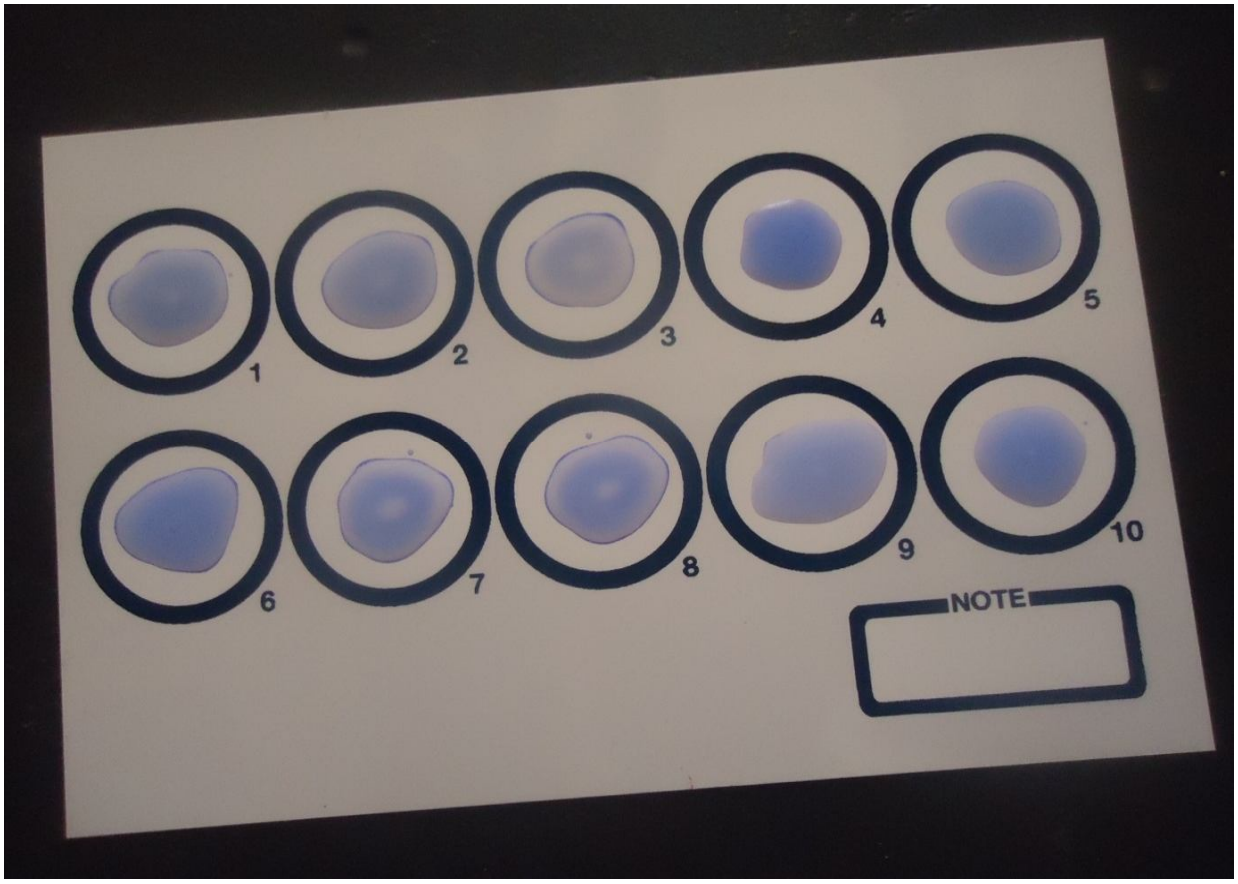
APPENDIX V: A questions and answers session during participatory epidemiology.



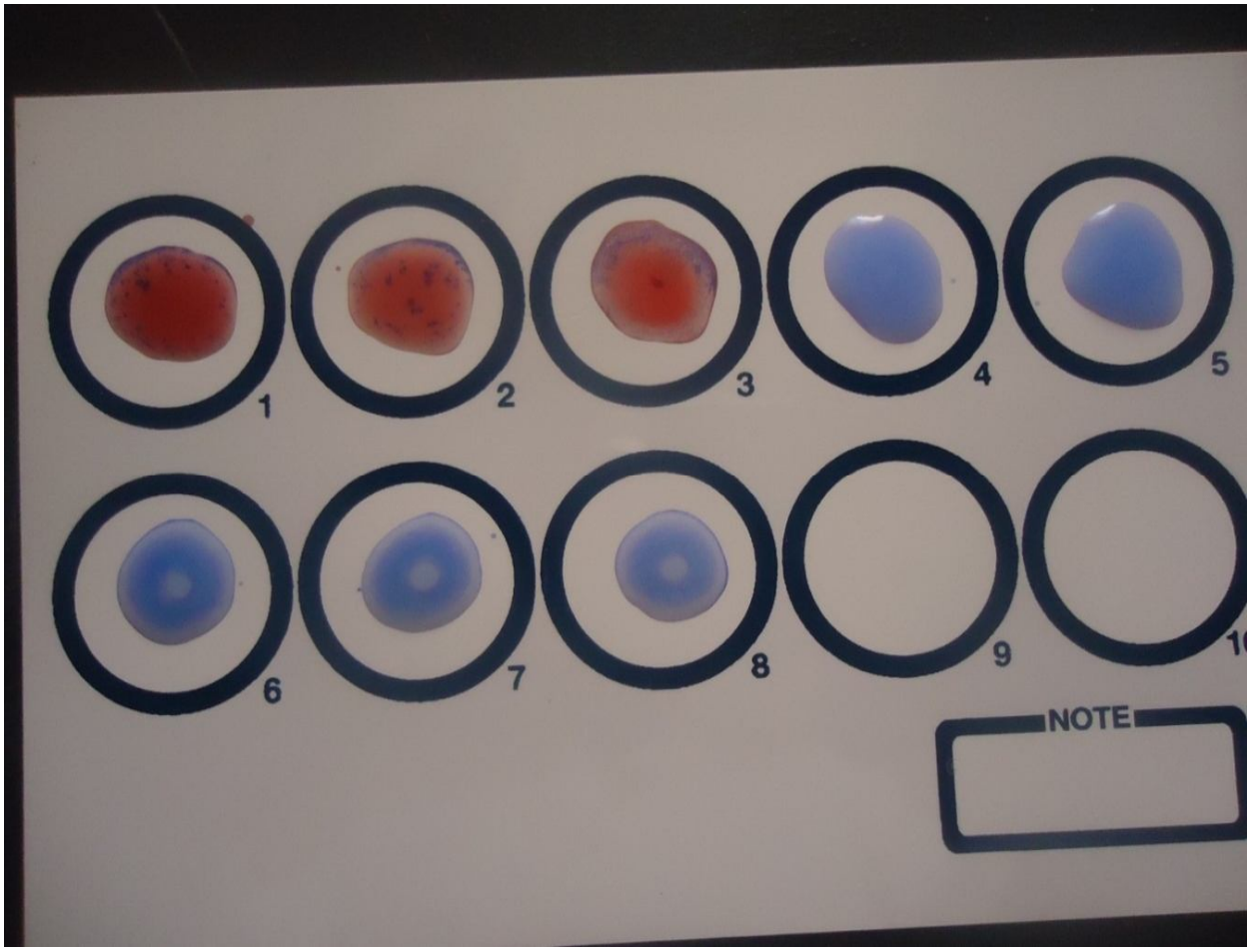
APPENDIX VI: The card agglutination test kit for the serological diagnosis of *T. b.gambiense.*



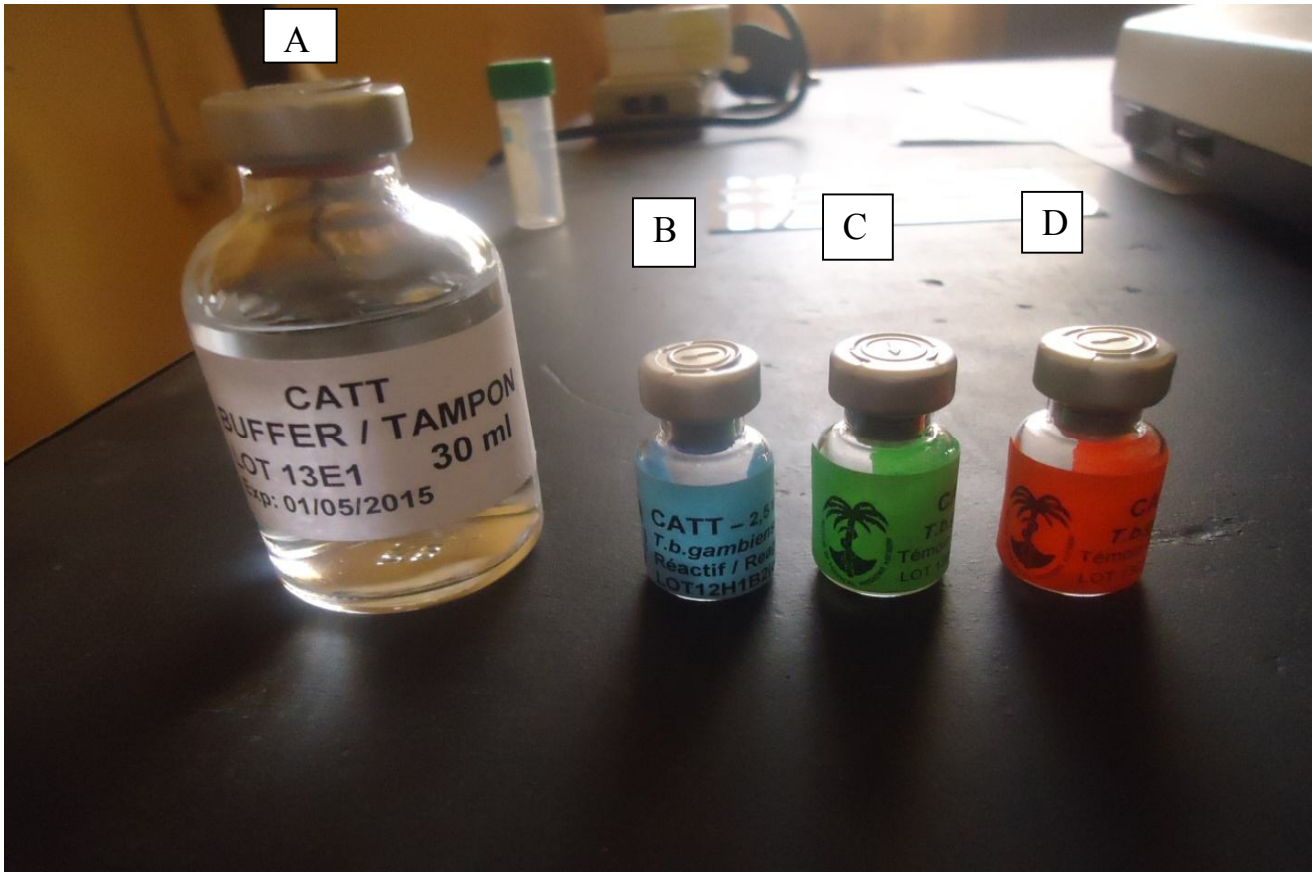
APPENDIX VII: Plain CATT agglutination card showing ten test areas.



APPENDIX VIII: CATT test card showing; HAT positive result on 1:4 serum dilution (1-3), negative result on 1:4 serum dilution (4 and 5), positivity using positive control (6-8) and negativity using negative control (9 and 10).



APPENDIX IX: CATT test card showing; positive result on whole blood (1-3), negativity using negative control (4 and 5) and positivity using positive control (6-8).



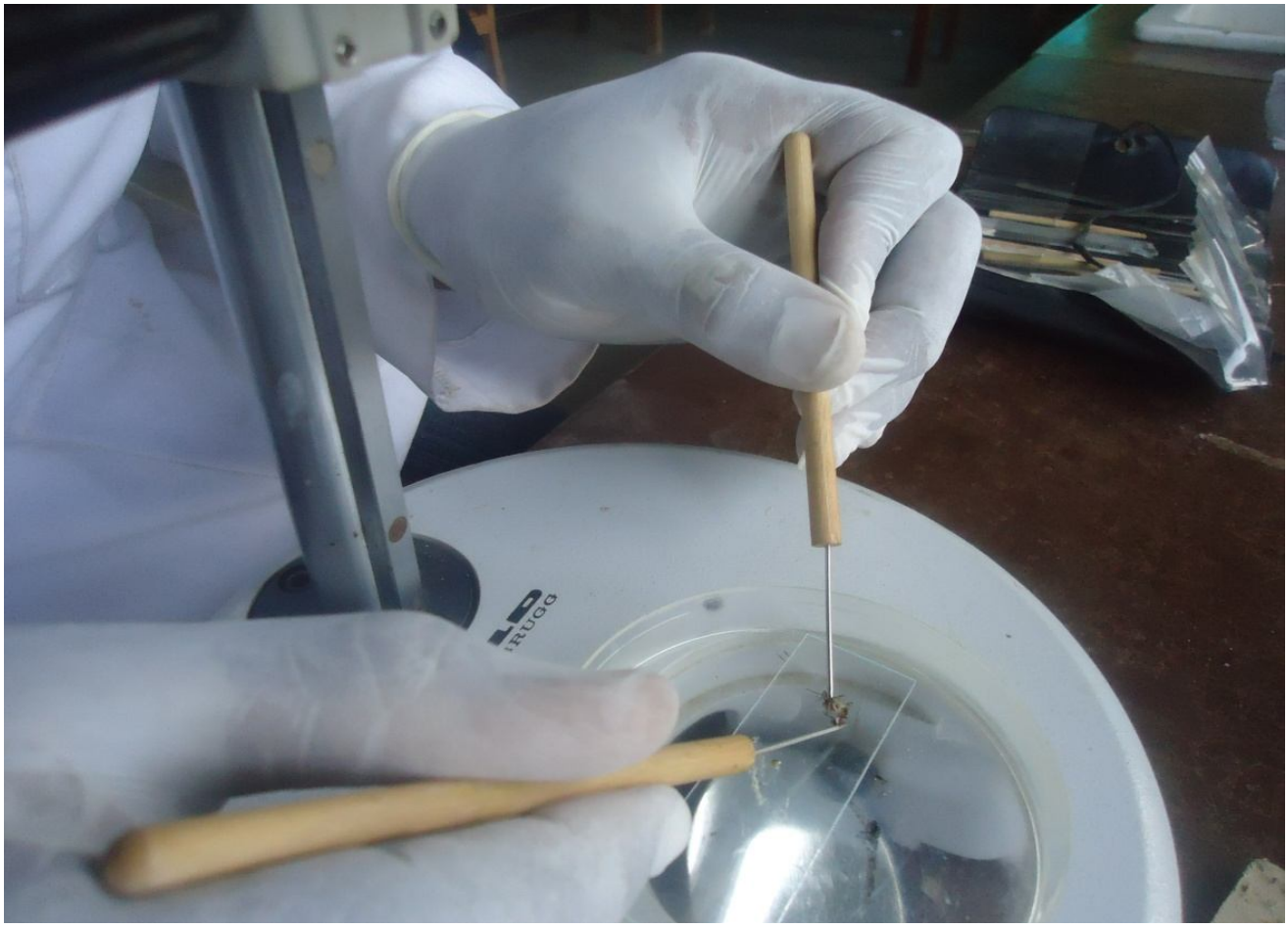
APPENDIX X: CATT/*T. b. gambiense* reagents; (a) CATT buffer, (b) CATT test reagent, (c) CATT negative control, and (d) CATT positive control.



APPENDIX XI: Mounted biconical trap for tsetse catch.



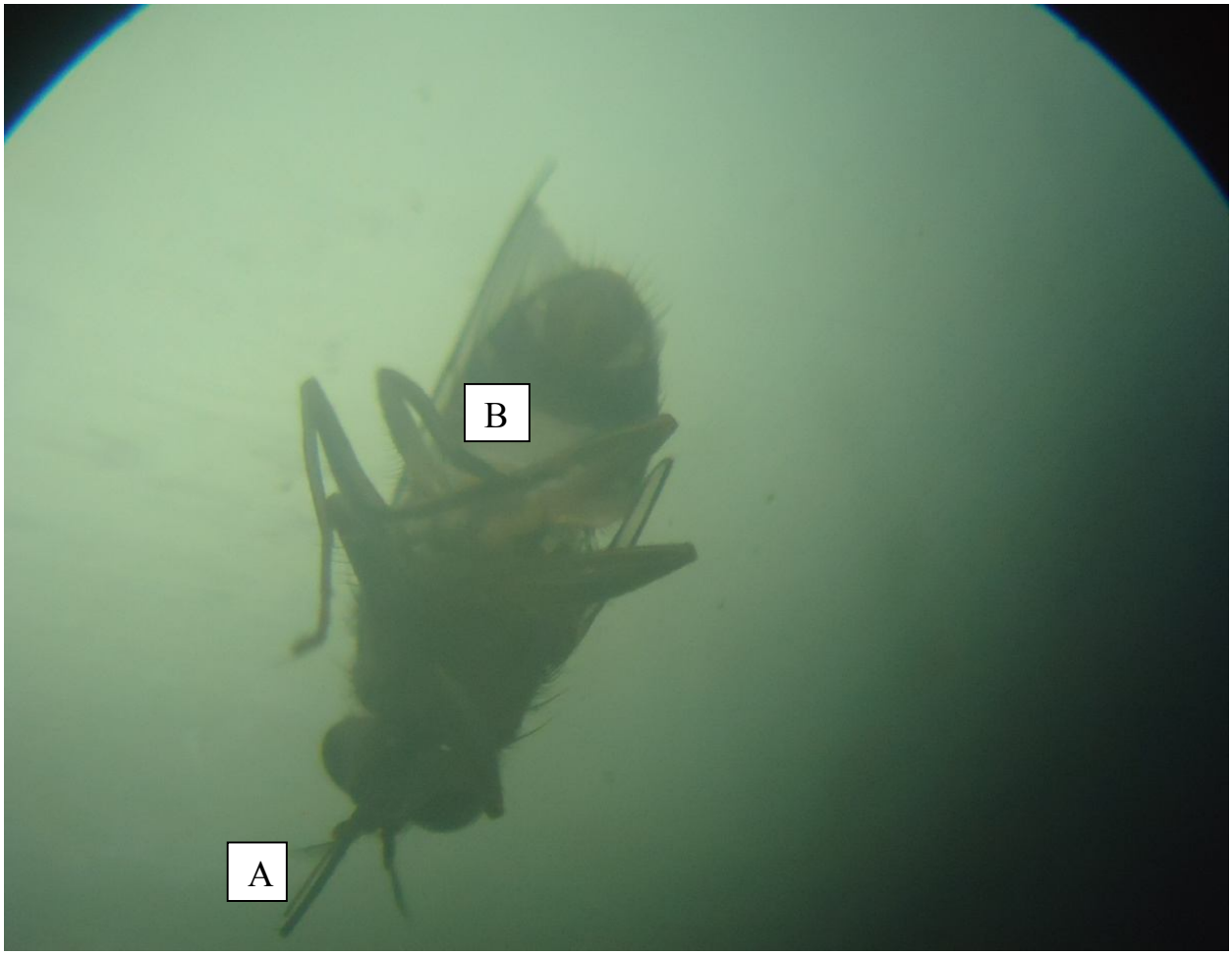
APPENDIX XII: A mounted Nitse trap beside a stream for tsetse catch.



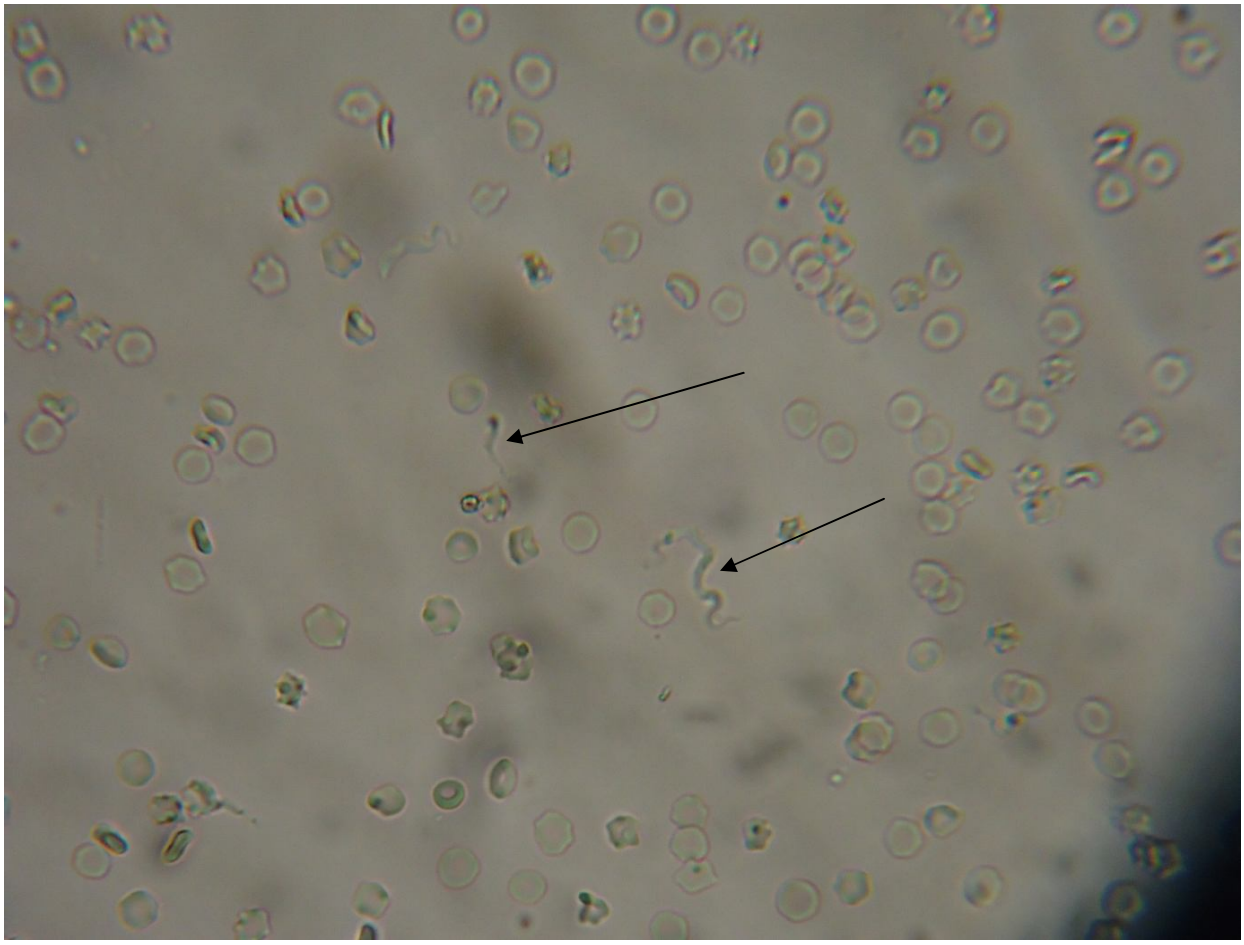
APPENDIX XIII: Dissection of trapped tsetse flies for vector stages of trypanosomes.



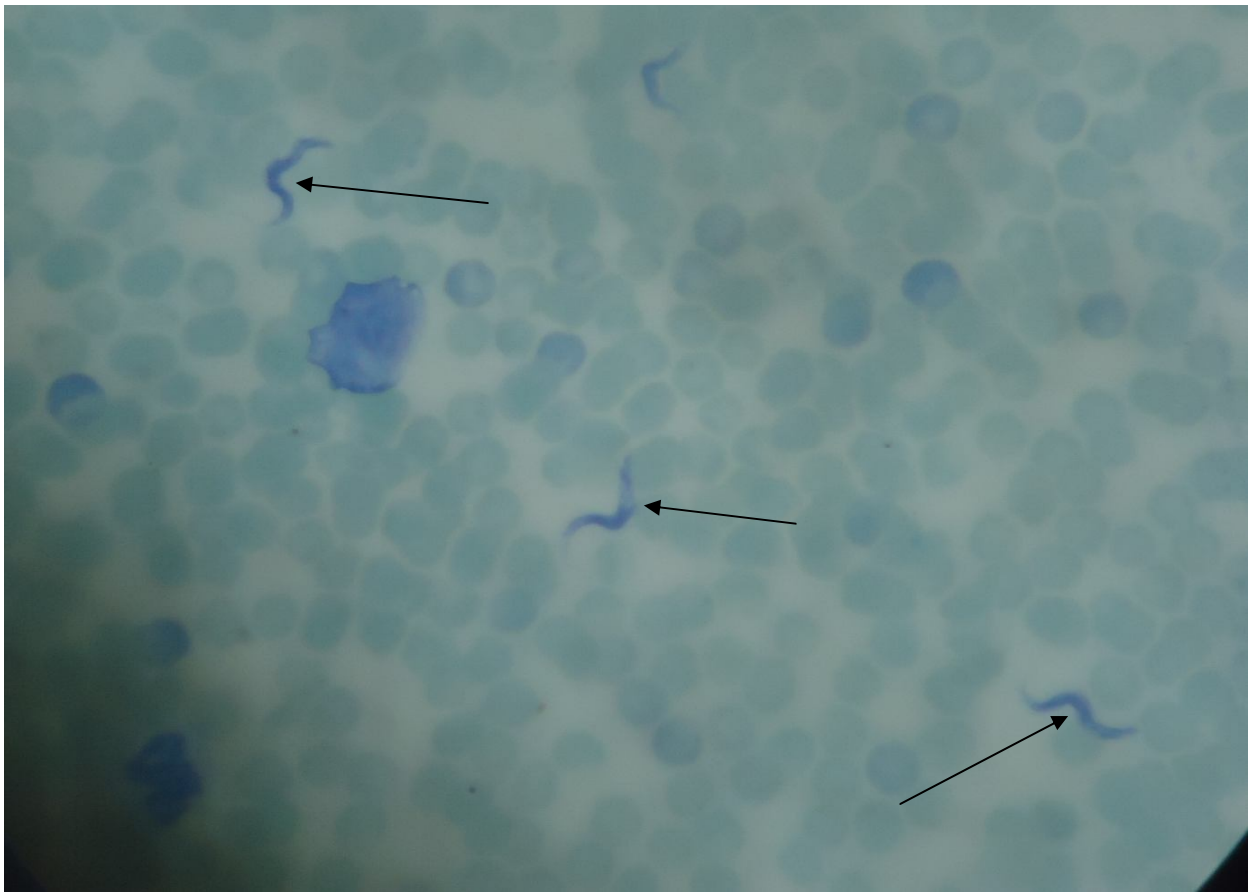
APPENDIX XIV: Some of the trapped tsetse flies during the study



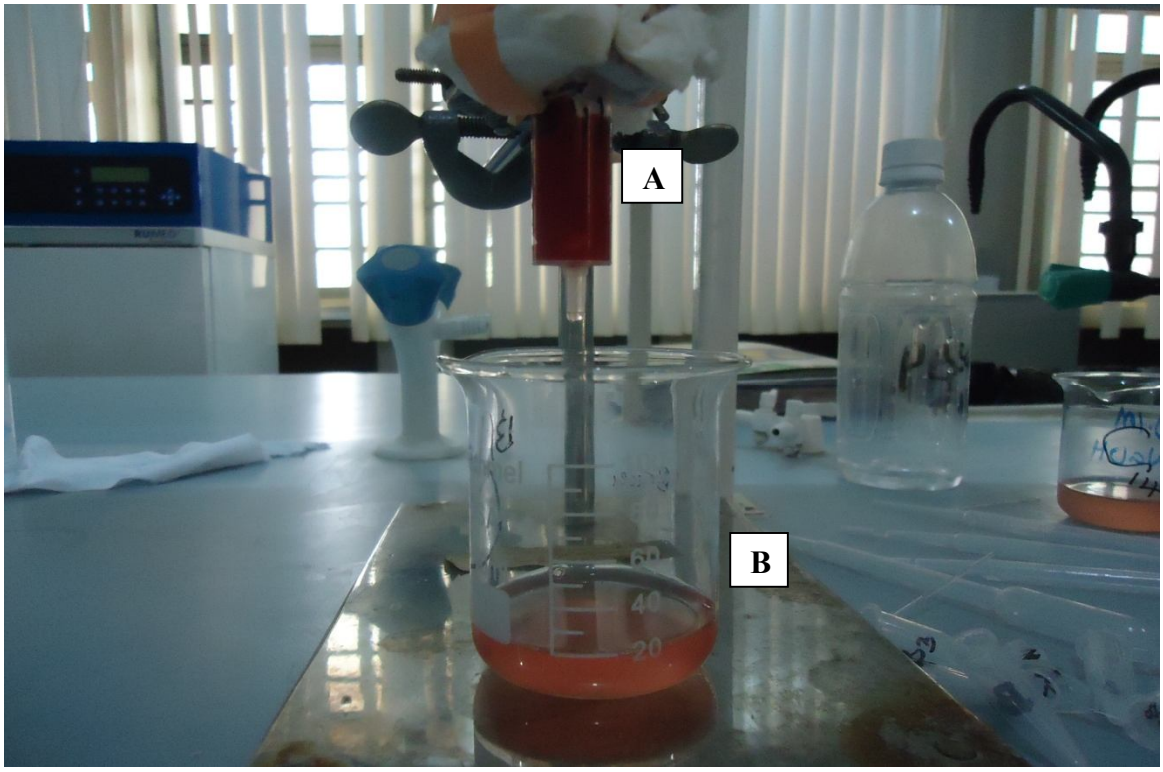
APPENDIX XV: A caught *Glossina palpalis* showing; (a) forwardly projecting proboscis, (b) black socked hind limbs.



APPENDIX XVI: Wet mount reaveling motile trypanosomes at × 40 magnification



APPENDIX XVII: Blood stream form of trypanosomes in mice blood 23 days post inoculation with blood from serologically positive individuals (Giemsa stained thin film at × 100 magnification).



APPENDIX XVIII: Purification of trypanosomes from mice blood: (A) DAE 52 packed column containing trypanosomes expanded in mice blood, (B) purified trypanosomes collected in a beaker.