PREVALENCE AND MOLECULAR CHARACTERISATION OF TRYPANOSOMES OF CATTLE IN OGUN AND KADUNA STATES, NIGERIA

by

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CERTIFICATION

I certify that this work was carried out by Dr. M. I. Takeet at the Department of Veterinary Microbiology and Parasitology, University of Ibadan, Ibadan, Nigeria, under our supervision.

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Dedication

This work is dedicated to God almighty

Abstract

African animal trypanosomosis is a complex infectious disease responsible for production losses in livestock herds. The severity of the disease has been associated with different species and strains of trypanosomes which have not been characterized molecularly in Nigeria. Many of the previous studies conducted in Nigeria were based on microscopy and serology. This study accessed the prevalence of trypanosomes in the forest (Ogun State) and Savannah (Kaduna State) zones, being major ecological belts of tsetse flies infestation in Nigeria and also carried out molecular characterization and phylogenetic analysis of identified trypanosomes.

Blood samples were collected from the jugular veins of 411 cattle (129 males and 282 females), studied between May and October 2010 using systematic random sampling method. These included Muturu (112), N'dama (31), Sokoto Gudali (68) and White Fulani (200) breeds of ages 6 months to 3 years. Trypanosomes were detected by microscopy and species-specific Polymerase Chain Reaction (PCR) techniques. Sequencing of the ITS-1 region of ribosomal DNA gene were carried out for phylogenetic analysis using Molecular Evolutionary and Genetic Analysis software. Data were analyzed using descriptive statistics and Chi-square test at P = 0.05.

The overall prevalence of trypanosomes in cattle were 15.1% and 63.7% for microscopy and PCR, respectively. By microscopy, *T. brucei* (3.6%), *T. congolense* (5.2%) and *T. vivax* (6.3%) were detected, while PCR detected *T. congolense* savannah (48.7%), *T. congolense* forest (0.5%), *T. vivax* (26.0%), *T. brucei* (4.4%) and *T. evansi* (0.5%). Prevalence by microscopy and PCR was significantly higher in Ogun State (18.1% and 75.1%) than in Kaduna State (8.9% and 35.6%). *Trypanosoma congolense* savannah and

forest types were detected in Ogun State but only *T. congolense* savannah was detected in Kaduna State. The strains of *T. brucei* in Kaduna were different from those in Ogun State due to common nucleotide deletion points at loci 186, 279, 280, 281 and 325 of the sequences from Ogun State and nucleotide alterations from G ⇢ T, C ⇢ T and G ⇢ A at loci 124, 197 and 201, respectively. Sequence polymorphism of the ITS-1 region was highest in *T. brucei brucei* compared to *T. congolense* and *T. vivax*. Phylogenetic analysis revealed that *T. vivax* from cattle in Kaduna and Ogun States clustered together and had 100% and 96% homology to those detected in cattle in Venezuela and Zambia, respectively. However, *T. brucei* from Kaduna and Ogun States clustered into two separate clades and both had 98% and 95% homology respectively, to those detected in cattle in Zambia.

This is the first report of the molecular characterization of *T. congolense* savannah and forest types in Nigeria. The genetic relatedness of trypanosomes from Nigeria to that of Venezuela and Zambia implies that the treatment regime known to be successful in the two countries can be adopted in Nigeria. It is recommended that regular surveillance and screening for new strains of trypanosomes be conducted on Nigerian cattle for effective prevention and control of the disease.

Keywords**:** Cattle, Molecular characterisation, Phylogenetic, Prevalence, Trypanosomes

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Michael Irewole Takeet

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ABREVIATIONS

W.H.O	World Health Organization
AAT	African Animal Trypanosomosis
OIE	Oficina International de Epizootias (Spanish: World Organization for Animal Health)
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction
ITS-1	Internal transcribed spacer-1
rDNA	Ribosomal deoxyribonucleic acid
IFN-γ	Interferon gamma
IL-10	Interleukin – 10
Mb	Mega base
PFGE	Pulse field gel electrophoresis
kb	Kilo base
bp	Base pair
VSG	Variant surface glycoprotein
cDNA	Complementary deoxyribonucleic acid

RNA	Ribonuceic acid
ES	Expression site
ESAG	Expression site associated gene
ORF	Open reading frame
NADH	Nicotinamide adenine dinucleotide
RBC	Red blood cell
PCV	Packed cell volume
ELISA	Enzyme-linked immunosorbent assay
CAT	Card agglutination test
CFT	Complement fixation test
IFAT	Indirect fluorescent antibody test
kDNA	Kinetoplastid deoxyribonucleic acid
FFLB	Fluorescent fragment length barcoding
NCBI	National center for biotechnology information
gGAPDH	Glycosomal glyceraldehydes phosphate dehydrogenase
EDTA	Ethylenediaminetetra-acetic acid
HCT	Haematocrit centrifuge technique
gDNA	Genomic deoxyribonucleic acid
UV	Ultraviolet
TAE	Tris-acetate ethylenediaminetetraacetic acid
CI	Confidence interval
SEM	Standard error of mean

SNP	Single nucleotide polymorphism
UPGMA	Un-weighted pair group method using average link
ML	Maximum likelihood
cTnI	Cardiac troponin-I
ANOVA	Analysis of variance

DEFINITIONS OF SOME TERMS AS USED IN THIS THESIS

Single nucleotide polymorphism: A Single nucleotide polymorphism or SNP (pronounced snip) is a DNA sequence variation occurring when a single nucleotide - A, T, C, or G - in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual).

Strain: This is one or more organisms within a species or a variety, characterized by some particular quality.

Gene: It is a unit of heredity that can be defined as a specific segment of DNA, usually in order of 1000 nucleotide, that specifies a single polypeptide.

Cyclical transmission: This is a mode of transmission found in trypanosomes in the parasites undergo a cycle of development and multiplication in the digestive tract of the fly until the infective metacyclic trypanosomes (metatrypanosomes) are produced.

Mechanical transmission: This is a mode of transmission found in trypanosomes in which the parasites undergo no cycle of development and multiplication in the digestive tract of the fly until the infective metacyclic trypanosomes (metatrypanosomes) are produced.

Vector: An organism, often an invertebrate arthropod, that transmits a pathogen from reservoir to host

Pathogenesis: The cellular events and reactions and other pathological mechanisms occurring in the development of disease**.**

Chemotherapy: The administration of a chemical, including antibiotics, to treat disease conditions.

Chemoprophylaxis: The administration of a chemical, including antibiotics, to prevent the development of an infection or the progression of an infection to active manifest disease, or to eliminate the carriage of a specific infectious agent to prevent transmission and disease in others

Trypanotolerance: Resistance to infection with trypanosomes, inherent in some breeds of cattle, e.g. the N'dama, Nigerian shorthorn, Lagune and others.

Prevalence: The total number of case of a specific disease in existence in a given population at a certain time.

Phylogenetic: The study of evolutionary history of a race or group of organisms.

CHAPTER 1

1.0 INTRODUCTION

Cattle are a major source of income and animal protein to Nigerians. Nigeria has about 14 million cattle, which represent 60% of the total livestock population in the country (Lamorde, 1998). Majority of the cattle herds belong to the *Bos indicus*, dominated mainly by Zebu breeds such as White Fulani (Bunaji) Sokoto Gudali (Bokoloji) and the N'Dama. White Fulani and Sokoto Gudali form about 51% and 12% respectively of the Nigerian cattle breeds and the remaining 37% consist of various crosses and muturu. Majority of the cattle is in the hand of nomadic Fulani and very few commercial cattle producing farms are available in Nigeria. Hence, this results to inadequate protein supply to the Nigerian populace.

The cattle production and management systems are such that the Fulani herdsmen migrate from the drier Northern part of the country to the more humid and wetter southern part of the country during the month of October to January in search of greener pasture for their cattle. As they migrate, the cattle are exposed to the vectors of various parasitic diseases, especially tsetse fly, the vector of salivarian trypanosomes.

Trypanosomes, the causative agents of animal trypanosomosis (nagana), are protozoan parasites that cause considerable loss to livestock farmers in sub-Saharan Africa. Though, Nzima (1985) reported babesiosis and anaplasmosis to be the major cause of death in dairy cow in Nigeria,

trypanosomosis is a serious threat to both human and animal health in sub-Saharan Africa as more than 60 million people living in 250 foci are at risk of contracting the disease with 500,000 new cases reported annually (WHO, 2005) and in animal the disease is one of the greatest hindrance to profitable African agricultural development (Ngure *et al.,* 2008). While the annual losses directly attributed to trypanosomosis, in terms of reduced meat and milk production and in terms of cost related to treating the disease and controlling the vector, has recently been estimated at US $1.2 billion (₦180,000,000,000). This figure may rise to over US $4.5 billion per year, if losses in potential crop production attributable to the disease are considered. Such indirect effects are seen in form of reduction in crop production as a result of inadequate number of oxen for animal traction (Swallow, 2000).

African animal trypanosomosis (AAT) is a disease complex caused by tsetse transmitted *Trypanosoma brucei brucei*, *Trypanosoma congolense* or *Trypanosoma vivax*, and occasionally *Trypanosoma evansi*, a non-tsetse-transmitted species in cattle. While simultaneous infections of cattle with one or more of these trypanosomes have been reported (Omotainse *et al.,* 2000), *T. congolense,* is considered less pathogenic to cattle than *T. T.vivax* and *T. brucei*, it is never the less the most important cause of African Animal Trypanosomosis (AAT) in West Africa.

1.2: Justification for the research

Diagnosis of trypanosomosis in Nigeria basically depends on microscopical, serological and animal inoculation methods.

The microscopical method lacks the sensitivity and precision required for purpose of adequate epidemiological, therapeutic and prophylactic control measures (Takeet *et al.,* 2013). Some members of the sub-class trypanozoon are pleomorphic, and the slender form of these members could be mistaken for other members of sub-group duttonella and nanomonas species which can lead to mis diagnosis. Low parasitaemic situation also gives high proportion of false negative results.

Serological techniques are particularly useful in detecting presence of the parasite antigens but will not distinguish the on-going infection from past infection and hence leads to false positive results. Cross reactions between the antigens of various trypanosome species have been reported (Ferenc *et al*., 1990), this may also lead to misdiagnosis of trypanosomal infections.

Animal inoculation such as sub-inoculation of rodents is particularly useful especially in revealing sub-patent infection. Apart from the fact that many *Trypanosoma vivax* do not grow in rodents (Gibson, *et al.,* 2012) the method is expensive, time consuming and many species will be missed using this diagnostic technique.

In the recent years, DNA based techniques especially polymerase chain reaction (PCR), have been used for the diagnosis of trypanosomosis. The

technique, though expensive and relatively new to certain parts of Africa, is very sensitive and precise. It could detect parasitaemia as low as 10 trypanosomes per milliliter of blood (Delespaux *et al*., 2003; and Desquensnes and Davila, 2002). Because of its sensitivity it has been used in some parts of Africa to ascertain the incidence and prevalence of trypanosoma species and characterization of Trypanosoma strains. Balmer and Caccone (2008) reported prevalence of multiple strain of *Trypanosoma brucei* in Africa using modern microsatellite markers, Mugittu *et al.* (2001) characterized Trypanosoma species of cattle in Tanzania, Solano *et al*. (1999) described the prevalence of Trypanosoma species of cattle in Burkina Faso. Cordon-Obras *et al.* (2009) reported prevalence of various species and strain in Equitoria Guinea, Simukoko *et al.* (2007) reported comparative prevalence of various *Trypanosoma species* in Zambia and Nyeko *et al.* (1990) characterized trypanosome species of cattle in Uganda, but no molecular characterization of *Trypansoma species* has been carried out in Nigeria except Y58 *T. vivax* strain, which was isolated in 1976 from a cattle in Yakawada in Zaria, Nigeria (Morlais *et al*., 2001).

1.3 Main objective of this study

- To carry out survey and characterization of *Trypanosoma species* of cattle in Nigeria using polymerase chain reactions techniques..

1.4: Specific objectives are to:

- Determine the prevalence of *Trypanosoma species* using microscopical techniques
- Determine the prevalence of *Trypanosoma species* using species-specific Polymerase Chain Reaction method (ss-PCR)
- Dompare the traditional (microscopy) and molecular diagnostic (PCR) approaches in bovine trypanosomoses.
- Devaluate and compare the genetic diversity of various species of Trypanosoma detected in naturally infected cattle in Nigeria using hyper-variable region, internal transcribe spacer 1 (ITS1), of ribosomal DNA (rDNA) gene.

CHAPTER 2

LITERATURE REVIEW

2.1 Trypanosomes:

2.1.1 Classification

Trypanosomes are extra-cellular flagellated protozoa belonging to the class mastigophora, order kinetoplastidae. Trypanosomes have been classified into two groups, the salivarian and stercoraria. (Soulsby, 1982)

The stercoraria group includes those trypanosomes that develop and multiply in the gut, then move to the rectum for the metacyclic form to be passed in the feaces. The stercoraria is divided into three sub-genera; megatrypanum in which *Trypanosoma theileri* is the most important, Herpetosoma, this include mostly the trypanosomes of rodents; *Trypanosoma lewisi* of rat, *Trypanosoma musculi* of mouse and *Trypanosoma nabiasi* of rabbit, Schizotrypanum has highly pathogenic trypanosomes, *Trypanosoma cruzi* that causes chagas disease in man (Hoare, 1972). The parasite is confined to America where it parasitizes not only man but various domestic and wild animals. The vector is bug of family Tritominae (Soulsby, 1982)

The salivarian group includes those trypanosomes that are pathogenic in Africa and are mainly transmitted by tsetse flies (*Glosina spp*). Based on the life cycle pattern and morphological characteristics of the

trypanosomes in this group they have been classified into four main sub-genera which include the Duttonella, Nanomonas species, Trypanozoon and Pycnomonas (Hoare, 1972). The sub-genus Duttonella contains the highly pathogenic genus *Trypanosoma vivax*, the sub-genus Nannomonas contains the *Trypanosoma congolense* and *Trypanosoma simiae* and the sub-genus Trypanozoon contain *Trypanosoma brucei.* Two sub-species; *Tryanosoma brucei gambiensse* and *Trypanosoma brucei rhodesiense* are responsible for sleeping sickness in man in Africa and one sub-species *Trypanosoma brucei brucei* is responsible for infection in domesticated animals. The sub-genus Pycnomonas contains *Trypanosoma suis* which causes chronic infection in pigs and it is transmitted by *Glossina brevipalpis*.

2.1.2 Morphology of trypanosome

Generally, trypanosomes are spindle-shaped protozoan ranging from 8-39µm long. They all possess flagella which arise at the posterior ends of trypanosomes from a basal body at the foot of a flagellar pocket. The flagellum runs to the anterior end of the body and is attached along its length to the pellicle to form an undulating membrane. Thereafter, the flagellum may continue forward as a free flagellum. Within a stained specimen a single centrally placed nucleus can be seen and adjacent to the flagellar pocket, a small structure, the kinetoplast, which contains the

DNA of the single mitochondrion. The size and location of the kinetoplast, the shape of the posterior end and the movement of trypanosomes in wet mount have been used in differentiating the species (Figure 2.1)

2.1.3 Molecular Structure

The African trypanosomes have two genomes, one within the nucleus and the other within the single mitochondrion (the kinetoplast) with the later accounting for about 10 – 20% of the total DNA content (Maudlin *et al.*, 2004). The nuclear genome is essentially diploid with the exception of the variant surface glycoprotein genes, its expression sites and mini-chromosomes which are haploid (size of about 35 Mb) in nature

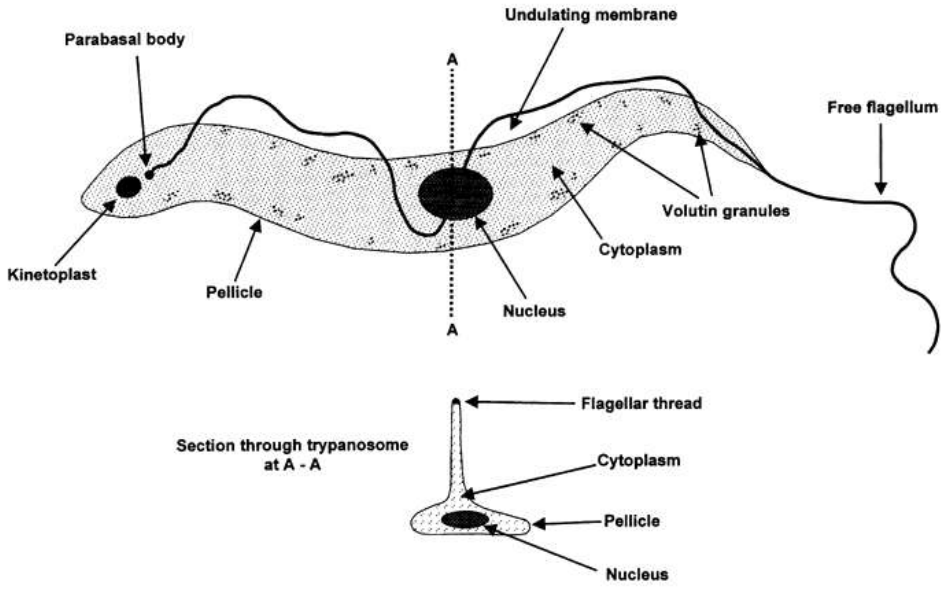

Figure 2.1: A diagrammatic illustration of the fundamental features of a trypanosome (trypomastigote) as seen in a stained preparation from the blood of an infected cattle (FAO, 2006)

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2.1.3.1 Nuclear chromosomes

Nuclear chromosomes, based on pulse field gel electrophoresis (PFGE) analysis, can be classified into three: mega-base chromosomes (≥ 1Mb), the intermediate chromosomes (> 100kb) and mini-chromosomes (30 – 100 kb). The latter two classes are considered to be aneuploid. The mini chromosomes consist of about 100 linear units ranging from 50 to 100 kb and account for about 20% of the nuclear DNA content. Based on the sequence analysis of a subset of these chromosomes, they are primarily composed of a 177 bp repeat sequence (90% of the mini-chromosomes), telomere repeats and one or two variant surface glycoprotein (VSG) genes (Weiden *et al.,* 1991). The intermediate chromosomes which represents about 0.2 – 1% of the nuclear genome, can be differentiated from the mini-chromosomes by their size (100 -700 kb) and the absence of the 177 bp repeats. Based on hybridization with cDNAs or markers for the VSG expression sites (ES), the intermediate chromosomes have been shown to contain expression sites (ESs) and telomeric sequences (Berriman *et al*., 2002). The mega-based chromosomes, also called housekeeping chromosomes (because they carry most of the genes involve with the basic functions of the trypanosomes) constitute about 80% of the nuclear genome. Based on pulse field gel electrophoresis (PFGE) analysis, Southern blotting and hybridization of cDNA probes 11 pairs (numbers I – XI) of diploid chromosomes have been identified that range in size from 1 to 6 Mb (Melville *et al.,* 2000).

2.1.3.2 Mitochondrial genome:

Mitochondrial genome (kinetoplast genome) occurs as a concatenated structure called the kinetoplast. It contains two classes of circular genome: the maxicircles and minicircle. About 50 maxicircles (identical) occur in each cellwhich are polymorphic in different species while the minicircle are highly heterogeneous with up to 200 different sequence classes per cell (Shapiro and Englund, 1995). The maxicircle encodes the 9S and 12S ribosomal RNA gene required for mitochondrial ribosomes and protein synthesis. Of the 17 open reading frames (ORF) present in the maxicircle, 11 encode the genes of known functions which include subunits of NADH dehydrogenase, cytochrome oxidase complexes I, II and III, ATPase 6 and cytochrome b. While sequence analysis of a number of minicircles has revealed that the minicircles do not encode any proteins, but encode guide RNAs (50 -70 bp) that play a central role in editing.

2.1.3.3 Variable surface glycoprotein (VSG)

The African trypanosomes species can change their surface coat continuously, the coat is composed of a single protein layer commonly referred to as variable surface glycoprotein (VSG).The spontaneous variation of this surface coat blocks the efficiency of the specific host antibodies (Gerrits *et al.*, 2002).Antigenic variation in trypanosomes is dependent on this protective protein coat that covers the entire surface of

the trypanosome (Ferrante *et al.,* 1983). An infecting population expresses a series of VSGs from a large reservoir of VSG sequences in the genome (Morrison *et al.,* 2005, Barriman *et al.,* 2005).

Trypanosoma brucei VSGs are composed of a combination of one N-terminal domain of ~340 residues and one or two C-terminal domains of 30 to 50 residues each (Carrington *et al.,* 1991). The N-terminal domains have been categorized into three types, A, B and C, according to two features of the primary structure: the location of conserved cysteine residues and the presence of a heptad repeat in a region known to form a coiled coil (Blum *et al.,* 1993 and Carrington, 1991). The C-terminal domains have been divided into six types, 1 to 6, based on the location of conserved cysteine residues and the sequence of the C-terminal glycosylphosphatidylinositol-anchor addition signal. The structure of the genomic reservoir of VSGs has been determined and consists between 1000 and 2000 potential VSG sequences in the genome; however, only 7 % encode functional VSG open reading frames (ORFs) and of the remainder 9 % encode an ORF for a VSG with atypical primary structure; 62 % are disrupted VSG ORFs containing frame shifts and/or stop codons and the remainder are fragmentary VSG ORFs. Around 10 % of the VSG sequences lie at telomeres of large, intermediate and mini-chromosomes but the majority are present in sub-telomeric tandem arrays (Berriman *et al.,* 2005 and Barry *et al., 2007)*

2.2 Transmission:

With the exception of *Trypanosoma equiperdum* of equines which is a venereal disease, all trypanosomes have arthropod vectors in which transmission is either cyclical or acyclical (Urquhart, 2006).

2.2.1 Biological (cyclical) transmission

In cyclical transmission the arthropod is a necessary intermediate host in which the trypanosomes multiply, undergo a series of morphological transformations before form infective for the next mammalian host are produced. When multiplication occurs in the digestive tract and proboscis, so that the new infection is transmitted when feeding, the process is known as anterior station development and the various species of Trypanosomes which use this process are often considered as a group salivaria and all are trypanosomes transmitted by tsetse fly, the main species being *T. congolense, Trypanosoma vivax and Trypanosoma brucei* (Maudlin *et al*., 2004).

In other trypanosomes, multiplication and transformation occurs in the gut and the infective form migrates to the rectum where they are passed out with feaces. This development is referred to as the posterior station development and the trypanosomes species are grouped together as the stercoraria. In the domestic animals, this group, stercoraria, is all relatively non-pathogenic trypanosomes such as *Trypanosoma theileri* and

Trypanosoma melophagium transmitted by tabanid flies and sheep ked respectively. An exception to this is the *Trypanosoma cruzi*, the cause of chagas' disease in South America that is transmitted in the feaces of reduviid bugs.

2.2.2 Mechanical (non-cyclical) transmission:

Non-cyclical transmission (acyclical) is essentially mechanical transmission in which the Trypanosomes are transferred from one mammalian host to another by the interrupted feeding of haematophagus flies of the genus *Tabanus* and *Stomoxys.* In this case there is no multiplication of the parasites on or in the contaminated proboscis and hence cross-transmission is only possible for a few hours. *Trypanosoma evansi* is transmitted mechanically by vampire bat in which the parasites undergo multiplication but without morphological transformation before they migrate into the buccal cavity. (Urquhart, 2006)

It is important to note that the salivarian trypanosomes are normally transmitted cyclically in tsetse flies , may on occasion be transmitted mechanically as *Trypanosoma vivax* has established itself in south America which is believed to have been transmitted mechanically from imported cattle by biting flies, also, apart from the classical cyclical and non-cyclical transmission, dogs, cats and wild carnivores may become

infected by eating fresh carcass or organ of animals which have died of trypanosomosis, the parasites penetrating oral abrasions (Urquhart, 2006).

2.2.3 Veneral transmission

Venereal transmission is the normal means by which dourine of equines, caused by *Trypanosoma equiperdum*, is propagated. Because of its presence in the mucous exudate of penis and sheath of the stallion and the vaginal mucus of the mare, *T. equiperdum* is easily transmitted directly during copulation from an infected to a healthy animal and its geographical distribution is not restricted to specific climatic conditions. This species is essentially a tissue parasite and causes at most very low parasitaemias in the circulating blood of equines (Brun et al., 1998).

2.3 Vector of African trypanosomes:

Vectors of African Trypanosomes are dipterans which include the biting flies, tsetse flies, Stomoxys and Tabanus while *Trypanosoma cruzi* the cause of chagas disease in South America is transmitted by *Reduviid* bug, a triatominae (Schmunis, 1999).

Tsetse flies (Glossina) are extremely important vector of African trypanosomoses. These flies are confined to a belt of tropical Africa extending from the southern Sahara (Lat 15^0N) in the north to Zimbabwe

and Mozambique in the south (Lat 20-30^0 S) (Zaria, 2005).Various species are restricted to various geographical areas according to habitat the three main groups include Fusca, Palpalis and Morsitans, found respectively in forest, riverine and savannah areas. The last two are the most important vectors of trypanosomes in domestic animals in Africa (Seifert, 1996).

2.4 Life cycle/ Reproduction:

Trypanosomes reproduce by longitudinal division whereby they double their basal apparatus and kinetoplast. After ingestion of trypanosomes by the vector in the blood or the lymph while feeding on an infected host ,the trypanosomes lose their glycoprotein surface coat , and in the case of *Trypanosoma brucei* and *Trypanosoma congolense* become elongated and multiply in the mid-gut before migrating to the salivary gland(*T. brucei*) and the proboscis (*T. congolense*). There they undergo a transformation losing their typical trypanosomes form (trypomastigote) and acquire epimastigote form characterized by the fact that the kinetoplast lie just in front of the nucleus. After further multiplication of the epimastigote they transform to a typical small trypomastigote form with a glycoprotein surface coat. These are the infective forms for the next host and are called the metacyclic trypanosomes. The entire process takes at least two to three weeks and the metacyclic trypanosomes are inoculated into the new host when the tsetse fly feeds (Soulsby, 1982). With *Trypanosoma vivax* a

similar process of cyclical development takes place except that it occurs entirely within the proboscis.

At the site of inoculation, the metacyclic forms multiply locally as the typical blood forms, producing within a few days a raised cutaneous inflammatory swelling called a chancre. There after they enter the blood stream, multiply and a parastaemia detectable in the peripheral blood usually becomes apparent 1-3 weeks later (Urquart, 2006).

2.5 Pathogenesis:

With the exception of *Trypanosoma vivax* which produces a hyperacute and fatal infection characterized by the high parasitaemia, fever ,severe anaemia and haemorhages on the mucosal and serosal surface , the pathogenesis of trypanosomoses is rather complex and depends on the species of the transmitting vector as well as the resistance of the host. The real cause which leads to the death of the infected animal is as ever not fully understood. On the one hand it is believed that the parasites released in to the circulation, toxic substances when it is destroyed in the circulatory system which damage the lining of the blood vessel. In some cases the released of large amount of toxic substances trigger a chain reaction which produces a shock like syndrome (Tizard *et al.*, 1978). With this theory, the typical symptoms of trypanosomoses such as emaciation, oedema, anaemia and nervous symptoms can be explained. Generally, the

pathogenic effect of the parasite may be understood as a syndrome, the components of which are as follow:

a. Pancytopenia as a result of the direct influence of the parasite on the cells or the phagocytic defence reaction of the organism which may be on an auto immunological basis. The resulting anaemia which appears with the progressing parasitaemia is the classical symptom of the disease.

b. Metabolic effects of the Trypanosomes which withdraw essential nutrients and produce toxic metabolites. The consumption of glucose, production of pyruvate and deaminization of the amino acids tyrosine and tryptophan seem to be especially important (Verstegen *et al.*, 1991).

c. The action of the secretions, such as acid phosphotase which activate the compliment system, and pharmacokinetic active substances like serotonine and kinine also have a direct pathogenic effect (Coombs and Mottram, 1997)

d. A trypanosome-induced hypothyroid status which may play a vital in the impairment of mitochondrial ATPase activity, a key enzyme in energy metabolism (Seifert 1996).

e. The action of biologically active lipids which are released when the pathogens are autolysed, and which together with free fatty acids, lead to cell damage and immunosuppressive effects (Seifert 1996).

f. Mechanical cell and tissue damage caused through active mechanical invasion of the extraordinary strong and mobile pathogens (Seifert 1996).

g. Immunological mechanisms, characterized by the ability of the parasites to change its surface-coat-antigen continuously, thus already exhausting the antibody production of the host during the IgM phase (only with the salivaria). The phenomenon known as antigenic variation seems to be the major reason why the pathogenic trypanosomes evade the host defense mechanisms and thus secondary infections from other haematozoans and other pathogen are activated.

2.6 Clinical signs:

Trypanosomoses develop in ruminants depending on species and strain of the trypanosomes, the vectors and the resistance of the affected animal either as an acute or chronic infection (Valli and Forsberg, 1979).Animals exposed to infection by tsetse flies develop patent infection after incubation period of variable length of time not only depending on the strain the species but also on the number of infective trypanosomes introduced by the tsetse flies. Trypanosomes appear in the blood of most animals exposed to infection with *Trypanosoma vivax* after 8 – 10 days and with *Trpanosoma congolense* after 12 -16 days. The prepatent period for *Trypanosoma brucei* is less well defined but the parasites can be detected in as early as 3 – 4 days after exposure to the infection. The major

signs are anaemia, enlargement of superficial lymph nodes, lethargy and progressive loss of condition. Fever and loss of appetite occur intermittently during parasitaemia peak and the latter becoming marked in the terminal stage of the disease. Trypanosomoses in the ruminants are commonly seen as chronic diseases with intermittent fever, increasing degree of anaemia and progressive loss of condition. Infected animals are listless, coat lack luster, lose weight and become easily exhausted and hence lag behind the herd (Desquesnes *et al.*, 2013). Superficial lymph nodes are enlarged and prominent. Cattle infected with *Trypanosoma vivax* often show sign of photophobia and excessive lacrimation. Trypanosomosis is not invariably a fatal disease, occasionally animal recovered more slowly over period of weeks or months from more severe trypanosomes infections which progress through a state of pre-immunity to complete self-cure Reproductive disorders are a common occurrence in human and animal trypanosomoses and include irregular oestrus, abortion, neonatal death and infertility.

2.7 Forms and characteristic of trypanosomoses:

2.7.1 Nagana

Nagana is a group of diseases caused typically by Africa Trypanosomes (*Trypanosoma vivax, Trypanosoma brucei, Trypanosoma congolense, Trypanosoma simiae and Trypanosoma suis).* They all are transmitted

cyclically by the tsetse flies. Nagana is wide spread in Africa South of the Sahara with exception of South Africa and Namibia. The causal trypanosomes develop mainly in the blood plasma and *Trypanosoma brucei* which develops additionally in the tissues. It is characterized by bout of fever, anaemia, pica, eye disorder, emaciation and death (Seifert, 1996).

2.7.2 Surra

Surra is a trypanosomosis of camelidae and equine due to *Trypanosoma evansi* infection, transmitted by biting insects other than tsetse flies. The disease could be acute or chronic in nature depending on the species or breed of the animal and the region. The disease is endemic throughout the northern Africa and sahelian Africa where Nigeria is found. The acute form of the disease is characterized by general debility with hyperthermia and rapid emaciation, Lacrimation, petechial hemorrhage on the conjunctiva and reduced milk yield, abortion, oedema, pulmonary complication and nervous signs while the chronic form is characterized by intermittent fever, anaemia and emaciation. Gradually, signs of general weakness appear including diarrhorea, irregular appetite, anaemia, abortion, pica and nervous symptoms (Desquesnes *et al.*, 2013).

2.7.3 Dourine:

This is a contagious trypanosomosis of equine due to *Trypanosoma equiperdum*. It is a venereal disease that is solely transmitted by coitus and is sometimes called horse syphilis or ´mal du coit´. The disease is now confined to part of Africa, Asia and South and Central America. The disease is characterized by genital and ventral abdominal oedema, transient urticarial plague and progressive emaciation (Urquhart, 2006).

2.7.4 Chagas disease

Chagas disease, also known as American trypanosomiasis, is a potentially life-threatening illness caused by the protozoan parasite, *Trypanosoma cruzi*. It is found mainly in Latin America, where it is mostly transmitted to humans by the faeces of triatomine bugs, known as 'kissing bugs. Chagas disease could be acute or chronic. The acute phase of Chagas disease, which may last for weeks or months, may be symptom-free. The clinical signs may include, swelling at the infection site, fever, fatigue, body rash, body aches, headache, loss of appetite, nausea, diarrhea or vomiting, swollen glands, spleno- and hepatomegally. These signs and symptoms usually go away on their own. However, if untreated, the infection persists and advances to the chronic phase that is characterized by irregular heartbeat, inflamed, enlarged heart (cardiomyopathy), congestive heart

failure, sudden cardiac arrest, difficulty swallowing due to enlarged esophagus and abdominal pain or constipation due to enlarged colon.

2.8 Diagnosis

The diagnosis of bovine Africa trypanosomoses is mostly based on the demonstration and identification of the parasites in the blood of the host even though the history of rapid loss of condition and death in a few acute cases may be an indication of the disease (Nantulya, 1990). Clinically, there are no pathognomonic signs of trypanosmoses, which, especially in their chronic forms resemble any other parasitic or infectious conditions causing physiological stress or anaemia, oedema, lymph node hypertrophy, conjunctivitis and or keratitis.

2.6.1 Parasitological diagnosis

Parasitological diagnosis enables the demonstration of the trypanosomes either directly or indirectly after concentration. The simplest direct examination techniques are the examination of wet, thick or thin films of fresh blood, usually obtained from the ear vein, jugular vein or the tail. Amongst the direct examination techniques, stained thin blood films are generally regarded as more specific but less sensitive than the other two (Nantulya, 1990).

2.8.1 Wet blood films:

These are made by placing a droplet of blood (about 2µl) on a clean microscope slide and covering with a cover slip (22 x 22mm). The blood is examined microscopically at x 40 magnification of light microscope or at x 400 total magnification with condenser aperture, phase-contrast or interference contrast. Approximately 50 -100 fields should be examined. Trypanosomes can be recognized by their movement among the red blood cells (RBCs) (Murray *et al.*, 1977). The advantage of this technique is that, it is faster and cheaper to carry out but may not be appropriate in a low parasitaemia situation.

2.8.2 Thick blood films:

These are made by placing a drop of blood (5 – 10µl) on a clean glass slide and spreading it over an area of about 2cm in diameter, using the corner of another slide. The thickness of the resultant film should be such that, when dry, the figure on wristwatch dial can be read through it. The film is air-dried and, without fixation, is dehaemoglobinized by immersion in distilled water for a few seconds and dry before staining for 30 minutes with 4% diluted Giemsa stain in phosphate buffered saline, pH 7.2. Staining time and stain dilution may vary with stain and individual. The

stained smear is washed with buffered water and examined at x1000 magnifications (Murray *et al.*, 1977).

2.8.3 Thin blood smears:

Thin blood smears are made by placing a small drop of blood (about 5µl), for example from mirohaematocrit tube, on a clean microscope slide approximately 20mm from one end (allowing for space to apply the thick smear) and spreading with the edge of another slide. This slide is placed at an angle of about 30^{o} to the first slide and drawn back to make contact with the blood droplet. The blood is allowed to run along the edge of the spreader, which is then pushed to the other end of the slide in a fairly rapid but smooth motion. The slide is dried quickly by waving in the and fixed for three minutes in methanol, and stained as for thick smear. After staining the slide is washed gently under slow running tap water and allowed to dry. Approximately 50 – 100 field of the stained smear should be examined with x100 oil-immersion objective lens.

Usually, both a thin and thick blood smears can be made from the same blood sample and on the same slide. Thick blood smear contain more blood than thin smears and hence, have a higher diagnostic sensitivity. Thin smears on the other hand allow *Trypanosoma species* identification.

2.8.4 Parasites concentration techniques:

The probability of detecting trypanosomes in a sample from an infected animal depends largely on the amount of blood and the level of parasitaemia. The amount of the blood examined with direct examination techniques is low and parasites are often very scanty in the blood of an infected animal. Both of these factors contribute to the low sensitivity of direct examination techniques. Sensitivity can be increased by increasing the volume of the blood to be examined and by concentrating the trypanosomes.

2.8.5 Microhaematocrit centrifugation technique (Woo, 1970):

This is based on the separation of various components of the blood sample depending on their specific gravity; i, fresh blood (about 70µl) is collected into heparinized capillary tube.(75 x 1.5mm), ii, one end of the capillary tube is sealed with plasticin or by heating ensuring that the column of the blood is not charred by the flame, iii, centrifuge in microhaematocrit centrifuge at 3000 g for 5 minutes, iv, a tube carrier is made from a slide on which two pieces of glass, 25 x10 x1.2mm have been fixed at 1.5mm apart to form a groove, v, the tube is placed in the groove with a cover slip placed on top and the interface flooded with water and the plasma/white blood cell interface (buffy coat) is examined by slowly rotating the the haematocrit tube. Trypanosomes movement can first be detected using the x10 objective lens with reduced condenser

aperture; the trypanosomes can be seen clearly using the x40 objective lens. This method is more sensitive than the direct examination technique (Kratzer *et al.,* 1989) because the trypanosomes have been concentrated at the buffy coat zone.

2.8.5.1 Dark-ground/phase contrast buffy coat technique (Murray method):

It is an improved technique for the detection of trypanosomes. It is carried out following steps i to iv above after which the capillary tube is cut, with diamond pencil, 1mm below the buffy coat, to include the top layer of RBCs. The buffy coat and uppermost layer of RBCs are extruded on to a clean microscope slide and cover with a cover slip and then examined under x 40 objective lens for trypanosome movement. The microhaematocrit centrifugation and buffy coat techniques are particularly useful in that the packed cell volume (PCV) can also be assessed at same time.

2.8.6 In-vitro cultivation of trypanosomes:

In-vitro cultivation is based on the cultivation of procyclic form of trypanosomes using a kit (KIVI). The kit has proven ability in isolating all species of *Trypanosoma brucei* in human and domesticated animal, though

the techniques value is in isolating *Trypanosoma congolense* but *Trypanosoma vivax* is still unknown (Mc Namara et al 1995a).

2.8.7 Animal inoculation

Animal inoculation is useful in revealing sub clinical infections. This method involves the sub inoculation of laboratory animals such as mice or rats and rabbits though not all Trypanosomes are infectious for these species (Eisler *et al.,* 1998). The laboratory animals are injected intraperitoneally with 0.2 -5ml (depending on the size of the rodent) of freshly collected blood. This method is more sensitive than direct examination of the wet blood film but the method is expensive and diagnosis is not immediate. This is the best method to detect *Trypanosoma brucei brucei* infection and also to detect the non-tsetse transmitted animal *Trypanosoma species* such as *Trypanosoma evansi*. However, some stains of *T. vivax* rarely grow in laboratory rodents.

2.8.8 Serological techniques

Serological methods have been developed for the detection of *Trypanosoma species* infection but field applications of these methods have given inconsistent results (Desquesnes, 1996). Serological techniques can not distinguish between on-going and previous infections. Among the

methods are; Enzyme-linked immunosorbent assay (ELISA) (Luckins 1992), Card agglutination test (CAT) and Compliment fixation test (CFT) (Luckins, 1993).

2.8.9 Molecular techniques

The various techniques available for DNA identification are based on the sequence of the four DNA nucleotides namely: adenine, thiamine, guanine and cytosine. These techniques include:

2.8.9.1 Species-specific identification techniques DNA probes

This is the first molecular method with sufficient sensitivity for direct identification of the small numbers of organisms found in naturally infected vertebrate and invertebrate hosts. The method involves preparing dot blots of unpurified samples of tissues collected directly from infected vertebrate and invertebrate hosts follows by hybridization with radioactively-labeled DNA fragments. This technique can detect up to 1000 trypanosomes per milliliter of blood sample. Each DNA probe being DNA fragment identified in a particular species, hence to increase sensitivity, each trypanosome species of interest requires an individual DNA sequence to be identified and tested for specificity. Generally, non-

coding, repetitive DNA elements are good for DNA probes as they are less conserved than coding region (Salim, 2011)

2.8.9.2 Species specific PCR

The application of PCR-based techniques greatly aided molecular species identification, as their high sensitivity allowed identification from low numbers of organisms from the tissues or blood samples. This technique is based on the use of pair of primers (Table 2) designed to amplify a region specific to each species in which, at least, 18-30 bases of the extremity sequences are known. Following initial denaturation of DNA at higher temperature ($94^{o}C$ – $95^{o}C$), 30-40 PCR cycles (denaturation, anealling and extension) and final extension of the amplified product, the DNA will have been amplified approximately 270 million times (figure 2). The resulting PCR product can be visualized on an agarose or polyacrylamide gel, after staining with fluorescent dye (ethidium bromide or gelred) and exposing under ultraviolet light. The specific sizes of the PCR products are evaluated by simultaneous migration of molecular size marker and a positive control. A negative control is run together to evidence any DNA contamination. The sample is said to be positive when, at least, a band of expected size is obtained.The actual sequence amplified can be analyzed by sequencing, or checked by DNA probing. However, the number of species-specific PCR required for each DNA sample can make this

method time consuming and expensive. For instance, within the tsetse-transmitted group, about 11 separate PCRs would be needed on each DNA sample collected from an individual tsetse fly to cover all the species and subgroups for which there are tests (Mekata *et al.*, 2009)

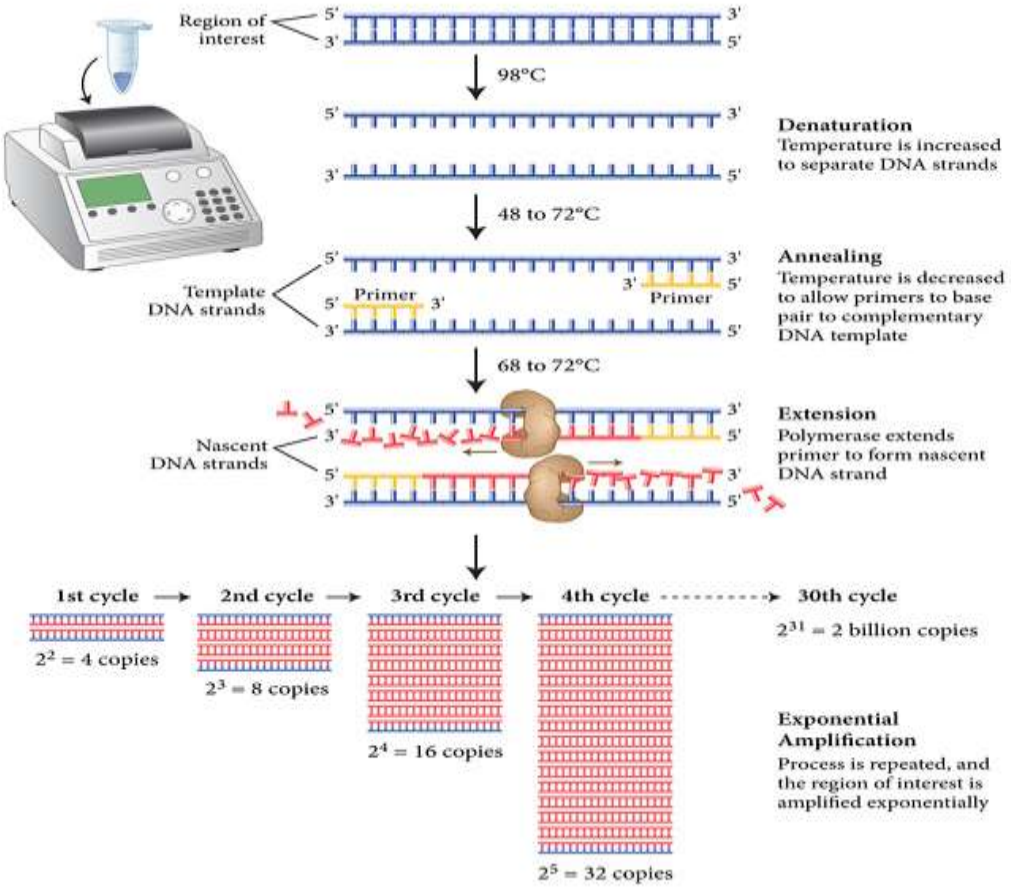

Figure 2.2: Schematic presentation of the three steps involved in polymerase chain reaction (PCR) for detection of trypanosomal genes (Cann, 2005)

2.8.9.3 Multiple species detection by PCR:

To overcome the limitation of the species-specific approach, a range of methods have been developed.The use of generic primers in conserved areas of the genome to amplify sections of DNA from any, or specific groups of trypanosome species. The trypanosome species are then identified by the length of one or more of the PCR products, either directly or after restriction digestion of the PCR-amplified fragment. Identification can also be achieved by sequencing the amplified PCR fragments (Adams and Hamilton, 2008).The regions commonly used in the generic PCR test include the ribosomal DNA spacer called internal transcribe spacer (ITS)region and the splice leader of kDNA minicircles (Adams *et al.*, 2006).

The internal transcribe spacer (ITS) region of the ribosomal RNA locus of eukaryotes have a high copy number and vary both in sequence and length between species. This region can be amplified using primers set that are complimentary to the conserved region of the 18S, 28S and or 5.8S rRNA gene. The internal transcribe spacer (ITS) consist of both internal transcribe spacer I and 2 (ITS 1 and ITS 2) found between 18S rRNAand 5.8S rRNA, and 5.8S rRNA and 28S rRNA respectively (figure 3). Though the use of a generic PCR test could reduce analysis to a single test per sample, the sizes of the ITS1 PCR bands for some species cannot be distinguished(Cox et al., 2005, Adams et al., 2006). On the other hand ITS PCR could identify new or unknown species (Adams *et al.*, 2006)

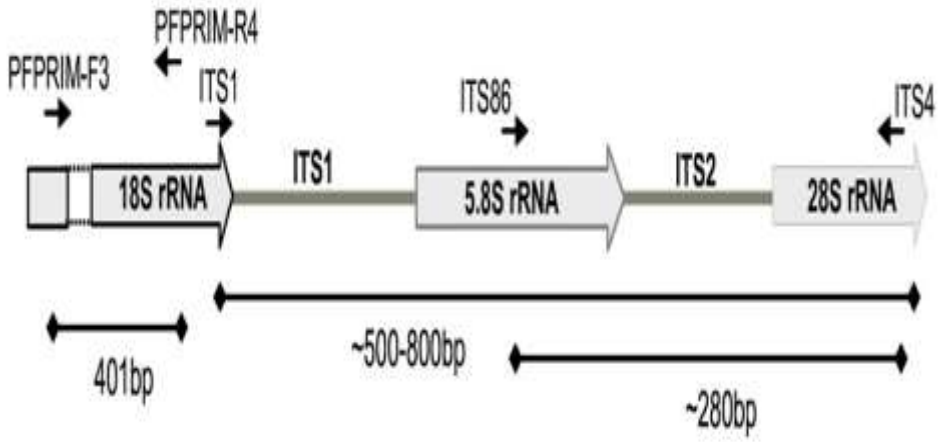

Figure 2.3: Schematic representation of the trypanosomal ribosomal 18S rRNA gene and Internal transcribed spacer (ITS) regions with primer binding locations.

2.8.9.4 PCR restriction fragment length polymorphism

This method, also known as ribotyping, has proven useful when only a few species are present, which differ in their restriction digestion profiles. This involved the amplification of the most variable region of 18S rDNA with generic primers, followed by restriction analysis using one or several restriction enzymes. The technique has been used to differentiate between DNA from four species of trypanosome infecting African cattle (*T. congolense, T. b. brucei, T. theileri and T. vivax*) in single and mixed infections (Delespaux *et al.*, 2003)

2.8.9.5 Fluorescent fragment length barcoding (FFLB)

This is a new method that was devised to overcome many of the limitations of other methods and provide an accurate and high-throughput means of species identification. FFLB uses an approach similar to that used for microsatellite analysis using fluorescently tagged primers and an automated sequencer to read the sizes of the PCR-amplified DNA fragments (Hamilton *et al.*, 2008). For trypanosomes, four sets of generic primers, with one of each pair fluorescently tagged, were designed to amplify small (<400 bp) fragments of 18S and 28S ribosomal DNA with known interspecies size variation. In contrast to other length-based methods, such as the ITS method, in which PCR products are analyzed by agarose gel electrophoresis, the sizes for FFLB are determined accurately

using an automated DNA sequencer. This enables PCR products to be sized to within 1 bp, allowing far greater resolution than conventional agarose gel electrophoresis. The PCR products of the four separate reactions on each DNA sample are pooled for simultaneous sequencer analysis. A barcode unique to each species is generated by using four regions and different fluorochromes (Hamilton *et al.,* 2008). Apart from the fact that this technique is more sensitive than the ITS-PCR, the greatest advantage of the technique is its ability to recognize new species by showing a unique barcode, even in mixed infections.

2.8.9.6 Sequence Analysis:

DNA sequences are increasingly being used for species identification of a wide range of organisms. The strengths of this approach are that sequences can be directly compared with those from previous studies that are held on publicly accessible databases, such as GENBANK (NCBI, 2005) , and no two unrelated species will have the same sequence. There are now very large data sets for sequences of rRNA genes and some genes encoded by mitochondrial DNA, such as the cytochrome oxidase subunit genes. For trypanosomes, over 100 18S rDNA sequences are available representing trypanosome species from a variety of hosts and geographical areas. There are also substantial data sets resulting from the choice of particular protein-coding genes for phylogenetic studies, for example, over 60

sequences of the glycosomal glyceraldehyde phosphate dehydrogenase (gGAPDH) gene from different trypanosomes (Hamilton *et al.*, 2007). *Trypanosome species* can be identified by sequencing one of these genes and either using BLAST analysis or sequence alignment and construction of phylogenetic trees. The most variable region of 18S rDNA is commonly used for this purpose, and can be sufficient for identification if sequences are identical (Hamilton *et al.*, 2003). However, since there is no consensus on the degree of similarity necessary to identify a known species, identification can be more difficult if the new and reference sequences differ slightly. The most variable region of the 18S rDNA by itself is often insufficient for precise phylogenetic placement. Sequencing of the gGAPDH gene can be more economical, as it is shorter than full-length 18S rDNA gene, no gaps are required to align trypanosomal gGAPDH gene sequences and both genes give similar phylogenetic resolution (Hamilton *et al.*, 2007). This method is particularly useful when there is no prior knowledge of the *Trypanosoma species* present, as it allows novel trypanosomes to be placed accurately on the phylogenetic tree.

However, for routine identification of trypanosome species, sequence analysis is time consuming and expensive, although the development of new high-throughput technologies, such as 454 sequencing, will make it a more attractive option (Metzker, 2005). In addition, if mixed infections are frequent, cloning of the PCR product and subsequent sequencing of multiple clones will be required prior to sequencing, adding time and

expense. For well-studied groups of trypanosomes, such as the African tsetse fly-transmitted trypanosomes, it is more efficient to use ITS or FFLB for identification and use sequence analysis for precise phylogenetic placement.

2.9 Treatment of trypanosomosis

2.9.1 Chemoterapy and chemoprophylaxis

Chemotherapy and chemoprophylaxis of the animal trypanosomes in Nigeria depends on three drugs, diminazene aceturate, isomethamedium chloride and homidium chloride.The approach to effective prophylaxis and chemotherapy in cattle especially depends on routine block treatment of the entire herd. This is generally carried out using prophylactic drugs, notably isometamedium chloride, at predetermined interval based on the perceived duration of the prophylaxis (Seifert, 1996).

Diminazene aceturate (Berenil®) has remarkable property. It is very active, stable and easy to use, and has very low toxicity. These advantages make it practical, risk-free trypanocide, at least for cattle but toxic to camel. Berenil I a yellow powder, easily soluble in water at up to 7% and the solution can only be kept for 2 – 3 days. Berenil is administered intramuscularly in ruminants and in addition to this route, subcutaneous in other animals at adose rate of 3.5mg per kilogram body weight for treating *Trypanosoma vivax* and *Trypanosoma congolense* infection while

infection due to *Trypanosoma brucei* in horse can be treated with a dose rate of 7mg per kilogram body weight. *Trypanosoma evansi* in dromeries can be treated with Berenil provided the dose rate of 3.5mgper kilogram body weight is not exceeded, since fatal toxic reactions were reported with higher doses (Eisler *et al*., 2001).

Dogs are apparently sensitive to Berenil which may sometimes cause oedematous or hemorrhagic encephalitis, and hepatic and renal lesion. The drug is administered intramuscularly in dog. It must be noted that few trypanosomal resistant to Berenil has been reported especially in Nigeria, Chad and Uganda (Geerts and Holmes, 1998)

Isomethamidium chloride hydrochlorate, suramin^{R}, is a red powder that is easily soluble in water. It is used in 1 or 2% aqueous solution administered by deep intramuscular route at the rate of 0.25 -1mg per kilogram body weight, depending on drug resistant risk. At 0.25 – 0.8mg/kg body weight dose, the drug is very active against cattle trypanosomosis due to *Trypanosoma vivax* and *Trypanosoma congolense*, and *Trypanosoma brucei* at 0.5 -0.8mg/kg.This drug is tolerated in low doses administered intramuscularly but at the dose higher than 1mg/kg body weight it can cause transient systemic symptoms in herbivores especially local reaction with persistent hardening of tissue that is usually invisible externally, but make the meat unfit for consumption , and therefore the drug should be given in divided doses in different sites. Isomathmedium can be used as a

curative and prophylaxis drug for horses, donkeys and dogs at the same as in cattle and under the same condition.

Homidium chloride (Novidium) is in form of purple tablet that dissolve easily in boiling water. It is administer to cattle in1 or 2.5% solution at the rate of 1mg/kg body weight. It is active against *Trypanosoma vivax* and slightly less active against *Trypanosoma congolense*. The drug is generally recommended to be administered subcutaneously at the dew lap. Homidium bromide (Ethidium), which is used the same way as Novidium has the same action as the Novidium.

Quinapyramine sulphate (Antrycide) is an old drug which is today manufactured as Trypacide or novoquin. It is a white powder that dissolves easily in water. It is a curative drug for both cattle and small ruminants at 5mg/kg body weight of 10% aqueous solution subcutaneously but because of its toxicity in it is contraindicated in cattle (Ndoutamia *et al.*, 1993). It can be used in *Trypanosoma brucei* and *Trypanosoma equiperdum* infections in horses and *Trpanosoma brucei* infection in dogs at the dose rate of 3 – 5mg/kg body weight respectively.

Other drugs that have been used with some degree of success are suramin sodium, a white crystalline powder that is soluble in cold water. It is effective in treating human sleeping sickness and cattle infections due to *Trypanosoma vivax a*nd *Trypanosoma congolense*.The minimum dose of 3 – 4g/animal in 10% aqueous solution given intramuscularly or

intravenously. The dose for horse is 7 -10mg /kg body weight (Ndoutamia *et al.*, 1993).

2.10 Control of African trypanosomosis

This currently depends on the control of tsetse flies, chemoprophylaxis and keeping of trypano-resistant breed.Chemoprophylactically, in cattle and if necessary in sheep and goats isomethamidium (samorin) is the drug of choice sine it remain in the tissue and has prophylactic effect for up to six months. An alternative is diminazene aceturate or homidium salts. To reduce the possibility of drug resistance it may be advisable to change from one trypanocidal drug to another periodically (Seifert, 1996)

2.10.1 Keeping trypanotolerant breeds

Keeping trypanoresistant breed has given a promising result on the field. The introduction and keeping of trypan-tolerant breed in West Africa taurine cattle breed seem to be an alternative biological method to preventing African trypanosomosis and thus economic losses for the animal holder (Horchner, 1983). N´Dama cattle and other West African non-descript local breeds possess an increase titer of resistance factors such as lysozymes, haemolytic complement C9 and the third complement

C3 and are better able to stabilize the balance of the host-parasites relationship known as premmunity.

2.10.2 Vector control

The control of vector of Trypanosomes is the most effective way of controlling trypanosomosis. This method can be divided into two; destruction of the habitat where the vectors rest or breed and destruction of the vectors on their hosts when feeding or resting (Schofield and Kabayo, 2008).

2.10.2.1 Destruction of breeding-resting places

The removal or destruction of the resting and the breeding places used to be considered the most important but removal of the habitat only displaced the tsetse flies but not their eradication is achieved but to achieve this total clearing of their resting place is required, since tsetse are unable to exist or survive in an open place without trees and bushes which provide shade. Today people are conscious of the importance of protecting their natural environment, therefore large scale clearing of forest is avoided. Partial clearing however may be carried out. To this end, the lower branches of trees and the under growth are removed especially along the rivers (Schofield and Kabayo, 2008). This allows the hot winds which blow from

the savannah to penetrate the forest and change the environment in such a way that the habitat of the *Glossina* is destroyed.

2.10.2.2 Removal of game and wild animals

Removal of game and the wild animals that served as reservoir for most of the blood parasites and from which Glossine can obtain blood meal as an alternative source will definitely reduce the population of tsetse flies (Wall and Doane, 1980).

2.10.2.3 Fly traps

The construction of which is based on the simple principle or knowledge that tsetse flies are attracted by the contrast between light and shade, and seek shade or dark spot. Tsetse flies prefer to feed in the shade (on the under belly).Consequently, the traps have been constructed in such away that the entrance is placed on the underside and made out of dark-coloured materials. Various modifications of the traps are haris trap, biconical trap, pyramidal traps etc. Generally the traps are often combined with odorous substances which are attractive to tsetse flies (Schofield and Kabayo, 2008).

2.10.2.4 Biological methods

Vectors may also be controlled biologically. This becomes very important because of the direct and indirect effects of bush clearing and chemical methods on the animals and the environment. Among these biological measures are the increasing and releasing of predators. Arthropod such as Aslidae flies prefer to attack the fully engorged tsetse flies which has difficulty flying. Dragon flies and spiders are also predators of adult glossines but none of these predators has been multiplied artificially in order to carry out organized tsetse controll programe.The flagellates, *Blastocrithidia triatomae* invade the triatomae, the vectors of chagas disease and delay the development of the larvae of the triatomes and increase their mortality rate (Schaub, 1986).

Vector control has been achieved by interfering with the hormonal physiology and the process of development during metamorphosis. Methods used for this purpose include: Application of insect growth regulators, application of juvenile hormone analogues, the sterile male techniques, genetic manipulation, chemo-sterilization and the use of attractants.

At present, almost all the methods used for the large scale control of tsetse flies depend on application of chemical compounds.These insecticides are applied by spraying either from the ground or air using plane or helicopter. For the treatment of large area fixed wing aircraft or helicopter are used.

Application with plane has to be carried out in windless condition. Because of economic reasons, chlorinated hydrocarbon is still used against tsetse flies in Africa because they have long-lasting effect. Organic phosphoric acid (OPAE) and carbamate are much better tolerated by the environment since they become catabolized rather quickly. Presently, pyrethroids which are also very effective against tsetse flies and only toxic to fish are a good alternative which the environment can better tolerate.

CHAPTER THREE

3.0 THE PREVALENCE OF *TRYPANOSOMA SPECIES* IN NIGERIAN CATTLE BREEDS USING MICROSCOPY.

3.1 INTRODUCTION

Trypanosomosis is a complex infectious disease of animals caused by a range of extra-erythrocytic protozoan parasites of the genus *Trypanosoma*, responsible for production losses, morbidity and sometime mortality in infected herds (Abenga *et al.*, 2002).The clinical signs of trypanosomosis depend on the species and strain of the parasite, breed of the animal involved (Anene *et al*., 1991a,b; Matioli *et al*., 1998) and the presence of vectors (Leak *et al*., 1990; Onyiah, 1997; Merkuria and Gadissa, 2011).

Clinical signs include anemia, intermittent fever, parasitaemia, lymphadenopathy, jaundice, progressive emaciation, loss of production, weakness and death, if left untreated (Akinwale *et al*., 1999; Merkuria and Gadissa, 2011). While Muturu and N'dama are considered trypanotolerant breeds because they strive well under the pressure of trypanosome infections, they act as reservoirs of the infection for other animals (Moloo *et al.*, 1992).

In Nigeria, diagnosis of bovine trypanosomosis largely depends on parasitological more often than immunological methods. Parasitological techniques, though with limitations, are very specific.

The prevalence of trypanosomosis has been extensively studied in Eastern and Northern parts of Nigeria (Abenga *et al.*, 2004; Oluwafemi *et al.*, 2007; Ahmed et al., 2007; Ezeani *et al.*, 2008; Qadeer *et al.*, 2008; Enwezor *et al*., 2009; Kamani *et al*., 2010; Samdi *et al.*, 2011). The only recent prevalence records of trypanosomosis in the Western part of the country were in Oyo and Ogun states (Ameen *et al*.,2008; Sam-Wobo *et al*., 2010).

The aim of this work therefore was to provide more information on the prevalence of trypanosomes of nomadic cattle herds and those slaughtered in abattoirs in Ogun and Kaduna States using parasitological techniques.

3.2 Materials and methods

3.2.1 Study area:

The study was conducted in two states in Nigeria. Ogun state, a transitional zone between the tropical rain forest and derived savannah zone in the south-west of Nigeria, lies between latitude 7^{o} 10'N and 3^{o} 21'E. It has two pronounced seasons, the dry season (November to March) and the wet season (April to October); and Kaduna state, located within the Northern Guinea Savannah zone of Nigeria lies between 11^{o} 10'N and 7^{o} 38'E (Figure 3.1)

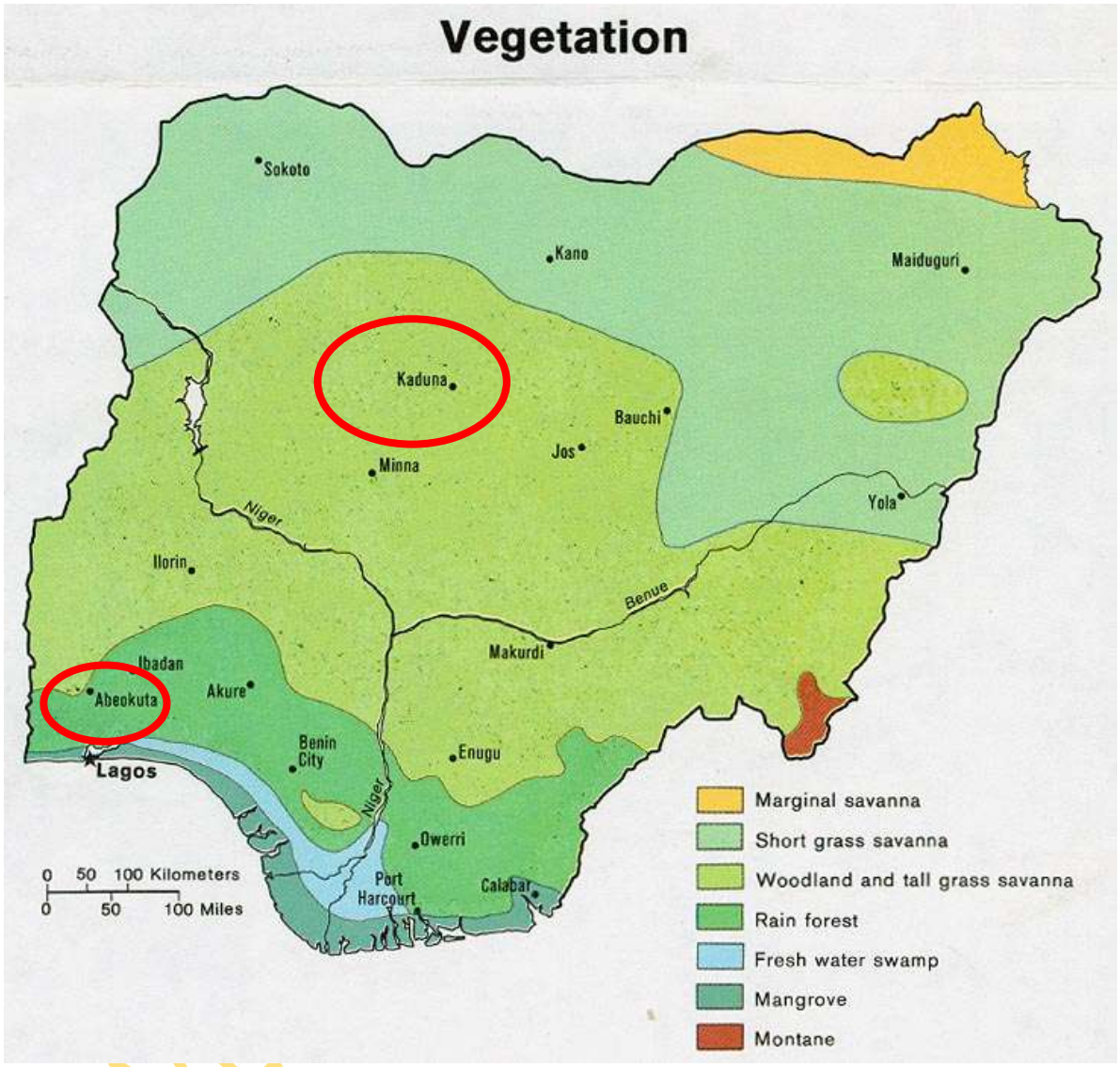

Figure 3.1: Map of Nigeria showing the two states where samples were collected

3.2.2 Study population:

A total of 411 cattle (282 females and 129 males) of ages ranging from 9 months to 6 years were sampled. They consisted of Muturu, N'Dama, Red Bororo, Sokoto Gudali and White Fulani breeds. Three hundred and eight cattle kept under traditional management system of free grazing (nomadic) and 103 from various abattoirs and slaughter slabs were randomly selected for sampling. The samples from the abattoir and various slaughter slabs were collected during students ambulatory and meat inspection exercise. Animals of one year and under were considered young calves, whereas those above this age were regarded as adults. Animals with history of recent trypanocidal treatment and those from institutional farms were excluded from this study. The age of selected cattle were determined using their dentition (Lasisi *et al.*, 2002) and for the purpose of this study, the body conditions were assessed and scored good (1) if the body skin is smooth, well covered with heavy deposit of fat and poor (2) if they show two of the following: marked emaciation, transverse process project prominent, spine appear sharply, individual dorsal spines are pointed to the touch, hips, tail, head and ribs are prominent as described by Nicholson and Butterworth (1986)

3.2.3 Sample collection:

Five milliliter (5ml) of blood samples were collected from the jugular vein of each cattle into 5ml tubes containing ethylene diamine tetraacetic acid

(EDTA) as anticoagulant (1mg/ml of blood) and 5ml tubes without the EDTA for serum analysis. The samples were transported in mobile refrigerator to the laboratory within 3 hours of collection. The blood samples without the anticoagulant were set on tray slanted and allowed to stay for 24 hours in the laboratory for serum harvest. Sera were collected in clean and sterile bottle and stored in -20^{o}C freezer until use while the blood, in the EDTA bottles were stored at 4^{o}C prior to DNA extraction.

3.2.4 Microscopical screening of the blood samples:

3.2.4.1 Haematocrit centrifugation technique

From each tube containing anticoagulant, blood was transfer into three capillary tubes which were sealed at one end with plasticin. The capillary tubes were spun in microhaematocrit centrifuge at 3000 rpm for 5 minutes. After centrifugation, the packed cell volume (PCV) was determined by haematocrit centrifugation technique. The buffy coat and upper most layer of red blood cells of one capillary tube was extruded onto a microscope slide and examined with a phase-contrast microscope at x 400 magnification (Murray *et al.*, 1977) for the presence motile trypanosomes. Not less than 50 microscopical fields were examined before each sample was declared positive or negative.

3.2.4.2 Thin and thick blood smear

The haematocrit centrifugation technique (HCT) positive samples were further processed as thin smear and stained with Giemsa for trypanosome species identification. Thick blood smear was also prepared, stained with Giemsa and all examined under x 100 oil immersion objective lens (x1000 magnification).

3.2.4.3 Determination of parasitaemia

A wet film of the blood from the sample cattle was made under a 7 x 22-mm cover glass as described by Herbert and Lumsden, (1973). The quantity of blood was just sufficient to fill the whole space under the cover glass when it was pressed down gently. The film is examined under x400 magnification, and a field is chosen in which the cells are evenly distributed. The examined fields were compared with the chart of Lumsden as shown below to estimate the number of trypanosomes per milliliter of blood sampled.

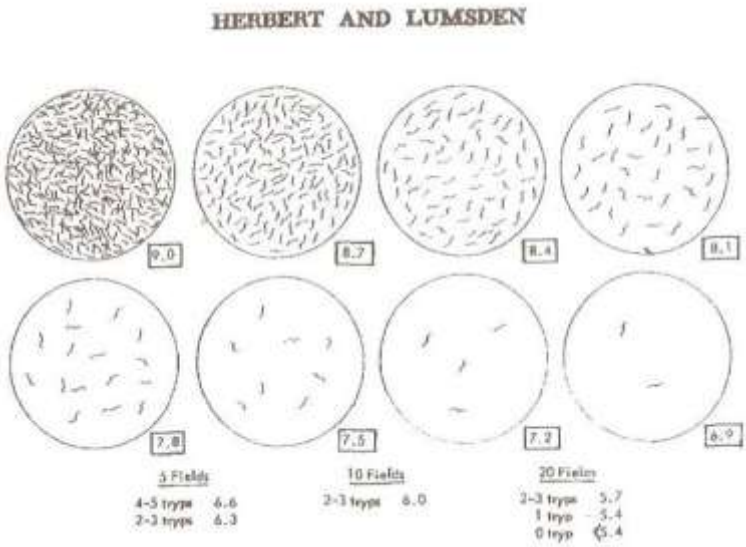

Figure 3.2: Schematic diagram of the trypanosomes concentration fields used in the rapid estimation of parasitaemia in infected animals. Diagram was copied from the paper of Herbert and Lumsdeen, 1975.

3.3 Result

3.3.1 Parasitaemia and PCV:

The mean ± SEM parasitaemia of infected cattle were 2.31 x 10^6 ± 1.02 x 10^6, 5.13 x 10^5 ± 1.45 x 10^6 and 1.37 x 10^6 ± 6.30 x 10^5 for *T. brucei*, *T. congolense* and *T. vivax*-infected cattle, respectively. The mean ± SEM PCV of *T. vivax*-infected cattle (30.8 ± 2.08%) was relatively lower than *T. brucei* (32.8 ± 3.25%) and *T. congolense*-infected cattle (33.1 ± 2.59%)(Table 4), but generally, there was significant difference ($p < 0.05$) between the PCV of non-infected cattle (37.3 ± 1.53%) and infected cattle (31.11 ± 3.04%) (Table 3.1)

3.3.2 Prevalence of trypanosomes using microscopy

Observation on the parasite detection using microscopy showed that 62 (15.1%) samples were infected with one or more species of trypanosomes. Of this, 14 (3.4%), 19 (4.6%) and 23(6.1%) samples were single infection of *T. brucei*, *T. congolense* and *T. vivax*, respectively. Six samples were positive for mixed infections of *T. brucei* and *T. congolense*, *T. brucei* and *T. vivax*, *T. vivax* and *T. congolense* and, *T. brucei*, *T. congolense* and *T. vivax* at prevalence rates of 1(0.2%), 1(0.2%), 2 (0.4%) and 2 (0.4%), respectively (Table 3.2)

Table 3.1: Parasitaemia and the Packed Cell Volume of trypanosomal infected cattle detected by microscopy

Infection	Parameter	
	Parasitaemia (mean ± SEM)	PCV (%) (mean ± SEM)
Trypanosoma brucei	$2.31 \times 10^5 \pm 1.02 \times 10^6$	32.8 ± 3.25
Trypanosoma congolense	$5.13 \times 10^5 \pm 1.45 \times 10^6$	33.1 ± 2.59
Trypanosoma vivax	$1.37 \times 10^6 \pm 6.30 \times 10^5$	30.8 ± 2.08

.

Table 3.2: Prevalence of Trypanosoma species using Microscopy

Trypanosoma species	Number and Percentage detected
Trypanosoma brucei	14 (3.4)
Trypanosoma congolense	19 (4.6)
Trypanosoma vivax	23 (6.1)
T. brucei and T. congolense	1 (0.2)
T. brucei and T. vivax	1 (0.2)
T. vivax and T. congolense	2 (0.4)
T.brucei, T.congolense and T. vivax	2 (0.4)
Total	62 (15.1)

3.3.3 Prevalence of *Trypanosoma species* based on the sex, age, body condition.

Out of 129 males and 282 females cattle examined, 22 (17.05%) and 40 (14.18%) were positive but the difference was not significant $p < 0.05$). However, prevalence between body condition score were significantly different (between the cattle with poor body condition 46 (13.52%) and those with good body condition 16 (22.61%), there was no statistical difference in the prevalence of trypanosomes of cattle less than a year 15 (17.44%) and those greater than a year 47 (14.46%). The prevalence of *Trypanosoma species* in those cattle sampled from Ogun State 49 (18.49%) were significantly higher than those sampled from Kaduna State 13 (8.90%) (Table 3.3).

3.3.4 Effect of breed on the prevalence of Trypanosoma species in Ogun and Kaduna States

The breed of cattle sampled include Muturu (112), N'Dama (11), Sokoto Gudali (68) and White Fulani (220). The prevalence of Trypanosoma species in White Fulani cattle 29(7.0%) was significantly higher ($p < 0.05$) than Muturu (20 (4.6%), N'Dama 2(0.5%) and Sokoto Gudali 11(2.7%) (Table3.4).

3.3.5 Effect of parasitaemia on the PCV of the infected cattle

Correlation analysis of the parasitaemia and the PCV of trypanosomes infected cattle revealed negative correlation (r = -0.2020) between the two parameters. Among the species of *Trypanosoma species* detected, the negative effect of *Trypanosoma vivax* was more on the PCV of infected cattle (r = -0.7786), followed by *T. congolense* (r = -0.4868) and *T. brucei* (r = -0.3461) (Table 3.5).

3.3.6 Effect of microscopical detection technique on the prevalence of Trypanosoma species in cattle.

The microscopical techniques employed in this study include wet mount(wet smear), haemtocrit centrifuge technique (HCT), thin smear and thick smear. The rate at which the laboratory techniques used detected the parasites were not significantly different but HCT 56 (13.0%) was more sensitive than wet mount, thin and thick smears which had 56(13.0%), 42 (10.2%) and 52 (12.6%) prevalence, respectively (Table 3.6).

Table 3.3: Effect of sex, age, body condition and sample location on prevalence of *Trypanosoma species*.

Variable		Number and percentage positive	Number and percentage negative	Total number sampled
Location	Ogun	49 (18.49)**	216 (81.91)	265
	Kaduna	13 (08.90)*	133(91.10)	146
Body Condition	Good	46 (13.52)*	294 (86.48)	340
	Poor	16 (22.61)**	55 (77.39)	71
Age	> 1 year	47 (14.46)*	278 (85.54)	325
	< 1 year	15 (17.44)*	71 (82.56)	86
Sex	Male	22 (17.05)*	107 (82.51)	129
	Female	40 (14.18)*	242 (85)	262

Value with different superscript are significantly different ($p < 0.05$)

Table 3.4: Effect of breed on the prevalence of *Trypanosoma species* in some part of Ogun and Kaduna States

Cattle breed	Number and percentage sampled	Number and percentage positive
Muturu	112 (27.3)	20 (4.6)*
N'Dama	11 (2.7)	02 (0.5)*
Sokoto Gudali	68 (16.5)	11(2.7)*
White Fulani	220 (53.6)	29 (7.0)**
Total	411	62 (15.1)

Value with different superscript are significantly different ($p < 0.05$)

Table 3.5: Correltion between the parasitaemia and the PCV of infected cattle sampled from Ogun and Kaduna States (correlation analysis of parasitaemia and PCV)

Trypanosoma zpecies			***T. brucei***			***T. congolense***			***T. vivax***		
	A	**B**		**A**	**B**		**A**	**B**		**A**	**B**
A	**1.0**		**A**	**1.0**		**A**	**1.0**		**A**	**1.0**	
B	**-0.2020**	**1.0**	**B**	**-0.3461**	**1.0**	**B**	**-0.4868**	**1.0**	**B**	**-0.7786**	**1.0**

Key:

A= PCV, B= parasitaemia

Table 3.6: Relative sensitivity of microscopical detection technique on the prevalence of Trypanosoma species in samples collected from Ogun and Kaduna States.

	Wet mount	HCT	Thin smear	Thick smear
T. brucei	14	15	08	13
T.congolense	18	21	15	19
T. vivax	24	25	19	20
Total (%)	56	61	42	52

Note: The mix infections were not put into consideration.

3.4 Discussion

This study has shown that animal Trypanosoma infection is prevalent in the study areas in Nigeria.

The results of microscopy screening 15.1% prevalence in the present study falls within the range of 5.3–18.57% reported by other wokers (Kalu and Lawani, 1996; Abenga *et al*.,2002; Enwezor *et al*., 2009) in Nigeria and elsewhere in Africa (Mamoudou *et al*., 2006; Merkuria and Gadissa, 2011). It was generally known that trypanosomosis was more prevalent in the derived savannah zonethan the forest zone (Ogunsanmi *et al.*, 2000). Consequently, the observed higher prevalence in Ogun than Kaduna State differ from the observation of Ogunsanmi *et al.* (2000). The study shows that T. vivax was more prevalent by microscopy while T. brucei was the least. This finding agrees with the reports of some authors in Nigeria and elsewhere (Abenga *et al.*, 2004; Ahmed *et al.*, 2007; Enwezor *et al.*, 2012 and Fasanmi *et al*., 2014) but does not agree with the report of Ogunsanmi *et al.*, (2009), Samdi et al., (2010) and Samdi *et al.*, (2011). The variation in results and findings may be related to diagnostic skill of individual identifying the trypanosomes. It is well documented that *T. brucei* is pleomorphic (Urquhart *et al.*, 2006) and the long, slender form of this species may be mistaken for *T. congolense* and *T. vivax* because of their similar morphology. While the increasing migration and importation of cattle to the forest zone of Nigeria may be responsible for higher prevalence of trypanosomes in Ogun State due to increasing number of

cattle on which the *Glossina* and non-biological vectors can feed, it is also likely that the condition (breeding habitat) is favorable to the multiplication of trypanosomes in the gut of tsetse flies. Significantly higher prevalence of Trypanosoma infection in cattle with poor body condition observed in the study may imply that the immune mechanism of those cattle was compromised. Microscopical methods of Trypanosoma species identification have different advantage and limitation. The higher prevalent of Trypanosoma species in the White Fulani than other breeds may be connected with the fact that more of the white Fulani breed was sampled. Parasitaemia was higher in *T.vivax* infection than *T. congolense* and *T. brucei* infections. This may be associated with ease of transmission of *T. vivax* by the *Glossina* vector in which development from the typical trypomastigote to the effective metacyclic stage is confined mostly, to the proboscis which make their transmission easier (Moloo and Gray, 1989). The significant effect of *T. vivax* parasitaemia on the mean PCV values of infected cattle in this study may be due to greater mechanical effect of trypanosomes on the red blood cells. Also, report of Paim *et al.* (2011) indicated that reduction of 24% in the PCV of murine model trypanosomosis was attributed to the increase of IFN-γ, TNF-α and IL-1 therefore, indicating that increased levels of cytokines are associated with anemia that is directly affected by the level of parasitaemia of infected animals. It could also implies that *T.vivax* is more pathogenic in cattle than *T. congolense* and *T. brucei,* as reported by Anosa (1983) and Saidu

et al. (1984), it does not agree with the findings of Sekoni *et al.* (1990) who reported *T. congolense* to more pathogenic in sheep.

Prevalence of trypanosomes by microscopy in Ogun and Kaduna States support the previous report of various authors in Nigeria. The advantages of HCT, wet mount and thin smear techniques clearly show that these three laboratory techniques should be conducted for effective detection and identification of *Trypanosoma species.*

4.0 MOLECULAR SURVEY OF TRYPANOSOMES OF CATTLE IN OGUN AND KADUNA STATES USING SPECIES SPECIFIC OLIGONUCLEOTIDES POLYMERASE CHAIN REACTION

4.1 Introduction

Trypanosomes, the agents of human and animal trypanoaomosis are unicellular organisms. The sub-class Duttonella and Nanomona are monomorphic while some species in the Trypanozoon sub-group are pleomorphic in nature which has made the accurate diagnosis of trypanosomes to a large extent very difficult.

In Nigeria, diagnosis of bovine trypanosomosis largely depends on parasitological and immunological methods. Parasitological techniques have significant limitations exemplified by inability to differentiate between some species in the sub-groups, for example, *Trypanosoma brucei* and *Trypanosoma evansi* except through the molecular composition of their kinetoplastDNA (kDNA) (Artama *et al.*, 1992; Feng-Jun *et al.*, 2007). Within species, parasitological methods can identify *Trypanosoma congolense* but not other members of the sub-groups. Hence, parasitological technique lacks the sensitivity and the precision required for accurate epidemiological survey of *Trypanosoma* species and sub-species for the purpose of adequate therapeutic and prophylactic control measures. Immunological techniques (i.e. enzyme linked immunosorbent assays, card agglutination and fluorescent antibody tests) on the other

hand are good for large scale epidemiological studies (Greiner *et al.*, 1997) but not sensitive enough to detect and differentiate between current and previous infections, also leading to false positive results (Desquesnes and Tresse, 1996).

Molecular technique such as polymerase chain reaction (PCR) has shown to be more sensitive and precise than the aforementioned techniques (Moser *et al.*, 1989; Pinchbeck *et al.*, 2008). The technique, though expensive and relatively new to certain parts of Africa, is so sensitive that parasitaemia as low as10 parasites per milliliter of blood can be detected using PCR (Desquesnes and Davila, 2002; Delespaux *et al.*, 2003). Due to its sensitivity, it has been used in some parts of Africa to ascertain the incidence, prevalence and characterization of trypanosome strains (Solano *et al.*, 1999; Mugittu *et al.*, 2001; Simukoko *et al.*, 2007; Balmer and Caccone, 2008; Cordon-Obras *et al.*, 2009). However, only *Trypanosoma vivax* Y58 strain, a field isolate that was isolated from cattle in Yakawada, Zaria in 1976, has been characterized from Nigeria (Morlais *et al.*, 2001).

The use of PCR as a better diagnostic tool to ascertain the incidence and prevalence of *Trypanosoma* infection has been advocated (Desquesnes and Tresse, 1996; Miyamoto *et al.*, 2006; El-Metanaway *et al*., 2009) but has not yet been applied in Nigeria. The present study was designed to determine the prevalence of *Trypanosoma* species and sub-species in Nigerian cattle using species specific PCR.

4.2 Materials and methods

4.2.1 Study area, population and sample collection

The study areas, sampled population and sample collection were as stated in Chapter three.

4.2.2 DNA extraction:

DNA was extracted from the blood in EDTA bottle using Quick-gDNA™ MiniPrep (Zymo Research Corporation, Irvine, CA 92614, U.S.A) as described by the manufacturer. The protocol involves: 400µl of genomic lysis buffer was added to 100 µl of blood, thoroughly mixed by vortexing for 4-6 seconds and incubated at room temperature for 5 – 10 minutes. The mixture, blood and the lyses buffer, was transferred to a spin column in a collection tube and centrifuge at 10,000 x g for 60 seconds after which the collection tube with the flow through was discarded and the spin column transferred to a new collection tube. Two hundred microliter of prewash buffer was added to the spin column and centrifuged at 10,000 x g for 60 seconds after which 500 µl of genomic DNA wash buffer was added to the spin column and centrifuged at 10,000 x g for 60 seconds. To elute the DNA, the spin column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease free water was added to the spin column and incubated at room temperature for 2-5 minutes, centrifuged at 16,000 x g for 30 seconds. Quantification of DNA yield and assessment of quality were done using Nanodrop ND-100 UV/Vis Spectrophotometer

(Nanodrop Technologies, Inc., DE, U.S.A). The eluted DNA was stored at -20^{o}C until use.

4.2.3 Primer sets and optimization:

Eight sets of primers were selected for optimization based on the published evaluations. The sets of primers were optimized with DNA extracted from the blood of cattle parasitologically positive for *T. vivax, T. congolense, T. brucei* and *T.evansi* that were obtained from National Veterinary Research institute, Vom, Nigeria which led to final selection of six sets of primers for the screening.

Table 4.1 shows primer sets names, sequences, sizes of the expected amplicon and references. The target DNA for amplification in *T. vivax, T. brucei, T. evansi* and *T. congolense* savannah strain genome are the gene encoding the diagnostic antigen (400bp fragment) (Masake *et al.*, 1997), 177bp repetitive element (Sloof *et al.*, 1983), 164bp repetitive element (Masiga *et al*., 1992) and 316bp repetitive element (Masiga *et al*., 1992) respectively. Animals were checked blindly for the presence of *T. congolense* Forest and Kilifi strains because of unavailability of known or identified strains of this species.

Table 4.1 Sequences of the species specific oligonucleotide used in this study and their expected fragment sizes.

Primer set	Species	Sequences	Expected sizes	References
TBR 1	*T. evansi*	GAATATTAAACAATGCGCAG	164bp	Masiga *et., al* (1992)
TBR 2		CCATTTATTAGCTTTGTTGC		
TBR 1*	*T. brucei*	CGAATGAATAAACAATGCGCAGT	177bp	Sloof *et al.,* (1983)
TBR2*		AGAACCATTTATTAGCTTTGTGC		
TCS 1	*T. congolense* savannah-type	CGAGCGAGAACGGGCAC	316bp	Majiwa and Otieno, 1990
TCS 2		GGGACAAACAAATCCCGC		
TCF 1	*T. congolense* forest-type	GGACACGCCAGAAGGTACTT	350bp	Masiga *et al*., (1992)
TCF 2		GTTCTCGCACCAAATCCAAC		
TCK 1	*T. congolense* kilifi-type	GTGCCCAAATTTGAAGTGAT	294bp	Masiga *et al*., (1992)
TCK 2		ACTCAAAATCGTGCACCTCG		
ILO1264	*T. vivax*	CAGCTCGCCGAAGGCCACTTGGCTGGG	400bp	Masake *et al.*, (1997)
ILO1265		TCGCTACCACAGTCGCAATCGTCGTCTCAAGG		

TBR 1* & TBR 2*; the primer set has different sequences from TBR1&2

4.2.4 Detection of Trypanosoma species by PCR:

Polymerase chain reaction amplification was performed in 20µl final reaction volume containing equivalent of 20ng of genomic DNA, 10mM Tris-HCl, pH 8.3, 1.5mM MgCl2, 50µM KCl, 200µM each of dNTPs, 40ng of each of the primers and 1unit of Thermus aquaticus DNA polymerase (Bioneer USA). The reactions were placed in C 1000 Series thermocycler (BIORAD, USA).

The reaction conditions were as follows:

T. brucei and *T. evansi*; Initial denaturation at 94^{o}C for 4 min followed by 35 cycles of 94^{o}C for 1 min, 55^{o}C for 1 min, and 72^{o}C for 1 min; and final extension at 72^{o}C for 5 min.

T. congolense; initial denaturation at 94^{o}C for 4 min followed by 35 cycles of 94^{o}C at 30 sec, 60^{o}C for 30 sec and 72^{o}C for 30 sec with final extension at 72oC for 5min.

T. vivax; initial denaturation at 94^{o}C for 4min followed by 35 cycles of 94^{o}C for 30sec, 60^{o}C for 45 sec and 72^{o}C for 30sec followed by final extension at 72^{o}C for 5min.

4.2.4.1 Gel electrophoresis:

Ten microliter of the PCR products were electrophoresed through 1% agarose gel in 1 x TAE (40 mM TRIS-acetate and 1 mM EDTA) at 90 V

for 80 min. along with 10µl of biological marker, GENEMate Quanti-Marker 100 bp DNA ladder (BioExpress, UT, USA). Gels were stained with GelRedR Nucleic Acid Stain (PHENIX Research Product, Candler, NC, U.S.A) at 5µl/100ml of the agarose gel suspension. After electrophoresis, the PCR products were visualized using ultra violet transilluminator (Spectroline^{R} TC 312 E) before gels were photographed using (Alpha Imager). Table 2 shows the thermocyling programs used for various species of Trypanosoma species.

4.2.4.2 Sequencing and sequence analysis:

To confirm and validate our results, five positive samples each of *T. vivax*, *T. congolense* (savannah), *T. brucei* and two positive samples each for *T. evansi* and *T. congolense* forest were selected. The PCR products of *T. vivax* and *T. congolense* (savannah and forest) strains were sequenced using Big dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the forward amplification PCR primers and AmpliTaq-FS DNA Polymerase while 30 µl of the PCR products of *T. brucei* and *T. evansi* were purified from the agarose gel using a ZymocleanTM Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA 92614, U.S.A) and then sequenced as described above. The sequences obtained were viewed and compared on Finch TV and Sequence Scanner (Applied Bioscience), manually cleaned, before they

were aligned with each other and published gene sequences of various *Trypanosoma species* using the Molecular Evolutionary Genetic Analysis (MEGA 5.05) software.

4.2.5 Statistical analysis:

Raw data were entered into a Microsoft excel spread-sheet and descriptive statistic used to summarize data. IBM SPSS Statistic 19 software (Student trial version) was used for data analysis. Prevalence of trypanosomes in studied cattle breeds using molecular technique were compared statistically using Chi-square test, the sensitivity and specificity of microscopy calculated using PCR as gold standard while the PCV was compared between the infected and non-infected cattle using Students' t-test (Paired t-test) analysis and the mean packed cell volume (PCV) values within the breeds of cattle studied were compared using one way ANOVA.

4.3 RESULTS:

4.3.1 Gel electrophoresis

Band sizes of 166bp, 316bp, 350bp and 400bp corresponding to expected band sizes of Trypanozoon group, *T. congolense (*savannah), *T. congolense* (forest) and *T, vivax*, respectively were obtained (Figure 3.1 – 3.4)

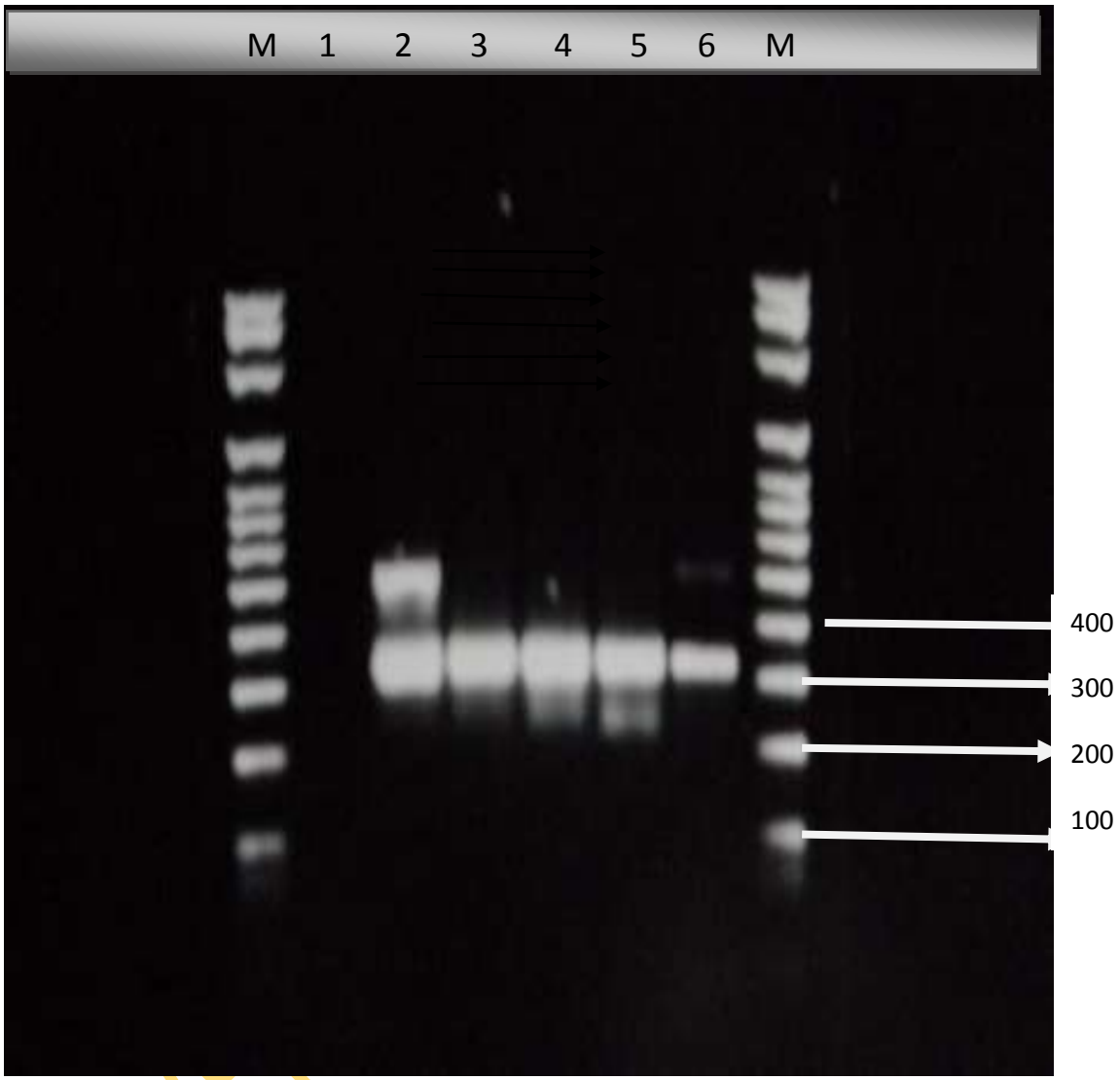

Figure 4.1: Gel electrophoresis showing *T. congolense* Savannah-type bands (316bp). M; Molecular weight marker, 1; Negative control, 2-5; *T. congolense* savannah-type, 6; positive control.

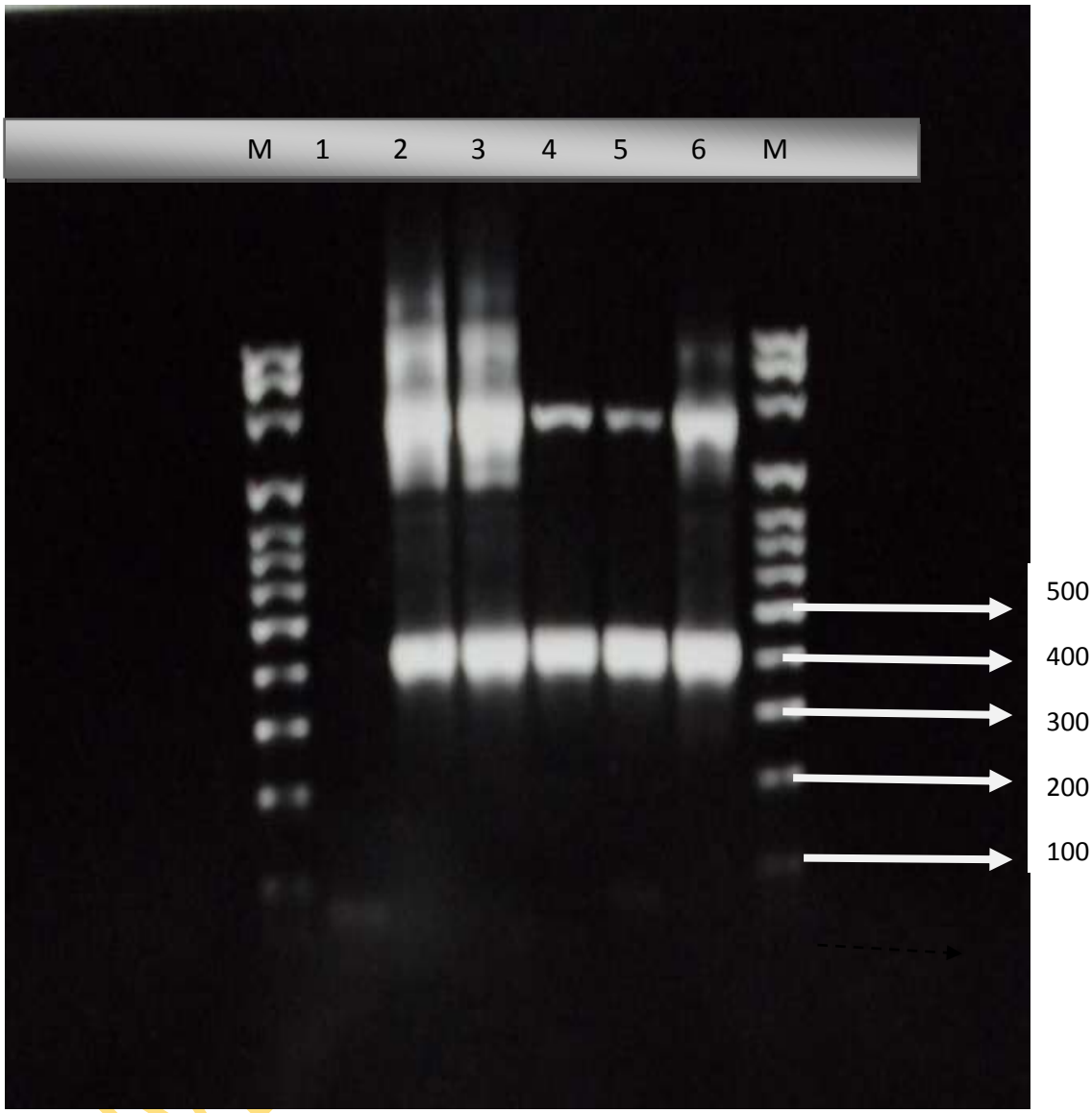

Figure 4.2: Agar rose gel electrophoresis showing *T. vivax* bands (400bp), M; Molecular marker, 1; negative control, 2 - 5; *T. vivax*, 6; positive control.

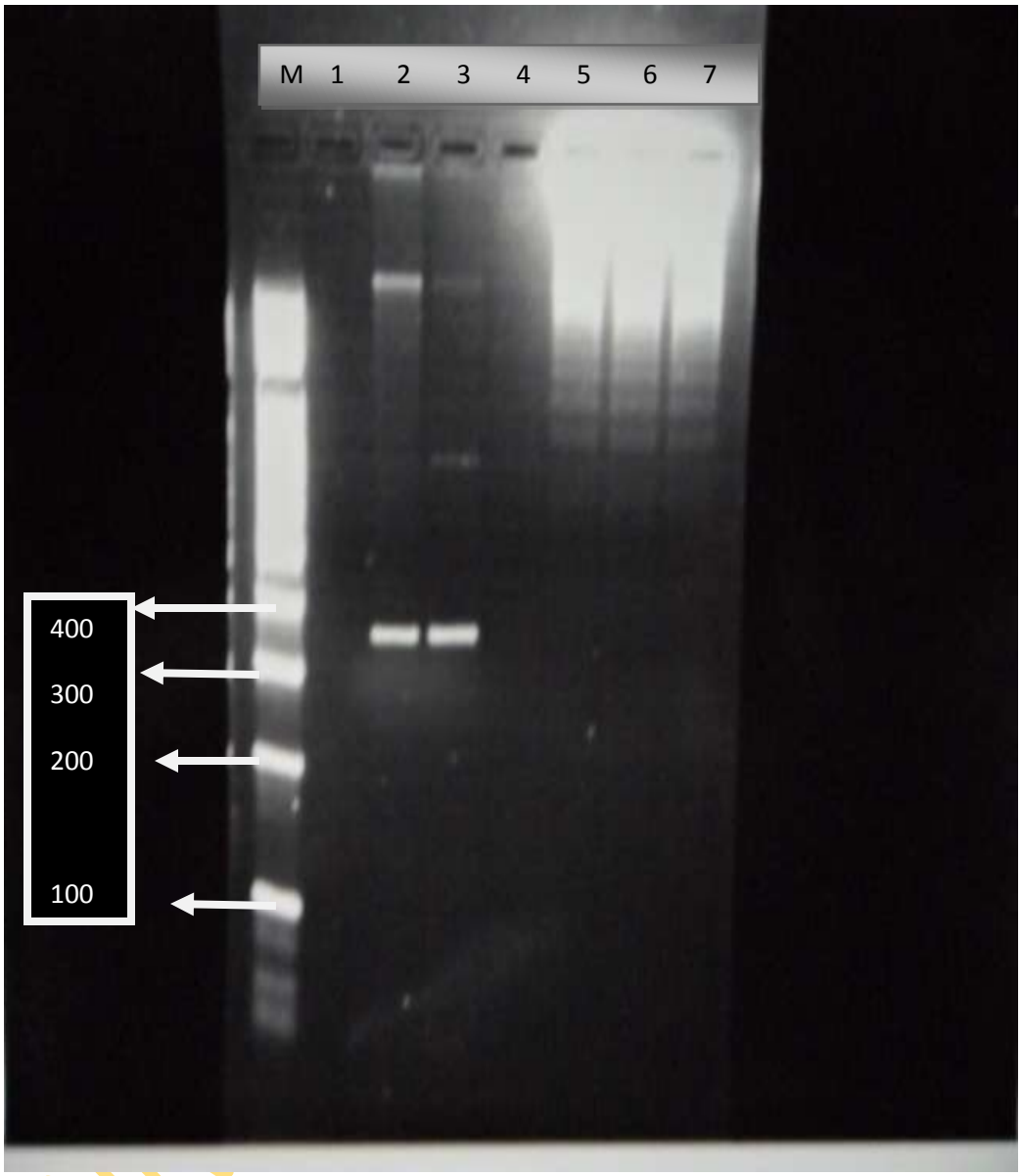

Figure 4.3: Agar rose gel electrophoresis showing *T. congolense*- forest type bands (350bp). M; 100bp molecular marker, 1; negative control, 2-3; *T. congolense- forest* type, 4 – 6; DNA samples that tested negative.

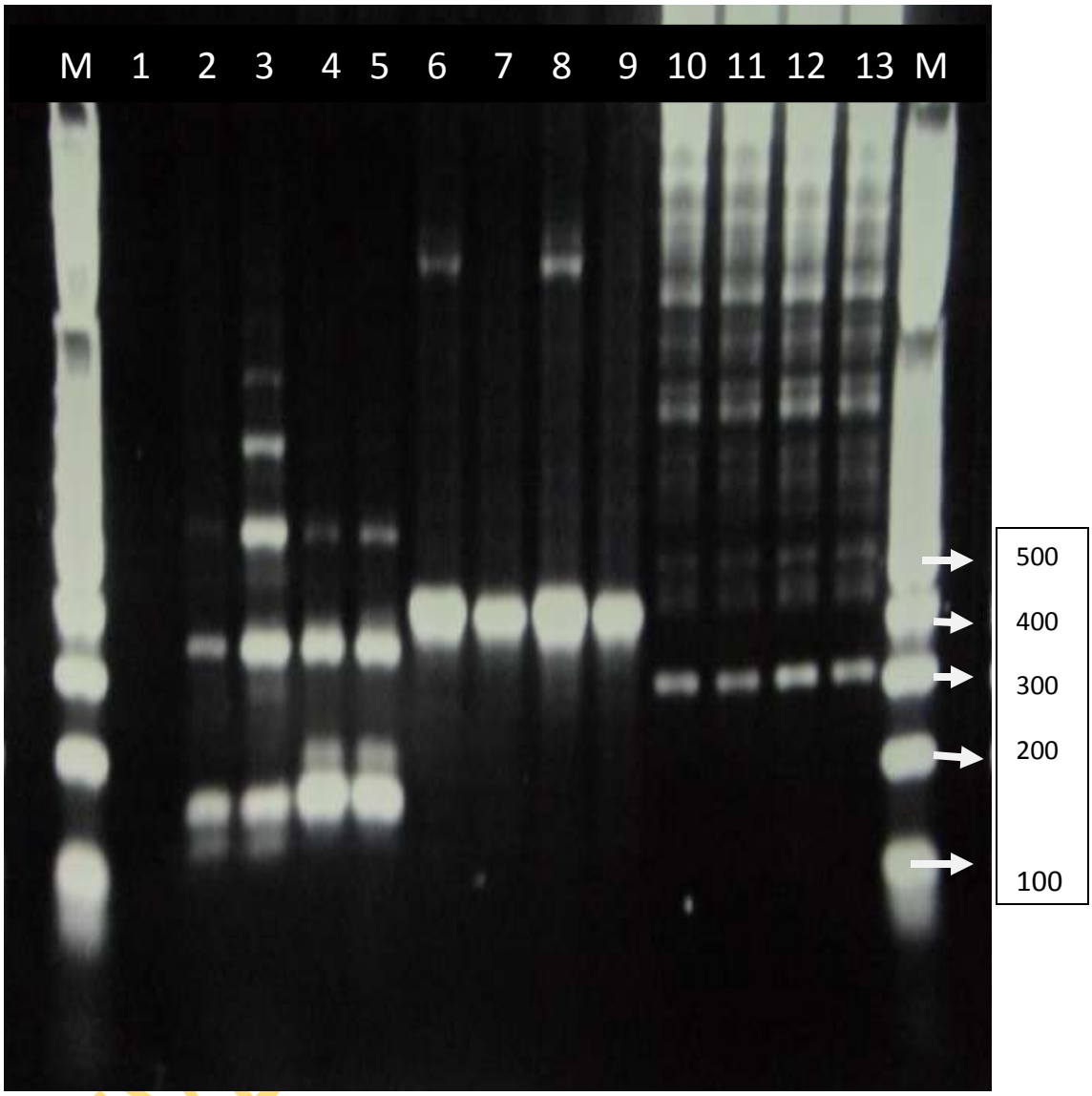

Figure 4.4: Agar rose gel electrophoresis showing *T. brucei*, *T. evansi*, (166bp) *T. vivax* and *T. congolense*-savannah type, M; molecular weight marker, 1; negative control for *T. evansi*, 2-3; *T. brucei*, 4-5; *T. evansi*, 6-9; *T. vivax*, 10-13; *T. congolense*- savannah type.

4.3.2 Sequence analysis of the amplified PCR products:

Using sequences retrieved from GenBank, the aligned *T. brucei* sequence had 97% homology with the sequence of *T. brucei* satellite DNA (K00392.1), *T. congolense* savannah-type had 100% homology with *T. congolense* IL300 (HE578911.1), *T. vivax* had 98 and 99%homology with the *T. vivax* Y486 and *T. vivax* diagnostic antigen(HE573027.1) and U43183.1), respectively, *T. congolense* forest-type had 94% homology with *T. congolense* (F) TSW 10 (S50876.1) and *T. evansi* had 94% homology with *T. brucei gambiense* (FN554966.1) (Table 4.2)

Table 4.2: Percentage homology of the detected *Trypanosoma species* from Nigeria with those available in the GenBank

Trypanosome	Homology (%)	Accession No
T. brucei	97	K00391.1
T. congo-s	100	HE57891.1
T. congo-f	94	S50876.1
T. vivax	98 -99	HE573027.1
T. evansi	94	FN554966.1

Note: *T. congo-s: Trypanosoma congolense* savannah type, *T. congo-f; Trypanosoma congolense* forest type.

4.3.3 Molecular detection of trypanosomes:

Polymerase Chain Reaction detection showed 262 samples infected by one or more species of Trypanosoma species, for an overall prevalence of 63.7% (95% CI, 59.4–68.8%).*T. congolense* was the most prevalent 48.7% (95% CI, 44.2–54.3%), followed by *T. vivax*26% (95% CI, 21.8 – 31.1%), and *T. brucei*4.4% (95 CI, 3.3 – 7.1%). All of the *T. congolense* detected were savannah-type, except for 2 samples which were single infections of *T. congolense* forest-type with prevalence of 48.2% and 0.5%, respectively. Additionally, we found 2 samples infected with *T. evansi* (0.5%). Prevalence of mixed infections was 13.9% (95% CI, 10.6–17.4%), being co-infection by *T. congolense* and *T. vivax.* Six samples were co-infected with *T. brucei, T. vivax* and *T. congolense* savannah-type, and one sample co-infected by *T. evansi, T. vivax* and *T. congolense* savannah-type. No infections by *T. congolense* Kilifi-type were detected (Table 4.3)

Table 4.3: Prevalence of *Trypanosoma species* in Ogun and Kaduna State based on PCR.

	PCR	
Trypanosoma species	Infected	Percentage
T. brucei	07	1.7
T. congolense	144	35
T. evansi	02	0.5
T. vivax	54	13.1
T.brucei and T.congolense	04	1.0
T. brucei and T. vivax	01	0.2
T. congolense and T. vivax	46	11.2
T. brucei/T.congo/T.vivax	06	1.5
Total	262	63.7

4.3.4 Effects of sex on prevalence of *Trypanosoma species* in Ogun and Kaduna States

Of the of 129 males and 282 females cattle examined, 60.5% and 63.5%, were positive for trypanosomes respectively, however, there was great difference between the infection of the female (60.5%) and male (63.5%) cattle that tested positive by PCR but not significantly different (Figure 4.5).

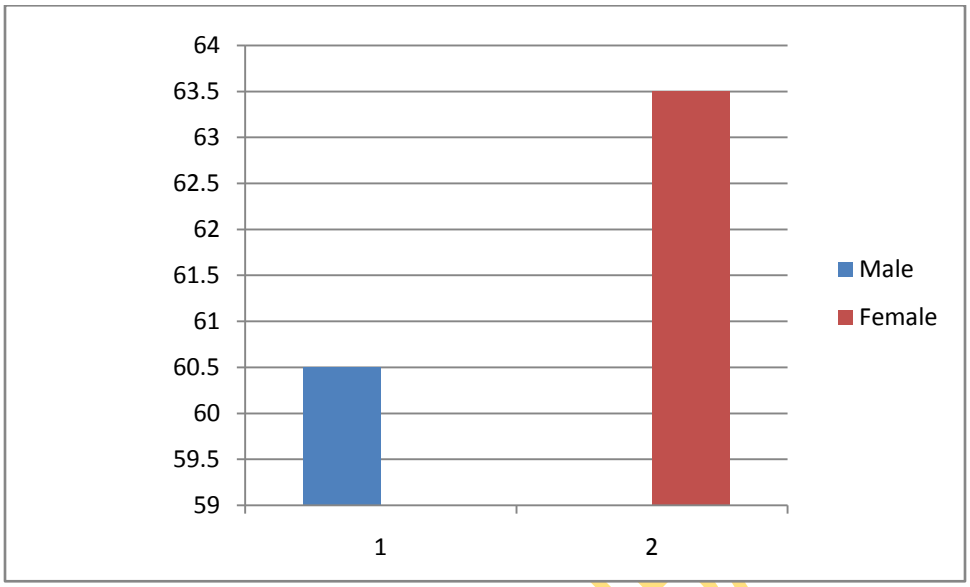

Figure 4.5: Effect of sex on the prevalence of *Trypanosoma species* in some Nigerian cattle breeds

4.3.5 Effects of age and body condition on prevalence of *Trypanosoma species* in Ogun and Kaduna State:

Of the 411 cattle sampled, 325 (79.07%) and 86(20.92%)e were below and above one year, respectively. The rate of infections among these age group were 59.3% and 63.4% for cattle below and above one year, respectively, but their means were not statistically significantly different ($p > 0.05$). While 340 and 71 sampled cattle were of good and poor body condition, respectively, 209(60.7%) and 53(71.4%) of animal with good and poor body condition were infected (Figures 4.6 and 4.7).

4.3.6 Effect of breeds on the prevalence of *Trypanosoma species* in Ogun and Kaduna State

A total of 262(63.7%) cattle consisting of 75(67%) Muturu, 29(9%) N,Dama, 35(51.5%) Sokoto Gudali and 131(65.5%) White Fulani breeds were confirmed positive for single and mixed infections of various species and strains of trypanosomes by PCR as opposed to 19(16.9%) Muturu, 2(6.5%) N,Dama, 11(16.21%) Sokoto Gudali and 30(13.2%) White Fulani detected by parasitological method (Table 4.4)

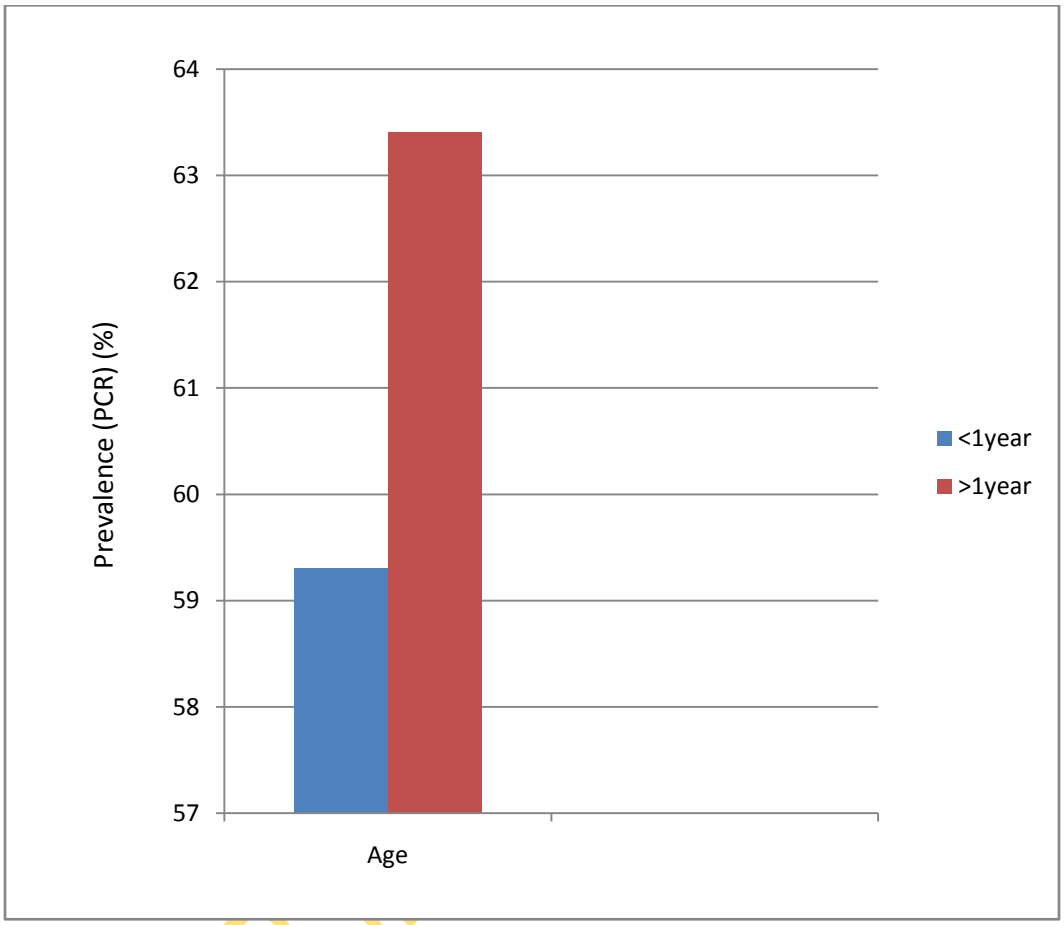

Figure 4.6: Effect of age on the prevalence of *Trypanosoma species* in some Nigerian cattle breeds

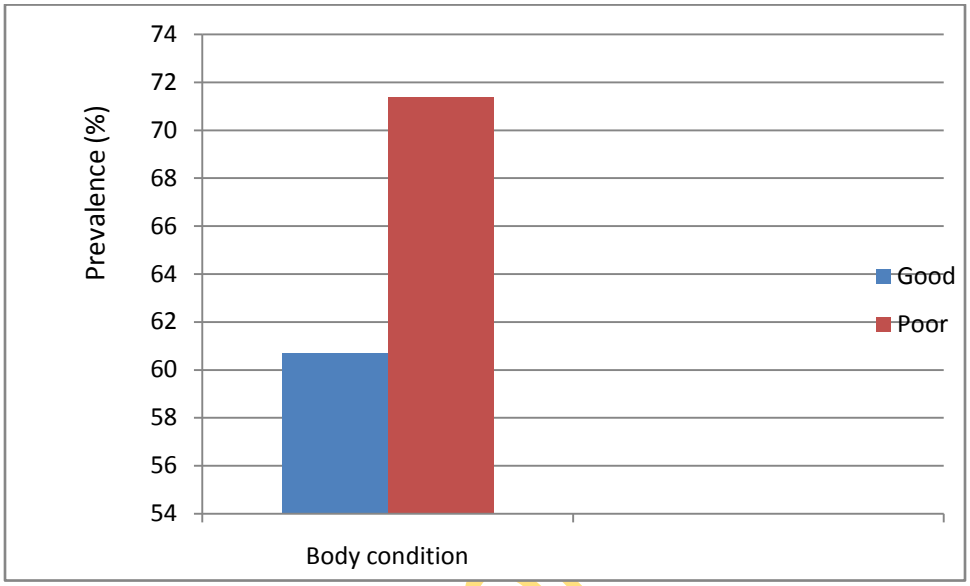

Figure4.7: Effect of body condition on the prvalense of *Trypanosoma species* in some Nigerian cattle breeds

Table 4.4: Effect of breed on the prevalence of *Trypanosoma species* in some Nigerian cattle breeds using PCR method

Breed	Prevalence	
	Number and percentage	Number and percentage positive
Muturu	112(27.3)	81(72.32)**
N'Dama	11(2.7)	04(36.36)*
Sokoto Gudali	68(16.5)	36(52.94)*
White Fulani	220(53.6)	141(64.09)**
Total	411(100)	262(63.7)

*Not significantly different; **Significantly different ($P < 0.05$)

4.3.7 PCV values of infected and non-infected Nigerian cattle breeds:

Generally, there was no significant different ($p > 0.05$) between the mean PCV of infected (32.2%) and non-infected (32.9%) cattle detected by PCR but the difference was significant ($p < 0.05$) between the *T. vivax* infected (32.95%) and non-infected (35.13%) cattle. Considering individual species infection on the PCV of infected and non-infected cattle, *T. brucei-*, *T. congolense-*, *T. vivax-* and non-infected cattle had mean PCV of 34.19 ± 1.36%, 33.97 ± o.45%, 32.95 ± 0.69% and 35.13 ± 0.45%, respectively. The cattle with mixed infection had 33.15 ± 0.80% (Table 4.5).

The mean ± SE PCV of Muturu, N'Dama, Sokoto Gudali and White Fulani infected and their non-infected counterpart were 33.38 ± 0.56% and 34.47 ± 0.99%, 33.33 ± 1.36% and 36.00 ± 1.31%, 33.84 ± 0.89% and 34.75 ± 1.05% and, 33.88 ± 0.49% and 35.51 ± 0.61% , respectively (Table 4.6).

Table 4.5: Comparison of the mean PCV values of different *Trypanosoma species*-infection in Nigerian cattle. Cattle infected with *T. evansi* was not included because it was found only in two animals infected with other species of *Trypanosoma species*. N: number of animal sampled.

Type of infection	Mean PCV (%) ± SE (N)
Non-infected	35.13 ± 0.45 (149)[a]
Trypanosoma brucei	34.19 ± 1.36 (7)[a,b]
Trypanosoma congolense	33.97 ± 0.45 (144)[a,b]
Trypanosoma vivax	32.95 ± 0.69 (54)[b]
Mixed infections	33.15 ± 0.80 (57)[b]

Values with different superscripts are significantly different ($P < 0.05$).

Table 4.6: Comparison of the mean PCV values of some infected and non-infected Nigerian cattle breeds. N: number of animals sampled.

	Non-infected	Infected
Cattle breed	Mean ± SE (N)	Mean ± SE (N)
Muturu	34.47 ± 0.99 (37)[a]	33.38 ± 0.56 (75)[a]
N'Dama	36.00 ± 1.31 (22)[a]	33.33 ± 1.36 (9)[b]
Sokoto Gudali	34.75 ± 1.05 (33)[a]	33.84 ± 0.89 (35)[a]
White Fulani	35.51 ± 0.61 (69)[a]	33.88 ± 0.49 (131)[b]

Values with different superscripts are significantly different ($P < 0.05$).

4.3.8 PCV values of some infected and non-infected Nigerian cattle breeds in relation to their body conditions:

In general, the PCV values of animals with good and poor body conditions were different. Packed Cell Volume in animals with poor body condition (28.79% ± 0.61) was on average 16.8% lower than animals with good body condition (34.50% ± 0.28). In addition, animals in good condition and with *Trypanosoma* infection had a significantly lower PCV value ($p < 0.05$) than those not infected (34.11 ± 0.34 and 35.13% ± 0.45,respectively), but animals with poor body condition did not showed any difference in PCV values between the groups of infected and non-infected (28.79% ± 0.68 and 28.80% ± 0.46, respectively) (Table 4.7).

Table 4.7: Comparison of mean PCV values of trypanosomes infected and non-infected cattle with good and poor body conditions

	PCV values
Body condition	Mean ± SE (N)
Good body condition	28.79% ± 0.61(340)[a]
Poor body condition	34.50% ± 0.28(71)[a]
Good body condition infected	34.11 ± 0.34 (116)[b]
Good body condition non-infected	35.13% ± 0.45(224)[a]
Poor body condition infected	28.79 ± 0.68(20)[a]
Poor body condition non-infected	28.80 ± 0.46(51)[a]

Values with different superscripts are significantly different ($P < 0.05$).

4.3.9 Sensitivity and specificity of microscopy in the detection of *Trypanosoma species* using molecular (PCR) methods as gold standard in some Nigeria cattle.

The levels of association between the two methods employed in the detection of *Trypanosoma species* of cattle in this study were subjected to sensitivity and specificity analysis using the PCR as a gold standard. The true negative and positive detection for microscopy and PCR were 138 and 48, respectively while the false negative and positive were 214 and 14 respectively (Table 4.7)

Based on the formula:

Sensitivity (True positive rate) = TP/P = TP/TP-FN

Specificity (True negative rate) = TN/N = TN/FP+TN

Where TP = True Positive, P = Number Positive, FN = False Negative, TN = True Negative, N = Number Negative, FP = False Positive

Therefore:

Sensitivity = 48/262 = 0.81

Specificity = 135/149 = 0.91

Table 4.8 Molecular detection (PCR) and Microscopy cross tabulation for sensitivity and specificity calculation.

	Microscopy detection		
Polymerase chain reaction	No Positive	No negative	Total
No Positive	48	14	62
No negative	214	135	349
Total	262	149	411

4.4 DISCUSSION

Existing parasitological and serological diagnostic techniques for screening blood samples to detect and differentiate bovine trypanosomes are not suitable for large-scale epidemiological analysis and precise species identification (El-Metanaway *et al*., 2009; Fernándeza *et al*., 2009). The use of PCR showed a much higher 63.7% prevalence of *Trypanosoma* infection in this study, a remarkably higher percentage than (5.3-18.57%) previously reported in Nigeria. This underscores the sensitivity of molecular screening based on PCR, and related to the difficulty of microscopic detection of parasites and especially with low levels of parasitaemia in subclinical infections. This corroborates the results of earlier workers (Desquesnes and Davila, 2002; Delespaux *et al.*, 2003; Karimuribo *et al*., 2011). The significantly higher prevalence of trypanosomal infections in Ogun State (Southern Nigeria) is consistent with heavy infestation of both biological (*Glossina spp*) and mechanical (Tabanids) vectors of trypanosomes (Ahmed, 2004). In western Kenya and Uganda, *T. b. brucei* and *T. b. rhodesiense* were found in the CNS of native cattle and were associated with significant mortality (Wellde *et al.*, 1989), hence the detection of *T. brucei* and *T. evansi* in Nigerian cattle might portend serious danger not only to cattle and other livestock but also to livestock owners and the communities at large as *T. evansi* infection has been reported in cattle and humans in India (Laha and Sasmal, 2009; Joshi *et al*., 2005). The prevalence of *Trypanosoma species* by microscopy

reported in our study falls within the previously reported in Nigeriabut higher prevalence of *T. congolense* savannah-type followed by *T.vivax* and *T. brucei* in this study using molecular methods could not be compared with any report in Nigeria due to paucity of data on molecular epidemiology of trypanosomosis but contrasts with other reports in which *T. vivax* was reported to have higher prevalence, followed by *T. congolense* and *T. brucei*, when using parasitological methods in Nigeria (Anene *et al.*, 1991a,b; Kalu, 1995;Omotainse *et al.*, 2000). B ut the present findings are in consonance with the report of Ogunsanmi *et al*. (2000) who reported higher incidence of *T. congolense* in a survey carried out in Southwestern Nigeria and Merkuria and Gadissa (2011) in Northwestern Ethiopia. This could be related to lower parasitaemia of *T. congolense* infections compared to those infected with *T. vivax* in this study.

The agreement of our parasitological findings with the low level of mixed infections reported by other workers in the country (Kalu, 1995; Abenga *et al*., 2002; Enwezor *et al.*, 2009) is an indication that parasitological method may not be suitable for epidemiological survey of trypanosomes. However, PCR results revealed higher levels of mixed infections, consistent with the findings of other researcher that used PCR elsewhere in Africa (Pinchbeck *et al*., 2008). We detected *T. congolense* riverine forest-type in two of the animals sampled and to the best of our knowledge this may be the first report of the existence of subgroup of *T. congolense*

in Nigeria. The apparently higher prevalence of *T. congolense savannah sub-type* could be an indication that its transmission is highly favored by the obligate cyclical vector , *Glossina*or the *T. vivax* and *T. brucei* respond better to the trypanocidal drugs, diminazene aceturate and homidium chloride, respectively. It could also be due to over-representation of *T. vivax* infections through more serious symptoms that induces producers to seek diagnosis and treatment, compared to infections by *T. congolense*. The highest prevalence of *T. congolense* savannah type in this study is in partial agreement with Solano *et al*. (1995) and De La Rocque *et al.* (1999) that the savannah type was predominant in tsetse flies as well as in cattle, but disagrees with their observations that the riverine/forest-type was only present in the vectors since we detected this in two of the cattle sampled.

While morphological identification error could be responsible for the inability of the PCR assay to detect two *T. congolense* and four *T. vivax* – positive animals detected by microscopy in this study, it could also be attributed to high concentration of template DNA that result in inhibition of the PCR amplification processes and or due to primers sets used. Similar observations were reported by Desquenses (1997) and Gonzales *et al*. (2003) who after diluting sera and blood spot eluate samples, respectively, obtained improvement in PCR detection rates and Gonzales *et al*. (2003) who were able to amplify *T. vivax* DNA using a set of new

primers (TVW A/B) from four of the samples that were classed as PCR negative when primer sets (TWJ1/2) were used.

The PCV results for infected and non-infected cattle are in accordance with the reports of Van den Bossche and Rowland (2001) and Simukoko *et al.* (2011) who reported that factors such as nutrition affect the PCV of rural cattle. Anemia, one of the cardinal signs of trypanosomosis (Getachew, 2005), could also be caused by other haemoprotozoan parasites and helminthes (Radostits *et al*., 2007). As a result of this, PCV values alone should not be used as a diagnostic parameter for trypanosomosis, except where diseases causing anemia are inapparent, then low PCV may be a good indicator of trypanosomal infection (Marcotty *et al*., 2008). Mean PCV of *T. vivax*-infected and mixed infected were significantly higher than non-infected cattle. Trail *et al*. (1994) and Rowlands *et al*. (2001) reported significantly low infection rate in calves below 15 months, similar to our findings in which there were significant differences in the prevalence of *T. vivax* and mixed infections between calves, below12 months and those above one year. This could be due to longer exposure of older animals to the disease vectors and higher chances of being infected and possession of stronger immunity. Although we found lower prevalence in N'dama and Muturu cattle, a reportedly trypanotolerant breeds (Mattioli *et al*., 1998). This contrasts the higher prevalence recorded in Kaduna state where Sokoto Gudali and White Fulani were the main animals sampled. Since some of the animals sampled

may be cross bred with trypanotolerant breeds, this could play a role in low parasitaemia levels since crossbred offspring may display appreciable levels of low parasitaemia (Orenge *et al.*, 2011) because there are no structured breeding programs by small cattle producers in Nigeria. In conclusion, this study has shown that PCR can be used effectively in extensive epidemiological surveys to validate carrier status of animal *Trypanosoma* infection in Nigerian cattle. The finding of new subgroup of *Trypanosoma spp* in Nigeria and the relatively high abundance of mixed infections are of clinical significance. This study also suggests that *T. congolense* may be the most prevalent species in Nigeria.

CHAPTER 5

5.0 EVALUATION AND COMPARISON OF GENETIC DIVERSITY OF *TRYPANOSOMA* SPECIES DETECTED IN NATURALLY INFECTED CATTLE IN NIGERIA USING HYPER-VARIABLE REGION, INTERNAL TRANSCRIBE SPACER 1 (ITS1), OF RIBOSOMAL DNA (rDNA) GENE.

5.1 INTRODUCTION

Trypanosomes are the most important vector borne protozoan parasites of cattle in Sub-Saharan African countries (FAO, 2002) including Nigeria (Enwezor *et al.,* 2006). Trypanosomosis of cattle, a disease complex caused by *Trypanosoma species*, has caused annual loss of about 1.0 – 1.2 billion dollar in sub-Saharan Africa (FAO, 2002). The pathogenic species in cattle are *T. vivax*, *T. congolense* and *T. brucei* (Radostits 2007), while *T. evansi* the causative of surrain equine has also been reported in cattle in Nigeria and elsewhere around the world.

The pathogenicity of these *Trypanosoma spp* varies (Gardiner and Mahmoud, 1992) depending on parasite strain, endemicity and host species (Batista *et al*., 2007). *Trypanosoma vivax*, in addition to anemia, causes predominantly a severe wasting disease of cattle in West Africa and East Africa, where in addition it also causes haemorrhagic syndrome in cattle (Magona *et al.,* 2008) while the clinical signs in South America varies from asymptomatic to chronic conditions with rarer outbreak of

severe disease (Osorio *et al.,* 2008).Though the African *T. vivax* shares the same morphological structure with the new world (South America) *T. vivax,* the latter has lost the ability to grow and multiply in tsetse fly and can only be mechanically transmitted by blood-sucking flies (Osorio *et al.*, 2008). Currently, the nannomonas subgenus contains two major clades with one containing the *T. congolense*: Savannah, Forest and Kilifi subgroups and the other containing *T. simiae, T. godfreyi* and *T. simiae* Tsavo (Gibson, 2003). While *T. congolense* riverine/forest strains are generally said to be less pathogenic than the savannah strain, there exist a variation in the pathogenicity of the savannah strains from different geographical locations (Bengaly *et al.,* 2002; Bengaly *et al.,* 2002; Masumu *et al.,* 2006; Van Den Bossche *et al.,* 2011). Infections with *T. brucei,* on the other hand, have been described as being chronic and sub-patent with minimal impact on the health of the animal (Van den Bossche *et al.,* 2004).

It is an established fact that different species of trypanosomes could infect a single tsetse vector and thus a single mammalian host therefore a genetic recombination could occur, leading to a novel genotype which may elicit completely different clinical manifestation in different hosts. During the last decade, researchers have employed a variety of molecular techniques to address questions about phylogeny, evolution, and population diversity among the pathogenic trypanosomes in African and South American (Fasogbon *et al.,* 1990; Cortez *et al.,* 2006; Rodrigues *et al.,* 2008; Hirhisa

et al., 2009; Adams *et al.*, 2010). Analysis of 18S (small subunit) rRNA, internal transcribed spacer (ITS) and, more recently, 5.8S rRNA gene sequences (Cortez et al., 2006) has figured heavily in these studies. While these studies have been extensively carried on *T. brucei* (Jess *et al.*, 1990; Stevens *et al.*, 1998; Parson *et al.*, 2007 and Jackson, 2007), minimal studies have been done on *T. congolense* and *T. vivax* around Africa and South America where the disease is prevalent but not in Nigeria. While *T. vivax*, especially Y486 strain, an isolate from Nigeria has been used as a reference for West African strains, the sample sizes of most studies, apart from Duffy et al. (2009), were very small and could barely be used to extrapolate any meaningful molecular epidemiological conclusion.

In Nigeria where single and mixed infections of animal trypanosomosis are frequently reported, the genetic relatedness or diversity and population dynamics within and between species of these parasites have not been explored. Hence, this study analyzed and compared the inter and intra-species genetic diversity in the ITS1 gene of ribosomal RNA among the *T. brucei*, *T. vivax* and *T. congolense* from naturally infected Nigerian cattle breeds and compared them with previously reported variants from other countries.

5.2 MATERIAL AND METHODS

5.2.1 Study area:

The animals sampled were predominantly from two locations in Nigeria. Ogun state, which is one of the two states where the study was conducted, is a transitional zone between the tropical rain forest and derived savannah zone in the south-west of Nigeria. It lies between latitude 7^{o} 10'N and 3^{o}21'E and has two pronounced seasons, the dry season (November to March) and the wet season (April to October). Kaduna state, is the second state and it's located within the Northern Guinea Savannah zone of Nigeria and it lies between 11^{o} 10'N and 7^{o}38'E.

5.2.2 Study population and sample collection:

Four hundred and eleven samples were collected between September and December 2010 from the two locations of about 1000km apart with different vegetations that could significantly affect the breeding of the obligate vector, tsetse flies. The cattle sampled kept under the traditional management system of free grazing (nomadic) and various abattoirs and slaughter slabs which were randomly selected for sampling. The ages of selected cattle were determined using their dentition as described by Lasisi *et al.* (2002) Blood samples were collected from the jugular vein of each cattle into 5ml tubes containing 5mg ethylenediaminetetraacetic acid (EDTA) as anticoagulant and 5ml tubes without the EDTA for serum

analysis. The samples were transported in mobile refrigerator to the laboratory within 3 hours of collection. The blood samples without the anticoagulant were set on tray slanted and allowed to stay for 24 hours in the laboratory for serum harvest. Sera were collected in clean and sterile bottle and stored in -20^{o} freezer until use while the blood in the EDTA bottles were stored at 4^{o}C prior to DNA extraction.

5.2.3 Parasitological diagnosis:

From each tube containing anticoagulated blood, three capillary tubes were filled and sealed at one end with plasticin, centrifuged at 3000 rpm for 5 minutes in micro-hematocrit centrifuge. After centrifugation, the packed cell volume (PCV) was determined. The buffy coat and upper most layers of red blood cells of one capillary tube was extruded onto a microscope slide and examined with a phase-contrast microscope at x 400 magnification as described by Murray *et al*. (1977) for the presence of motile trypanosomes. Not less than 50 fields were examined before positive or negative was declared for each sample. While the haematocrit centrifugation technique (HCT) positive samples were further processed as thin smear stained with Giemsa for trypanosome species identification, thick blood smear was also prepared, stained with Giemsa and all examined under x 100 oil immersion objective lens (x 1000 magnification).

5.2.4 DNA extraction:

DNA was extracted from the blood in EDTA bottle using Quick-gDNA™ MiniPrep (Zymo Research Corporation, Irvine, CA 92614, U.S.A) as described by the manufacturer. Briefly, 400µl of genomic lysis buffer was added to 100 µl of blood, thoroughly mixed by vortexing for 4-6 seconds and incubated at room temperature for 5 – 10 minutes. The mixture, blood and the lyses buffer, was transferred to a spin column in a collection tube and centrifuge at 10,000 x g for 60 seconds after which the collection tube with the flow through was discarded and the spin column transferred to a new collection tube. 200 µl of prewash buffer was added to the spin column and centrifuged at 10,000 x g for 60 seconds after which 500 µl of genomic DNA wash buffer was added to the spin column and centrifuged at 10,000 x g for 60 seconds. To elute the DNA, the spin column was transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease free water was added to the spin column and incubated at room temperature for 2-5 minutes, centrifuge at 16,000 x g for 30 seconds. Quantification of DNA yield and assessment of quality were done using Nanodrop ND-100 UV/Vis Spectrophotometer (Nanodrop Technologies, Inc., DE, U.S.A). The eluted DNA was stored at -20^{o}C until use.

5.2.5 Primer sets and optimization:

All the samples were initially tested by species-specific primers directed against multi-copy satellite repeats of each species and strains. Eight sets

of primers were selected for optimization based on the published work. These sets of primers were optimized with DNA extracted from the blood of cattle parasitologically positive for *T. vivax, T. congolense, T. brucei* and *T. evansi* that were donated by the National Veterinary Research Institute, Vom, Nigeria which led to final selection of six sets of primers for the screening. Primer set ITS1 CF & BR was also used to compare the sensitivity and specificity of species specific and multiplex primers in detecting trypanosome species. The names of primer sets, sequences, sizes of the expected amplicon and references are presented in Table 5.1.

Table 5.1: Sequences of the oligonucleotide primers (ITS1 CF & BR) used in this study and the expected fragment sizes of each species.

Primer set	Sequences	Expected band size(bp)			Reference
		Tb	Tc	Tv	
ITS1 CF	CCGGAAGTTCACCGATATTG	480	700	250	Njiru, et al (2005)
ITS1 BR	TTGCTGCGTTCTTCAACGAA				

Tb; *Trypanosoma brucei*, Tc; *Trypanosoma congolense*, Tv; *Trypanosoma vivax*

5.2.6 Trypanosomes detection by ITS1 PCR:

Polymerase Chain Reaction amplification using universal primer sets, ITS1 CF & BR, was performed in 20µl final reaction volume containing equivalent of 20ng of genomic DNA, 10mM Tris-HCl, pH 8.3, 1.5mM MgCl2, 50µM KCl, 200µM each of dNTPs, 40ng of each of the primers and 1unit of Thermus aquaticus DNA polymerase (Bioneer USA) with initial denaturation of 95^{o}C for 5 min followed by 35 cycles of 95^{o}C for 1 min, 58^{o}C for 1 min and 72^{o}C for 1 min with final extension at 72^{o}C for 10 min. Ten microliter of the PCR products were electrophoresed through 1.0% agarose gel in 1 x TAE (40 mM TRIS-acetate and 1 mM EDTA) at 90 V for 60 min along with 10µl of biological marker, GENEMate Quanti-Marker 100 bp DNA ladder (BioExpress, UT, USA). Gels were stained with GelRed^{R} Nucleic Acid Stain (PHENIX Research Product, Candler, NC, U.S.A) at 5µl/100ml of the agarose gel suspension. After electrophoresis, the PCR products were visualized using ultra violet transilluminator (Spectroline^{R} TC 312 E) before gels were photographed using (Alpha Imager).

5.2.7 Sequencing of PCR fragment:

The PCR products of the samples that were positive as single infection of both *T. vivax* and *T. congolense* were selected for sequencing. Twenty PCR products each of *T. vivax* and *T. congolense* (savannah and forest)

strains, comprising ten samples each from the northern and southern part of Nigeria, were sequenced directly using Big dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the forward amplification PCR primers and AmpliTaq-FS DNA Polymerase. The sequences obtained were viewed and compared on Finch TV and Sequence Scanner (Applied Bioscience) before they were aligned with each other and with published sequences of various *Trypanosoma spp* using the Molecular Evolutionary Genetic Analysis software (MEGA 5.05).

5.2.8 Sequence alignment and analysis:

The ITS1 sequences of *T. brucei*, *T. vivax* and *T. congolense* were used to do blast search from the NCBI database. The other sequences of *T. congolense* and *T. vivax* from South America and East Africa available in GenBank were selected. The alignment was done using Clustal W method of Molecular Evolutionary Genetic Analysis (MEGA) software version 5.05 (Tamura *et al.*, 2011). A phylogenetic tree was constructed using Unweighted pair group method (UPGMA) and maximum likelihood (ML) algorithm of the phylogeny program of MEGA 5.05 (Tamura *et al.*, 2011) which included forty-two sequences of the forty-eight PCR products sent for sequencing and the South America and East Africa strains of *T. congolense* (FJ712718 and U22319) and *T. vivax* (DQ316041and

DQ316051) obtained from GenBank with *Herpetmonas muscarum* (AY180151.1) as the out group. The bootstrap confidence interval of the tree was determined based on l000 replicates.

5.3 RESULT

5.3.1 Trypanosomes detection by PCR (Species-specific and ITS1) techniques:

One hundred and sixty-two (45.26%) of the 411 examined cattle herds were found to be positive for one or more trypanosomes using the internal transcribe spacer 1 (ITS1) as against 262 (63.7%) positive cases detected by species specific primer sets. While the ITS1 PCR detected more *T. vivax* 74(18.01%) than *T. congolense* 71(17.27%) and *T. brucei* 3 (0.72%) as single infections and 14 (3.4%) as mixed onfections, species specific PCR did not detect those animals with mixed infections (Table 5.2)

Table 5.2: Prevalence of trypanosomes in Nigerian cattle breeds as detected by ITS 1 PCR

	Cattle				
Trypanosoma species	Muturu	N,Dama	S. Gudali	W.Fulani	Total(%)
Tryapanosoma brucei	01	00	00	02	03(0.72)
Trypanosoma congolense	30	01	05	35	71(17.27)
Trypanosoma vivax	37	02	07	32	74(18.01)
T. brucei and T. vivax	01	00	00	02	03(0.72)
T. congo and T. vivax	04	00	01	06	11(2.68)
Total	69	03	13	77	162(45.26)

5.3.2 Amplification and gel electrophoresis of ITS1 rDNA gene:

Upon amplification of the *T. brucei*, *T. congolense* and *T. vivax* genomic DNA of cattle from Northern and Western part of the country, agarose gel electrophoresis revealed amplified product sizes of about 480bp, 700bp and 250bp, respectively. No contamination was observed in any amplification assay (Figure 5.1)

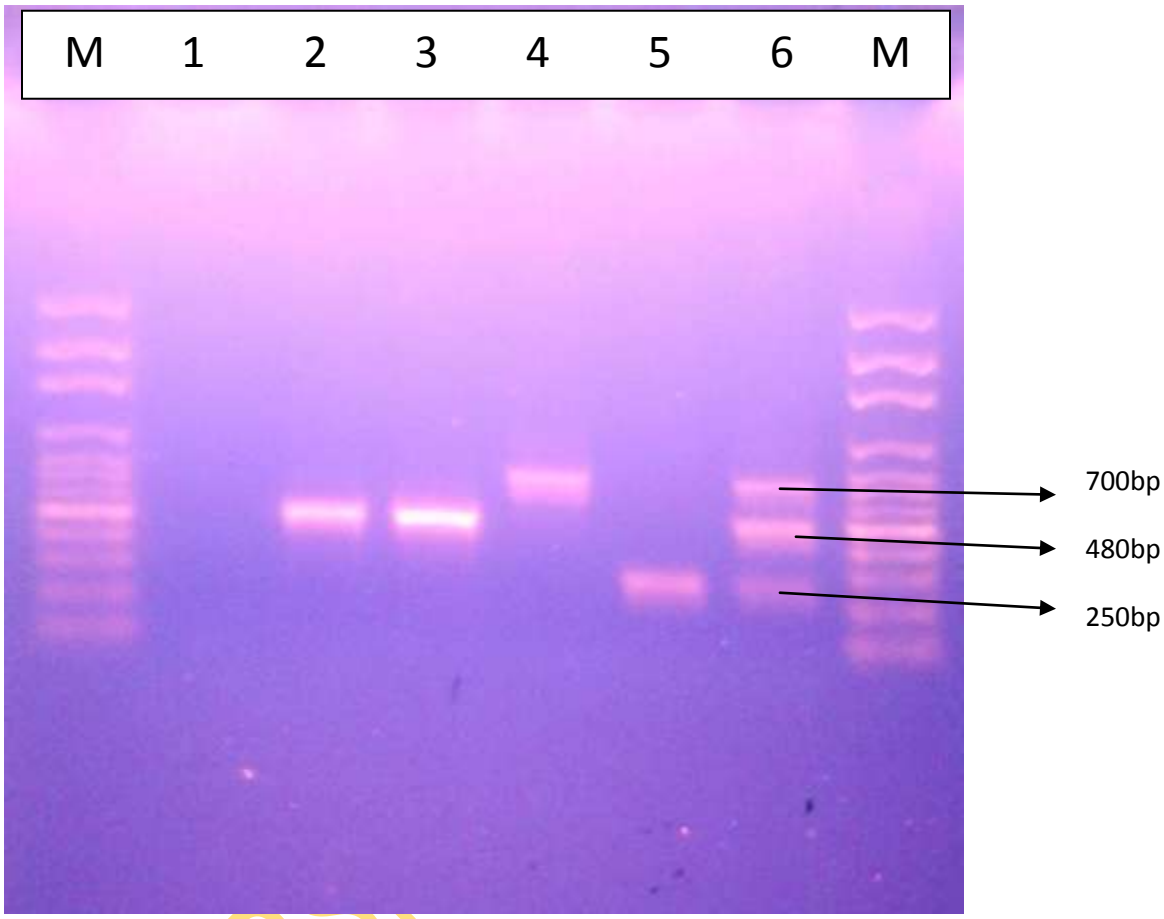

Figure 5.1: Gel electrophoresis showing *Trypanosoma species* detected by ITS1-PCR. M; 100bp molecular marker, 1; negative control, 2 and 3; *T. brucei*, 4; *T. congolense* and 5; *T. vivax*, 6; positive control.

5.3.3 Sequence alignment and phylogenetic analysis:

5.3.3.1 *T. brucei*:

The overall sizes of ITS1 rDNA of *T. brucei* sequences range from 299 to 369bp. The aligned sequences of *T. brucei* revealed it to be highly polymorphic while the analysis of the sequence revealed a mean G/C content of 33.57%. Single nucleotide polymorphism (SNP) characterized by base alteration or insertion was also revealed at various points. Common deletion points for sample 274, 294 and 269 (*T. brucei* strain from Ogun State) were observed at loci 186, 279, 280, 281 and 325. Nucleotides alterations from G ⇢ T, C ⇢ T and G ⇢ A at loci 124, 197 and 201, respectively were also observed. Insertion at locus 94 for sample 64 and 74 (Appendix 10 and 11), The genetic distance between the detected population of *T. brucei* in Nigeria range from 0.00 – 0.01 while the distant between Nigerian species and those of East Africa range from 0.00 – 0.03 (Table 5.3).

Phylogenetic analyses using the un-weighted pair group method using average linkage (UPGMA) and maximum-likelihood (ML), all yielded trees with almost identical topologies and relatively high bootstrap or nodal support values for *T. brucei* sequences. The phylogenetic analysis showed that *T. brucei* ITS1 rDNA gene sequences fell into four main groups, designated A, B, C and D (Fig 5.2, 5.3 & 5.4). Group A and B

contained the sequences those from Nigeria, group C contains those sequences from East Africa and group D contains the out-group.

5.3: Estimates of evolutionary divergence between sequences of *T. brucei* detected in naturally infected cattle from Nigeria and those sequences of *T. brucei* from Zambia.

		1	2	3	4	5	6	7	8	9	10
1	64 T. *brucei* ITS1-Nig	0.00									
2	294 T. *brucei* ITS1-Nig	0.00	0.00								
3	74 *T. brucei* ITS1-Nig	0.00	0.00	0.00							
4	274 *T. brucei* ITS1-Nig	0.01	0.01	0.01	0.00						
5	269 *T. brucei* ITS1-Nig	0.00	0.00	0.00	0.01	0.00					
6	AB569250 *T. theileri*ITS1	1.82	1.82	1.82	1.77	1.82	0.00				
7	DQ316051 *T. vivax* ITS1 Vene	2.12	2.12	2.12	2.06	2.12	0.54	0.00			
8	22 *T. vivax* ITS1 –Nig	2.08	2.08	2.08	2.02	2.08	0.59	0.02	0.00		
9	*T. brucei* AF306771-Zambia	0.03	0.03	0.03	0.03	0.03	1.91	2.20	2.16	0.00	
10	*T. brucei* AF306770-Zambia	0.02	0.02	0.02	0.03	0.02	1.87	2.20	2.16	0.01	0.00

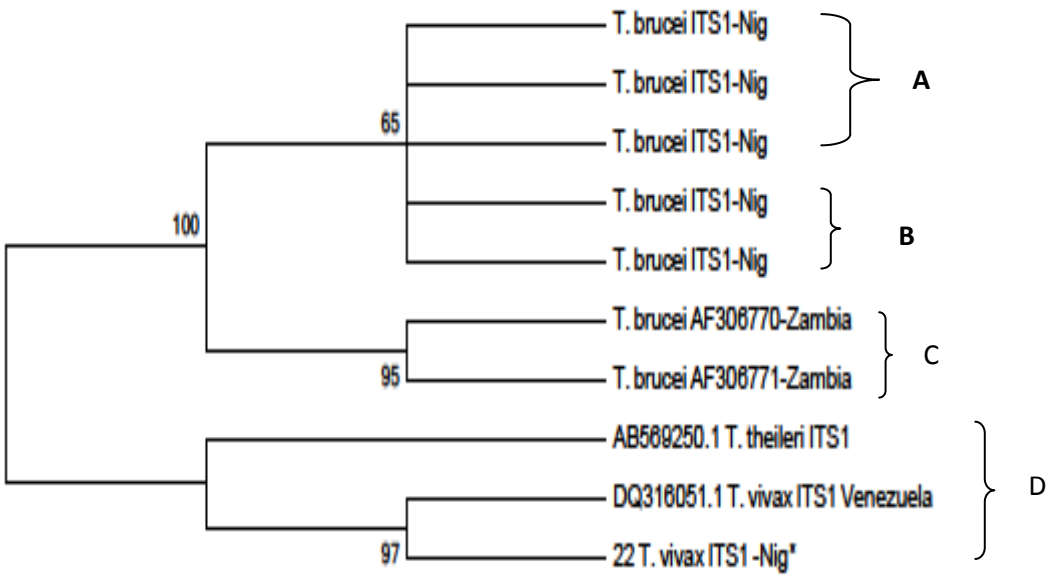

Figure 5.2: Fig. 1.Evolutionary relationships of strains of *T. brucei* found in this study compared to other sequences from the GenBank, using using ITS-1 rDNA sequences analyzed by the ML method. The percentage of replicate trees above 50% in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in those. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

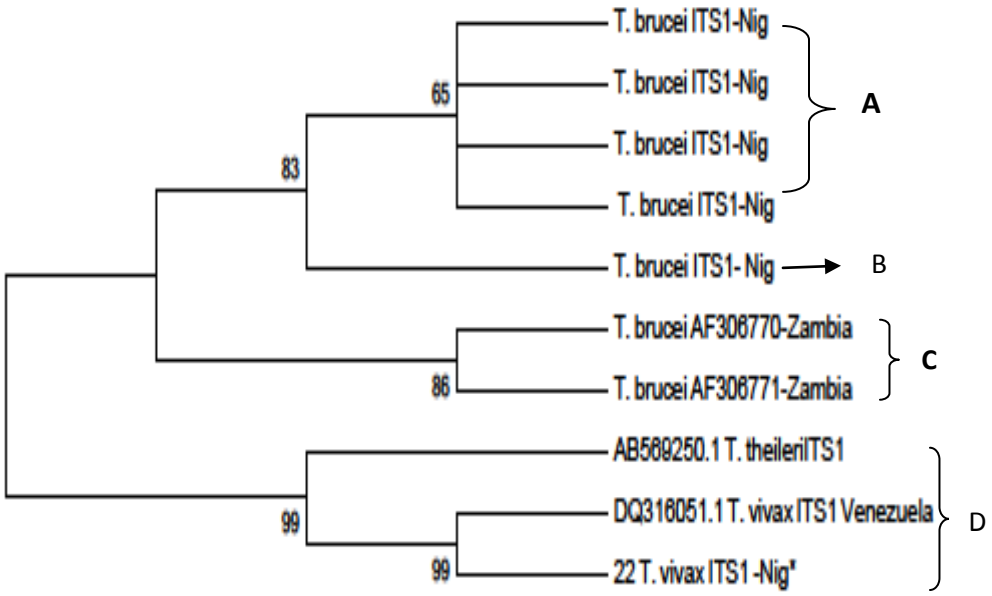

Figure 5.3: Evolutionary relationships of strains of *T. brucei* found in this study compared to other sequences from the GenBank, using using ITS-1 rDNA sequences analyzed by the UPGMA method. The percentage of replicate trees above 50% in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in those. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

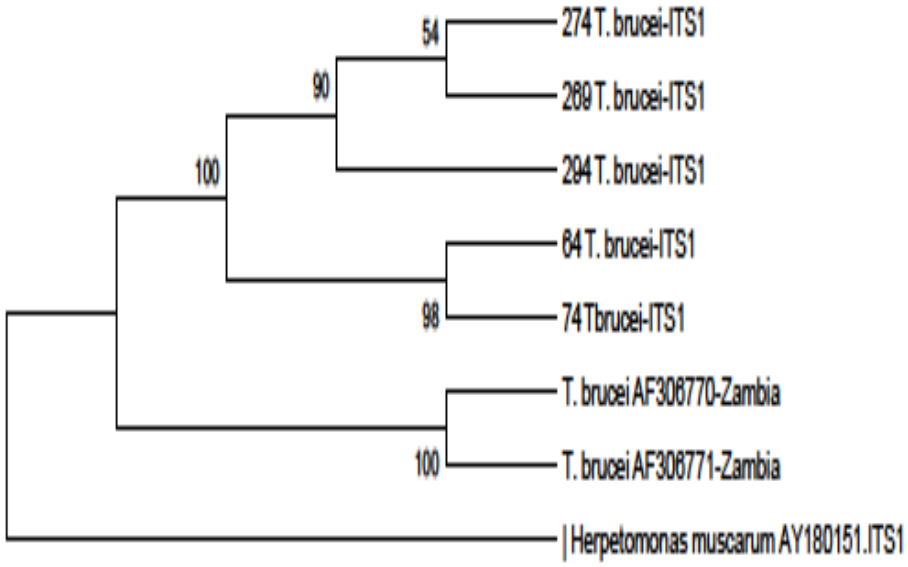

Figure 5.4: Evolutionary relationships of strains of *T. brucei* found in this study compared to other sequences from the GenBank, using ITS-1 rDNA sequences analyzed by the UPGMA method. The percentage of replicate trees above 50% in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in those. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The outgroup used is *Herpatomonas muscarum.*

5.3.3.2 *T. congolense*:

The overall sizes of the ITS1 rDNA of *T. congolense* sequences range from 313bp. While the aligned sequences are highly polymorphic, analysis of the sequences revealed a mean G/C content of 49.4%. Single nucleotide polymorphisms (SNPs), characterized by base alteration or insertion was also revealed as sequence (#02) showed A/T changes in position 77, #04 sequence showed A/T change at 62 and 64, C/T in position 86 with unique insertion (G) in position 84. #31 showed C/T changes at positions 10 and 56 and A/C changes at position 58, 60 and 65, A/T changes at 61, 62 and 64, and G/A changes at position 95. All the 12 samples sequenced except #05 showed a unique mononucleotide deletion in position 84(Appendix 12 and 13)

The average genetic distance between the detected population of *T. congolense* in Nigeria was 0.01 – 0.33 while the distant between Nigerian species and those of East Africa range from 0.18 – 0.45 (Table 5.4).

Phylogenetic analyses using the un-weighted pair group method using average linkage (UPGMA) and maximum-likelihood (ML), all yielded trees with almost identical topologies and relatively high and low bootstrap or nodal support values for *T. congolense* sequences. The phylogenetic analysis showed that *T. congolense* ITS1 rDNA gene sequences fell into four main groups, designated A, B, C and D (Figure 5.5). Group A contained the sequences of *T. congolense* ITS1 rDNA

comprising those from Northern and western part of Nigeria. Group B contained a lonely sequence of *T. congolense*-killifi type. Group C contained the sequences of *T. congolense*-savanah type from Tanzania and Kenya (East Africa) and group D contained four sequences of *T. congolense*-savanah type detected in the Northern part of Nigeria while the out group, *T. theileri*, was clearly separated from the sequences of *T. congolense.*

Table 5.4 Estimates of evolutionary divergence between sequences of *T.congolense* detected in naturally infected cattle from Nigeria and those sequences of *T. congolense* from GeneBank. Analyses were conducted using the Maximum Composite Likelihood model.

01_T._congolense_ITS_1														
02_T._congolenese_ITS_1	0.01													
04_T._congolense_ITS_1	0.21	0.19												
05_T._congolense_ITS_1	0.07	0.08	0.23											
07_T._congolense_ITS_1	0.07	0.05	0.19	0.08										
31_T._congolense_ITS_1	0.29	0.29	0.31	0.35	0.31									
32_T._congolense_ITS_1	0.06	0.05	0.24	0.11	0.08	0.29								
33_T._congolense_ITS_1	0.01	0.00	0.19	0.08	0.05	0.29	0.05							
34_T._congolense_ITS_1	0.21	0.19	0.07	0.22	0.19	0.28	0.24	0.19						
35_T._congolense_ITS_1	0.19	0.17	0.04	0.20	0.17	0.30	0.22	0.17	0.08					
36_T._congolense_its_1	0.07	0.05	0.27	0.14	0.11	0.38	0.11	0.05	0.26	0.24				
39_T._congolense_ITS_1	0.04	0.03	0.19	0.11	0.07	0.29	0.08	0.03	0.19	0.17	0.08			
40_T._congolense_ITS_1	0.03	0.01	0.17	0.10	0.05	0.28	0.06	0.01	0.17	0.15	0.07	0.01		
43_T._congolense_ITS_1	0.17	0.16	0.08	0.22	0.16	0.26	0.21	0.16	0.04	0.09	0.23	0.16	0.14	
FJ712718.1_T._congolense_Kenya	0.39	0.39	0.42	0.34	0.37	0.18	0.40	0.39	0.38	0.40	0.45	0.39	0.38	0.39

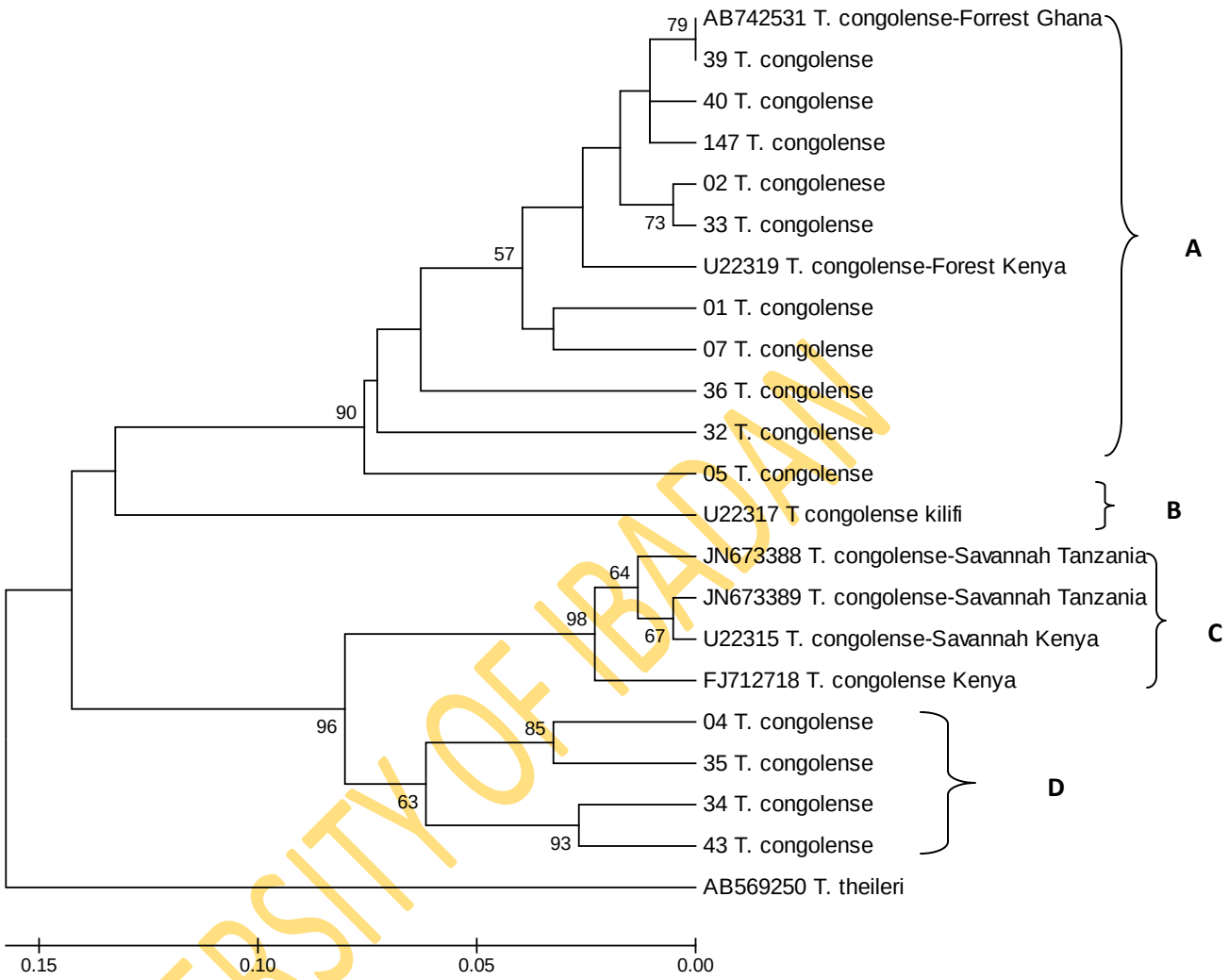

Figure 5.5: Evolutionary relationships of strains of *T. congolense* found in this study compared to other sequences from the GenBank, using using ITS-1 rDNA sequences analyzed by the UPGMA method. The percentage of replicate trees above 50% in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in those. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

5.3.3.3 *T. vivax*:

The overall sizes of the ITS1 rDNA of *T. vivax* sequenced were 215bp. While the aligned sequences were less polymorphic (Figure 5.11 and 5.12), analysis of the sequences revealed a mean G/C content of 61.9% and both size and sequences polymorphism as *T. vivax* sequence (#71) showed G/A changes in position 32, 34 and 83, T/A and T/C changes in positions 33 and 101, respectively. Sample #20, 50 and 71 showed mononucleotide insertion at position 30(A), 57(T) and 100(T) while sample #94 showed mononucleotide insertion at position 100(G), 118(A), 132(G) and 267(A). The average genetic distance between the detected population of *T. vivax* in Nigeria was 0.01 – 0.64 (Table 5.5)

Phylogenetic analyses using the un-weighted pair group method using average linkage (UPGMA) yielded tree with topologies of relatively higher bootstrap or nodal support for *T. vivax* sequences. The phylogenetic analysis showed that *T. vivax* ITS1 rDNA gene sequences fell into four main groups, designated A, B, C and D (Figure 5.6). While group A contained the sequences of *T. vivax* detected in Nigeria within which the South American sequences (DQ316041) clustered tightly, group B contained the sequences of *T. vivax* from Kenya (DQ316041) and C and D contained the sequences of those from Tanzania (JN673392 and JN673393). The out group, *T. theileri* (AB569250) was clearly separated from the rest of the sequences.

Table 5.5: Estimates of Evolutionary Divergence between Sequences of *T. vivax* detected in naturally infected cattle in Nigeria. The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Maximum Composite Likelihood model of MEGA5.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	44 T. vivax ITS1																												
2	14 T. vivax ITS1	0.00																											
3	85 T. vivax ITS1	0.00	0.00																										
4	86 T. vivax ITS1	0.02	0.02	0.02																									
5	68 T. vivax ITS1	0.01	0.00	0.01	0.01																								
6	69 T. vivax ITS1	0.02	0.02	0.03	0.01	0.01																							
7	12 T. vivax ITS1	0.02	0.02	0.02	0.00	0.01	0.01																						
8	11 T. vivax ITS1	0.01	0.00	0.01	0.01	0.00	0.01	0.01																					
9	13 T. vivax ITS1	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01																				
10	18 T. vivax ITS1	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.01	0.02																			
11	16 T. vivax ITS1	0.00	0.00	0.04	0.02	0.01	0.02	0.02	0.01	0.02	0.02																		
12	15 T. vivax ITS1	0.01	0.00	0.04	0.02	0.01	0.02	0.02	0.01	0.02	0.02	0.01																	
13	17 T. vivax ITS1	0.01	0.00	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02																
14	17 T. vivax ITS1	0.02	0.02	0.03	0.00	0.02	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.01															
15	18 T. vivax ITS1	0.06	0.04	0.03	0.05	0.03	0.05	0.05	0.03	0.04	0.04	0.06	0.06	0.05	0.07														
16	19 T. vivax ITS1	0.01	0.01	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.05													
17	20 T. vivax ITS1	0.02	0.02	0.05	0.04	0.02	0.03	0.03	0.02	0.03	0.03	0.02	0.02	0.01	0.00	0.07	0.03												
18	21 T. vivax ITS1	0.01	0.01	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.05	0.03	0.03											
19	22 T. vivax ITS1	0.03	0.03	0.03	0.02	0.01	0.02	0.02	0.02	0.01	0.01	0.03	0.03	0.02	0.03	0.05	0.02	0.03	0.03										
20	23 T. vivax ITS1	0.04	0.04	0.00	0.02	0.03	0.03	0.02	0.03	0.02	0.02	0.04	0.04	0.03	0.09	0.03	0.03	0.05	0.03	0.03									
21	24 T. vivax ITS1	0.02	0.02	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.02	0.01	0.03	0.05	0.01	0.03	0.01	0.02	0.03								
22	25 T. vivax ITS1	0.03	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.03	0.02	0.05	0.02	0.03	0.02	0.01	0.01	0.01	0.02							
23	26 T. vivax ITS1	0.03	0.02	0.02	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.02	0.02	0.01	0.04	0.05	0.01	0.03	0.01	0.02	0.02	0.01	0.01						
24	27 T. vivax ITS1	0.05	0.04	0.04	0.04	0.04	0.04	0.03	0.04	0.03	0.03	0.05	0.05	0.05	0.08	0.08	0.05	0.08	0.05	0.04	0.04	0.05	0.03	0.03					
25	28 T. vivax ITS1	0.03	0.03	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.03	0.02	0.04	0.05	0.02	0.03	0.02	0.01	0.01	0.02	0.00	0.01	0.04				
26	29 T. vivax ITS1	0.02	0.02	0.02	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.02	0.02	0.01	0.04	0.05	0.01	0.03	0.01	0.02	0.02	0.01	0.01	0.00	0.05	0.01			
27	30 T. vivax ITS1	0.05	0.04	0.04	0.03	0.04	0.04	0.03	0.04	0.03	0.03	0.05	0.05	0.05	0.08	0.08	0.05	0.08	0.05	0.04	0.04	0.05	0.03	0.03	0.00	0.03	0.03		
28	44 T. vivax ITS1	0.02	0.02	0.02	0.00	0.01	0.01	0.00	0.01	0.01	0.01	0.02	0.02	0.01	0.03	0.05	0.01	0.03	0.01	0.03	0.02	0.01	0.01	0.00	0.04	0.01	0.00	0.03	

127

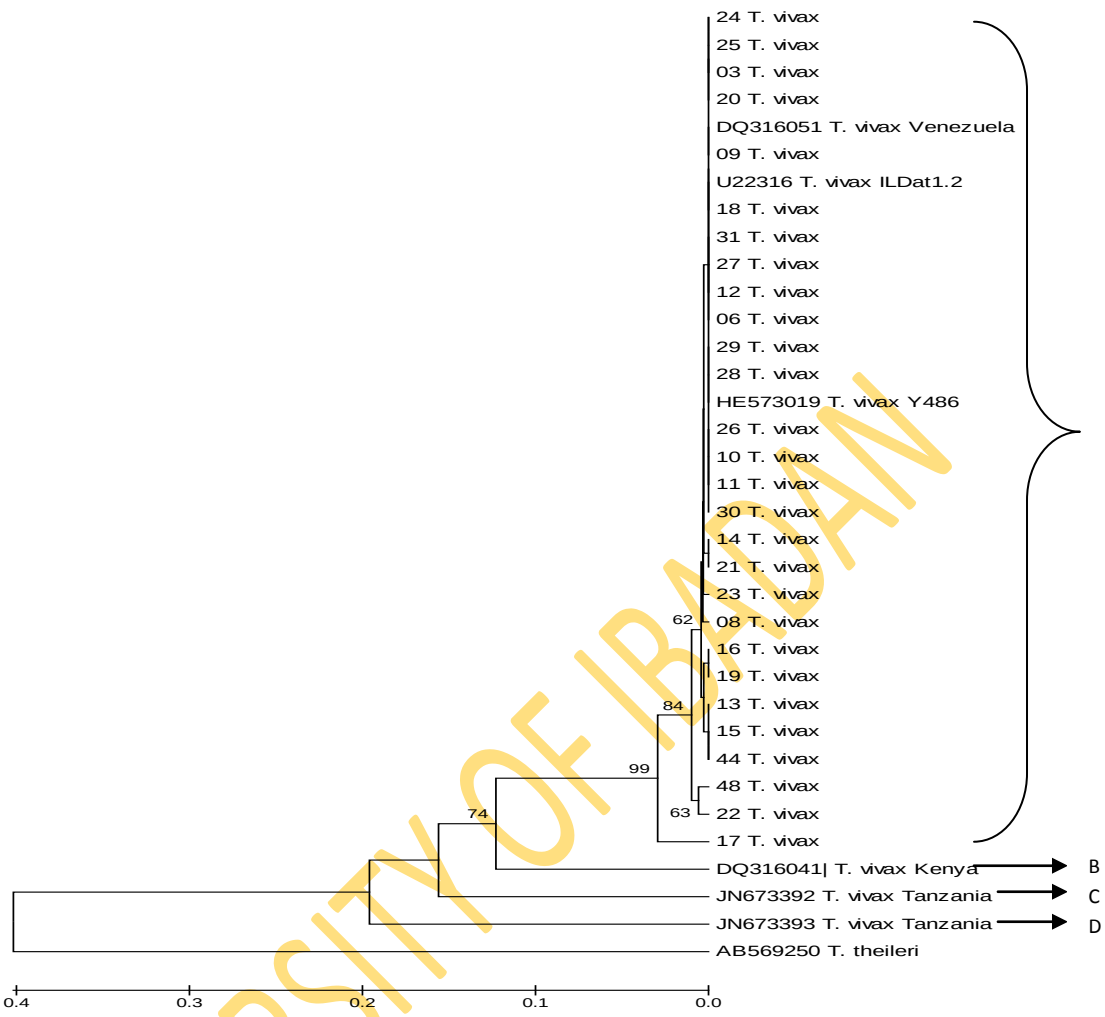

Figure 5.6: Evolutionary relationships of strains of *T. vivax* found in this study compared to other sequences from the GenBank, using using ITS-1 rDNA sequences analyzed by the UPGMA method. The percentage of replicate trees above 50% in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches in those. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

5.3.3.4 Comparative phylogenetic analysis of the sequences of *T. brucei, T. congolense* and *T. vivax*:

Alignment and phylogenetic analysis of all the *T. brucei*, *T. congolense* and *T. vivax* sequences and those from East Africa and South America obtained from GenBank revealed that the three species formed three separate clades A, B and C (Figure 5.7 and Appendix 14). While the South American *T. vivax* (DQ316051.1) clustered within the *T. vivax* sequences clade (A) detected in Nigeria, the East African species (DQ316041) was placed at the periphery of the clade. The *T. brucei* ITS 1 sequences formed a separate clade, B together with the East African strains. Also, the *T. congolense* sequences from Nigeria cattle cluster tightly together in a clade C but the sequences of the East Africa species (FJ712718) clustered tightly within the Nigerian species of *T. congolense*. The *T. theileri* is peripherally placed with *T. brucei* detected in Nigeria. The dendogram did not clearly separate the *Herpetomonas muscarum*, used as an out group from the clades.

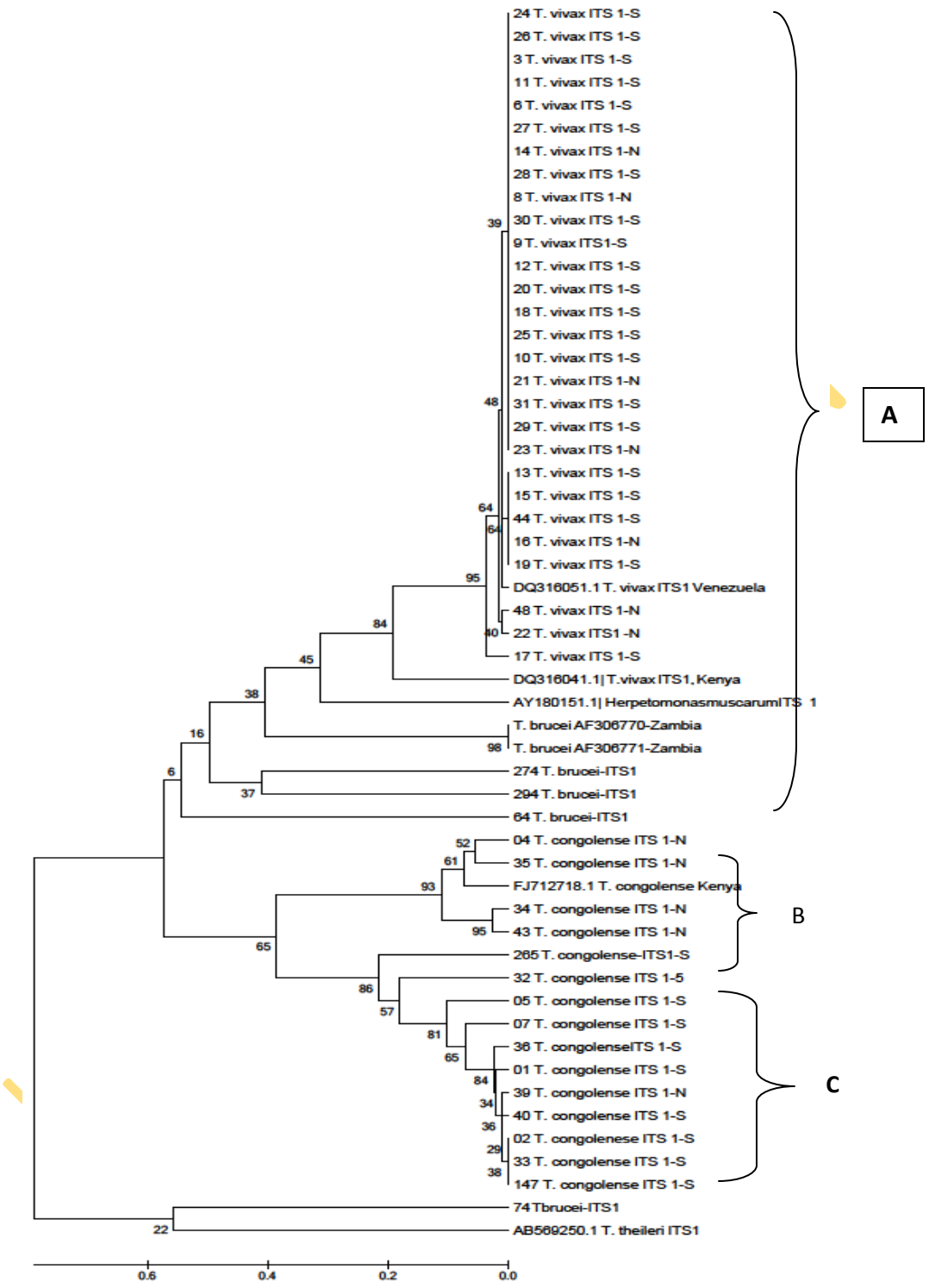

Figure 5.7: Comparative phylogenetic analysis of the sequences of *T. brucei, T. congolense* and *T. vivax* as inferred by un-weighted pair group method using average linkage UPGMA method.

5.4 DISCUSSION

The genetic diversity of *T. brucei*, *T. congolense* and *T. vivax* infecting cattle in two different vegetation zones in Nigeria is poorly understood. This is mainly due to lack of a sensitive tool to analyze and differentiate between sub-groups and strains of trypanosomes in Nigeria. In this study, we used ITS1 rDNA gene sequences, a highly variable portion between *Trypanosoma species* but constant within each species (Njiru, et al., 2005), to determine the genetic diversity within and among *T. brucei*, *T. congolense* and *T. vivax* detected in the Northern (savannah zone) and Western (forest zone) part of Nigeria.

The aligned sequences of *T. congolense* are more polymorphic, followed by the sequences of *T. brucei* but the ITS1 region seems more conserved in *T. vivax*. The high polymorphic nature of *T. congolense* and *T. brucei* is supported by their high nodal values which may be an indication that the reproduction in these species are likely more of sexual reproduction as against *T. vivax* with highly conserved ITS 1 region as indicated by the nodal values on the phylogenetic tree inferred by UPGMA and ML bootstrapping. The lower nodal values in the dendrogram of *T. vivax* may suggest less genetic exchange between this species and that their reproduction may be more clonal in nature. Our findings could not be compared with any work in Nigeria because of paucity of information on the molecular characterization of trypanosomes but disagrees with the report of Koffi *et al.,* (2009) who used microsatellite marker-based

analysis to investigate polymorphism of *T. b. gambiense* in Ivory coast and Guinea. The lower diagnostic sensitivity showed by ITS1 CF and BR based primer set in this study has been described by Njiru, et al., (2005) but the specificity and capability of detecting all the pathogenic trypanosomes in a single PCR indicate a greater potential for these primers as a universal test for pathogenic trypanosomes. More *T. vivax* infections were detected by the ITS1 CF and BR primer than *T. congolense* infections in this study. This might be due to the fact that ITS1 rDNA sequence designed for *T. vivax* detection involved an isolate from Nigeria as against that designed for the detection of *T. congolense* which is based on isolates from other part of African other than Nigeria. This assertion is supported by our findings in which the homology of the sequenced PCR products of *T. vivax* from this study was 97% with ITS1 rDNA of Y486 isolate from Nigeria as against 91- 95% sequence homology of *T. congolense* sequences obtained .. While 344 bp and 37% G-C content of ITS1 region reported by Agbo *et al.,* (2001) for *T. brucei* did not agree with the 443 bp – 780 bp range of sequences length and 50.9% G-C content of ITS1 rDNA obtained in our study, the variations in sequences length and the G-C content of these trypanosomes may be due to the fact that the *T. brucei* sequenced in our study were detected from different cattle hosts in which some levels of genetic exchanges and assortment may have occurred as opposed to those isolates analyzed by Agbo *et al*., (2001), all which were isolates maintained in laboratory animals or liquid

nitrogen. This report shows that intra-specific size changes is not restricted to the non-transcribed spacer region alone as reported by Sturm *et al.* (1998) but could also occur in internal transcribed spacer 1 region.

The inability of ITS1 rDNA-PCR to differentiate members of trypanozoon may question the fitness of ITS based PCR to analyse the genetic relatedness of the members of the group. Though, conflicting reports exist on the suitability of ITS genomic region for the study of evolutionary relationship between and within trypanosomal species (Khuchareontaworn *et al.*, 2007 and Areekit *et al.*, 2008) as well as the application of ITS1 and ITS2 (Tian *et al.*, 2011; Areekit *et al.*, 2008), this region (ITS1 rDNA) clearly separated *T. congolense* and *T. vivax* into distinct clades. The relatively higher G-C content of *T. vivax* than that of *T. brucei* and *T. congolense* may explain why the *T. vivax* is more pathogenic than the *T. congolense* and *T. brucei*. The G-C pair is bound to three hydrogen bonds and A-T two, it is expected that species with higher G-C content will be more thermostable.

This study has shed more light on population dynamic and genetic diversity within and among *T. brucei*, *T. congolense* and *T. vivax* in Nigeria. The *T. brucei* strain in Kaduna is different from those detected in Ogun and three starins of *T. congolense* savannah were detected in the study area.

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

This investigation revealed high prevalence of trypanosomosis in Nigeria using PCR technique. The species detected include *T. brucei*, *T. congolense (s)*, *T. congolense (f)*, *T. evansi* and *T. vivax* with *T. congolense* (savannah type) being the most prevalent. The specificity of microscopy was higher than PCR which shows higher sensitivity. The species specific primers used for detection of trypanosomes in this study were more sensitive than universal (ITS1) primer set.

The *Trypanosoma species* detected in this study were clearly separated into three different clades but distantly separated from their counterpart from East Africa except *T. vivax* from South America and Zambia that clustered within the clade formed by Nigerian *T. vivax* species. The *T. brucei* ITS1 sequences sizes are longer than the sequences of *T. vivax* and *T. congogolense* while the G-C content of *T. vivax* sequence is higher than that of *T. brucei* and *T. congolense.*

This is the first report of the molecular characterization of *T. congolense* savannah and forest types in Nigeria. The genetic relatedness of trypanosomes from Nigeria to that of Venezuela and Zambia implies that the treatment regime known to be successful in the two countries can be adopted in Nigeria. It is recommended that

regular surveillance and screening for new strains of Trypanosoma species be conducted on Nigerian cattle for effective prevention and control of the disease.

6.2 SUGGESTED AREAS OF FURTHER STUDIES

In this study, we detected four species of trypanosomes (*T. brucei brucei, T. congolense (s), T. congolense (f) T. evansi and T. vivax*) and two of the species (*T. brucei* and *T. congolense*) have different strains. It is known that propagation of trypanosomes is aided by uptake of iron through transferin receptor that is encoded by expression site-associated gene (ESAG). ESAG, especially ESAG 6 and 7 genes are located in variant surface glycoprotein expression sites. Futher researches are needed to see if the amplification of the ESAG 6 and 7 in the major *Trypanosoma species* of cattle and their insertion into a common vector can give a complex clone that will be immunogenic enough to protect aginst all pathogenic trypanosomes in the cattle.

6.3 RECOMMENDATIONS

The presence of trypanolytic factor in human serum has made the infection of human by animal trypanosomes impossible. But new cases of human trypanosomiasis due to non-convectional human trypanosomes have been reported in India Joshi *et al.* (2005) and Kunz *et al.* (2009). It is therefore important to carry out surveillance using modern techniques to detect animal Trypanosoma species that can be pathogenic to human so that farmers and livestock owners can be educated on the possible zoonotic implication of animal infective *Trypanosoma species.*

Since pathogenicity in trypanosomosis is associated with species and strains of infecting trypanosomes, we recommend that the pathogenicity of various strains of trypanosomes detected in Nigeria be investigated for effective treatment and control.

The following specific actions are recommended:

- Improvement of the existing diagnostic machine and equipment in our national diagnostic centers
- National periodic surveillance for both animal and human trypanosomes.
- Quarantine, examination and molecular detection of trypanosomes in imported animals from trypanosomes

infested countries for possible presence of pathogenic trypanosomes

- Establishment of gene bank where indigenous trypanosomal gene sequences can be archived.
- Research aiming at production of vaccine against all animal trypanosomosis which should be funded by the gorverment.

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8.0 APPENDICES

Appendix 8.1: Publication from this work

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Molecular survey of pathogenic trypanosomes in naturally infected Nigerian cattle

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ABSTRACT

Microscopy and polymerase chain reaction (PCR) were used to survey pathogenic trypanosome infection in naturally infected Nigerian cattle. In 411 animals sampled, microscopy detected 15.1% positive infection of at least one of *Trypanosoma brucei*, *Trypanosoma congolense* or *Trypanosoma vivax*, while PCR detected 63.7% positive infections of at least one of those species and *Trypanosoma evansi*. PCR detected 4.4%, 48.7%, 26.0% and 0.5% respectively of *T. brucei*, *T. congolense*, *T. vivax* and *T. evansi* infections. All of the *T. congolense* detected were savannah-type, except for two forest-type infections. Prevalence of mixed infections was 13.9%, being primarily co-infection by *T. congolense* and *T. vivax* while prevalence of mixed infections by *T. evansi*, *T. vivax* and *T. congolense* was 1.5%. Microscopy showed poor sensitivity but specificity greater than 94%. Infection rates were much higher in Southern than in Northern Nigeria. Infections were lowest in N'dama compared to Muturu, Sokoto Gudali and White Fulani breeds. Animals with *T. vivax* monoinfection and mixed infections showed significantly lower packed cell volume (PCV) values. Those infected with any *Trypanosoma* species with <200 parasites/μl showed higher PCV values than those infected with >200 parasites/μl. The new finding of savannah- and forest- type *T. congolense* in Nigeria and the relatively high abundance of mixed infections are of significant clinical relevance. This study also suggests that *T. congolense* is the most prevalent species in Nigeria.

1. Introduction

Trypanosomosis is a complex infectious disease of animals caused by a range of extra-erythrocytic protozoan parasites of the genus *Trypanosoma*, responsible for production losses, morbidity and sometime mortality in infected herds (Ahenga et al., 2002). The clinical signs of trypanosomosis depend on the species and strain of the infecting trypanosome, breed of the animal involved (Anene et al., 1991a,b; Matiolo et al., 1998) and the prevalence of vectors (Leak et al., 1990; Onyiah, 1997; Merkuria and Gadisa, 2011). Clinical signs include anemia, intermittent fever, parasitaemia, lymphadenopathy, jaundice, progressive emaciation, loss of production, weakness and death, if left untreated (Akinwale et al., 1999; Merkuria and Gadissa, 2011). While Muturu and N'dama are considered trypanotolerant breeds because they strive well under the pressure of trypanosome infections, they act as reservoirs of the infection for other animals (Moloo et al., 1992).

In Nigeria, diagnosis of bovine trypanosomosis largely depends on parasitological and immunological methods. Parasitological techniques have significant limitations exemplified by inability to differentiate between *Trypanosoma brucei* and *Trypanosoma evansi* except through the molecular composition of their kinetoplast DNA (kDNA) (Artama et al., 1982; Feng-Jun et al., 2007). Within species, parasitological methods can identify *Trypanosoma congolense* but not sub groups of the parasite. Hence, this technique lacks the sensitivity and the precision required for the purpose of adequate therapeutic and prophylactic control measures, exacerbated by a high proportion of false negative results. Immunological techniques (i.e. enzyme linked immunosorbent assays, card agglutination and fluorescent antibody tests) on the other hand are good for large scale epidemiological studies (Greiner et al., 1997) but not sensitive enough to detect and differentiate between current and previous infections, also leading to false positive results (Desquesnes and Tresse, 1996). Molecular technique such as polymerase chain

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reaction (PCR) has shown to be more sensitive and precise than the aforementioned techniques (Moser et al., 1989; Pinchbeck et al., 2008). This technique, though expensive and relatively new to certain parts of Africa, is so sensitive that parasitaemia as low as 10 parasites per milliliter of blood can be detected using PCR (Desquesnes and Davila, 2002; Delespaux et al., 2003). Due to its sensitivity, it has been used in some parts of Africa to ascertain the incidence, prevalence and characterization of trypanosome strains (Solano et al., 1999; Mugittu et al., 2001; Simukoko et al., 2007; Balmer and Caccone, 2008; Cordon-Obras et al., 2009). However, only *Trypanosoma vivax* Y58 strain, a field isolate with unknown isolation year (Feng-Jun et al., 2007) has been characterized in Nigeria (Morlais et al., 2001).

The prevalence of trypanosomosis has been extensively studied using parasitological and immunological methods in Eastern and Northern parts of Nigeria (Daniel et al., 1993; Kalu, 1995; Kalu and Lawani, 1996; Abenga et al., 2004; Oluwafemi et al., 2007; Ezeani et al., 2008; Qadeer et al., 2008; Enwezor et al., 2009; Kamani et al., 2010). The only recent records of trypanosomosis in the Western part of the country are 3.9% and 36.8% prevalence in Ogbomoso, Oyo and Ogun states, respectively (Ameen et al., 2008; Sam-Wobo et al., 2010). The use of PCR as a better diagnostic tool to ascertain the incidence and prevalence of trypanosomosis has been advocated (Desquesnes and Tresse, 1996; Miyamoto et al., 2006; El-Metanaway et al., 2009) but has not yet been applied in Nigeria. The present study was designed to determine the prevalence and characteristics of trypanosome species and strains in Nigerian cattle using PCR for the first time.

2. Materials and methods

2.1. Study population and sample collection

Random sampling was not possible due to lack of data on national reference census of nomadic herds. Therefore, working with owners, sampling was carried out on selected cattle herds in areas where cattle converge to rest during migration. Two major abattoirs were used for sampling. The animals to be sampled were selected by systematic random sampling technique whereby the sampling interval (I) is computed as the study population size divided by the required sample size and the first study subject is chosen randomly from among the first I study subjects, then every Ith study subject after that is included in the sample (Dohoo et al., 2009).

A total of 411 cattle (129 males and 282 females) ranging in age from 9 months to 6 years consisting of Muturu (112), N'dama (31), Sokoto Gudali (68) and White Fulani (200) breeds were sampled in Ogun and Kaduna states (Southern and Northern regions respectively). Animals aged one and under were considered young calves, while those over one year were regarded as adults. Animals with histories of recent trypanocidal treatment and those from institutional farms were excluded from the study. Age was determined by dentition and for the purpose of this study the body conditions were assessed and scored as described by Nicholson and Butterworth (1986). Blood samples were collected from the jugular vein of each animal into 5 ml tubes containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The samples were transported in a mobile refrigerator to the laboratory within 3 h of collection, and were stored at 4°C prior to DNA extraction.

2.2. Parasitological diagnosis

From each tube containing anticoagulant, blood was transferred into three capillary tubes which were sealed at one end with plasticine. The capillary tubes were spun in a microhaematocrit centrifuge at 10,000 rpm for 3 min. After centrifugation, the packed cell volume (PCV) was determined. The buffy coat and upper most layers of red blood cells of one capillary tube were extruded onto a microscope slide and examined with a phase-contrast microscope at 400× magnification (Murray et al., 1977) for the presence of motile trypanosomes. At least 50 fields were examined before positive or negative was declared for each sample. Positive samples were further processed as thin smear stained with Giemsa for trypanosome species identification. Thick blood smears were also prepared, stained with Giemsa and examined with 100× oil immersion objective lens (1000× magnification). Parasitaemia was determined as described by Hebert and Lumsden (1976).

2.3. DNA extraction

DNA was extracted from the blood using Quick-gDNA™ Mini-Prep (Zymo Research Corporation, Irvine, CA, USA) as described by the manufacturer. Briefly, 400 µl of genomic lysis buffer was added to 100 µl of blood, thoroughly mixed and incubated at room temperature for 5–10 min. The mixture was transferred to a spin column in a collection tube and centrifuged at 10,000 × *g* for 60 s after which the collection tube with the flow through was discarded and the spin column transferred to a new collection tube. A volume of 200 µl of prewash buffer was added to the spin column and centrifuged at 10,000 × *g* for 60 s, after which 500 µl of genomic DNA wash buffer was added to the spin column and centrifuged at 10,000 × *g* for 60 s. The soluble DNA was eluted into 50 µl nuclease free water from the spin column into a clean 1.5 ml microcentrifuge tube, incubated at room temperature for 2–5 min and centrifuged at 16,000 × *g* for 30 s. Quantification of DNA yield and assessment of quality were done using Nanodrop ND-100 UV/Vis Spectrophotometer (Nanodrop Technologies, Inc., DE, USA). The eluted DNA was stored at −20 °C until use.

2.4. Primer sets and optimization

Polymerase chain reaction (PCR) primers were selected for optimization based on previously published work. These primers were optimized with DNA extracted from the blood of cattle parasitologically positive for *T. vivax*, *T. congolense*, *T. brucei* and *T. evansi* which led to final selection of six primer sets for this study. Details of primer sets are presented in Table 1.

2.5. Trypanosome detection by PCR

PCR amplification was performed in 20 µl final reaction volume containing equivalent of 20 ng of genomic DNA, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 µM KCl, 200 µM each of dNTPs, 40 ng of each of the primers and 1unit of *Taq* DNA polymerase (Bioneer, Inc. Alameda, CA USA). The reactions were placed in a C-1000 series thermocycler (Biorad, Hercules, CA, USA). The reaction conditions were as follows: *T. brucei* and *T. evansi*; initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and final extension at 72 °C for 5 min. *T. congolense*; initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C at 30 s, 60 °C for 30 s and 72 °C for 30 s with final extension at 72 °C for 5 min. *T. vivax*; initial denaturation at 94 °C for 4 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 30 s followed by final extension at 72 °C for 5 min. Ten microliters of the PCR products were electrophoresed through 1% agarose gel in 1× TBE (89 mM Tris, 89 mM boric acid 1 mM EDTA) at 90 V for 80 min, along with 10 µl of GENEMate Quanti-Marker 100 bp DNA ladder (BioExpress, Kaysville, UT, USA). Gels were stained with GelRed® Nucleic Acid Stain (Phenix Research Products, Candler, NC, USA) at 5 µl/100 ml of the agarose gel suspension. After electrophoresis, PCR products were

Table 1
Sequences of the oligonucleotide primers used in this study and their expected fragment sizes.

Primer set	Species	Sequences	Expected sizes	References
TBR 1	*T. evansi*	GAATATTAAACAATGCGCAG	164 bp	Masiga et al. (1992)
TBR 2		CCATTTATTAGCTTTGTTGC		
TBR 1*	*T. brucei*	CGAATGAATAAACAATGCGCAGT	177 bp	Ilouf et al. (1883)
TBR 2*		AGAACCATTTATTAGCTTTGTGC		
TCS 1	*T. congolense* savannah-type	CGAGCGAGAACGGGCAC	318 bp	Majiwa and Otieno (1990)
TCS 2		GGGACAAACAAATCCCGC		
TCF 1	*T. congolense* forest-type	GGACACGCCAGAAGGTACTT	350 bp	Masiga et al. (1992)
TCF 2		GTTCTCGCACCAAATCCAAC		
TCK 1	*T. congolense* Kilifi-type	GTGCCCAAATTTGAAGTGAT	294 bp	Masiga et al. (1992)
TCK 2		ACTCAAAATCGTGCACCTCG		
ILO1264	*T. vivax*	CAGCTCGCCGAAGGCCACTTGGCTGCG	400 bp	Masake et al. (1997)
ILO1265		TCGCTACCACAGTCGCAATCGTCGTCTCAAGG		

TBR 1* & TBR 2*: the primer set has different sequences from TBR1&2.

visualized on a UV transilluminator and were photographed using an Alphalmager HP System (Protein Simple, Santa Clara, CA, USA). All positive samples were tested twice to confirm the PCR diagnosis and positive and negative (no DNA) samples were used as controls in each run.

2.6. DNA sequencing and sequence analysis

To confirm and validate our results, five positive samples each of *T. vivax*, *T. congolense* savannah, *T. brucei* and the two positive samples each for *T. evansi* and *T. congolense* forest were selected. The PCR products of *T. vivax* and *T. congolense* (savannah and forest subgroups) were sequenced directly using Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the forward amplification PCR primers and AmpliTaq-FS DNA Polymerase while 30 μl of the PCR products of *T. brucei* and *T. evansi* were purified from the agarose gel using a Zymoclean™ Gel DNA Recovery Kit (Zymo Research Corporation, Irvine, CA, USA) and then sequenced as described above. The sequences obtained were viewed and compared on Finch Trace Viewer and Sequence Scanner (Applied Biosystems, Foster City, CA, USA) before they were aligned with each other and with published sequences of various *Trypanosoma* species from GenBank using the Molecular Evolutionary Genetic Analysis software (MEGA 5.05).

2.7. Statistical analysis

The data were summarized using descriptive statistics. Prevalence of trypanosomes in studied cattle using parasitological and molecular techniques were compared statistically using Student's *t*-test (paired *t*-test) while the difference in the mean PCV values and the prevalence of infections within breeds of cattle were compared using one-way ANOVA using SPSS version 19 software.

3. Results

3.1. Sequence analysis of the amplified PCR products

Using sequences retrieved from GenBank, the aligned *T. brucei* sequence had 97% homology with the sequence of *T. brucei* satellite DNA (K00392.1), *T. congolense* savannah-type had 100% homology with *T. congolense* IL300 (HE578911.1), *T. vivax* had 98 and 99% homology with the *T. vivax* Y486 and *T. vivax* diagnostic antigen (HE573027.1 and U43183.1), respectively, *T. congolense* forest type had 94% homology with *T. congolense* (F) TSW 10 (550876.1) and *T. evansi* had 94% homology with *T. brucei gambiense* (FN554966.1).

3.2. Parasitological and molecular (PCR) detection of parasites

Parasite detection by microscopy observation showed 62 samples infected by one or more species of *Trypanosomes*, for a prevalence of 15.1% (95% CI, 12–18%). *T. vivax* was seen more frequently, followed by *T. congolense* and *T. brucei* (Table 2). However, PCR detection showed 262 samples infected by one or more species of *Trypanosoma*, for an overall prevalence of 63.7% (95% CI, 59.4–68.8%) and *T congolense* was the most prevalent 48.7% (95% CI, 4.2–54.3%), *T. congolense* was also the species most often missed by microscopy observation, followed by *T. vivax* 26.0% (95% CI, 21.8–31.1%) and *T. brucei* 4.4% (95% CI, 3.3–7.1%) (Table 3). All of the *T. congolense* detected were savannah-type, except for 2 samples which were single infections by *T. congolense* forest-type (48.2% and 0.5%, respectively). Of those 2 samples, only one was detected by the parasitological method. Additionally, we found 2 samples infected with *T. evansi* (0.5%), one was also infected with *T. vivax* and the second was also infected with *T. vivax* and *T. congolense* savannah-type. Prevalence of mixed infections was 13.9% (95% CI, 10.6–17.4%) being co-infection by *T. congolense* and *T. vivax*. We found 6 samples co-infected by *T. brucei*, *T. vivax* and *T. congolense* savannah-type, and one sample co-infected by *T. evansi*, *T. vivax* and *T. congolense* savannah-type. No infections by *T. congolense* Kilifi-type were detected.

Parasitaemia in the samples detected by microscopy observation ranged between 1 and 5,600 parasites per μl. Infection by *T. congolense* had the lowest number of parasites with 60% of the samples showing <10 parasites/μl, compared to *T. brucei* and *T. vivax* with only 33.3% and 8.9% of the samples showing <10 parasites/μl, respectively. Mixed infections also showed <10 parasites/μl in 60% of the samples as well. Two *T. congolense* and 4 *T. vivax* samples detected by the parasitological method were not detected by PCR. Compared to PCR method as the gold standard of parasite diagnosis, microscopy shows poor sensitivity for detection of all *Trypanosoma* species (Table 3), but specificity was high in all cases (>94%).

3.3. Effect of sex, age and body condition on prevalence of trypanosomosis

Prevalence was similar in both sexes, regardless of the type of infection (Table 4). However, age affected the infection rate of *T. vivax* and mixed infections, being more prevalent in younger animals (less than 1 year old). Prevalence for all *Trypanosoma* infections were much higher in Ogun state (Southern Nigeria) than in Kaduna state (Northern Nigeria). Cattle with poor body condition showed higher infection rate than cattle in good condition, regardless of the type of infection. N'dama cattle had lower prevalence than

Table 2
Comparison of the diagnostic results obtained in this study by parasitological and molecular methods.

Microscopy	PCR								
	Tb	Tc	Tv	Tb/Tc	Tb/Tv	Tc/Tv	Tb/Tc/Tv	Negatives	Totals (%)
Tb	1	5	4	0	0	3	1	0	14(3.4)
Tc	1	10	5	0	0	1	0	2	19(4.6)
Tv	1	10	7	1	0	2	0	4	25(6.1)
Tb/Tc	0	0	0	0	0	1	0	0	1(0.2)
Tb/Tv	0	0	0	0	0	0	0	0	0(0.0)
Tc/Tv	0	0	2	0	0	0	0	0	2(0.5)
Tb/Tc/Tv	0	0	0	1	0	0	0	0	1(0.2)
Negatives	4	119	36	2	1	39	5	143	349(84.9)
Totals (%)	7(1.7)	144(35.0)	54(13.1)	4(1.0)	1(0.2)	46(11.2)	6(1.5)	149(36.5)	411

Tb: *Trypanosoma brucei*, Tc: *T. congolense* savannah-type, Tv: *T. vivax*. The two samples infected with *T. evansi* were not included in this analysis.

Table 3
Prevalence of *Trypanosoma* species in naturally infected cattle in Nigeria, and the sensitivity and specificity of the microscopic detection, using PCR as a gold standard.

Parasite		Estimated	95% CI Lower	95% CI Upper
T. brucei	Prevalence	4.4	3.3	7.1
	Sensitivity	0.17	0.04	0.42
	Specificity	0.97	0.94	0.98
T. congolense	Prevalence	48.7	44.2	54.3
	Sensitivity	0.07	0.04	0.11
	Specificity	0.95	0.91	0.98
T. vivax	Prevalence	26.0	21.8	31.1
	Sensitivity	0.10	0.06	0.18
	Specificity	0.94	0.91	0.97

the other breeds of cattle. Non-significant differences were found in parasitaemia levels among breeds.

3.4. PCV values of infected and non-infected animals

PCV values were affected by the type of infection so that animals with *T. vivax* monoinfection and mixed infections showed significantly lower PCV values than non-infected animals (Table 5). Animals with *T. brucei* and *T. congolense* monoinfections showed no differences in PCV values, compared to non-infected and those infected with *T. vivax* or mixed infections. Non-infected N'dama cattle showed slightly higher PCV values than the other breeds of cattle without infection. In general, PCV values were lower in infected cattle but only significantly lower in *Trypanosoma* infected N'dama and White Fulani cattle, compared to non-infected animals of those breeds, respectively (Table 6), while the differences were non-significant between Muturu and Sokoto Gudali cattle.

In general, PCV values were different depending on the body condition. PCV in animals with poor condition (28.79% ± 0.61) was on average 16.8% lower than animals with good body condition (34.50% ± 0.28). In addition, animals in good condition and with *Trypanosoma* infection had a significantly lower PCV value ($p < 0.05$) than those not infected (34.11 ± 0.34 and 35.13% ± 0.45, respectively), but animals with poor condition did not showed any difference in PCV values between the groups of infected and non-infected (28.79% ± 0.68 and 28.80% ± 0.46, respectively). In addition, PCV values were significantly different in animals infected with *Trypanosoma* at different levels of parasitaemia, with higher PCV (33.68% ± 0.97) in animals with <200 parasites/μl compared to those with >200 parasites/μl (30.19% ± 1.55).

4. Discussion

Existing parasitological and serological diagnostic techniques for screening blood samples to detect and differentiate bovine trypanosomes are not suited to large-scale epidemiological analysis and precise species identification (El-Metanaway et al., 2009; Fernández et al., 2009). The results of microscopy screening in this study was 15.1%, which falls within the range of 5.3–18.57% reported in other studies (Kalu and Lawani, 1996; Abenga et al., 2002; Enwezor et al., 2009) in Nigeria and elsewhere in Africa (Mamoudou et al., 2006; Merkuria and Gadissa, 2011). The use of PCR showed a much higher 63.7% prevalence of trypanosome infection in this study, a remarkably higher percentage than previously reported in Nigeria. This underscores the sensitivity of molecular screening based on PCR, and related to the difficulty of microscopic detection of parasites and low levels of parasitaemia in subclinical infections. This corroborates the results of earlier

Table 4
The prevalence of trypanosomosis detected by PCR according to sex, geographical regions, age groups, body condition and breed.

Factor		N	Overall prevalence	*T. congolense*	*T. vivax*	Mixed infections
Sex	Male	129	60.5[a]	48.1[a]	26.4[a]	17.1[a]
	Female	282	63.5[a]	48.2[a]	24.8[a]	12.8[a]
Age group	≤1 year	86	58.3[a]	41.9[a]	18.6[a]	4.7[a]
	>1 year	325	63.4[a]	49.8[a]	27.1[b]	16.6[b]
Zone	Kaduna	146	37.0[a]	39.0[a]	15.9[a]	8.2[a]
	Ogun	265	76.6[b]	53.2[b]	28.3[b]	17.4[b]
Body condition	Good	340	60.7[a]	46.0[a]	23.2[a]	11.7[a]
	Poor	71	71.4[b]	58.6[b]	35.7[b]	25.7[b]
Breed	Muturu	112	67.0[a]	45.5[a]	33.0[a]	15.2[a]
	N'Dama	31	29.0[b]	19.4[b]	9.7[a]	0.0[a]
	S. Gudali	68	51.5[a]	42.6[a]	22.1[a]	14.7[a]
	W. Fulani	200	65.5[a]	52.5[a]	23.0[a]	14.0[a]
Average		411	62.5	48.2	25.3	14.1

Values with different superscripts are significantly different ($P < 0.05$).

Table 5
Comparison of the mean PCV values of the Nigerian cattle infected or non-infected with *T. brucei*, *T. congolense* and *T. vivax*. Data from cattle infected with *T. evansi* was not included because it was found only in two animals infected with other species of Trypanosome. N: number of animal sampled.

Type of infection	Mean (%) ± SE (N)
Non-infected	35.13 ± 0.45 (349)[a]
T. brucei	34.19 ± 1.36 (7)[a,b]
T. congolense	33.97 ± 0.45 (344)[a,b]
T. vivax	32.95 ± 0.69 (54)[b]
Mixed infections	33.15 ± 0.80 (57)[b]

Values with different superscripts are significantly different ($P < 0.05$).

Table 6
Comparison of the mean PCV values of the different Nigerian cattle breeds infected or not infected with *Trypanosoma* spp. *N*: number of animal sampled.

Cattle breed	Non-Infected Mean ± SE (*N*)	Infected Mean ± SE (*N*)
Muturu	34.47 ± 0.09 (37)[a]	33.38 ± 0.56 (75)[a]
N'Dama	36.00 ± 1.31 (22)[a]	33.33 ± 1.36 (9)[a]
Sokoto Gudali	34.75 ± 1.05 (33)[a]	33.84 ± 0.89 (35)[a]
White Fulani	35.51 ± 0.61 (89)[a]	33.88 ± 0.49 (131)[b]

Values with different superscripts are significantly different ($P < 0.05$).

workers (Desquesnes and Davila, 2002; Delespaux et al., 2003; Karimuribo et al., 2011). The significantly higher prevalence of trypanosomal infections in Ogun State (Southern Nigeria) is consistent with heavy infestation of both obligate (*Glossina* spp) and mechanical (*Tabanids*) vectors of trypanosomes (Ahmed, 2004). In western Kenya and Uganda, *T. b. brucei* and *T. b. rhodesiense* were found in the CNS of native cattle and were associated with significant mortality (Welde et al., 1989), hence the detection of *T. brucei* and *T. evansi* in Nigerian cattle might portend serious danger not only to cattle and other livestock but also to livestock owners and the communities at large as *T. evansi* infection has been reported in cattle and humans in India (Laha and Saimal, 2009; Joshi et al., 2005).

Higher prevalence of *T. congolense* savannah-type followed by *T. vivax* and *T. brucei* in this study using molecular methods contrasts with other reports in which *T. vivax* was reported to have higher prevalence, followed by *T. congolense* and *T. brucei*, when using parasitological methods in Nigeria (Anene et al., 1991a,b; Kalu, 1995; Omotainse et al., 2000). But the present findings are in consonance with the report of Ogunsanmi et al. (2000) who reported higher incidence of *T. congolense* in a survey carried out in Southwestern Nigeria and Merkuria and Gadissa (2011) in Northwestern Ethiopia. This could be related to lower parasitaemia of *T. congolense* infections compared to those infected with *T. vivax* in this study.

Our parasitological findings agree with the low level of mixed infections detected by parasitological techniques by earlier workers in the country (Kalu, 1995; Abenga et al., 2002; Enwezor et al., 2009). However, PCR results revealed higher levels of mixed infections, consistent with elsewhere in Africa (Pinchbeck et al., 2008). We detected *T. congolense* riverine forest-type in two of the animals sampled and to the best of our knowledge this is the first report of this subgroup of *T. congolense* in Nigeria. Lefrancois et al. (1998) also reported this in three animals in Sideradougou, Burkina Faso. The apparently higher prevalence of *T. congolense* could be an indication that its transmission is highly favored by the obligate cyclical vector or the *T. vivax* and *T. brucei* respond better to the trypanocidal drugs, diminazene aceturate and homidium chloride, respectively. It could also be due to over-representation of *T. vivax* infections through more serious symptoms that induces producers to seek diagnosis and treatment, compared to infections by *T. congolense*. The highest prevalence of *T. congolense* savannah type in this study is in partial agreement with Solano et al. (1995) and De La Rocque et al. (1998) who indicated that the savannah-type was predominant in tsetse flies as well as in cattle, but disagrees with their observations that the riverine/forest-type was only present in the vectors since we detected this in two of the cattle studied.

While morphological identification error could be responsible for the inability of the PCR assay to detect two *T. congolense* and four *T. vivax* – positive animals detected by microscopy in this study, it could also be attributed to high concentration of template DNA that result in inhibition of the PCR amplification processes and or due to primers sets used. Similar observations were reported by Desquesnes (1997) and Gonzales et al. (2003) who after diluting sera and blood spot eluate samples, respectively, obtained improvement in PCR detection rates and Gonzales et al. (2003) who were able to amplify *T. vivax* DNA using a set of new primers (TVW A/B) from four of the samples that were classed as PCR negative when primer sets (TW)1/2) were used.

The PCV results for infected and non-infected cattle are in accordance with the reports of Van den Bossche and Rowland (2001) and Simukoko et al. (2011) who reported that factors such as nutrition affect the PCV of rural cattle. Anemia, one of the cardinal signs of trypanosomosis (Getachew, 2005), could also be caused by other haemoprotozoan parasites and helminthes (Radostits et al., 2007). As a result of this, PCV values alone should not be used as a diagnostic parameter for trypanosomosis, except where diseases causing anemia are unapparent, then low PCV may be a good indicator of trypanosomal infection (Maroufy et al., 2008). Mean PCV of *T. vivax*-infected and mixed infected were significantly higher than non-infected cattle. *T. vivax* infection also had more cases of high levels of parasitaemia than infections by *T. congolense*. While this could be an indication that *T. vivax* is more pathogenic in cattle than *T. congolense* and *T. brucei*, as reported by Authie (1983) and Sabbu et al. (1984), it does not agree with the findings of Sekoni et al. (1990). The high percentage of cases of mixed infections with low levels of parasitaemia may be explained by parasite interactions similar to mixed plasmodium infections of more than one species in human, where some form of cross-species regulation of parasitaemia exists (Bruce et al., 2000; Bruce and Day, 2002, 2003).

Trail et al. (1994) and Rowlands et al. (2001) reported significantly low infection rate in calves below 15 months, similar to our findings in which there were significant differences between the prevalence of *T. vivax* and mixed infections between calves below 12 months and those above one year. This could be due to longer exposure of older animals to the disease vectors and higher chances of being infected and possession of stronger immunity. Although we found lower prevalence in N'dama cattle, a reportedly trypanotolerant breed (Mattioli et al., 1998), similarly low prevalence in trypanotolerant Muturu may be due to high numbers of this breed in Ogun state. This contrasts with twice the prevalence in Kaduna state where Sokoto Gudali and White Fulani are found in higher numbers. Since some of the animals sampled may be cross-breeds with trypanotolerant breeds, this could play a role in low parasitaemia levels since crossbred offspring may display appreciable levels of low parasitaemia (Orenge et al., 2011) because there are no structured breeding programs by small cattle producers in Nigeria. In conclusion, this study has shown that PCR can be used effectively in extensive epidemiological surveys to validate carrier status of animal trypanosoma infection in Nigerian cattle. The finding of new subgroup of *Trypanosoma* spp. in Nigeria and the relatively high abundance of mixed infections are of clinical significance. This study also suggests that *T. congolense* is the most prevalent species in Nigeria.

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Appendix 8.2: Partial sequences of diagnostic antigen gene of *T. vivax* obtained from samples submitted for sequencing

Sample 08: *T. vivax* – Kaduna State

CATGTGAAACAATGCACTGTGGGTAACAAAAGTTGCTTTGGACACAAG
GGCAGTGCGTGTAGTCGCCGCAGCACGCCANNTAGNCGGGGAACAGA
GCAGTCTCGGCGCGCCCCATGTTTCACCTCCAGGAGCCCCGGCACCGT
GTCGCGCAGGCGCTTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGGT
TGGCCGCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAG
ATCTTGGGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGTGTG
TGTGTGCGTATAATCTGTGTGTCTGTGTNCG

Sample 52: *T. vivax* – Kaduna State

TTGANCAGCCTGGAAAGCGGCGGCATCGAACGTTGCTTTGGACACAA
GGCAGTGCGTTGGTAGTCGCCGCAGCACGCCACATTAGCCGGGGAAC
AGAGCAGTCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACC
GTGTCGCGCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGG
TTGGCCGCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAG
ATCTTGGGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGTGTG
TGTGTGTGTGTGTNTGTNTGTATGTGTGTGNGT

Sample 02: *T. vivax*- Kaduna State

GTGAAAGCCTGGAAGCGGCGGCATCGACGTTGCTTGGACACAAGGCA
GTGCGTGTAGTCGCCGCAGCACGCCACATAGCCGGGGAACAGAGCAG
TCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACCGTGTCGC
GCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGGTTGGCCG
CCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAGATCTTGG
GAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGTGTGTGTGTGT
GTNTGTCTGTGTGTCTGTGTGTGNGTGTGAGAG

Sample 9N: *T. vivax*-Ogun State

TGAAAACATGGAAAGGGGCGGAATCAACGTGCTTTGGACACAAGGCA
GTGCGTTGGTAGTCGCCGCAGCACGCCACATAGCCGGGGAACAGAGC
AGTCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACCGTGTC
GCGCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGGTTGGC
CGCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAGATCTT
GGGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGTGTGTGTGT
GTGTNTGTNTGTGTGTNTGTGTGCGNGTGTGAG

Sample 32N: *T. vivax*-Ogun State

GTGTTGAAAAGCATGGAAAGCGGGCGGCATCAAACGTGCTTGGGACA
CAAGGCAGTGCGTTGTTAGTCGCCGCAGCACGCCACATAGCCGGGGA
ACAGAGCAGTCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCA
CCGTGTCGCGCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCA
GGTTGGCCGCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGC
AGATCTTGGGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGCG
TGTGTGTCCNTATNTNTGTGTGTGCGTGTACGA

Sample 75N: *T. vivax* –Ogun State

GATGTGAAAAAATGGGAAGGGGGGAAATAAAAGTGCTTTGGGACACA
AGGCAGTGCGGTGTTAGTCGCCGCAGCACGCCACATAGCCGGGGAAC
AGAGCAGTCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACC
GTGTCGCGCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGG
TTGGCCGCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAG
ATCTTGGGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGCGTG
TGTGTNNGCATGTNTGTGTGTNCGAGTACGA

Sample 90N: *T.vivax*- Ogun State

AATTGCTTTGGACACAAGGCAGTGCGTGTAGTCGCCGCAGCACGCCAT
TTAGGCGGGGAACAGAGCAGTCTCGGCGCGCCCCATGTTCACCTCCAG
GAGCCCCGGCACCGTGTCGCGCAGGCGCTTGCAGGTTCTCCTCCACCG
CGTTTACCCGGCAGGTTGGCCGCCATCTTTTCGGGGTCCAGCGAGAAG
AAGACAGCGTGGCAGATCTTGGGAGCCATGGTTCCTGTGGTGTCTGTC
TGTCTTTGTGTGCGTGTGTGTNCGTATATA

Sample 94N: *T. vivax*-Ogun State

GTTGACAGCCTGGAAAGCGGCGGCATCGAACGTTGCTTTGGACACAA
GGCAGTTGCGTGTAGTCGCCGCAGCACGCCACATAGCCGGGGAACAG
AGCAGTCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACCGT
GTCGCGCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGGTT
GGCCGCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAGA
TCTTGGGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGTGTGT
GTGTGTGTGTGTCCGTATGTCTGTGTGTCCGTGT

Sample 96N: *T. vivax*-Ogun State

GGTGAAAAACATTGGAAGCGGCGGGAATCAACGTGCTTGGACACAAG
GCAGTGCGTTGTAGTCGCCGCAGCACGCCACATAGCCGGGGAACAGA
GCAGTCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACCGTG
TCGCGCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGGTTG

GCCGCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAGAT
CTTGGGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGCGTGTG
TGTCCGTATGTNTGTGTGTCCGTGTACGAGNGGG

Sample 98N: *T. vivax*-Kaduna State

GGTGACAGCCTGGAAGCGGCGGCATCGAACGTTGCTTTGGGAACACA
AGGCAGTGCGTGTAGTCGCCGCAGCACGCCACATAGCCGGGGAACAG
AGCAGTCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACCGT
GTCGCGCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGGTT
GGCCGCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAGA
TCTTGGGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGTGTGT
GTGTGTGTGTGTNTGTNTGTNTGTGTGTGAGTG

Sample 235: *T.vivax*-Ogun State

TTTGNCAGCCTGGAAGCGGCGGCATCGAACGTGCTTGGAACACAAGG
CAGTGCGTGTAGTCGCCGCAGCACGCCACATAGCCGGGGAACAGAGC
AGTCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACCGTGTC
GCGCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGGTTGGC
CGCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAGATCTT
GGGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGTGTGTGTGT
GTGTGTGTNTGTNTGTNTGTGTGTNAGTGTNA

Sample 234: *T.vivax*-Ogun State

GTGTGACAGCCTGGAAGCGGCGGCATCGACGTGCTTGGACACAAGGC
AGTGCGTGTAGTCGCCGCAGCACGCCACATAGCCGGGGAACAGAGCA
GTCTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACCGTGTCG
CGCAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGGTTGGCC
GCCATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAGATCTTG
GGAGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGTGTGTGTGTG
TGTNTGTNTGTGTGTNTGTGTNTGAGTGTGAGN

Sample 136: *T. vivax*- Ogun State

CGCCTGGTAAGCGGCGGCAATCGAACGTGCTTGGAACACAAGGGCAG
TGCGTGTAGTCGCCGCAGCACGCCACATAGCCGGGGAACAGAGCAGT
CTCGGCGCGCCCCATGTTCACCTCCAGGAGCCCCGGCACCGTGTCGCG
CAGGCGCTGCAGGTTCTCCTCCACCGCGTTACCCGGCAGGTTGGCCGC
CATCTTTTCGGGGTCCAGCGAGAAGAAGACAGCGTGGCAGATCTTGGG
AGCCATGGTTCCTGTGGTGTCTGTCTGTCTTTGTGTGNGTGTGTGTGTG
TNTGTNTGTNTGTCTGTGTGGNGTGTGAGAGC

Appendix 8.3: Partial sequences of satellite repeats of *T. congolense Savannah type* obtained from samples submitted for sequencing

Sample 168: *T. congolense* Savannah type -Kaduna State

CGCATCCCTTTTGGGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGT
CAAAAATGTCAAAAACGCACAAATTCGAAAAACGCGTATTTGGCACG
TATTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAAT
TTTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTG
AAAATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAAT
TTTTTCTGTTCCAAAAATGGTTGTGCGGGATTTGATTTGCCCC

Sample 99N: *T. congolense* Savannah type – Ogun State

CGGGCTAACCATTTTTGGGCCAAATGGCCAAAAACCGGTTTTTTTGAA
AATGGTCAAAAATGTCAAAAACGCCCCCTTAGGGNAAACGCGTATTTT
GCACGTATTTGTCGTTTTTGGACCTTTTTGACGCGCATAGTGGTTTTTC
AAAATTTTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAAT
TTTTGAAAATTTTTTTGTAAAAAATATTTTTTTTTGACTTTTTGGGCGA
AAATTTTTTCTGTTCCAAAAATGGTNGTGCGGGATTTGTTT

Sample 156: *T. congolense* Savannah type-Ogun State

CATCCCTTTTGGGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGTCA
AAAATGTCAAAAACGCACAAATTCGAAAAACGCGTATTTGGCACGTA
TTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTT
GCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGAAA
ATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAATTTTT
TCTGTTCCAAAAATGGTTGTGCGGGATTTGGTTTGNCCCAA

Sample 80: *T. congolense* Savannah type- Ogun State

CATCCTTTTGGGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGTCAA
AAATGTCAAAAACGCAAAAATTCGAAAAACGCGTATTTGGCACGTATT
TGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTTG
CAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGAAAA
TTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTNGGGCGAAAATTTTTT
CNGTTCCAAAAANGGTNGTGCGGGATTTGTTTGCCCCANA

Sample 78N: *T. congolense* Savannah type

CCCTTTTGGGCCAATGGCCAAAAACCGGTTTTTTTGAAAATGGTCAAA
AATGTCAAAAACGCANCAATTCGAAAAACGCGTATTTGGCACGTATTT
GTCGTTTTCGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTTGC
AAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGAAAAT
TTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAATTTTTTC
TGTTCCAAAAATGGTTGTGCGGGATTTGTTTGNCCCAAATT

Sample 5N: T. *congolense* Savannah type –Kaduna Sate

GCATCCTTTTGGGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGTCA
AAAATGTCAAAAACGCACCAATTCGAAAAACGCGTATTTGGCACGTAT
TTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTT
GCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGAAA
ATTTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAATTTTT
TCNGTTCCAAAAATGGTTGTGCGGGATTTGTTTTGTCCCAA

Sample 52N: *T. congolense* Savannah type-Kaduna State

CATCCCTTTTGGGCCAATGGCCAAAAACCGGTTTTTTTGAAAATGGTC
AAAAATGTCAAAAACGCACCAATTCGAAAAACGCGTATTTGGCACGT
ATTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATT
TTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGA
AAATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTNGGGCGAAAATT
TTTTCNGTTCCAAAAANGGTTGTGCGGGATTTGTTTTGNCCCA

Sample85N: *T. congolense Savannah*-Kaduna State

CATCCCTTTTGGGCCAATGGCCAAAAACCGGTTTTTTTGAAAATGGTC
AAAAATGTCAAAAACGCACAAATTCGAAAAACGCGTATTTGGCACGT
ATTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATT
TTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGA
AAATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAATT
TTTTCTGTTCCAAAAATGGTTGTGCGGGATTTGTTTGNCCCAA

Sample 89N: *T. congolense Savannah*-Ogun State

GCATCCTTTTGGGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGTCA
AAAATGTCAAAAACGCAAAAATTCGAAAAACGCGTATTTNGCACGTA
TTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTT
GCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGAAA
ATTTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAATTTTT
TCTGTTCCAAAAATGGTTGTGCGGGATTTGGTTTGNCCCAA

Sample 42: *T. congolense Savannah- Ogun State*

GCATCCTTTTGGGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGTCA
AAAATGTCAAAAACGCAAAAATTCGAAAAACGCGTATTTGGCACGTA
TTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTT
GCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGAAA
ATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAATTTTT
TCTGTTCCAAAAANGGTTGNGCGGGATTTGGTTTGNCCCAA

Sample 51: *T. congolense Savannah-Ogun State*

GCATCCTTTTGGGCCAATGGCCAAAAACCGGTTTTTTTGAAAATGGTC
AAAAATGTCAAAAACGCACCAATTCGAAAAACGCGTATTTGGCACGT
ATTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATT
TTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGA
AAATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAATT
TTTTCTGTTCCAAAAANGGTTGTGCGGGATTTGATTTGNCCCA

Sample 61 *: T. congolense Savannah-Kaduna State*

GCAATTTTGAGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGTCAAA
AATGTCAAAAACGCCCCCTTTACGGAAAACGCGTATTTGGCACGTATT
TGTCGTTTTCGGGCCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTTG
CAAAAAATTGTGTCAAAAACTTTTTCAATTTTTGCAAATTTTTGAAAAT
TTTTTTGTAAAAAAATATTTTTTTTGGCCTTTTGGGGGAAAAATTTTTC
CGGTCCCAAAAAGGGTGGGCCGGAATTGGTTGGCCCAANCC

Sample 65 *: T. congolense Savannah-Ogun State*

GCAGCCATTTTGGGCCCAATGGCCAAAACCGGTTTTTTTGAAAATGGT
CAAAAATGTCAAAAACGCACNAATTCGAAAAACGCGTATTTTGCACGT
ATTTGTCGTTTTTGGACCTTTTTGACGCGCATAGTGGTTTTTCAAAATT
TTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTNGA
AAATTTTTTTGTAAAAAAATATTTTTTTTTGNCTTTTTGGGCGAAAATT
TTTTCTGTCCCAAAAATGGTNGTGCGGGATTTGTTTGCCCCA

Sample 121: *T. congolense Savannah-Ogun State*

CGCATCCCTTTTGGGCCAAATGGCCAAAAACCGGTTTTTTTGAAAATG
GTCAAAAATGTCAAAAACGCACCAATTCGAAAAACGCGTATTTGGCA
CGTATTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAA
ATTTTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTT
TGAAAATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAA
ATTTTTTCTGTTCCAAAAATGGTTGTGCGGGATTTGGTTTGTC

Sample 150: *T. congolense Savannah-Ogun State*

GCATCCTTTTGGGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGTCA
AAAATGTCAAAAACGCACAAATTCGAAAAACGCGTATTTGGCACGTA
TTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTT
GCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGAAA
ATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAATTTTT
TCTGTTCCAAAAATGGTTGTGCGGGATTTGGTTTGNCCCAA

Sample 152: *T. congolense Savannah- Kaduna State*

AACCCTATTTGGGCCAAATGGCCAAAAACCGGTTTTTTTGAAAATGGT
CAAAAATGTCAAAAACGCACCAATTCGAAAAACGCGTATTTTGCACGT
ATTTGTCGTTTTTGGGCCTTTTTGACGCGCATAGTGGTTTTTCAAAATT
TTGCAAAAAATTGNGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGA
AAATTTTTTTGTAAAAAAATATTTTTTTTTNGNCTTTTNGGGCGAAAATT
TTTCCGGTCCCAAAAAGGGTTGTGCGGGATTTGTTNGCCCA

Sample 165: *T. congolense Savannah-Kaduna Sate*

CATCCCTTTTGGGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGTCA
AAAATGTCAAAAACGCAAAAATTCGAAAAACGCGTATTTGGCACGTA
TTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTT
GCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGAAA
ATTTTTNTGTAAAAAAATATTTTTTTTTNGACTTTTNGGGCGAAAATTTT
TCCGGTCCCAAAAAGGGTGGGGCGGAATTGGTTTTGCCCAA

Sample 166: *T. congolense Savannah-Ogun State*

GCCATCCTGTTTGGGCCAAATGGCAAAAACCGGTTTTTTTGAAAATGG
TCAAAAATGTCAAAAACGCACCCAATTCGAAAAACGCGTATTTTGCAC
GTATTTGTCGTTTTTGGACCTTTTTGACGCGCATAGTGGTTTTTCAAAA
TTTTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTT
GAAAATTTTTTTGTAAAAAAATATTTTTTTTTNGCCTTTTGGGGCGAAAA
TTTTTCCGGTCCCAAAAAGGGTGGNGCGGGATTTGTTNGCC

Sample 273: *T. congolense Savannah-Kaduna State*

TATCCCTTTTGGGCCAAATGGCCAAAAACCGGTTTTTTTGAAAATGGT
CAAAAATGTCAAAAACGCCCCAATTCGAAAAACGCGTATTTGGCACGT
ATTTGTCGTTTTTGGACCTTTTTGACGCGCATAGGGGTTTTTCAAAATT
TTGCAAAAAATTGNGTCAAAAACTTTTTCTAATTTTTGCAAATTTTNGA
AAATTTTTTTGTAAAAAAATATTTTTTTTTNGACTTTTNGGGCGAAAATT
TTTTCTGTCCAAAAATGGTNGTGCGGGATTTGTTNGNCCAA

Sample 270: *T. congolense Savannah-Ogun State*

GCGCATCCTTTTGGGCCAATGGCCAAAAACCGGTTTTTTTGAAAATGG
TCAAAAATGTCAAAAACGCAACAATTCGAAAAACGCGTATTTGGCAC
GTATTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGGGGTTTTTCAAAA
TTTTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTNGCAAATTTTN
GAAAATTTTTTNGTAAAAAAATATTTTTTTTNGACTTTTNGGGCGAAA
ATTTTTCCNGTCCCAAAAANGGTNGNNCGGGATTTGTTTGCCCA

Sample 82N: *T. congolense Savannah-Ogun State*

CATCCCTTTTGGGCCAAATGGCCAAAAACCGGTTTTTTTGAAAATGGT
CAAAAATGTCAAAAACGCACCAAATTCGAAAAACGCGTATTTGGCAC
GTATTTGTCGTTTTCGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAA
TTTTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTT
GAAAATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTNGGGCGAAAA
TTTTTNCNGTTCCAAAAANGGTNGNGCGGGATTTGTTTGCCCC

Sample 273: *T. congolense Savannah-Ogun State*

GGTTTCAATTTTGGGCCAATGGCCAAAACCGGTTTTTTTGAAAATGGT
CAAAAATGTCAAAAACGCACCCATTCGAAAACGCGTATTTGGCACGTA
TTTGTCGTTTTTGGACCTTTTTGACGCGCATAGTGGTTTTTCAAAATTTT
GCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGAAA
ATTTTTTTGTAAAAAAATATTTTTTTTNGACTTTTNGGGCGAAAATTTT
TTCNGTCCCAAAAATGGTTGGGCGGGATTNGTTTGTCCCA

Sample 294: *T. congolense Savannahtype-Ogun State*

CAATCCCTTTTGGGCCAAATGGCCAAAACCGGTTTTTTTGAAAATGGT
CAAAAATGTCAAAAACGCACCAATTCGAAAAACGCGTATTTGGCACG
TATTTGTCGTTTTTGGNCCTTTTTGACGCGCATAGTGGTTTTTCAAAAT
TTTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTG
AAAATTTTTTTGTAAAAAAATATTTTTTTTTGACTTTTTGGGCGAAAAT
TTTTTCTGTCCCAAAAATGGTTGTGCGGGATTTGTTTGCCCCA

Sample 127: *T. congolense Savannah* type-Ogun State

TCCCTTTTGGGCCAAATGGCCAAAAACCGGGTTTTTTTGAAAATGGTC
AAAAATGTCAAAAACGCACCAATTCGAAAAACGCGTATTTGGCACGT
ATTTGTCGTTTTTGGACCTTTTTGACGCGCATAGTGGTTTTTCAAAATT
TTGCAAAAAATTGTGTCAAAAACTTTTTCTAATTTTTGCAAATTTTTGA
AAATTTTTTTGTAAAAAAATATTTTTTTTTTGACTTTTTGGGCGAAAATT
TTTTCTGTTCCAAAAATGGTTGTGCGGGATTTGTTTGNCCCAA

Appendix 8.4: Partial sequences of satellite repeats of *T. congolense* Forest type obtained from samples submitted for sequencing.

Sample 09: *Trypanosoma congolense*-forest type-Ogun State

CAGCCAACGCCTTTTTTTGAAATGCTCAAAAACGTGAAAAACGCCAAA
ATTCGGAAAATGCGTGTTTTTCACCAATTGGTCGTTTTTGGGCGTTTTT
GACCCGCATAGTGGATTTTTGAAATTTTTCAAAAAAAAAGGCAAAATT
TTTTTCAAATTTTTGNGTAAAAAACAACTTTTTTTNCGACTTTTNGGGG
AAAATTTTTNCGGCGCAAAAAATGAATAGGCGGGTCAAAANGGCCCC
TTTGAGACGTTTAAAANGCTTTTNGAAATTTGAAATTTGGTTGGATTN
GGGGCNAAAACAACCCCCTA

Sample 80 : *Trypanosoma congolense*-forest type-Ogun State

GGGAAAAACGCCAAAATTCCGGAAAATGCGTGTTTTTCCCCAATTGGG
CGTTTTTGGGCGTTTTTGACCCGCATAGTGGATTTTTGAAATTTTTCAA
AAAAAAAGGCCAAAATTTTTTTCAAATTTTNGNGTAAAAAACAACTTT
TTTTNCGACTTTTNGGGGAAAATTTTTNCGGCCCAAAAAATGAATAGG
CGGGCCAAAATGGCCCCTTTGAGACGTTTAAAANGCTTTTTGAAATTT
GAAATTTGGTTGGATTNGGGACAAAAANAAAAACTGTTCGGGCGGCC
CCCCGCCCGA

Appendix 8.5: Partial sequences of satellite repeats of *T.brucei* obtained from samples submitted for sequencing

Sample 64: T. brucei

ATGTGTGCCATATTAATTACAAGTGTGCACCATTAAATACAAGTGTGT
AACATTAATTTGCANAGTTTGCAACGCTGTTCTTTAGNGTTTAANGGG
NGCAACAAAGCTATAAATGGTCCTAATCCGAATGAATATTAAACAATG
CGCAGTTTAACGCTATT

Sample 74: *T. brucei*

TTTTAATGTGTGCCATATTAATTACAAGGTGTGCAACAATTAAATACA
AGTGTGTAACATTAATTTGCAAGGTTTGCAACAATGTTCTTTAGTGTTT
AATGGGTGCAACAAAGCTAATAAATGGTTCTAATCCGAACCGAATATT
AAACAATGCGCGGCTA

Sample 274: T. brucei

TTAATGTGTGCCATATTAATTACAAGTGTGCAACAATTAAATACAAGT
GTGTAACACCCATTTGCAAGTTTGCAACAATGTTCTTTAGGTGTTTAAT
GGGTGCAACAAAGCTAATAAATGGTTCTAATACGAATGAATATTAAAC
AATGCGCAGTNAACA

Sample 294: *T. brucei*

TTTTAATGTGTGCCATATTAATTACAAGTGTGCAACAATTAAATACAA
GGTGTGTAACACAAATTTGCAAGTTTGCAACAATGTTCTTTAGGTGTTT
AATGGGTGCAACAAAGCTAATAAATGGTTCTAATACGAATGAATATTA
AACAATGCGCAGNTA

Sample 269: *T. brucei*

GAGACAGGGGTTTTAGGGNGCCACATTATTCAAGAGTCACATTTAAAT
ACAAGATGTGTAACAGTTAACCCCGTTAAGAGTGGAAAACAGGGTTCT
TTTAGGTGGTTTAGTNGGGGGGCAACAACAGCTAATAAAATGAGAAT
GCGGGGGCGNAGAGCTAGANAATTGTNCAGGTAATGTCCNCTGTGGG
GGGGGANAAAACTTTGTGGGTTGGTCCGCTGCGGTGAAGGAGGTGGA
GAGAAGC

Appendix 8.6: Partial sequences of satellite repeats of *T. evansi* obtained from samples submitted for sequencing

Sample 73: *T. evansi*

CAATANCATACTTTTAATGTGTGCCATATTAATTACAAGTGTGCAACA
TTTAAATACAAGTGTGTAACATTACCCTTGCAAGTTTGCAACAATGTTC
TTTAGTGTTTAATGGGTGCAACAAAGCTAATAAATGGACCTTATACGA
ATGAATATTAAACAATGCGCAGTTAACGCTATTATACACAATAACTTT
TAATGTGTGCCATATTAATTACAAGGGTGCAACATTAAATACAAGT

Sample 269: *T. evansi*

TTTCCATACTTTTAATGTGTGCCATATTAATTACAAGGTGTGCAACAAT
TTAAATACAAGTGTGTAACATTACCCTTGCAAGTTTGCAACAATGTTCT
TTAGTGTTTAATGGGTGCAACAAAGCTAATAAATGGACCTTATACGAA
CGAATATTAAACAATGCGCAGTTAACGCTATTATACACAATAACTTTT
AATGTGTGCCATATTAATTACAAGTGTGCAACATTAAATACAAGGG

Appendix 8.7: Partial sequences of Internal Transcribed Spacer I (ITS-1) of ribosomal DNA (rDNA) gene of *T. brucei* obtained from samples submitted for sequencing

Sample 64: *T. brucei-ITS1-*

TGTGCTCCTATCCTCTGGGAGGTGGTGGAGGTGCTCTAATGAGAATAG
CAGGTGAGTGTGCGGGTATATTTCGCATTTTCTGCACGCATATAATGTC
ACCTTTATCTTGATTGTAGCTGTTCCCTCTACCGCCTCGATTGGGGTAA
TTATGGAGGATGTCCTTTTTGTGGTCATTATACTCATAGAATTTCTGTT
TGATGACCTCTGTGGATATGCTTGCAAATGTGCGCTCACTGCCGCCCTT
GTATGCCTGGTTAAGATTGATCTCTTTGACGGCGTACCTATCTGAAAA
ACCTTGATGAACTATATCATTGTATAAGTCCTGGATGTATATCCTCGCT
GTTCCGGCATAACCACATCAGGGGGGTTGCGGGGCAAGACACGCCAT
AACCTCCGTTTCCTCTTTTTACATCACTCTGATTTTTAATACCAATCCCT
TGATGGTGTTACACGTGTTGGCATACCTGAAGATTGCATAGGATGGGT
TCTTAATACGAAGATCTACCGACCACATTAACATCCGTAGGTCTCCTTC
TTACAGTTAAATATGATTATTTTTATAGGGGGAGGGTTATCCGCTCCCA
CTTCCCCCCAGCGAGACTGATCAAATTCCCTGGATCGACATGTCGATC
CTCTATGGTCTGACACATACTAGGCAGAATCGCCGACGCCACACGAGA
ACGGTTGATGGCCCTACACCCCCACTCTCCCATGGGGAAGATCGAGTA
CTCGCACTACAGGCTCAAGAGCGCTTGAACGCGCGACC

Sample 74: *T. brucei-ITS1-*

TCATCTTGCTTTTGGAGGGGGTGTACGGGTGATTATTTGACCGTTAGAT
ATTGGTATTATGAACCCTTCCTATGCTATCCTCGCCTATAATATGTCCT
CAAACACCATTGAGGTGATTGCTACTACCTCCCCTGTGGGGGCAATTA
ACTGGGAACGCAGGTTTTTGATCCTGGTATTCCCAGGAGTTTTGTTGA
AAAACTCCTGCATGCGTCGATGCTTTGTGTCAACGAATTCTGCCTTTTT
ATACTTCATCTTGGGTTTCTTCAAAAGTCCAGAACCAAGTTGATATTAC
CTACCCCGCCATTCAAAGGCTGCAGCTATTGTAGTCCTCAAAAACTGT
TCCTGCCGTTTTCATCCTGGGTGTTTGTAATATACGGATTACCGAAACT
CCCTCCTGAACTCTTACTCCAAACTGAAGATTATCTCGAATACCTTCTG
CCTGGGTTTGACCTACATGGAAACCTTGCTAAAAAATTGAATAACCTC
CAAACAATAAAAACTTGCGGCCCCACTCAACCACGACCGCCTCACCTC
TGACATCCGGCTGCTAACTAAGCCCGATAGAAGGCTGAGTTGGCTGCT
CTGACCCCTCCTCAATACCAAATATAACCCCTTGGGCCGGTCTAATCG
AGCTCTTGTTGGGTTTGTGGTTGAAGGCAGGACCAATATCCGGATTGG

GGAAAGGTGACGGGCCCTGTCCTCCGATCTTACTCTTGGGGAAGGGGG
GAGGATCGCACATCAGGCACCACTACCTGGAACGTCCTTACGCCAGCT
CCTTGC

Sample 274: *T. brucei-ITS1-*
CTGCAGTGGGAGGAGACAGAAGTTAAGCCCGATTGGGATTTAGTAATT
CTCCTTGGAAGTTGCCCTTTTTGAACTAGGGTCTTGGTTAATTCTCTGC
CTCACACAATGGATCAACTTACCTTGCCCCCCGGAGCAAAGATCTGGG
AGCTGTGCAAGGTGCGGATTTAGGGCGCCCATGGTCTGGGGGTGTCGC
CTGTGCCCGCCCCTTACTTTGTGTTGCTCTGTTGGGCCCTCTGGAAAGT
GACTCGACCTACCTCACCGGGGCAGGACCTATGTCACAAAACCCCTGT
CACAAAATCTAAGTTTAAACTCTTCCTTGTGCAGACGCCCTGTTCCTCG
GAGATCCCCCGCTACACACAAGAAGGGGGGAGGCATCACAAGATCGT
CTTCACTTTTGCTACCCCAATGAGGATTCCGGCGCAGAGCTCTGGATT
GGGGGCACA

Sample 294: *T. brucei-ITS1-*

TTCGTGACCGACGGCTGGGAGATTGGAGGATGATCGAAGGTGAAAGA
AAGAGGGACTTTAAGTGACACGATTGTGAATGGAATGTTGATGAGGA
GTACTCGGGAGGCCATCAACTCTTCTCCACAGCGTCACCCTCGGCACT
GAGCTGGGAGCGCTGAGTGATGTGCCGTTTGCATGCTCATGCTGTCGA
GGCAAGTTGCTGCGAGGCATGTACTTGCTGTTGCATCTTTGGCATCTTA
TGTTGTCTCCATACCCTGAATGACAGGTGTAGGAGCTAGCATCAAGAA
CACGGTCAGCAAACCTAATTATAAATGAGTGGTCGGCCACGCGCGCTG
GTCCTTGATAATCGCCCCCTCGAAATAGGAAGGGGGGGGGGGGGAGA
CGGAGGGGAAAGACTTCTTATACCCCATAAAGGTTGACGAGGATCCG
CTGCCATGGAGGCAACT

Sample 269: *T. brucei*-ITS1-

GTGAACCTGCAGCTGGAGAATTTCCGATGATAAAAAATGGAGTAGGG
CTGTTTCCCGTGAGCCTGTGGGTTCCAAAGGACGGTTTTGGTGAGGGA
CTGTGTGCCCATCCCGCACGCGTATCCACATCCCGCCCCCCCCTATGTA
CTTGGTGCCGCCGCGATGCGATGGGTGTTGGGATGCCATGTTGTCGCTA
CAGTTCTGCGCTCACCGACAATTTTATGCTCCACAGTTAACCCCTCGA
AAAAAAGATCACTAAACACACTGGTCCCATCGGCGTTGCTATCGAGTC
CTGTGTCAAAAATACAATTGATGAGATAACGGCAGCGCGCGGGGCGT
GGTCCCGGCTGATCCCCCCCAAATGGAAGGCGGGGGGGAGAAAGCGG
AAAGGTCTGTCCCTTGTTGGCGTGCAATTTGGGATGAGTATGGGGAAG
TCTGGCATGGTGGGTTCTCAGATTGCATCTCTTGCTAATCCAATTCATA
AGCTTCTTTTTCAATAGATAAAGGTCAAAAAGAACTGGTGTAGGGACC
CTACGACCTAGGAGTGAAAGGGCAAGGTAGGGGGAATGGGAGCCTGA
CGCTATCATGTTCTCCGAAGCTCTCAAGGCGCTTGGGCGCACTCTCGG
TCCTATAAGGCGGCAAGACTCAAGCGGAACCTTCCGCGCAACGGTTCA
GGTT

Appendix 8.8: Partial sequences of Internal Transcribed Spacer I (ITS-1) of ribosomal DNA (rDNA) gene of *T. congolense* Savannah type obtained from samples submitted for sequencing

Sample 01: *T. congolense* ITS 1-Ogun State

AAAAAAGCGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTT
TCCGATGATAAAAAAGTATACCTNCAAGGGTGTACGTGTAGTGTAGGT
GTGTCTAACGAAGGTTGTTGTTGTGTGCTTGTGTCCCCNTCGCTCATGG
GCATCCCCATCCCGCACGCNCCATGTTTTGTGTGCTGTACGACGCGTG
GGTNGGGGTGGCGAACTCTCNTGGGCGGGCGTGGGGTGTGCCGACCN
CTAAAAAAAAAAAGAAAAGCCGGGGAAAACAAATGTACGAGACCAA
GCCCCCTCGCGGTGTCNCTCTCT

Sample 02: *T. congolenese* ITS 1-Ogun State

AGAAAAGCGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTT
TCCGATGATAAAAAAGTATACCTTCATAGGTGTACGTGTAGTGTAGGT
GTGTNCTATCGAAGGTTGTTGTTGTGTGCTTGTGTGCCCTTCGGTCATG
CGCATCCCCATCCCGCACGCCCCAGTGTTTTGTGTGCTGTGCGATGCG
GCGGTNGGTGTGCGATCTGTCNTTGGCNGNCGTNGTGAGGCGGCGACC
ACTAATCATGAAAACGTAGAAGCACGNANNGANAAATANACGAGTAC
AAGCCCGCTTCTCGATGTCGCTGTCT

Sample 04: *T. congolense* ITS 1-Kaduna State

AAAAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTT
CCGATGATAATATATATACCCATATGGTGTGTGTACCATATGGTGTGT
GTATGAGAGNGNGTTGTTGTGTGCTCGCGTGCGTACGGGCCCCCGT
GCATGCGAATTATTCCCATCCCCCCCCNCCCCGGGGGGGGGTGGGTGT
GGGTNGGGAACCCCGCGGGGGGGGGGGCGGNTTTGACCNGACNCCAG
AAACAAAATCGCCGCGGAACACGCACCGTCCAAACACCCGCNCCCCG
NGGACGTTCTCTTGTGT

Sample 05: *T. congolense* ITS 1-Ogun State

AAAAAAGCGTAACAGGTAGCTGTAGGTGACCTGCAGCTGGATCATTTT
CCGATGATAAAAANGGACCTTAAAATGGCACGACGTAGCGGGGTAGG

GGTCCTGACGAAAGGTTGTTGTTGGGGTCCTTTGGGTCCCCGTTCGCTC
ATGTGCATCCGCCATCCCGCACGCCCCAGATGTTTTGNGTGCTTGTGC
GACGCGGCGATGGGTGAGTGACGAATCTCGGCGGTGGGTGAGGTGCC
GACCACCAACCATAAAAACTAGAANAACNCCGAGAAACAATGNGAAC
AAGCCCGCGT

Sample 07: *T. congolense* ITS 1-Ogun State

AGAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTC
CGATGATAAAAAAGGATACCTTTATAGTGTACGTGNAGGGTGGGNGT
GTCCGATCCAAAGGTGTGGTGTGTGCTCCGTGTGCCCGTNGCNCACTG
NGCACCCGCCAGCCCGCACGCCCCAGAGTTTTGTGGGCTAGGNGTACG
CGGGCGAGGGGGTGTCTCGCAGTATCGCGGTGGGGGGNGGGNCGGAC
AC

Sample 31: *T. congolense* ITS 1-Ogun State

CTNGAAAGTGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTT
TCCGACCCTCTTCTCTTCTCGTCTNCGCCCGTCTCCCGGCCACCGGGGC
GGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAG
GTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCC
GGTTCGTTGAAGAACGCAGCAAA

Sample 32: *T. congolense* ITS 1-Ogun State

AAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCG
ATGATAAAAAAGACCCTTTAAGGGTGTACGNGTAGAGGAGGGGGTGC
AAAAGAAGGTTGTTGTAGGTGCTTGGGGCGCCCGCTCGATCATGGCCC
CCTCAACGGCACACGTGGTGTGTTGTGTGTTGCACGCGGCGNNNGTTG
GGATCGCGATTNTCATGCGCTGGAACGTTGCTGCCACGAACCTTGAAA
AATANATGAAAGTTCGGAAAGACACATCCAAGCACGGGCCCNCATGT
CGGTGTCTCTCTCTT

Sample 33: *T. congolense* ITS 1-Ogun State

GAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCC
GATGATAAAAAAGTATACCTTNATAGGTGTACGTGTAGTGTAGGTGTG
TGCTATCGAAGGTTGTTGTTGTGTGCTTGTGTGCCCTTCGCTCATGCGC
ATCCCCATCCCGCACGCCCCAGTGTTTTGTGTGCTGTNCGATGCGGCG
GTGGGTGTTGNATCTCNCNTTGGCGGCAGCGTGATGCGCCGACCACAA
ACATGAAAACGTAGAAGCACGNNTCGAAAAATACACGAGACAAGCNC
GCGTCTCGATGTCGCTGTCTCG

Sample 34: *T. congolense* ITS 1-Kaduna State

AAGGACAAGTGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATT
TTCCGATGATAATATATATACCCCTAAGGGTGTGTGTACGCATATGGT
GTGTGTTTAGAGAGAGTGGTTGTTGTGTGCGCGCGCGCGTGCGGGGNG

CCCCCCTCGCGCGTGTNATTTTNANCCGCGCCCACNCCCCCGTGGGGG
GCGTGGTGTGTGGGGAGCAACACGCGNGGGGGGGGTGTTGTTGTTTCT
CCCACACTCTCTATTGAAGACCGAAACACNCACGCGTGTGTACANAAG
CCCCCCCCCGGTTCTCTCTC

Sample 35: *T. congolense* ITS 1-Kaduna State

GAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCC
GATGATAATATATATACCCCTTAAGGTGTGTATACGCATATGGGTGTG
TGTCATGAGAGGGTGNTGGTGGTGTGTGCGCGCGTGCGNACNGTGCCC
CCCGCTCATGCGAATTAATCCCAGTCGCATCCACCCCGGTGGGGGGNG
GGGTGTGGGGTGGGAACACACCGGGGGGGNGNCGGTCTGTGGAC

Sample 36: *T. congolense* ITS 1-Ogun State

CGGGAACACAAACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTT
TCCGATGATAAAAAAGTACCCCTTTAAAGGGTACGTGTAGTGTAGGTG
TGTGCTATCGAAGGTTGTTGTTGTGTGCTTGTGTGCCCTTCGCTCATGC
GCATCCCCATCCCGCACGCCCCAGTGGTTTGTGTGCTGTGCGATGCGG
CGTGTGGTGGNATCGCGCATTGTCGGGCGGTGTAGTGTGGTGGCCACT
AACCNTGAAAACCATGAAGCATGTCCGTGAAACACAGTGGTCAAGCA
CGCGTCGCCATGNCGTGTCTCT

Sample 39: T. congolense ITS 1-Kaduna State

GAAAGCGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTC
CGATGATAAAAAAGTATACATACATATGTGTACGTGTAGTGTAGGTGT
GTGCTATCGAAGGTTGTTGTTGTGTGCTCGTGTGCCCTTCGCTCATGCG
CATCCCCATCCCCGCACGCCCCAGTGTTTTGTGTGCTGTGCGATGCGGC
GGTGTGTGTTGTGATCGCGCATTGTCGGGCGCTGTGATGTGCCGACCA
CGAACCTNNAAAACCTGAAGCACGTCCGAGAAACACACGTGTCCAAG
CNCGCGTCTCTANGNCGCTGCCT

Sample 40: *T. congolense* ITS 1-Ogun State

AGAAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTT
CCGATGATAAAAAAGTATACCTACAATGTGTACGTGTAGAGTGGTGTG
TGCTATCGAAGGTTGTTGTTGTGTGCTTGTGTGCCCTTCGNTCATGCGC
ATCCCCATCCCGCACGCCCCAGTGTTTTGTGTGCTGTCGATGCGGCGG
TGGGTGTGNAC

Sample 43: *T. congolense* ITS 1-Ogun State

GACAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTC
CGATGATAATATATATACCTATACGTGTGTNTNTACNCATATGNGTGT
GTGNTAAGAGAGAGTGGTTGTNGTGTGCGCGCGCGCGTGCGAGCCCC
CCCCTCNCGCGAGNAATTTTCACCCGCCCCACCCCCCCGTGGGGGGCG
TGGTGTGTGGNGAACA

Appendix 8. 9: Partial sequences of Internal Transcribed Spacer I (ITS-1) of ribosomal DNA (rDNA) gene of *T. vivax* obtained from samples submitted for sequencing

Sample 44 *T. vivax* ITS 1-Ogun State

CTGAAAAGTGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTT
TCCGACCCTCTTCTCTTCTCCCTTAAGGCGTCTCCCGGCCACCGGGGCG
GGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAGG
TGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCG
GTTCGTTGAAGAACGCAGCAA

Sample 147: *T. vivax* ITS 1-Ogun State

GAAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTC
CGATGATAAAAAAGTATACCTATATATGTGTACGTGTAGTGTAGGTGT
GTGCTATCGAAGGTTGTTGTTGTGTGCTTGTGTGCCCTCGCTCATGCGC
ATCCCCATCCCGCACGCCCCAGTGTTTTGTGTGCTGTGCGATGCGGCG
TGGG

Sample 48: *T. vivax* ITS 1-Kaduna State

CAGAAAGTGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTT
CCGACCCTCTTCTCTTCTCGTCTAGCCCGTNTCCAGGCCACCGGGGCG
GGACAGCAAACCACGCAGCTGCGGCTCGACCGCGCCCCGCGCGGAGG
TGGAGCACGGCCCGCACAACGTGACGCGATGGATGACTTGGCTTCNC
GGTTCGTTGAAGAACGCAGCAAAC

Sample 03: *T. vivax* ITS 1-Ogun State

CNAAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTT
CCGACCCTCTTCTCTTCTCGTTCGCCCGTCTCCCGGCCACCGGGGCGGG
ACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAGGTG
GAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCGGT
TCGTTGAAGAACGCAGCAAA

Sample 06: *T. vivax* ITS 1-Ogun Stae

CANAAAAAGNGTACAGGTAGCCTGTAGGTGAACCTGCAGCTGGATCA
TTTTCCGACCCTCTTCTCTTCTCCTCTNGGGCGTCTCCCGGCCACCGGG
GCGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGC
AGGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTC
CCGGTTCGTTGAAGAACGCAGCAA

Sample 08: *T. vivax* ITS 1-Kadun State

CCAAAAAAGCGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCA
TTTTCCGACCCTCTTCTCTTCTCTTAGGGCGCCCGTCTCCCGGCCACCG
GGGCGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGC
GCAGGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGC
TTCCCGGTTCGTTGAAGAAANCAGNAAATT

Sample 09 T. vivax ITS1-Ogun State

CAAAAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTT
TCCGACCCTCTTCTCTTCTCGTCGCGCCCGTCTCCCGGCCACCGGGGCG
GGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAGG
TGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCG
GTTCGTTGAAGAACGCAGCAAATCAAAT

Sample 10: *T. vivax* ITS 1-Ogun State

CCTAAAAAAGCGTACAAGGTAGCCTGTAGGTGAACCTGCAGCTGGAT
CATTTTCCGACCCTCTTCTCTTCTCCTTTGGGCGTCTCCCGGCCACCGG
GGCGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCG
CAGGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTT
CCCGGTTCGTTGAAGAACGCAGCAAAT

Sample 11: *T. vivax* ITS 1-Ogun State

CNNGAAAGCGTACAGGTAGCCTGTAGGTGAACCTGCAGCTGGATCATT
TTCCGACCCTCTTCTCTTCTCCTCTGCCGTCTCCCGGCCACCGGGGCGG
GACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAGGT
GGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCGG
TTCGTTGAAGAACGCAGCAAAACAA

Sample 12: *T. vivax* ITS 1-Ogun State

CTAGGAAAGTGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATT
TTCCGACCCTCTTCTCTTCTCCTTTAGACCGTCTCCCGGCCACCGGGGC
GGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAG
GTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCC
GGTTCGTTGAAGAACGCAGCAA

Sample 13 *T. vivax* ITS 1-Ogun State

CTGAAAAGTGTACAGGTAGCCTGTAGGTGAACCTGCAGCTGGATCATT
TTCCGACCCTCTTCTCTTCTCCCTTAGCCGTCTCCCGGCCACCGGGGCG
GGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAGG
TGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCG
GTTCGTTGAAGAACGCAGCAA

Sample 14: *T. vivax* ITS 1-Kaduna State

CTGGAAAAGNGTACAGGTAGCTGTAGGTGAACCCTGCAGCTGGATCA
TTTTCCGACCCTCTTCTCTTCCCCTTTAGAGGTCTCCCGGCCACCGGGG
CGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCA
GGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCC
CGGTTCGTTGAAGAACGCAGCAAATT

Sample 15: *T. vivax* ITS 1-Ogun State

CTGAAAAAGTGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCAT
TTTCCGACCCTCTTCTCTTCTCCCTCTAGGCGTCTCCCGGCCACCGGGG
CGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCA
GGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCC
CGGTTCGTTGAAGAACGCAGCAAA

Sample 16: *T. vivax* ITS 1-Kaduna State

AAAGGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCG
ACCCTCTTCTCTTCCCCCTTAAAGGGGTCTCCCGGCCACCGGGGCGGG
ACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAGGTG
GAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCGGT
TCGTTGAAGAACGCAGCAAA

Sample 17: T. *vivax* ITS 1-Ogun State

CCTGGAAAGTGTACAGGTAGCCTGTAGGTGAACCTGCAGCTGGATCAT
TTTCCGACCCTCTTCTCTTCTCCTCTAGACGGGCCCGTGTCCAGGGAAN
CGGGACGGGACAGCAAACACGGAGNTGCGGCTNGACCGCGCCCCGCG
GAGGTGGAGCACGGACCGCACAACGTGACGCNATNGATGACTTGGCT
TCCCG

Sample 18: *T. vivax* ITS 1-Ogun State

CTGGAAAAGTGTAACAGGTAGCCTGTAGGTGAACCTGCAGCTGGATC
ATTTTCCGACCCTCTTCTCTTCTCCTCGAGACCGTCTCCCGGCCACCGG
GGCGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCG
CAGGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTT
CCCGGTTCGTTGAAGAACGCAGCA

Sample 19: *T. vivax* ITS 1-Ogun State

GGAAAAGGTAACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATT
TTCCGACCCTCTTCTCTTCCCCCTTTAAGGGTCTCCCGGCCACCGGGGC
GGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAG
GTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCC
GGTTCGTTGAAGAACGCAGCA

Sample 20: *T. vivax* ITS 1-Ogun State

CTGGAAAGTGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTT
TCCGACCCTCTTCTCTTCTCCTCTAACCGTCTCCCGGCCACCGGGGCGG
GACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAGGT
GGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCGG
TTCGTTGAAGAACGCAGCAAA

Sample 21: *T. vivax* ITS 1-Kaduna State
CGGAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTT
CCGACCCTCTTCTCTTCCCCTTTAGGCGCGCCCGTCTCCCGGCCACCGG
GGCGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCG
CAGGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTT
CCCGGTTCGTTGAAGAACGCAGCA

Sample 22: *T. vivax* ITS1 –Kaduna State

CTAGGAAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATT
TTCCGACCCTCTTCTCTTCTCGTCGCGCCCGTNTCCNGGCCANCGGGGC
GGGACAGCAAACCACGNAGCTGCGGCTCGACCGCGCCCCGCGCGNAG
GTGGAGCACGGCCCGCACAACGTGACGCGATGGATGACTTGCCTTCCC
GGTTCGTTGAAGAACGAGCAA

Sample 23: *T. vivax* ITS 1-Kaduna State

CTAAAAACGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTT
CCGACCCTCTTCTCTTCTCCTTTAGCCCGTCTCCCGGCCACCGGGGCGG
GACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAGGT
GGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCGG
TTCGTTGAAGAACGCAGC

Sample 24: *T. vivax* ITS 1-Ogun State

TCTGAAAGTGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTT
TCCGACCCTCTTCTCTTCTCGTCGCGCCCGTCTCCCGGCCACCGGGGCG
GGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAGG
TGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCG
GTTCGTTGAAGAACGCAGCAA

Sample 25: *T. vivax* ITS 1-Ogun State

CTGAAAAGTGTACAGGTAGCCTGTAGGTGAACCTGCAGCTGGATCATT
TTCCGACCCTCTTCTCTTCTCGTCGCGCCCGTCTCCCGGCCACCGGGGC
GGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAG
GTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCC
GGTTCGTTGAAGAACGCAGCAAA

Sample 26: *T. vivax* ITS 1-Ogun State

CCTGGAAAGTGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCAT
TTTCCGACCCTCTTCTCTTCTCGTCGCGCCCGTCTCCCGGCCACCGGGG
CGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCA
GGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCC
CGGTTCGTTGAAGAACGCAGCAAAN

Sample 27: *T. vivax* ITS 1-Ogun State

CTAGAAAGTGTACAGGTAGCCTGTAGGTGAACCTGCAGCTGGATCATT
TTCCGACCCTCTTCTCTTCTCGTCGCGCCCGTCTCCCGGCCACCGGGGC
GGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAG
GTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCC
GGTTCGTTGAAGAACGCAGCAA

Sample 28: *T. vivax* ITS 1-Ogun State

CTAGAAAAGTCGTACAAGGTAGCCTGTAGGTGAACCTGCAGCTGGATC
ATTTTCCGACCCTCTTCTCTTCTCGTCTTAGCCCGTCTCCCGGCCACCG
GGGCGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGC
GCAGGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGC
TTCCCGGTTCGTTGAAGAACGCAGCAAAT

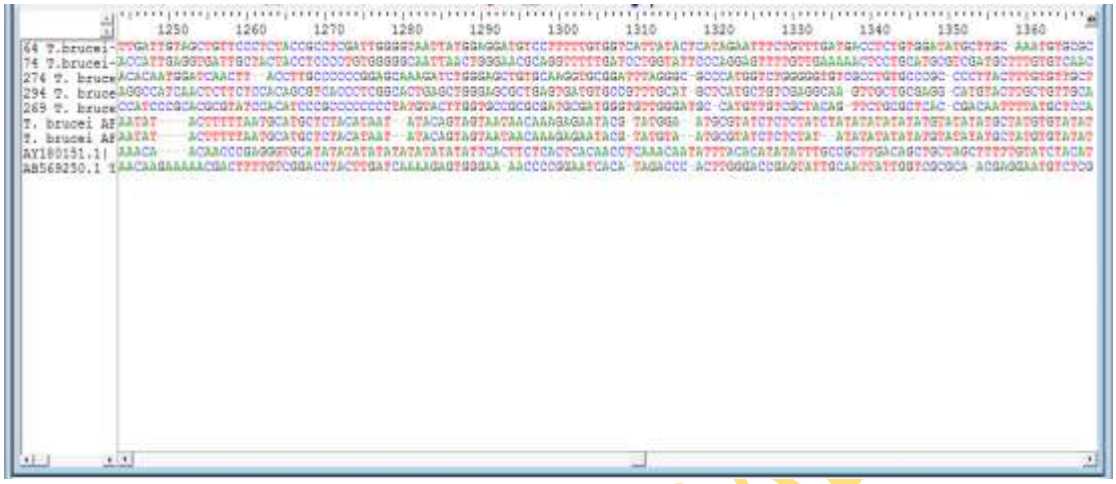
Sample 29: *T. vivax* ITS 1-Ogun State

CTGGAAAGTGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATT
TTCCGACCCTCTTCTCTTCTCGTCGCGCCCGTCTCCCGGCCACCGGGGC
GGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCAG
GTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCC
GGTTCGTTGAAGAACGCAGCAAAGC

Sample 30: *T. vivax* ITS 1-Ogun State

CTAGGAAAAGTGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCAT
TTTCCGACCCTCTTCTCTTCTCGTCGCGCCCGTCTCCCGGCCACCGGGG
CGGGACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGCA
GGTGGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCC
CGGTTCGTTGAAGAACGCAGCAA

9.0 APPENDIX 10: Aligned *T. brucei* ITS1 rDNA sequences showing various points of deletions, insertions and alterations



9.1 APPENDIX 11: Multiple alignment of internal transcribed pacer 1 of rDNA sequences of five *T. brucei* detected in naturally infected cattle in Nigeria.

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                          **.*. **************************************************
64 T.  brucei ITS1    GGACAG-CGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCATT 59
269 T. brucei ITS1    -GACAG-CGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCATT 58
274 T. brucei ITS1    GGACAG-CGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCATT 59
294 T. brucei ITS1    GGAAAG-CGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCATT 59
74 T.  brucei ITS1    GGACAAGCGTACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCTGATATCCATT 60

                        *****.***. **************************************************
64 T.  brucei ITS1    ATACAAAAAAAGAGCATATTTATGTGCATGTATAAATTGCACAGTATGCAACCAAAAATAT 119
269 T. brucei ITS1    ATACAAAAAAAGAGCATATTTATGTGCATGTATAAATTGCACAGTATGCAACCAAAAATAT 118
274 T. brucei ITS1    ATACACAAAG-AGCATATTTATGTGCATGTATAAATTGCACAGTATGCAACCAAAAATAT 118
294 T. brucei ITS1    ATACAAAAAAAGAGCATATTTATGTGCATGTATAAATTGCACAGTATGCAACCAAAAATAT 119
74 T.  brucei ITS1    ATACAAAAAAAGAGCATATTTATGTGCATGTATAAATTGCACAGTATGCAACCAAAAATAT 120

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64 T.  brucei ITS1    ACATGTATGTTTTACATGTATGTGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATG 179
269 T. brucei ITS1    ACATTTATGTTTTACATGTATGTGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATG 178
274 T. brucei ITS1    ACATTTATGTTTTACATGTATGTGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATG 178
294 T. brucei ITS1    ACATTTATGTTTTACATGTATGTGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATG 179
74  T. brucei ITS1    ACATGTATGTTTTACATGTATGTGTTTCTATATGCCGTTTGACATGGGAGATGAGGGATG 180

                      ************************************************************
64  T. brucei ITS1    CTATACATAGTTCTGTCATTGTCTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATA 239
269 T. brucei ITS1    CTATAC-TAGTTCTGTTATTATCTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATA 238
274 T. brucei ITS1    CTATAC-TAGTTCTGTTATTATCTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATA 238
294 T. brucei ITS1    CTATAC-TAGTTCTGTTATTATCTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATA 239
74  T. brucei ITS1    CTATACATAGTTCTGTCATTGTCTATCATGTATGTGTGTTAGAGTGTCTGTGTTAATATA 240

                      ************************************************************
64 T.  brucei ITS1    CTTTTTAATGCATGCTCTACATAATATACAGTAGTAATAACACAGAGAATACGTATGGAA 299
269 T. brucei ITS1    CTTTTTAATGCATGCTCTACATAATATACAGTAGTAA---CACAGAGAATACGTATGGAA 298
274 T. brucei ITS1    CTTTTTAATGCATGCTCTACATAATATACAGTAGTAA---CACAGAGAATACGTATGGAA 298
294 T. brucei ITS1    CTTTTTAATGCATGCTCTACATAATATACAGTAGTAA---CACAGAGAATACGTATGGAA 299
74  T. brucei ITS1    CTTTTTAATGCATGCTCTACATAATATACAGTAGTAATAACACAGAGAATACGTATGGAA 300


64 T.  brucei ITS1    TGCGTATCTCTCTATATATATTTATGTATATATGCTATGTGTGTATCACCCCCTCGCATT 359
269 T. brucei ITS1    TGCGTATCTCTCTATATATATTTA-GTATATATGCTATGTGTGTATCACCCCCTCGCATA 358
274 T. brucei ITS1    TGCGTATCTCTCTATATATATTTA-GTTTATATGCAATGTGTGTATATCCCCCTCGCATT 358
294 T. brucei ITS1    ------------------------------------------------------------
74  T. brucei ITS1    TGCGTATCTCTCTATATATATTTATGTATATATGCTATGTGTGTATAACCC--------- 351


64  T. brucei ITS1    TTATC 364
269 T. brucei ITS1    TT--- 360
274 T. brucei ITS1    T---- 359
294 T. brucei ITS1    -----
74  T. brucei ITS1    -----
```

9.2 APPENDIX 12: Aligned *T. congolense* ITS1 rDNA sequences showing variouspoints of deletions, insertions and alterations

9.3 APPENDIX 13: Complete Alignement of *T. congolense* savannah ITS1rDNA

sequences showing various points of deletions, insertions and alterations

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                                  .*.. .: ...* ************ ************************  .*.
04 T. congolense ITS       --AAAAAAGCGTACAGG-TAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 57
35 T. congolense ITS       ----GAAAGCGTACAGG-TAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 55
34 T. congolense ITS       AAGGACAAGTGTACAGG-TAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 59
43 T. congolense ITS       ---GACAAGCGTACAGG-TAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 56
02 T. congolense ITS       --AGAAAAGCGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 58
33 T. congolense ITS       ----GAAAGCGTACAGG-TAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 55
40 T. congolense ITS       --AGAAAAGCGTACAGG-TAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 57
36 T. congolense ITS       -CGGGAACACAAACAGG-TAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 58
39 T. congolense ITS       ----GAAAGCGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 56
01 T. congolense ITS       --AAAAAAGCGTACAAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 58
05 T. congolense ITS       --AAAAAAGCGTAACAGGTAGCTGTAGGTG-ACCTGCAGCTGGATCATTTTCCGATGATA 57
07 T. congolense ITS       ---AGAAAGCGT-ACAGGTAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 56
32 T. congolense ITS       -----AAAGCGTACAGG-TAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGATGATA 54
31 T. congolense ITS       -CTNGAAAGTGTACAGG-TAGCTGTAGGTGAACCTGCAGCTGGATCATTTTCCGACCCTC 58

                          ::.:     .                .*                .     .      *
04 T. congolense ITS       ATATATATACCCAT-ATGGTGTGTGTAC--CATATGG-TG--TGTGT-ATGAGAGNGNGT 110
35 T. congolense ITS       ATATATATACCCCTTAAGGTGTGTATACG-CATATGGGTG--TGTGTCATGAGAGGGTGN 112
34 T. congolense ITS       ATATATATACCCCTAAGGGTGTGTGTACG-CATATG-GTG--TGTGTTTAGAGAGAGTG- 114
43 T. congolense ITS       -ATATATATACCTATACGTGTGTNTNTACN-CATATGNGTG-TGTGNTAAGAGAGAGTG- 112
02 T. congolense ITS       AAAAAGTATACCTTCATAGG---TGTACG-TGTAGTGTAG-GTGTGTNCTATCG-AAGGT 112
33 T. congolense ITS       AAAAAGTATACCTTNATAGG---TGTACG-TGTAGTGTAG-GTGTGTGCTATCG-AAGGT 109
40 T. congolense ITS       AAAAAGTATACCTACAATG----TGTACG-TGTAGAGTGG--TGTGTGCTATCG-AAGGT 109
36 T. congolense ITS       AAAAAGTACCCCTTTAAAGG----GTACG-TGTAGTGTAG-GTGTGTGCTATCG-AAGGT 111
39 T. congolense ITS       AAAAAGTATACATACATATG---TGTACG-TGTAGTGTAG-GTGTGTGCTATCG-AAGGT 110
01 T. congolense ITS       AAAAAGTATACCTNCAAGGG---TGTACG-TGTAGTGTAG-GTGTGT-CTAACG-AAGGT 111
05 T. congolense ITS       AAAANGG--ACCTTAAAATG----GCACGACGTAGCGGGGTAGGGGTCCTGACGAAAGGT 111
07 T. congolense ITS       AAAAAGGATACCTTTATAGT----GTACG-TGNAGGGTGG-GNGTGTCCGATCCAAAGG- 109
32 T. congolense ITS       AAAAAGA--CCCTTTAAGGG---TGTACG-NGTAGAGGAG--GGGGTGCAAAAG-AAGGT 105
31 T. congolense ITS        TTCTCTT--CTCGTCTNCG------CCCG---------------TCTCCCGGCCACCGG- 94

                             *  *           *  * .    .    ..       **    .  .*.
04 T. congolense ITS       TGTTG----------TGTGTGCTCGC---GTGCGTACGGG--CCCCCGTGCATGCGAAT 154
35 T. congolense ITS       TGGTGG---------TGTGTGCGCGC---GTGCGNACNGTGCCCCCCCGCTCATGCGAAT 159
34 T. congolense ITS       -GTTGT----------TGTGTGCGCGC---GCGCGTGCGGGGGNGCCCCCCCTCGCGCGTGT 160
43 T. congolense ITS       -GTTGT----------NGTGTGCGCGC---GCGCGTGCGAG-CCCCCCCCCTCNCGCGAGN 157
02 T. congolense ITS       TGTTGTT-GTGTGCT-TGTGTGCCCTT--CGGTCATGCGCATCC-CCATCCCGCACGCCC 167
33 T. congolense ITS       TGTTGTT-GTGTGCT-TGTGTGCCCTT--CGCTCATGCGCATCC-CCATCCCGCACGCCC 164
40 T. congolense ITS       TGTTGTT-GTGTGCT-TGTGTGCCCTT--CGNTCATGCGCATCC-CCATCCCGCACGCCC 164
36 T. congolense ITS       TGTTGTT-GTGTGCT-TGTGTGCCCTT--CGCTCATGCGCATCC-CCATCCCGCACGCCC 166
39 T. congolense ITS       TGTTGTT-GTGTGCT-CGTGTGCCCTT--CGCTCATGCGCATCC-CCATCCCGCACGCCC 165
01 T. congolense ITS       TGTTGTT-GTGTGCT-TGTGTCCCCNT--CGCTCATGGGCATCC-CCATCCCGCACGCNC 166
05 T. congolense ITS       TGTTGTTGGGGTCCTTTGGGTCCCCGTT-CGCTCATGTGCATCCGCCATCCCGCACGCCC 170
07 T. congolense ITS       TGTGGT--GTGTGCTCCGTGTGCCCGTNGCNCAC-TGNGCACCCGCCAGCCCGCACGCCC 166
32 T. congolense ITS       TGTTGTA--GGTGCTTGGGGCGCCCGCT-CGATCATGG----CC-CCCTCAACGGCACAC 157
31 T. congolense ITS       -GGCGG-----------GACAGCAAACCACGCAGCTGCCGCTCGACCGCGCCCCGCGCGC 142

                          .          .  .       *
04 T. congolense ITS       TATT---CCCATCCCC--CCCCNCCCCGGGGGGGGGTGGG-TGTGGGTNGGGAACCCCGC 208
35 T. congolense ITS       TAAT---CCCAGTCGC--ATCCACCCCGGTGGGGGGNGGGGGTGTGGGGTGGGAACACACC 214
34 T. congolense ITS       NATT---TTNANCCGCGCCCACNCCCCCGTGGGGGGCGTGGTGTGTGGGGAGCAACACGC 217
43 T. congolense ITS       AATT---TTCACCCGC-CCCACCCCCCCGTGGGGGGCGTGGTGTGTGGNGAACA------ 207
02 T. congolense ITS       CAGT-GTTTTGTGTGCTGTG-CGATGCGGCGGTNGGTGTGCG-ATCTGTCNTTGGCNGNC 224
33 T. congolense ITS       CAGT-GTTTTGTGTGCTGTN-CGATGCGGCGGTGGGTGTTGN-ATCTCNCNTTGGCGG-C 220
40 T. congolense ITS       CAGT-GTTTTGTGTGCTGT--CGATGCGGCGGTGGGTGTGN------------------- 202
36 T. congolense ITS       CAGT-GGTTTGTGTGCTGTG-CGATGCGGCG---TGTGGTGGNATCGCGCATTGTCGGGC 221
39 T. congolense ITS       CAGT-GTTTTGTGTGCTGTG-CGATGCGGCGGTGTGTGTTGTGATCGCGCATTGTCGGGC 223
01 T. congolense ITS       CA-T-GTTTTGTGTGCTGTA-CGACGCGTGGGTNGGGGTGGCGAACTCTCNTGGGCGGGC 223
05 T. congolense ITS       CAGATGTTTTGNGTGCTTGTGCGACGCGG-CGATGGGTGAGTGACGAA-TCTCGGCGGTG 228
07 T. congolense ITS       CAGA-GTTTTGTGGGCTAGGNGTACGCGGGCGAGGGG-GTGTCTCGCAGTATCG-CGGTG 223
32 T. congolense ITS       GTGGTGTGTTGTGTGTTGCA-CGCGGCGNNNGTTGGGATCGCG-------ATTNTCATGC 209
31 T. congolense ITS       AGGT-----GGAGCACGGCCCGCACAACGTGTCGCGATGGATGACTTGGCTTCCCGGTTC 197
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9.4 APPENDIX 14: Aligned *T. vivax* ITS1 rDNA sequences showing various points of deletions, insertions and alterations

9.5 APPENDIX 15: Aligned *T. vivax* ITS1 rDNA sequences showing various points of deletions, insertions and alterations

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                              **.    *...*  :. ************* ********************
44 T. vivax ITS    -CTG-AAAAGT-GTACAGG-TAG-CTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 54
13 T. vivax ITS    -CTG-AAAAGT-GTACAGG-TAGCCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 55
16 T. vivax ITS    ------AAAGG-T-ACAAG-GTAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 50
19 T. vivax ITS   - --GGAAAAGG-TAACAAG-GTAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 54
14 T. vivax ITS   -C TGGAAAAGN-GTACAGG--TAGCTGTAGGTGAACCCTGCAGCTGGATCATTTTCCGAC 56
15 T. vivax ITS   -CT GAAAAAGT-GTACAAG-GTAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 56
12 T. vivax ITS   -CTA GGAAAGT-GTACAGG--TAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 55
11 T. vivax ITS    --CNNGAAAGC-GTACAGG-TAGCCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 55
20 T. vivax ITS    --CTGGAAAGT-GTACAGG-TAG-CTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 54
21 T. vivax ITS    ---CGGAAAGC-GTACAGG--TAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 53
23 T. vivax ITS    ---CTAAAAAC-GTACAGG--TAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 53
6  T. vivax ITS    -CANAAAAAGN-GTACAGG-TAGCCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 56
10 T. vivax ITS    CCTAAAAAAGC-GTACAAGGTAGCCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 58
18 T. vivax ITS    -CTGGAAAAGT-GTAACAGGTAGCCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 57
28 T. vivax ITS    -CTAGAAAAGTCGTACAAGGTAGCCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 58
25 T. vivax ITS    -CTGA--AAAGTGTACAGG-TAGCCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 55
27 T. vivax ITS    -CTAG--AAAGTGTACAGG-TAGCCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 55
30 T. vivax ITS    -CTAGGAAAAGTGTACAGG-TAG-CTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 56
26 T. vivax ITS    CCTGG--AAAGTGTACAAG-GTAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 56
29 T. vivax ITS    -CTGG--AAAGTGTACAAG-GTAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 55
24 T. vivax ITS    TCTG---AAAGTGTACAGG--TAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 54
3  T. vivax ITS    --CNA--AAAGCGTACAGG--TAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 53
8  T. vivax ITS    CCAAA--AAAGCGTACAAG-GTAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 56
9  T. vivax ITS    -CAAA--AAAGCGTACAGG--TAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 54
48 T. vivax ITS    -CAG---AAAGTGTACAGG--TAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 53
22 T. vivax ITS    -CTAGGAAAGC-GTACAGG--TAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 55
17 T. vivax ITS    CCTGG--AAAGTGTACAGG-TAGCCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAC 56
147T. vivax ITS    -----GAAAAGCGTACAGG--TAGCTGTAGGTGAACC-TGCAGCTGGATCATTTTCCGAT 52

                     .*.::.:. *.  .              *.*  * ..   .*. * *  :**:*:*
44 T. vivax ITS   CCTCTTCTCTTCTCCCTTAAG----GCG-TCTCCCGGCCACCGGGGCGGGACAGCAAACC 109
13 T. vivax ITS   CCTCTTCTCTTCTCCCTTAG-----CCG-TCTCCCGGCCACCGGGGCGGGACAGCAAACC 109
16 T. vivax ITS   CCTCTTCTCTTCCCCCTTAAA----GGGGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 106
19 T. vivax ITS   CCTCTTCTCTTCCCCCTTTAA----GGG-TCTCCCGGCCACCGGGGCGGGACAGCAAACC 109
14 T. vivax ITS   CCTCTTCTCTTCCCCTTTAGA----GGT--CTCCCGGCCACCGGGGCGGGACAGCAAACC 110
15 T. vivax ITS   CCTCTTCTCTTCTCCCTCTAG----GCG-TCTCCCGGCCACCGGGGCGGGACAGCAAACC 111
12 T. vivax ITS   CCTCTTCTCTTCTCCTTTAGA----CCG-TCTCCCGGCCACCGGGGCGGGACAGCAAACC 110
11 T. vivax ITS   CCTCTTCTCTTCTCC-TCTG-----CCG-TCTCCCGGCCACCGGGGCGGGACAGCAAACC 108
20 T. vivax ITS   CCTCTTCTCTTCTCC-TCTAA----CCG-TCTCCCGGCCACCGGGGCGGGACAGCAAACC 108
21 T. vivax ITS   CCTCTTCTCTTCCCCTTTAGGCGCGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 113
23 T. vivax ITS   CCTCTTCTCTTCTCCTTTAG-----CCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 108
6  T. vivax ITS   CCTCTTCTCTTCTCCTCTNGG----GCG-TCTCCCGGCCACCGGGGCGGGACAGCAAACC 111
10 T. vivax ITS   CCTCTTCTCTTCTCCTTT-GG----GCG-TCTCCCGGCCACCGGGGCGGGACAGCAAACC 112
18 T. vivax ITS   CCTCTTCTCTTCTCCTCG-AG----ACCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 112
28 T. vivax ITS   CCTCTTCTCTTCTCGTCTTAG----CCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 114
25 T. vivax ITS   CCTCTTCTCTTCTCGTCG-----CGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 110
27 T. vivax ITS   CCTCTTCTCTTCTCGTCG-----CGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 110
30 T. vivax ITS   CCTCTTCTCTTCTCGTCG-----CGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 111
26 T. vivax ITS   CCTCTTCTCTTCTCGTCG-----CGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 111
29 T. vivax ITS   CCTCTTCTCTTCTCGTCG-----CGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 110
24 T. vivax ITS   CCTCTTCTCTTCTCGTCG-----CGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 109
3 T.  vivax ITS   CCTCTTCTCTTCTCGTT------CGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 107
8 T.  vivax ITS    CTCTTCTCTTCTCTTAGGG---CGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 113
9 T.  vivax ITS    CTCTTCTCTTCTCGTCG-----CGCCCGTCTCCCGGCCACCGGGGCGGGACAGCAAACC 109
48 T. vivax ITS    CTCTTCTCTTCTCGTCTAG-----CCCGTNTCCAGGCCACCGGGGCGGGACAGCAAACC 108
22 T. vivax ITS   CCTCTTCTCTTCTCGTCGCG-----CCCGTNTCCNGGCCANCGGGGCGGGACAGCAAACC 110
17 T. vivax ITS   CCTCTTCTCTTCTCCTCTAGACGGGCCCGTGTCCAGGGAANCGGGACGGGACAGCAAAC- 115
147 . vivax ITS   GATAAAAAAGTATACCTATAT-----ATGTGTACGTGTAGTGTAGGTGTG--TGCTATCG 105
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                    *.*  :* **  *   *.  * *  *** **   * * * * *.* . .******..*
44 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 166
13 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 166
16 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 163
19 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 166
14 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 167
15 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 168
12 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 167
11 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 165
20 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 165
21 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 170
23 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 165
6  T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 168
10 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 169
18 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 169
28 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 171
25 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 167
27 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 167
30 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 168
26 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 168
29 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 167
24 T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 166
3  T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 164
8  T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 170
9  T. vivax ITS     ACG-CAGCTGCCGCTCGACCGCG-CCCCGCGCGCGCAGGTGGAGCACGG-CCCGCACAACGT 166
48 T. vivax ITS     ACG-CAGCTGCGGCTCGACCGCG-CCCCGCGCGGAGGTGGAGCACGG-CCCGCACAACGT 165
22 T. vivax ITS     ACG-NAGCTGCGGCTCGACCGCG-CCCCGCGCGNAGGTGGAGCACGG-CCCGCACAACGT 167
17 T. vivax ITS     ACG-GAGNTGCGGCTNGACCGCG-CCCCGCGG--AGGTGGAGCACGG-ACCGCACAACGT 170
147T. vivax ITS     AAGGTTGTTGTTGTGTGCTTGTGTGCCCTCGCTCATGCGCATCCCCATCCCGCACGCCCC 165

                              .  *  :*. .** **   * :  **
44 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAA------- 213
13 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAA------- 213
16 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAA------ 211
19 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCA-------- 212
14 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAATT---- 217
15 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAA------ 216
12 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAA------- 214
11 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAAACAA-- 217
20 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAA------ 213
21 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCA-------- 216
23 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGC--------- 210
6  T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAA------- 215
10 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAAT----- 218
18 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCA-------- 215
28 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAAT----- 220
25 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAA------ 215
27 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAA------- 214
30 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAA------- 215
26 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAAN----- 217
29 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAAGC---- 217
24 T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAA------- 213
3  T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAA------ 212
8  T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAAANCAGNAAATT---- 220
9  T. vivax ITS      GTCGCGATGGATGACTTGGCTTCCCGGTTCGTTGAAGAACGCAGCAAATCAAAT 220
48 T. vivax ITS      GACGCGATGGATGACTTGGCTTCNCGGTTCGTTGAAGAACGCAGCAAAC----- 214
22 T. vivax ITS      GACGCGATGGATGACTTGCCTTCCCGGTTCGTTGAAGAACG-AGCAA------- 213
17 T. vivax ITS      GACGCNATNGATGACTTGGCTTCCCG----------------------------- 196
147T. vivax ITS      AGTGTTTTGTGTG-CTGTGCGATGCGG--CGTGGG------------------- 197
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9.6 APPENDIX 16: Aligned sequences of *T. brucei*, *T. congolense* and *T. vivax* ITS-1 rDNA sequences detected from some Nigerian cattle.

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30 T. vivax ITS-1          ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 40
25 T. vivax ITS-1          ----------ACAGG---------------TAGCCTG------TAGGTGAACC-TGCAGC 39
27 T. vivax ITS-1          ----------ACAGG---------------TAGCCTG------TAGGTGAACC-TGCAGC 39
26 T. vivax ITS-1          ----------ACAAG---------------GTAGCTG------TAGGTGAACC-TGCAGC 40
29 T. vivax ITS-1          ----------ACAAG---------------GTAGCTG------TAGGTGAACC-TGCAGC 39
31 T. vivax ITS-1          ----------ACAGG----------------TAGCTG------TAGGTGAACC-TGCAGC 38
3  T. vivax ITS-1          ----------ACAGG----------------TAGCTG------TAGGTGAACC-TGCAGC 37
8  T. vivax ITS-1          ----------ACAAG---------------GTAGCTG------TAGGTGAACC-TGCAGC 40
9  T. vivax ITS-1          ----------ACAGG----------------TAGCTG------TAGGTGAACC-TGCAGC 38
17 T. vivax ITS-1          ----------ACAGG---------------TAGCCTG------TAGGTGAACC-TGCAGC 40
22 T. vivax ITS-1          ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 39
48 T. vivax ITS-1          ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 37
11 T. vivax ITS-1          ----------ACAGG---------------TAGCCTG------TAGGTGAACC-TGCAGC 39
20 T. vivax ITS-1          ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 38
21 T. vivax ITS-1          ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 37
23 T. vivax ITS-1          ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 37
6  T. vivax ITS-1          ----------ACAGG---------------TAGCCTG------TAGGTGAACC-TGCAGC 40
10 T. vivax ITS-1          ----------ACAAGG--------------TAGCCTG------TAGGTGAACC-TGCAGC 42
18 T. vivax ITS-1          ----------AACAGG--------------TAGCCTG------TAGGTGAACC-TGCAGC 41
28 T. vivax ITS-1          ----------ACAAGG--------------TAGCCTG------TAGGTGAACC-TGCAGC 42
13 T. vivax ITS-1          ----------ACAGG---------------TAGCCTG------TAGGTGAACC-TGCAGC 39
12 T. vivax ITS-1          ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 39
14 T. vivax ITS-1          ----------ACAGG---------------TAG-CTG------TAGGTGAACCCTGCAGC 40
15 T. vivax ITS-1          ----------ACAAGG--------------TAG-CTG------TAGGTGAACC-TGCAGC 40
44 T. vivax ITS-1          ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 38
16 T. vivax ITS-1          ----------ACAAGG--------------TAG-CTG------TAGGTGAACC-TGCAGC 34
19 T. vivax ITS-1          ----------ACAAGG--------------TAG-CTG------TAGGTGAACC-TGCAGC 38
FJ712718 T. congo ITS-1    ----------ACAAGG--------------TAG-CTG------TAGGTGAACC-TGCAGC 74
DQ316041 T. vivax ITS-1 GCGATGGATGACTTGG---------------CTTCCCGG----TTCGTTGAAGAACGCAGC 175
DQ316051 T. vivax ITS-1 GCGATGGATGACTTGG---------------CTTCCCGG----TTCGTTGAAGAACGCAGC 163
01 T. congolense ITS-1  GCGATGGATGACTTGG---------------CTTCCTAT----TTCGTTGAAGAACGCAGC 517
02 T. congolense ITS-1    ----------ACAAGG--------------TAG-CTG------TAGGTGAACC-TGCAGC 38
40 T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 37
147T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 36
33 T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 35
39 T. congolense ITS-1    ----------ACAAGG--------------TAG-CTG------TAGGTGAACC-TGCAGC 36
36 T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 38
01 T. congolense ITS-1    ----------ACAAGG--------------TAG-CTG------TAGGTGAACC-TGCAGC 38
07 T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 36
05 T. congolense ITS-1    ----------AACAGG--------------TAG-CTG------TAGGTG-ACC-TGCAGC 37
32 T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 34
04 T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 37
35 T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 35
34 T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 39
43 T. congolense ITS-1    ----------ACAGG---------------TAG-CTG------TAGGTGAACC-TGCAGC 36
74 T. brucei-ITS-1      G---------TCCTCA--------------AAAACTG------TTCCTGCCGTTTTCATC 356
274 T.brucei-ITS-1      -----CGGATTTAGGG--------------CGCCCATGGTCTGGGGGTGTCG-CCTGTGC 199
269 T.brucei-ITS-1      -----TGGGTGTTGGG--------------ATGCCATG--TTGTCGCTACAGTTCTGCGC 203
64 T. brucei-ITS-1      CCT-TGTATGCCTGGTTAAGATTGATCTCTTTGACGGCGTACCTATCTGAAAA-ACCTTG 298
294 T.brucei-ITS-1      AC--------CCT---------------------CGGC--ACTGAGCTGGGAG-CGCTGA 158
                                                        *           *.

24 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 53
30 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 55
25 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 54
27 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 54
26 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 55
29 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 54
31 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 53
3  T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 52
8  T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 55
9  T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 53
17 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 55
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22 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 54
48 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 52
11 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 54
20 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 53
21 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 52
23 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 52
6  T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 55
10 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 57
18 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 56
28 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 57
13 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 54
12 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 54
14 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 55
15 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 55
44 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 53
16 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 49
19 T. vivax ITS-1          TGG--------------ATCATTTTCCGA------------------------------ 53
FJ712718.T. cong ITS-1  TGG--------------ATCATTTTCCGATGATAATATGTATATATACATATGCGTGTAT 120
DQ316041 T.vivax ITS-1  AAAGCGCGATATGTGGTATGATCTGCAGAAC------------------------------ 206
DQ316051 T.vivax ITS-1  AAAGCGCGATAGTTGGTATGATCTGCAGAAC------------------------------ 194
01 T. congolense ITS-1   AAGTGCGATAAGTGGTATCAATTGCAGAAT------------------------------ 548
02 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 53
40 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 52
147 T.congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 51
33 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 50
39 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 51
36 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 53
01 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 53
07 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 51
05 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 52
32 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 49
04 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 52
35 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 50
34 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 54
43 T. congolense ITS-1   TGG--------------ATCATTTTCCGA------------------------------ 51
74 T. brucei ITS-1       CTGGGTGTTTGTAATATACGGATTACCGAAACT--------------------------- 389
274 T.brucei ITS-1       CCGCCC----------CTTACTTTGTGT------------------------------- 217
269 T.brucei ITS-1       TCACCG----------ACAATTTTATGC------------------------------- 221
64  T.brucei ITS-1       ATGAAC-TATATCATTGTATAAGTCCTGGATG---------------------------- 329
294 T.brucei ITS-1       GTG----------------ATGTGCCGTTTG---------------------------- 173
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24 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTCG---------------------- 72
30 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTCG---------------------- 74
25 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTCG---------------------- 73
27 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTCG---------------------- 73
26 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTCG---------------------- 74
29 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTCG---------------------- 73
31 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTCT-----N---------------- 73
3  T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTT----------------------- 70
8  T. vivax ITS-1          CCTC--------------------TTCTCTTCTCTTAG-----G---------------- 75
9  T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTCG---------------------- 72
17 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCTCTAGACG----------------- 79
22 T. vivax ITS-1          CCTC--------------------TTCTCTTCTC-----GTCG----------------- 73
48 T. vivax ITS-1          CCTC--------------------TTCTCTTCTC-----GTCT----------------- 71
11 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCTCTG---------------------- 74
20 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCTCTAA--------------------- 74
21 T. vivax ITS-1          CCTC--------------------TTCTCTTCCCCTTTAGGCG----------------- 76
23 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCTTTAG--------------------- 73
6  T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCTCTN---------------------- 75
10 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCTTT----------------------- 76
18 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCTCG---------------------- 75
28 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCGTCTT---------------------- 77
13 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCCTT----------------------- 73
12 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCTTT----------------------- 73
14 T. vivax ITS-1          CCTC--------------------TTCTCTTC-CCCTTT---------------------- 74
15 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCCTCT---------------------- 75
44 T. vivax ITS-1          CCTC--------------------TTCTCTTCTCCCTTA---------------------- 73
16 T. vivax ITS-1          CCTC--------------------TTCTCTTCCCCCTTA---------------------- 69
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19 T. vivax ITS-1          CCTC--------------------TTCTCTTCCCCCTTT---------------------- 73
FJ712718 T. congo ITS-1    CCTCGTTCATGCGAATTGTTCCCATCCGCATCCACCCTGGTGTGGTGTGCGTTGTGTGTT 240
DQ316041 T. vivax ITS-1    ACCCG-----------ATTACCCAATCTTTGAACGCAAACGGCG-------------CAT 243
DQ316051 T. vivax ITS-1    ACTCG-----------ATTACCCAGTCTTTGAACGCAAACGGCG-------------CAT 231
01 T. congolense ITS-1     ATTTC-----------ATTG----ATCTTTGAACGCAAACGGCG-------------CAT 581
02 T. congolense ITS-1     GATA--------------------AAAAAGTATACCTTCATAGG---------------- 78
40 T. congolense ITS-1     GATA--------------------AAAAAGTATACCTACAATG----------------- 76
147 T.congolense ITS- 1    GATA--------------------AAAAAGTATACCTATATATG---------------- 76
33 T. congolense ITS-1        ATA--------------------AAAAAGTATACCTTNATAGG---------------- 75
39 T. congolense ITS-1      GATA--------------------AAAAAGTATACATACATATG---------------- 76
36 T. congolense ITS-1      GATA--------------------AAAAAGTACCCCTTTAAAG----------------- 77
01 T. congolense ITS-1      GATA--------------------AAAAAGTATACCTNCAAGGG---------------- 78
07 T. congolense ITS-1      GATA--------------------AAAAAGGATACCTTTATAG----------------- 75
05 T. congolense ITS-1      GATA--------------------AAAANGGACCTTAAAATGGC---------------- 77
32 T. congolense ITS-1      GATA--------------------AAAAAG--ACCCTTTAAGGG---------------- 72
04 T. congolense ITS-1      GATA--------------------ATATAT-ATACCCAT-ATGG---------------- 75
35 T. congolense ITS-1      GATA--------------------ATATAT-ATACCCCTTAAGG---------------- 74
34 T. congolense ITS-1      GATA--------------------ATATAT-ATACCCCTAAGGG--------------- 78
43 T. congolense ITS-1      GATA--------------------ATATAT-ATACCTATACGTG---------------- 75
74 T. brucei ITS-1          CCTCCTG-----------------AACTCTTACTCCAAACTGAAG--------------- 418
274T. brucei ITS-1          GCTC--------------------TGTTGGGCCCTCTGGAAAGTGA------------- 244
269T. brucei ITS-1          CCAC--------------------AGTTAACCCCTCGAAAAAAAG-------------- 247
64 T. brucei ITS-1          ATCCT-------------------CGCTGTTCCGGCATAACCACAT-------------C 360
294T. brucei ITS-1          GCTCA-------------------TGCTGTCGAGGCA---------------------- 194


24 T. vivax ITS-1             ------CGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 96
30 T. vivax ITS-1             ------CGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 98
25 T. vivax ITS-1             ------CGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 97
27 T. vivax ITS-1             ------CGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 97
26 T. vivax ITS-1             ------CGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 98
29 T. vivax ITS-1             ------CGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 97
31 T. vivax ITS-1             ------CGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 97
3  T. vivax ITS-1             ------CGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 94
8  T. vivax ITS-1             -----GCGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 100
9  T. vivax ITS-1             ------CGCCCG-----------------TCTCCCGGCCACCGGG-------------GC 96
17 T. vivax ITS-1             ------GGCCCG-----------------TGTCCAGGGAANCGGG-------------AC 103
22 T. vivax ITS-1             ------CGCCCG-----------------TNTCCNGGCCANCGGG-------------GC 97
48 T. vivax ITS-1             ------AGCCCG-----------------TNTCCAGGCCACCGGG-------------GC 95
11 T. vivax ITS-1             ---------CCG-----------------TCTCCCGGCCACCGGG-------------GC 95
20 T. vivax ITS-1             ---------CCG-----------------TCTCCCGGCCACCGGG-------------GC 95
21 T. vivax ITS-1             - ------CGCCCG---------------TCTCCCGGCCACCGGG-------------GC 100
23 T. vivax ITS-1             -- ------CCCG----------------TCTCCCGGCCACCGGG-------------GC 95
6  T. vivax ITS-1             --- ---GGGCG-----------------TCTCCCGGCCACCGGG-------------GC 98
10 T. vivax ITS-1             ---- --GGGCG-----------------TCTCCCGGCCACCGGG-------------GC 99
18 T. vivax ITS-1             ----- -AGACCG----------------TCTCCCGGCCACCGGG-------------GC 99
28 T. vivax ITS-1             ------ AGCCCG----------------TCTCCCGGCCACCGGG-------------GC 101
13 T. vivax ITS-1             ------A GCCG-----------------TCTCCCGGCCACCGGG-------------GC 96
12 T. vivax ITS-1             ------AG ACCG----------------TCTCCCGGCCACCGGG-------------GC 97
14 T. vivax ITS-1             -------AG AGG----------------TCTCCCGGCCACCGGG-------------GC 97
15 T. vivax ITS-1             -------AGG CG----------------TCTCCCGGCCACCGGG-------------GC 98
44 T. vivax ITS-1             -------AGGC G----------------TCTCCCGGCCACCGGG-------------GC 96
16 T. vivax ITS-1             ------AAGGGG -----------------TCTCCCGGCCACCGGG-------------GC 93
19 T. vivax ITS-1             ------AAGGG-- ----------------TCTCCCGGCCACCGGG-------------GC 96
FJ712718 T. congo ITS-1       GGGAACCGCACGTGGTGGGGTGCTGTTGTTGTACCGGCCACAAGCTCTAAAACGCACCTC 300
DQ316041 T. vivax ITS-1       GGGAGCAGCCCCTCGGGG--------TCATCCCCGTGCATGCCGCAG---------TCTC 286
DQ316051 T. vivax ITS-1       GGGAGCAGCCCCCCGGGG--------TCATCCCCGTGCATGCCGCAG---------TCTC 274
01 T. congo ITS-1             GGGAGAAGCTCTCTCGAG--------CCATCCCCGTGCATGCCACATT--------TCTC 625
02 T. congo ITS-1             ------TGTACG-----------------TG-TAGTGTAGGTGTGTN----------CT 103
40 T. congo ITS-1             ------TGTACG-----------------TG-TAGAGTGG-TGTGTG----------CT 100
147T. congo ITS-1             ------TGTACG-----------------TG-TAGTGTAGGTGTGTG----------CT 101
33 T. congo ITS-1             ------TGTACG-----------------TG-TAGTGTAGGTGTGTG----------CT 100
39 T. congo ITS-1             ------TGTACG-----------------TG-TAGTGTAGGTGTGTG----------CT 101
36 T. congo ITS-1             ------GGTACG-----------------TG-TAGTGTAGGTGTGTG----------CT 102
01 T. congo ITS-1             ------TGTACG-----------------TG-TAGTGTAGGTGTGT-----------CT 102
07 T. congo ITS-1             ------TGTACG-----------------TG-NAGGGTGGGNGTGTC----------CG 100
05 T. congo ITS-1             ------ACGACG-----------------TAGCGGGGTAG--GGGTC----------CT 101
32 T. congo ITS-1             ------TGTACG-----------------NG-TAGAGGAGG-GGGTG----------CA 96
04 T. congo ITS-1             ------TGTGTG-----------------TAC-CATATGG-TGTGTG----------T- 98
35 T. congo ITS-1             ------TGTGTA-----------------TACGCATATGGGTGTGTG----------TC 100
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34 T. congo ITS-1          ------TGTGTG-----------------TACGCATATG-GTGTGTG-----------TT 103
43 T. congo ITS-1          ------TGTNTN-----------------TACNCATATGNGTGTGTG-----------NT 101
74 T. brucei ITS-1         ---ATTATCTCG--------------AATACCTTCTGCCTGGGTTTG-------ACCTAC 454
274T. brucei ITS-1         CTCGACCTACCT-----------------CACCGGGGCAG--GACCTAT------GTCAC 279
269T. brucei ITS-1         ATC-ACTAAACA-----------------CACTGGTCCCATCGGCGTTG------CTATC 283
64 T. brucei ITS-1         AGGGGGGTTGCG-----------------GGGCAAG-ACACGCCATAA--------CCTCC 395
294T. brucei ITS-1         --AGTTGCTGCG-----------------AGGCATGTACTTGCTGTTG--------CATCT 228


24 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 116
30 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 118
25 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 117
27 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 117
26 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 118
29 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 117
31 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 117
3  T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 114
8  T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 120
9  T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 116
17 T. vivax ITS-1        GGGACAG----------CAAAC-ACGGAGN------------------------------ 122
22 T. vivax ITS-1        GGGACAG----------CAAACCACGNAGC------------------------------ 117
48 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 115
11 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 115
20 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 115
21 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 120
23 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 115
6  T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 118
10 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 119
18 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 119
28 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 121
13 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 116
12 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 117
14 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 117
15 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 118
44 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 116
16 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 113
19 T. vivax ITS-1        GGGACAG----------CAAACCACGCAGC------------------------------ 116
FJ712718 T. congo ITS-1  GGAACACGCAC--GTGTCCAAACACGCGTCCCCATGTCGCTCTCTTTCTCTTGTGTTGCG 358
DQ316041 T. vivax ITS-1  AGTGTCG---------AACCAAAAACACGCCG------------------------------ 309
DQ316051 T. vivax ITS-1  AGTGTCG---------AACACAACACACGCCGCCCACGC---------------------- 304
01 T. congo ITS-1        AGTGTCGAATATAAAAACAAAACACACACCTATTTTTTGTGTTGTT--------------- 671
02 T. congo ITS-1        ATCGAAG----------GTTGTTGTTGTG------------------------------- 122
40 T. congo ITS-1        ATCGAAG----------GTTGTTGTTGTG------------------------------- 119
147T. congo ITS-1        ATCGAAG----------GTTGTTGTTGTG------------------------------- 120
33 T. congo ITS-1        ATCGAAG----------GTTGTTGTTGTG------------------------------- 119
39 T. congo ITS-1        ATCGAAG----------GTTGTTGTTGTG------------------------------- 120
36 T. congo ITS-1        ATCGAAG----------GTTGTTGTTGTG------------------------------- 121
01 T. congo ITS-1        AACGAAG----------GTTGTTGTTGTG------------------------------- 121
07 T. congo ITS-1        ATCCAAA----------GGTG-TGGTGTG------------------------------- 118
05 T. congo ITS-1        GACGAAAG---------GTTGTTGTTGGGG------------------------------ 122
32 T. congo ITS-1        AAAGAAG----------GTTGTTGTAGGT------------------------------- 115
04 T. congo ITS-1        ATGAGAG----------NGNGTTGTTG--------------------------------- 115
35 T. congo ITS-1        ATGAGAG----------GGTGNTGGTGG-------------------------------- 118
34 T. congo ITS-1        TAGAGAG----------AGTG--GTTGT-------------------------------- 119
43 T. congo ITS-1        AAGAGAG----------AGTG--GTTGT-------------------------------- 117
74 T. brucei ITS-1       ATGGAAACCTTG-----CTAAAAAATTGAATAACCT------------------------ 485
274 T. brucei ITS-1      AAAACCCCTG------TCACAAAATCTAAG------------------------------ 303
269 T. brucei ITS-1      GAGTCCTGTG------TCAAAAATACAATT------------------------------ 307
64  T. brucei ITS-1      GTTTCCT----------CTTTTTACATCACTCTGATTT---------------------- 423
294 T. brucei ITS-1      TTGGCAT----------CTTATGTTGTCTC------------------------------- 248
                  .


24 T. vivax ITS-1        -------------TGCCGCTCGAC------------------------------------ 127
30 T. vivax ITS-1        -------------TGCCGCTCGAC------------------------------------ 129
25 T. vivax ITS-1        -------------TGCCGCTCGAC------------------------------------ 128
27 T. vivax ITS-1        -------------TGCCGCTCGAC------------------------------------ 128
26 T. vivax ITS-1        -------------TGCCGCTCGAC------------------------------------ 129
29 T. vivax ITS-1        -------------TGCCGCTCGAC------------------------------------ 128
31 T. vivax ITS-1        -------------TGCCGCTCGAC------------------------------------ 128
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3  T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 125
8  T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 131
9  T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 127
17 T. vivax ITS-1        ------------TGCGGCTNGAC---------------------------------- 133
22 T. vivax ITS-1        ------------TGCGGCTCGAC---------------------------------- 128
48 T. vivax ITS-1        ------------TGCGGCTCGAC---------------------------------- 126
11 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 126
20 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 126
21 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 131
23 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 126
6  T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 129
10 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 130
18 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 130
28 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 132
13 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 127
12 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 128
14 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 128
15 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 129
44 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 127
16 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 124
19 T. vivax ITS-1        ------------TGCCGCTCGAC---------------------------------- 127
FJ712718 T. congo ITS-1  CGACGTGTTCTTATGCCGCCCGACGCTCAT-------------------------TGTGT 453
DQ316041 T. vivax ITS-1  TGCGCGC--CTCGTGCCGCAGCA---------------------------------- 331
DQ316051 T. vivax ITS-1  TGCGCACTGCACGTGCCGCGGCG---------------------------------- 329
01 T. congo ITS-1        CGCACGCACAAAATCCCGCCACCTCTTCTCCTCGTGTGGTGCATATTCATGTTTGTGAGT 734
02 T. congo ITS-1        -------TGCT-TGTGTGCCCTT---------------------------------- 137
40 T. congo ITS-1        -------TGCT-TGTGTGCCCTT---------------------------------- 134
147T. congo ITS-1        -------TGCT-TGTGTGCCCT----------------------------------- 134
33 T. congo ITS-1        -------TGCT-TGTGTGCCCTT---------------------------------- 134
39 T. congo ITS-1        -------TGCT-CGTGTGCCCTT---------------------------------- 135
36 T. congo ITS-1        -------TGCT-TGTGTGCCCTT---------------------------------- 136
01 T. congo ITS-1        -------TGCT-TGTGTCCCCNT---------------------------------- 136
07 T. congo ITS-1        -------TGCTCCGTGTGCCCGT---------------------------------- 134
05 T. congo ITS-1        -------TCCTTTGGGTCCCCGTT--------------------------------- 139
32 T. congo ITS-1        --------GCTTGGGGCGCCCGCT--------------------------------- 131
04 T. congo ITS-1        ------------TGTGTGCTCG----------------------------------- 125
35 T. congo ITS-1        ------------TGTGTGCGCG----------------------------------- 128
34 T. congo ITS-1        ------------TGTGTGCGCG----------------------------------- 129
43 T. congo ITS-1        ------------NGTGTGCGCG----------------------------------- 127
74 T. brucei ITS-1       AACAATAAAAACTTGCGGCCCCACT-----------------------------CAACC 518
274 T. brucei ITS-       ------------TTTAAACTCTTCC---------------------------------- 316
269 T. brucei ITS-1      ------------GATGAGATAACGG---------------------------------- 320
64  T. brucei ITS-1      -----TTAATACCAATCCCTTGATGGTGTT----------------------------- 448
294 T. brucei ITS-1      ------------CATACCCTGAATG---------------------------------- 261

24 T. vivax ITS-1         -CGCGC---------------------------------------------------- 132
30 T. vivax ITS-1         -CGCGC---------------------------------------------------- 134
25 T. vivax ITS-1         -CGCGC---------------------------------------------------- 133
27 T. vivax ITS-1         -CGCGC---------------------------------------------------- 133
26 T. vivax ITS-1         -CGCGC---------------------------------------------------- 134
29 T. vivax ITS-1         -CGCGC---------------------------------------------------- 133
31 T. vivax ITS-1         -CGCGC---------------------------------------------------- 133
3  T. vivax ITS-1         -CGCGC---------------------------------------------------- 130
8  T. vivax ITS-1         -CGCGC---------------------------------------------------- 136
9  T. vivax ITS-1         -CGCGC---------------------------------------------------- 132
17 T. vivax ITS-1         -CGCGC---------------------------------------------------- 138
22 T. vivax ITS-1         -CGCGC---------------------------------------------------- 133
48 T. vivax ITS-1         -CGCGC---------------------------------------------------- 131
11 T. vivax ITS-1         -CGCGC---------------------------------------------------- 131
20 T. vivax ITS-1         -CGCGC---------------------------------------------------- 131
21 T. vivax ITS-1         -CGCGC---------------------------------------------------- 136
23 T. vivax ITS-1         -CGCGC---------------------------------------------------- 131
6  T. vivax ITS-1         -CGCGC---------------------------------------------------- 134
10 T. vivax ITS-1         -CGCGC---------------------------------------------------- 135
18 T. vivax ITS-1         -CGCGC---------------------------------------------------- 135
28 T. vivax ITS-1         -CGCGC---------------------------------------------------- 137
13 T. vivax ITS-1         -CGCGC---------------------------------------------------- 132
12 T. vivax ITS-1         -CGCGC---------------------------------------------------- 133
14 T. vivax ITS-1         -CGCGC---------------------------------------------------- 133
15 T. vivax ITS-1          -CGCGC---------------------------------------------------- 134
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44 T. vivax ITS-1            -CGCGC---------------------------------------------------- 132
16 T. vivax ITS-1            -CGCGC---------------------------------------------------- 129
19 T. vivax ITS-1            -CGCGC---------------------------------------------------- 132
FJ712718 T. congo ITS-1      GCGCACTGGCTCGCTTTTCTCCCCCTTCTTCTTCTCCTCGTCCTCATCTTTTCTCTGCTT 513
DQ316041 T. vivax ITS-1      --GCGCA-----------------------------------------ACAAAAGAGCCTG 349
DQ316051 T. vivax ITS-1      --GCGCA-----------------------------------------CCAACG-AGCCTG 346
01 T. congo ITS-1            GTGCACATATACGATATCATTCAACTGTTTCTACT---------CGCACAATGGTGTATG 785
02 T. congo ITS-1            -CGGTCA---------------------------------------------------- 143
40 T. congo ITS-1            -CGNTCA---------------------------------------------------- 140
147T. congo ITS-1            -CGCTCA---------------------------------------------------- 140
33 T. congo ITS-1            -CGCTCA---------------------------------------------------- 140
39 T. congo ITS-1            -CGCTCA---------------------------------------------------- 141
36 T. congo ITS-1            -CGCTCA---------------------------------------------------- 142
01 T. congo ITS-1            -CGCTCA---------------------------------------------------- 142
07 T. congo ITS-1            -NGCNCAC--------------------------------------------------- 141
05 T. congo ITS-1            -CGCTCA---------------------------------------------------- 145
32 T. congo ITS-1            -CGATCA---------------------------------------------------- 137
04 T. congo ITS-1            -CGTGCG---------------------------------------------------- 131
35 T. congo ITS-1            -CGTGCG---------------------------------------------------- 134
34 T. congo ITS-1            -CGCGCG---------------------------------------------------- 135
43 T. congo ITS-1            -CGCGCG---------------------------------------------------- 133
74 T. brucei ITS-1           ACGACCG---------------------------------------------------- 525
274T. brucei ITS-1           TTGTGCA---------------------------------------------------- 323
269T. brucei ITS-1           CAGCGCG---------------------------------------------------- 327
64 T. brucei ITS-1           ACACGTG-----------------------------------------TTGGCATACCTGAA 469
294T. brucei ITS-1           ACAGGTG-----------------------------------------TAGG---------A 273
                             .

24 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 149
30 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 151
25 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 150
27 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 150
26 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 151
29 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 150
31 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 150
3  T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 147
8  T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 153
9  T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 149
17 T. vivax ITS-1            -CCCGCGG------------------------AGGTGGAG------------------- 153
22 T. vivax ITS-1            -CCCGCGCGN----------------------AGGTGGAG------------------- 150
48 T. vivax ITS-1            -CCCGCGCGG----------------------AGGTGGAG------------------- 148
11 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 148
20 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 148
21 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 153
23 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 148
6  T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 151
10 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 152
18 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 152
28 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 154
13 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 149
12 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 150
14 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 150
15 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 151
44 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 149
16 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 146
19 T. vivax ITS-1            -CCCGCGCGC----------------------AGGTGGAG------------------- 149
FJ712718 T. congo ITS-1      TCCCACGTGTGT--------------TGGGAGAGTGGAGGAGGAAGTGTGTGTGTGTTTG 559
DQ316041 T. vivax ITS-1      GCACACCCTGAA--------------------AAAGGGAAAAGAGAGA------------ 377
DQ316051 T. vivax ITS-1      GCACACACACAC--------------------GCAGG------------------------ 363
01 T. congo ITS-1            TCACGCATATACGTGTGTGTAGTGAGTGATATGGAAGAGAAATGGGAAAGGCATATATAT 845
02 T. congo ITS-1            -TGCGCATCC----------------------CCATCCCG------------------- 160
40 T. congo ITS-1            -TGCGCATCC----------------------CCATCCCG------------------- 157
147T. congo ITS-1            -TGCGCATCC----------------------CCATCCCG------------------- 157
33 T. congo ITS-1            -TGCGCATCC----------------------CCATCCCG------------------- 157
39 T. congo ITS-1            -TGCGCATCC----------------------CCATCCCG------------------- 158
36 T. congo ITS-1            -TGCGCATCC----------------------CCATCCCG------------------- 159
01 T. congo ITS-1            -TGGGCATCC----------------------CCATCCCG------------------- 159
07 T. congo ITS-1            -TGNGCACCCG---------------------CCAGCCCG------------------- 159
05 T. congo ITS-1            -TGTGCATCCG---------------------CCATCCCG------------------- 163
32 T. congo ITS-1            --TGGCCCCCT---------------------CAACGGCA------------------- 154
04 T. congo ITS-1            -TACGGG--CC---------------------CCCGTGCA------------------- 147
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35 T. congo ITS-1            -NACNGTGCCC----------CCCGCTCA---------- 152
34 T. congo ITS-1            -TGCGGGGNGC----------CCCCCTCG---------- 153
43 T. congo ITS-1            -TGCGAG-CCC----------CCCCCTCN---------- 150
74 T. brucei ITS-1           CCTCACCTCTG----------ACATCCGG---------- 544
274 T. brucei ITS-1          -GACGCCCTGT----------TCCTCGGAG---------- 342
269 T. brucei ITS-1          -CGGGGCGTGG----------TCCCGGCTG---------- 346
64  T. brucei ITS-1          GATTGCATAGGATGG------------GTTCTTAATACGAAG---------- 499
294 T. brucei ITS-1          GCTAGCATC----------AAGAACACG---------- 291



24 T. vivax ITS-1            ----------CACGGCCCGC---------- 159
30 T. vivax ITS-1            ----------CACGGCCCGC---------- 161
25 T. vivax ITS-1            ----------CACGGCCCGC---------- 160
27 T. vivax ITS-1            ----------CACGGCCCGC---------- 160
26 T. vivax ITS-1            ----------CACGGCCCGC---------- 161
29 T. vivax ITS-1            ----------CACGGCCCGC---------- 160
31 T. vivax ITS-1            ----------CACGGCCCGC---------- 160
3  T. vivax ITS-1            ----------CACGGCCCGC---------- 157
8  T. vivax ITS-1            ----------CACGGCCCGC---------- 163
9  T. vivax ITS-1            ----------CACGGCCCGC---------- 159
17 T. vivax ITS-1            ----------CACGGACCGC---------- 163
22 T. vivax ITS-1            ----------CACGGCCCGC---------- 160
48 T. vivax ITS-1            ----------CACGGCCCGC---------- 158
11 T. vivax ITS-1            ----------CACGGCCCGC---------- 158
20 T. vivax ITS-1            ----------CACGGCCCGC---------- 158
21 T. vivax ITS-1            ----------CACGGCCCGC---------- 163
23 T. vivax ITS-1            ----------CACGGCCCGC---------- 158
6  T. vivax ITS-1            ----------CACGGCCCGC---------- 161
10 T. vivax ITS-1            ----------CACGGCCCGC---------- 162
18 T. vivax ITS-1            ----------CACGGCCCGC---------- 162
28 T. vivax ITS-1            ----------CACGGCCCGC---------- 164
13 T. vivax ITS-1            ----------CACGGCCCGC---------- 159
12 T. vivax ITS-1            ----------CACGGCCCGC---------- 160
14 T. vivax ITS-1            ----------CACGGCCCGC---------- 160
15 T. vivax ITS-1            ----------CACGGCCCGC---------- 161
44 T. vivax ITS-1            ----------CACGGCCCGC---------- 159
16 T. vivax ITS-1            ----------CACGGCCCGC---------- 156
19 T. vivax ITS-1            ----------CACGGCCCGC---------- 159
FJ712718 T. congo ITS-1      GTGTGTACGCAGGTGTGTTGGTCACGGCTCTC---------- 632
DQ316041 T. vivax ITS-1      ----------CGGCACACCGCCCGCAGCTCGG---------- 406
DQ316051 T. vivax ITS-1      ----------CAGCG-------CGCAGCCCGG---------- 385
01 T. congo ITS-1            TGTGTATATACAGAGAGTCTGTGGCGGTTGGGACATGTGTATAAATATATATGTATATGT 965
02 T. congo ITS-1            ----------CACGCCCCAG---------- 170
40 T. congo ITS-1            ----------CACGCCCCAG---------- 167
147T. congo ITS-1            ----------CACGCCCCAG---------- 167
33 T. congo ITS-1            ----------CACGCCCCAG---------- 167
39 T. congo ITS-1            ----------CACGCCCCAG---------- 168
36 T. congo ITS-1            ----------CACGCCCCAG---------- 169
01 T. congo ITS-1            ----------CACGCNCCA---------- 168
07 T. congo ITS-1            ----------CACGCCCCAG---------- 169
05 T. congo ITS-1            ----------CACGCCCCAGA---------- 174
32 T. congo ITS-1            ----------CACG----TGG---------- 161
04 T. congo ITS-1            ----------TGCGAATTAT---------- 157
35 T. congo ITS-1            ----------TGCGAATTAA---------- 162
34 T. congo ITS-1            ----------CGCGTGTNAT---------- 163
43 T. congo ITS-1            ----------CGCGAGNAAT---------- 160
74  T.brucei ITS-1           ----------CTGCTAACTAAGCCCGATAG---------- 564
274 T.brucei ITS-1           ----------ATCCCCCGCTAC---------- 354
269 T.brucei ITS-1           ----------ATCCCCCCCAAA---------- 358
64  T.brucei ITS-1           ----------ATCTACCGACCACATTAACATCCGTAGGTCTCCTTC----- 535
294 T.brucei ITS-1           ----------GTCAGCAAACCTAATTA---------- 308
                                      .

24 T. vivax ITS-1            ---------ACAACGTG----------TCGCG----------ATG 175
30 T. vivax ITS-1            ---------ACAACGTG----------TCGCG----------ATG 177
25 T. vivax ITS-1            ---------ACAACGTG----------TCGCG----------ATG 176
27 T, vivax ITS-1            ---------ACAACGTG----------TCGCG----------ATG 176
26 T. vivax ITS-1            ---------ACAACGTG----------TCGCG----------ATG 177
29 T. vivax ITS-1            ---------ACAACGTG----------TCGCG----------ATG 176
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31 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 176
3  T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 173
8  T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 179
9  T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 175
17 T. vivax ITS-1              ---------ACAACGTG----------ACGCN----------------------ATN 179
22 T. vivax ITS-1              ---------ACAACGTG----------ACGCG----------------------ATG 176
48 T. vivax ITS-1              ---------ACAACGTG----------ACGCG----------------------ATG 174
11 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 174
20 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 174
21 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 179
23 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 174
6  T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 177
10 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 178
18 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 178
28 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 180
13 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 175
12 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 176
14 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 176
15 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 177
44 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 175
16 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 172
19 T. vivax ITS-1              ---------ACAACGTG----------TCGCG----------------------ATG 175
FJ712718 T. congo ITS-1        ---------ACAACGTG----------TCGCG----------------------ATG 648
DQ316041 T. vivax ITS-1        ---------CCAGCGG----------TCACACGCA-------------------ACG 425
DQ316051 T. vivax ITS-1        ---------CCAGCGG----------ACCCAC----------------------ACG 401
01 T. congo ITS-1            GTGTGTTCCCCTGTGGAGATTTTATATCTTACGGAGAGTGTTCATATATATGTTTGTACG 1025
02 T. congo ITS-1              ------TGTTTTGTGTG---------CTGTGCG----------------------ATG 191
40 T. congo ITS-1              ------TGTTTTGTGTG---------CTGT-CG----------------------ATG 187
147T. congo ITS-1              ------TGTTTTGTGTG---------CTGTGCG----------------------ATG 188
33 T. congo ITS-1              ------TGTTTTGTGTG---------CTGTNCG----------------------ATG 188
39 T. congo ITS-1              ------TGTTTTGTGTG---------CTGTGCG----------------------ATG 189
36 T. congo ITS-1              ------TGGTTTGTGTG---------CTGTGCG----------------------ATG 190
01 T. congo ITS-1              ------TGTTTTGTGTG---------CTGTACG----------------------ACG 189
07 T. congo ITS-1              ------AGTTTTGTGGG---------CTAGGNGT---------------------ACG 191
05 T. congo ITS-1              ------TGTTTTGNGTGC--------TTGTGCG----------------------ACG 196
32 T. congo ITS-1              ------TGTGTTGTGTG---------TTGCACG---------------------- 179
04 T. congo ITS-1              ------TCCCATCCCCCC--------CCNC--CC----------------------CGG 178
35 T. congo ITS-1              ------TCCCAGTCGCAT--------CCAC--CC----------------------CGG 183
34 T. congo ITS-1              ------TTTNANCCGCGC--------CCACNCCC----------------------CCG 186
43 T. congo ITS-1              ------TTTCACCCGC-C--------CCACCCCC----------------------CCG 182
74 T. brucei ITS-1             ------AAGGCTGAGTTGG-------CTGCTCTGACCCCTCCTCAATACCAAATATAACC 611
274T. brucei ITS-1             ------ACACAAGAAGGGG-------GGAGGCA----------------------TCA 377
269 T. brucei ITS-1            ------TGGAAGGCGGGGG-------GGAGAAAGCG-----------GAAAGGTCTGTCC 394
64  T. brucei ITS-1            --TTACAGTTAAATATGATTATTTTTATAGGGGGAGGGTTATCCGCTCCCACTTCCCCCC 593
294 T. brucei ITS-1            ---------TAAATG-------------AGTGGTCGG------------------CCACG 328


24 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 192
30 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 194
25 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 193
27 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 193
26 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 194
29 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 193
31 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 193
3  T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 190
8  T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 196
9  T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 192
17 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 196
22 T. vivax ITS-1                GATGACTT------------------------GCCTTCCCG-------------------- 193
48 T. vivax ITS-1                GATGACTT------------------------GGCTTCNCG-------------------- 191
11 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 191
20 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 191
21 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 196
23 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 191
6  T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 194
10 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 195
18 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 195
28 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 197
13 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 192
12 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 193
14 T. vivax ITS-1                GATGACTT------------------------GGCTTCCCG-------------------- 193
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15 T. vivax ITS-1              GATGACTT-----------------------GGCTTCCCG------------------- 194
44 T. vivax ITS-1              GATGACTT-----------------------GGCTTCCCG------------------- 192
16 T. vivax ITS-1              GATGACTT-----------------------GGCTTCCCG------------------- 189
19 T. vivax ITS-1              GATGACTT-----------------------GGCTTCCCG------------------- 192
FJ712718 T. congo ITS-1        GATGACTT-----------------------GGCTTCCTA------------------- 665
DQ316041 T. vivax ITS-1        CGTGCA-------------------------CGCACGCC------------------- 439
DQ316051 T. vivax ITS-1        CACGTG-------------------------CGCAGCGC------------------- 415
01 T. congo ITS-1              CATGTATTTT---------------------GGCGCCCCGTATAGAGATTAAAAAAGAA 106
02 T. congo ITS-1              CGGCGGTNGG---------------------TGTGCG-AT------------------- 209
40 T. congo ITS-1              CGGCGGTGGG---------------------TGTGN---------------------- 202
147T. congo ITS-1              CGGCG-TGGG---------------------------------------------- 197
33 T. congo ITS-1              CGGCGGTGGG---------------------TGTTGN-AT------------------- 206
39 T. congo ITS-1              CGGCGGTGTG---------------------TGTTGTGAT------------------- 208
36 T. congo ITS-1              CGGCGTGTGG---------------------TGGNAT---------------------- 206
01 T. congo ITS-1              CGTGGGTNGG---------------------GGTGGCGAA------------------- 208
07 T. congo ITS-1              CGGGCGAGGG---------------------GGTGT----------------------- 206
05 T. congo ITS-1              CGGCGATGGG---------------------TGAGTG--------------------- 212
32 T. congo ITS-1              CGGCGNNNGT---------------------TGGGAT--------------------- 195
04 T. congo ITS-1              GGGGGGGTGG---------------------G-TGTGGGT------------------ 196
35 T. congo ITS-1              TGGGGGGNGG---------------------GGTGTGGGG----------------- 202
34 T. congo ITS-1              TGGGGGGCGT---------------------GGTGTGTGG------------------ 205
43 T. congo ITS-1              TGGGGGGCGT---------------------GGTGTGTGG------------------ 201
74  T. brucei ITS-1            CCTTGGGCCG---------------------GTCTAATCG------------------ 630
274 T. brucei ITS-1            CAAGAT-------------------------CGTCT--------------------- 388
269 T. brucei ITS-1            CTTGTTGGCGTGCAATTTGGGATGAGTATGGGGAAGTCTGGCATGGTG---------- 442
64  T. brucei ITS-1            AGCGAGACTGATCA-----------------AATTCCCTGGATCGA------------ 622
294 T. brucei ITS-1            CGCG---CTGG--------------------TCCTTG------------------- 342
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