EXPRESSION PATTERN OF TRANS-SIALIDASE GENES IN THE LIFE CYCLE OF *TRYPANOSOMA CONGOLENSE*

BY

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BY

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DECLARATION

I declare that the work in this Dissertation entitled Expression of trans-sialidase genes in the life cycle of *Trypanosoma congolense* has been carried out by me in the Department of Biochemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other institution.

Gloria Dada CHECHET

Name of Student	Signature	Date

CERTIFICATION

This dissertation entitled EXPRESSION OF TRANS-SIALIDASE GENES IN THE INFECTION CYCLE OF *TRYPANOSOMA CONGOLENSE* by Gloria Dada CHECHET meets the regulations governing the award of the degree of PhD Biochemistry of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

In thankfulness, this work is dedicated to GOD Almighty.

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ABSTRACT

Trypanosoma congolense an agent of Nagana, the trypanosomiasis in African ruminants remains a threat throughout sub-Sahara Africa, and new approaches to disease prevention and treatment remain a priority. Trypanosomes express an enzyme called trans-sialidase (TS; E.C. 3.2.1.18), which is believed to play an important role in maintaining pathogenicity of the parasites. Data of Expressed Sequence Tag sequences obtained from *T.congolense* cultures made available through the Welcome Trust Sanger Institute web site (www.sanger.ac.uk/Projects/T_congolense/) have suggested that the TS-like genes identified in the genome are differentially expressed in procylic, metacyclic, epimastigote and blood stream parasites. In order to test the hypothesis that trans-sialidase (TS) and TS-like gene p roducts play a role in the infection cycle of *T. congolense*, the transcription pattern of TS genes during the entire life cycle of *Trypanosoma congolense*, i.e. during the manifestation of infection in a suitable host and the maturation of parasites in the flies has been investigated. To establish the infection cycle, an experimental rat carrying *Trypanosoma congolense* was made available for tsetse flies (*Glossina morsitans morsitans*) to feed on to aquire infection. These infected flies were fed on clean goats. Generic PCR analysis for identification due to the reason that inter-species size variation in the PCR product of the internal transcribed spacer (ITS-1) region of ribosomal RNA repeat region enables species identification. Species-specific tests were used to confirm these identifications. Due to it's improved sensitivity, this method facilitated identification of various trypanosoma species and monitoring the infection cycle of *Trypanosoma congolense* in the mammalian host and fly vector reflecting the parasitic status of the infected tsetse flies. Samples for analysis by Reverse transctiptase Polymerase chain

reaction (RT-PCR), Western blot and Quantitative polymerase chain reaction (qPCR) were obtained from fly tissues dissected 21 days post infection and blood collected daily from the goats. Contrary to previous reports,the RT-PCR, Western blot and qPCR results clearly show that Trans-sialidase genes are expressed in the blood stream and procyclic forms and are developmentally regulated.

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LIST OF ABBREVIATIONS

HAT	Human African trypanosomiasis
AAT	Animal African trypanosomiasis
FAO	Food and Agriculture Organization
Neu5Ac	*N*-acetylneuraminic acid
TS	Trans-sialidases
Sia	Sialic acids
TcTS	*Trypanosoma cruzi* Trans-sialidase
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction (Real-Time)
DALY	Disability Adjusted Life Year
VSG	Variant Specific Surface Glycoprotein
GPI-PLC	Glycosyl-phosphatidyl-inositol-specific phospholipase C
PARP	Procyclic Acidic Repetitive Proteins
GPI	Glycosyl-phosphatidylinositol
ES	Expression site
VAT	Variable antigenic type
Gal	ß-D-galactopyranosyl
GalNAc	ß -D-N-acetylgalactosaminyl
PSA	Polysialic acid
Kdn	2-keto-3-deoxynononic acid

ECM	Extracellular matrix
ER	Endoplasmic recticulum
CMP-Sia	Cytidine 5-monophosphate sialic acid
Neu5Gc	N-glycolylneuraminic acid
KO	Knockout
TconTS1	*Trypanosoma congolense* Trans-sialidase 1
TconTS2	*Trypanosoma congolense* Trans-sialidase 2
TconTS3	*Trypanosoma congolense* Trans-sialidase 3
TconTS4	*Trypanosoma congolense* Trans-sialidase 4
WTSI	Wellcome Trust Sanger Institute
MI	Maturation index
hpe	hours post-emergence
DMFO	Difluromethylornithine
SIT	Sterile Insect Technique
GMOs	Genetically modified organisms
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
HIV	Human immunodeficiency virus
NVRI	National Veterinary Research Institute
NITOR	Nigerian Institute for Trypanosomiasis and Oncocerchiasis research
PATTEC	Pan African Tsetse and trypanosomiasis Eradication Campaign
DFG	Deutsche Forschungsgemeinschaft (German Research Foundation)

ITS-1	Internal transcribed spacer 1
NCBI	National center for Biotechnology information
DEPC	Diethylpyrocarbonate
cDNA	Complementary DNA
mRNA	Messanger RNA
dNTP	Deoxynucleoside triphosphate
BLAST	Basic local alignment search tool
SDS-PAGE	Sodium dodecyl polyacrilamide gel electrophoresis
DTT	Dithiothreitol
TBST	Tris-Buffered Saline-Tween 20
mAB	Monoclonal anti-body
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HKG	House keeping gene
gDNA	Genomic DNA
PCR-RFLP	Polymerase Chain Reaction Restriction fragment length polymorphism
PCR-ELISA	Polymerase Chain Reaction Enzyme linked immunosorbent assay
mAEC	Mini-anion exchange columns
HTC	Haematocrit centrifugation technique
GlcN	D-glucosamine
HDP	Host defense peptide
GARP	Glutamic acid/Alanine Rich Proteins
cGMP	Cyclic guanosine monophosphate

CHAPTER I

1.0 INTRODUCTION

1.1 Background

Trypanosomes are single-celled, parasitic eukaryotes found worldwide and in all vertebrate classes. They have diagenetic (two-host) life cycles, which alternate between vertebrate and invertebrate hosts. Trypanosomes are transmitted by a wide range of blood – sucking invertebrates, such as aquatic or terrestrial leeches, tsetse flies, ticks and triatome bugs. Several trypanosome species cause diseases of major medicinal and veterinary importance. The disease causes morbidity and mortality, as well as widespread economic loss and social hardship, especially in Africa and South America. In tropical Africa, a range of trypanosome species are transmitted via the saliva of tsetse flies, of which two *Trypanosoma brucei* subspecies cause Human African trypanosomiasis (HAT) and a wider range of species cause Animal African trypanosomiasis (AAT), (Adams and Hamilton, 2008) with *T. vivax* and *T.congolense* are regarded as major pathogens of cattle and other ruminants (Aksoy, 2003). Although, a total number of deaths from HAT per year is unknown due to limited surveillance an estimated 60 million people are at risk and approximately, 500,000 people are currently infected (WHO, 2014). The Food and Agriculture Organization (FAO) estimates annual losses from livestock production owing to AAT are approximately US$ 1.0- 1.2 billion, and losses to Africa in terms of potential agricultural GDP maybe as much as US$4.75 billion (FAO, 2014). Nagana has restricted agricultural development and has a profound effect on the economy of tropical Africa. Approximately 70% of the humid and semi-humid zones of sub-Sahara are devoid of cattle and farming systems and only through

continued chemotherapy and tsetse control programs, cattle can graze on the fringe of the tsetse habitat. Shortages of the financial and human resources necessary to implement or sustain a comprehensive control program characterize all endemic countries (Aksoy, 2003).

Tsetse flies (Diptera: Glossinidae) are the sole vectors of several species of pathogenic trypanosomes in tropical Africa (Aksoy, 2003). The three main species of tsetse flies for transmission of trypanosomes are *Glossina morsitans*, which favors the open woodland of the savanna; *Glossina palpalis*, which prefers the shaded habitat immediately adjacent to rivers and lakes; and *Glossina fusca*, which favor the high, dense forest areas. These pathogens are transmitted through tsetse fly saliva when the fly feeds on an animal or other biting flies through the transfer of blood from one animal to another (Roder *et.al.*, 1984). However, other biting insects may mechanically transmit trypanosomes (Desquesnes and Dia, 2003).

Sialic acids (Sia) are 9-carbon backbone acidic monosaccharides found within the group of glycoconjugate containing glycans (Freire-de-Lima *et al.*, 2012). There are about 40 naturally occurring sialic acids with *N*-acetylneuraminic acid [Neu5Ac] and *N*-glycolylneuraminic acid [Neu5Gc] being the two major ones found in mammals (Kelm *et al*., 1998, Freire-de-Lima *et al.*, 2012). They are terminal acidic sugars of many glycoproteins and glycolipids especially of higher animals. In this exposed position they contribute significantly to the structural properties of these molecules, both in solution and on cell surfaces. Therefore, it is not surprising that Sia are important regulators of cellular and molecular interactions, where they play important roles in a myriad of biological processes. They can either mask recognition sites like subterminal

carbohydrate structures or proteins, or can serve as recognition determinants. They play a role in masking and in binding of pathogens to host cells and also in non-pathological cellular interaction (Kelm and Schauer, 1997, Tiralongo *et al*., 2003). Trypanosomes lack sialic acids but have been shown to express an enzyme which protects them from the immune system of the host (Engstler *et al*., 1995; Schenkman *et.al*., 1994, Montagna *et.al.*, 2002; Tiralongo *et.al*., 2003).

Trans-sialidases (TS) are unique glycosyltransferases, which are expressed by some trypanosomes (Tiralongo *et.al*., 2003). TS catalyse the transfer of preferably α-2,3-carbohydrate-linked sialic acids to another carbohydrate forming a new α-2,3-glycosidic linkage to galactose or N-acetylgalactosamine (Schauer and Kamerling, 1997). Although of crucial importance for their survival, cyclical transmission and hence pathogenicity, trypanosomes lack the biochemical metabolic machinery synthesizing Sia but use this unique enzyme to transfer Sia onto the parasites surface molecules from the environment. TS were first described in the blood stream form of American trypanosome, *Trypanosoma cruzi* (Schenkman *et. al.*, 1991) the pathogen of Chagas disease, afflicting millions of people in Latin America. Currently, TS for *Trypanosoma brucei brucei* have been cloned and studied (Montagna *et. al*., 2002) likewise the characterization of TS variants from *Trypanosoma congolense* has been reported (Tiralongo *et al*., 2003; Koliwer-Brandl *et. al*., 2011; Gbem *et al*., 2013). In African trypanosomes where TS is thought to be expressed only in the procyclic insect stages (Pontes de Carvalho *et.al*., 1993: Engstler *et.al.*, 1995), TS has been shown to increase the survival, maturation and hence establishment of the parasites in the vector midgut (Nagamune *et. al*., 2004).

1.2 Statement of Problem

In Africa, the wide occurrence of African trypanosomiasis in people and their livestock is a great constraint to development. Currently, chemotherapy and vector control strategies are principally used in the management of African trypanosomiasis. However, these methods are faced with problems ranging from imperfect diagnostic techniques (Checchi *et al*., 2012; Checchi *et al*., 2011), inadequate drug treatment, resistance to current trypanocides (Baker *et al*., 2013), high prevalence of drug resistance even in animal trypanosomes without a history of drug exposure (Simbarashe *et al*., 2012), ineffective vector control strategies (Eisen *et al*., 2009), wildlife reservoir of infection (Hamil *et al*., 2013; Anderson *et al*., 2011), difficulties in making a conventional vaccine due to antigenic variation of the parasites (La Greca and Magez, 2011; Baral, 2010), limitations of resistant livestock (www.ilri.org/InfoServ/Webpub/fulldocs/ilrad89/Trypano.htm). Hence, the need for new biochemical targets to fight against the disease.

1.3 Justification

Nagana is caused by the parasites *T.congolense*, *T.vivax* and to a lesser extent *T.b.brucei* transmitted from reservoir hosts to livestock by biting insects (Naessens, 2006). Most research on African trypanosomes has been focusing on *T.b.brucei.* Therefore, most of our current understanding of African trypanosomes and their life cycles is based on studies with *T.b.brucei* whereas relatively little research has been done with other African trypanosomes including *T. congolense* which seems to be the predominant causative agent of bovine trypanosomiasis (Ajayi *et al*., 1997; Onyiah, 1997; Oluwafemi

et al., 2007). However, recently the number of publications describing studies on the biology of *T. congolense* has increased indicating that this parasite attracting more interest in the scientific community (Peacock *et al*., 2012). This certainly will be further fostered by the progress finalizing the genome project at the Welcome Trust Sanger Institute (Helm *et al.*, 2009, Morrison *et al*., 2009).

A hallmark of most African trypanosomal diseases is the cycle of the parasites between the mammalian host and the insect vector. This is reflected by discrete morphologies and gene expression patterns necessary to prepare for the infection of flies (Matthews, 2005). The interactions between the trypanosomes and the defense mechanisms of the fly vector play a critical role for subsequent transmission to the mammalian host (reviewed in Roditi and Lehane, 2008) as the trypanosome can establish a stable infection and mature to transmissible metacyclic parasites only in a small proportion of flies. Studies have revealed that the sialylation of parasites by trypanosomal trans-sialidases is an essential factor for their survival, pathogenicity and virulence (Schenkman *et al*., 1992; Engstler *et al*., 1995; Montagna *et al*., 2002). Given the essential roles this enzyme is a good target for the development of alternative chemotherapy against the parasite.

Knowledge of the gene expression pattern of each developmental stage is a valuable contribution to the study of the parasite biology and the pathogenesis of Nagana disease. Consequently, information on some of these stage specific genes encoded enzymes that might help in identifying new candidate targets for vaccine studies and development against the disease.

1.4 Aims and objectives

1.4.1 Aim

The aim of this project is to test the hypothesis that trans-sialidase (TS) and TS-like proteins play a role in the infection cycle of *T. congolense* and related trypanosomes, particularly in the establishment of infection in the fly vector and the subsequent transmission to mammalian hosts.

1.4.2 Objectives

i. Optimization of Polymerase Chain Reaction (PCR)- based diagnosis of *Trypanosoma* species in the fly vector and host.

ii. Establishment of life cycle of *T.congolens*e between the fly vector (tsetse fly) and host (goat).

iii. Investigation of stage-specific expression of TS mRNA in *T. congolense* (bloodstream, procyclic, epimastigote and metacyclic stages) by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

iv. Investigation of stage-specific expression of TS proteins in *T. congolense* (bloodstream, procyclic, epimastigote and metacyclic stages) by immunoblot analysis.

v. Investigation of stage-specific quantitative TS gene expression by Real-Time PCR (qPCR).

CHAPTER 2

LITERATURE REVIEW

2.1 African Trypanosomiasis

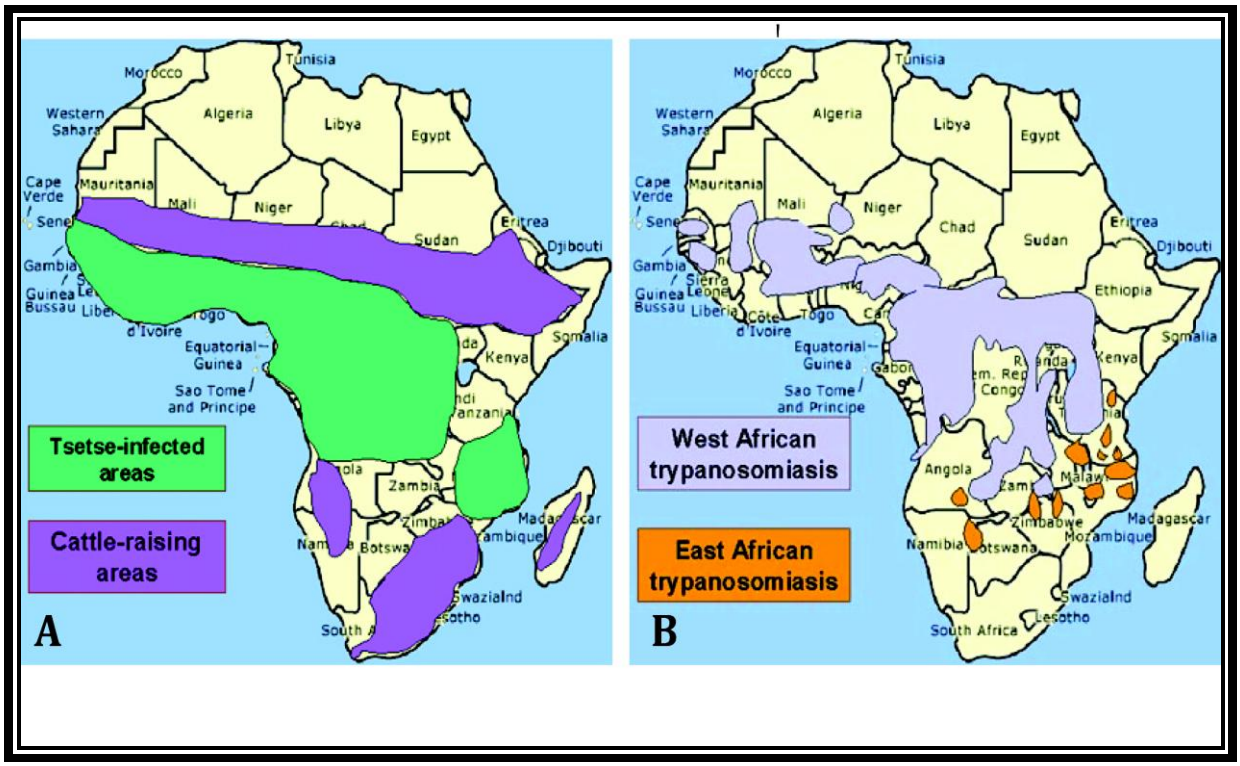
Trypanosomiasis is a fatal infectious disease in both human and livestock of similar aetiology and epidemiology. Besides death, it causes a heavy economic loss predominantly in Africa. (Baral, 2010). The etiological agent of the disease is a unicellular flagellated protozoan parasite of the genus *Trypanosoma* that lives and multiplies extracellularly in the blood and tissue fluids of their mammalian hosts and are transmitted by the bite of infected tsetse flies (*Glossina* sp.) *(*Stervading, 2008). The distribution of trypanosomiasis in Africa corresponds to the range of tsetse flies and comprises currently an area of 8 million km^2 between 14 degrees North and 20 degrees South latitude (Molyneux *et al*., 1996; Stervading, 2008) (Figure 2.1A). Throughout history, African trypanosomiasis has severely repressed the economic and cultural development of Africa. (Stervading, 2008).

African animal trypanosomiasis or nagana disease is caused by *T. congolense, T. vivax* and *T. brucei* species and carried with higher prevalence by a greater number of hematophagous glossina species commonly called tsetse flies which are the vectors of the African trypanosomes and are responsible for cyclical transmission of the parasitic protozoan between numerous vertebrate hosts (Baral, 2010). In wild animals, these parasites cause relatively mild infections while in domestic animals they cause a severe, often-fatal disease. All domestic animals can be affected by Nagana and the symptoms are fever, listlessness, emaciation, hair loss, discharge from the eyes, oedema, anaemia, and paralysis. As the illness progresses the animals weaken more and more and

eventually become unfit for work, hence the name of the disease "N'gana" which is a Zulu word that means "powerless/useless" (Winkle, 2005). On account of Nagana, stock farming is very difficult within the tsetse belt and this is invariably the greater epidemic across the African continent with direct economic consequences (Baral, 2010). In general, trypanosome infections that threaten livestock have a 100 to 150-fold higher prevalence than the Human African Trypanosomiasis (HAT) (Baral, 2010). Although trypanosomiasis is often referred to as African Trypanosomiasis, certain trypanosomes do cause infections outside this continent. *T. evansi*, the causative agent of "surra" occurs not only in Africa, but also in Central and South America, the Middle East, and Asia. It causes a disease in camels, horses, cattle, pigs, buffaloes, and dogs (Wuyts *et al*., 1994).

Trypanosomes are the causative agent of Human African trypanosomiasis (HAT), also known as "sleeping sickness". The term "sleeping sickness" describes the deregulation of the sleep-wake cycle and the intrasleep cycle, which is observed in the late stage of the disease (Buguet *et al*., 2005). The disease is caused by *Trypanosoma brucei rhodesiense* in Eastern and Southern Africa as well as *Trypanosoma brucei gambiense* in Western and Central Africa (Figure 2.1B). Both protozoan species are morphologically indistinguishable, but have drastically different epidemiological features. Both forms of the sleeping sickness affect the central nervous system. The typical East African form of trypanosomiasis is characterized by a rapid and acute development of the disease, and untreated patients can die within weeks or months of the infection, whereas the West African form of this disease is more chronic that can last for several years (Baral, 2010). According to WHO reports, African sleeping sickness is the third most important

contributor to the global burden of the parasitic diseases after malaria and schistosomiasis (Cattand *et al*., 2001). It is estimated that at least 300,000– 500,000 people are presently infected with at least 60 million people at risk of the infection (WHO, 1998).



Modified from: http://www.microbiologybook.org/lecture/images/africa2.jpg

Figure 2.1: A. Distribution of tsetse flies, which corresponds to the occurrence of Nagana (green) and Nagana-free, cattle raising areas (purple). B. Distribution of West (*T.b.gambiense*) and East (*T.b,rhodesiense*) African sleeping sickness.

2.2 Classification of Trypanosomes

The protozoan parasite trypanosomes are grouped in the order "kinetoplastida" because of the presence of a kinetoplast. Based on the mode of transmission by their insect vector, the genus *Trypanosoma* is divided in two main groups: stercoraria and salivaria (Hoare, 1966; W'ery, 1995). The development of stercoraria parasites takes place in the intestinal track of the invertebrate vector and the infection to the vertebrate is via feces. *T. cruzi*, the causative agent of Chagas' disease, is an example of the stercoraria group. On the other hand, salivarian parasites colonize the stomach of their invertebrate vector, but never pass to the intestinal track. Rather, they migrate towards the salivary gland of the vector where the infectious form for vertebrate host develops. Infection of the vertebrate occurs via saliva when the vector bites in order to take the blood meal.

The African parasites, *T. brucei*, *T.congolense, T.evansi*, and *T.equiperdum* all belong to the salavarian group. *T. brucei* has three subgenera; while *T. b. brucei* is the causative agent of Nagana, a cattle disease in Africa, *T. b. rhodesiense* and *T. b. gambiense* are the causative agents of the sleeping sickness in human (Figure 2.2). The other two species *T. congolense* and *T. vivax* are the major causative agents of animal trypanosomiasis in Africa. *T. equiperdum* is the causative agent of an equine venereal disease that is called "dourine" where the parasites are transmitted during coitus. *T. evansi* causes a livestock disease called "surra" (Baral, 2010).

Phylum	Protozoa						
Order	Kinetoplastida						
Suborder	Trypanosomatina						
Family	Trypanosomatidae						
Genus	Trypanosoma						
Subgenus	Herpetosoma	Megatrypanum	Schizotrypanum	Dutonella	Nannomonas	Trypanozoon	Pycomonas
Species	*T.theleri*	*T.rangeli* *T.lewisi* *T.musculi*	*T.cruzi*	*T.vivax* *T.uniforme*	*T.congolense* *T.simiae* *T.godfreyi*	*T.equiperdium* *T.evansi* *T.brucei*	*T.suis*
Subspecies	**Stecoraria**			**Salivaria**		*T.b.brucei* *T.b.rhodesiense* *T.b.gambiense*	

Figure 2.2: Classification of trypanosomes

2.3 Life Cycle of trypanosomes

Trypanosomes are the excellent examples of organisms that display an extreme adaptation to their environment, in many cases because they must evade the immune response of the host. African trypanosomes are transmitted between mammalian hosts by tsetse flies (Figure 2.3). However, in each host, the parasites undergo many life cycle stages involving forms with discrete morphologies, patterns of gene expression, and proliferation status. In each case, these developmental changes are programmed precisely (Mathews *et al*., 1999). Infection in the mammalian host begins when the infective stage, known as the metacyclic stage, is injected intradermally by the tsetse fly (Figure 2.3).

The organisms rapidly transform into the blood-stage trypomastigotes (long, slender forms), and divide by binary fission in the interstitial spaces at the site of the bite. The buildup of metabolic wastes and cell debris leads to the formation of a "chancre". In the mammalian host, the metacyclic parasites rapidly undergo cell cycle reentry and morphological changes (Barry and McCulloch, 2001). Once established in the mammalian host, the bloodstream parasite is heterogeneous (Vickerman, 1985; Vickerman, 1965) comprising the proliferative slender forms during the ascending phase of parasitemia and the non-proliferative stumpy forms at the peak of parasitemia (Mattews *et al*., 2004). The transition from the morphological extremes (i.e., the slender versus stumpy forms) involves a progression from proliferation to cell cycle arrest, accompanied by a series of biological and morphological transformations (Vassella *et al*., 1997; Tyler *et al*., 1997). Once stumpy forms develop during the course of parasitemia, the population is preadapted for transition to the procyclic forms, which

occupy and proliferate in the midgut of the tsetse. The key features of the stumpy formation are the cell cycle arrest, the elaboration of some mitochondrial activities, and a relative resistance to lysis by antibodies (McLintock *et a*l., 1993; Redpath *et al*., 2000) or to the proteolytic environment that might be encountered in the midgut of tsetse (Sbicego *et al*., 1999; Ziegelbauer *et al*., 1993). When the vector-fly (tsetse) bites to an infected individual, it takes the parasites with the blood meal. The parasites undergo metabolic changes in the midgut of the fly. They lose their surface coat, which consists of about 10^7 molecules of the Variant Specific Surface Glycoprotein (VSG), and transform into the proliferative procyclic forms. In this form, they express their own surface proteins called the Procyclic Acidic Repetitive Proteins (PARPs, or procyclins).

The defining events of the differentiation from the bloodstream forms to the procyclic forms are the loss of VSG and gain of the procyclins. VSG loss occurs very rapidly and involves the combined action of glycosyl-phosphatidyl-inositol-specific phospholipase C (GPI-PLC) and a proteolytic cleavage of the VSG via a zinc metalloprotease (Ziegelbauer *et al*., 1993; Gruszynski *et al*., 2003; Gruszynski *et al*., 2006). The transformation to procyclic form also changes the energy generation from being exclusively based on glycolysis in the bloodstream to a mitochondrion-based respiratory system, which requires a structural elaboration and the metabolic activation of organelle (Matthews *et al*., 2005). For successful transmission, the parasite undergoes two stages of differentiation in the fly: first, establishment in the midgut and then maturation in the mouthparts or the salivary gland. It is generally thought that during normal development in the fly, there are no intracellular stages and the parasites

do not cross an epithelial barrier to enter the fly. After proliferation in the tsetse midgut, the parasite migrates to the salivary gland. The epimastigote forms generated there attach to the gland through elaboration of the flagellar membrane. After further multiplication, the parasite undergoes division arrest, reacquires a VSG coat, and is released into the salivary gland lumen, in preparation for an inoculation into a new mammalian host (Matthews *et al*., 2005).

If the tsetse flies ingest more than one strain of trypanosome, there is the possibility of genetic exchange between the two strains, generating an increase in the genetic diversity in an organism that may not have a true sexual cycle. Indeed, it was shown by laboratory crosses that genetic exchange in the African trypanosome is possible (Sternberg *et al*., 1989; Sternberg *et al*., 1988; Sternberg and Tait, 1990; Turner *et al*., 1991; Jenni *et al*., 1986). Precisely at which stage of the life cycle this genetic exchange takes place is equivocal, suggesting at the midgut stage (Schweizer and Jenni, 1991), in the salivary gland of the fly (Bingle *et al*., 2001), and possibly at the proventriculus and foregut stage (Van den Abbele *et al*., 1999).

2.4 The Organism

Trypanosoma congolense (Nannomonas) is probably the most prevalent and widespread pathogenic trypanosome of the livestock trypanosomes in tropical Africa, being found in ruminants, pigs, dogs and other domestic animals throughout the tsetse belt (Buguet *et al.,* 2005, Desquesnes *et al*., 2012). In the mammalian bloodstream, *T. congolense* is a small trypanosome (10µm), shorter in length than *T. brucei* and without a conspicuous undulating membrane (Peacock *et al*., 2012). In the tsetse fly vector, both

species develop and multiply in the midgut initially, before onward migration to the mouthparts; infective metacyclics develop in the proboscis for *T. congolense* and in the salivary glands for *T. brucei*. This difference in vector developmental site led to the subgeneric classification in current use for the African tsetse-transmitted trypanosomes: trypanosomes that develop in the midgut and proboscis are in subgenus Nannomonas, while those that use the midgut and salivary glands are in subgenus Trypanozoon (figure 2.2) (Hoare, 1972).

Unlike other trypanosomes, *T.congolense* is monomorphic in the mammalian host. In the fly, it differentiates into elongated proliferative forms in the remains of the blood meal, which passes through the peritrophic matrix into the ectoperitrophic space before migrating anteriorly to colonize the proventiculus, where they cease division. The long proventicular trypomastigotes pass through the peritrophic matrix to enter the foregut lumen before migrating to the proboscis. Here they become epimastigotes and attach to the chitinous lining of the proboscis and cibarium (Peacock *et al*., 2012), where they proliferate and subsequently develop into infective metacyclics (Thevenaz and Hecker, 1980; Peacock *et al*., 2012).

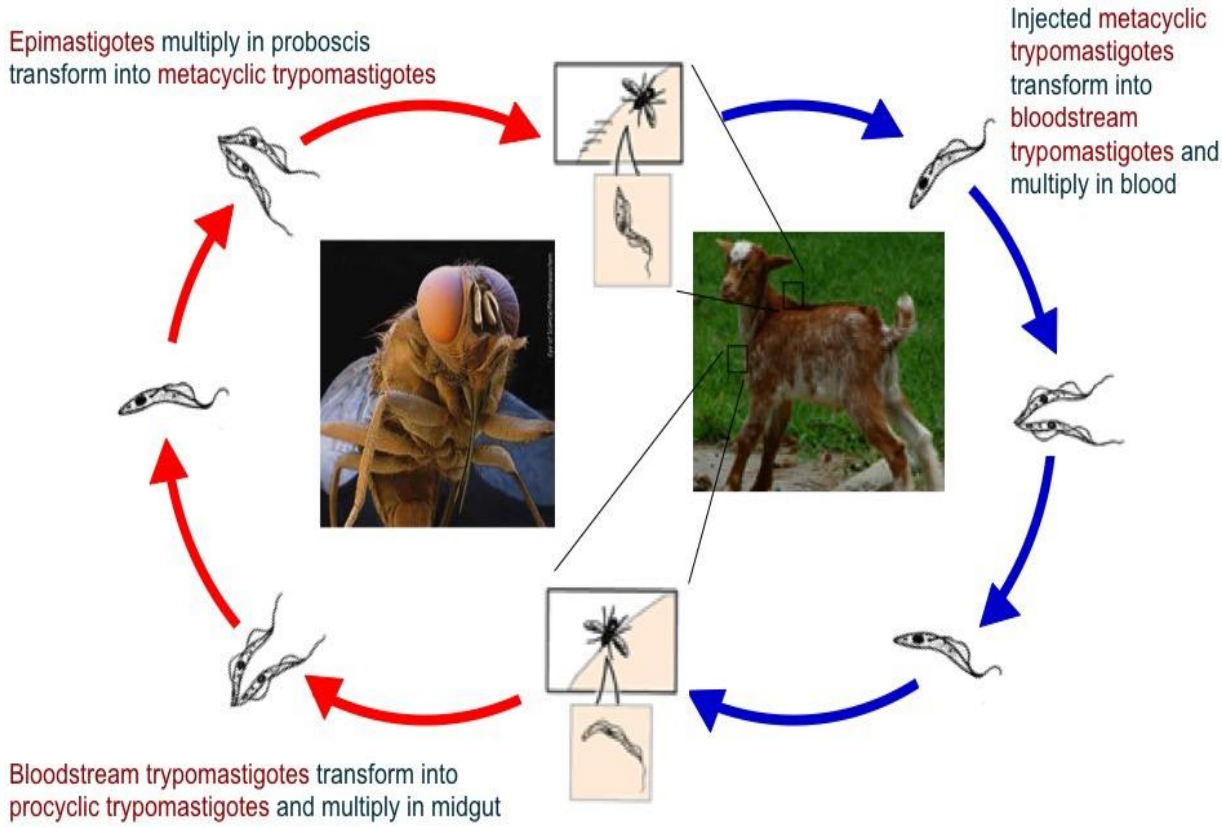


Figure 2.3: Life cycle of Trypanosomes (modified from: http://www.dpd.cdc.gov/dpdx)

2.5 Antigenic variation and immune evasion.

Trypanosomes growing in the bloodstream of mammalian host need mechanisms to circumvent the host immune response. Antigenic variation is one of the most spectacular adaptive mechanisms exhibited by the African trypanosomes. The main protective feature of these parasites is a thick and dense surface coat, which covers the entire plasma membrane. This coat consists of a monolayer of $\sim 10^7$ molecules of a single glycoprotein known as variant surface glycoprotein (VSG), which is a major antigen of the parasite whose antigenicity is in continuous changes (Pays, 2006). The VSG molecules are attached to the plasma membrane by glycosylphosphatidylinositol (GPI) anchors, and they form homodimers. VSGs undergo continuous variation, which leads to the periodic change of the VSG loops that are exposed at the surface of the parasite. These loops contain the only antigenic determinants of living intact trypanosomes that are recognized by the immune system. Therefore, such variation is known as antigenic variation, and it allows the parasite to evade antibody-mediated clearance (Cross, 1978; Barry, 1997; Borst, 2002; Pays *et al*., 2004).

Only one VSG variant is produced at any one time, because only one VSG allele at a time can be transcribed from the repertoire of VSG genes, which consists of more than 1,000 sequences. This transcription occurs at one of several VSG gene expression sites (ESs), and antigenic variation can result from two distinct mechanisms: transcriptional switching between VSG ESs, and homologous recombination between the active VSG gene and another VSG gene from the repertoire (Pays, 2006). During the ascending phase of the parasitemia, the majority of parasites are of the same antigenic type (called homotype). The host immune system recognizes this homotype and makes antibodies

against it. As the parasites of the major variable antigenic type (VAT) are eliminated the parasitemia goes in descending phase but at the same time, the parasites expressing the heterotype or the minor VATs are multiplying and one of them overgrows others. As a result this one becomes the new homotype, leading to a new wave of parasitemia and resulting in a long-lasting chronic infection. So expression of the VSG is central in the antigenic variation process and eventually for exhausting the host immune system in the benefit of the parasite. For the immune evasion, trypanosomes have also developed another method; macromolecular trafficking mechanism (Field *et al*., 2009) whereby the VSG-complexed with antibody are sorted and endocytosed (Engstler *et al*., 2007). This mechanism most probably protects the parasites from the complement-mediated killing and as such the trypanosomes escape from the host immune system. For the purpose of escaping from the host immune system, trypanosomes have more mechanisms such as capping of the surface bound immune factors, restriction of the invariant receptors in the flagellar pocket, rendering them inaccessible to the host immune effectors (Field and Carrington, 2009).

2.6 Sialic acids

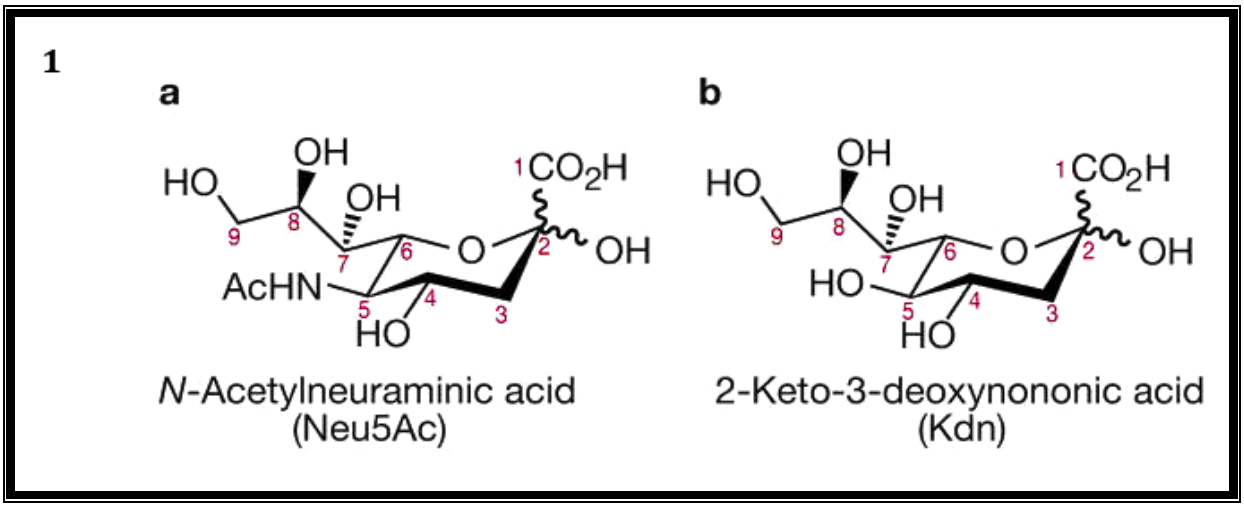
Sialic acids is a generic term to indicate a wide family of related nine-carbon sugar acids that feature prominently at terminal positions of many eukaryotic surface-exposed glycoconjugates, where they confer important properties upon the resulting cell surface (Severi *et al*., 2007) and can be subjected to numerous modifications generating more than 50 structurally distinct molecules (Schauer and Kamerling, 1997; Angata and Varki, 2002). Such a wide diversity is an outcome of evolutionary selection by host-pathogen interactions acting along with pressure to conserve critical endogenous

functions (Giacopuzzi *et al*., 2012). The name sialic acid was coined from the Greek word “sialos” partly because of it’s discovery in salivary mucins by Gunnar Blix, Ernst Klenk and other investigators in the late 1950s (Blix *et al*., 1957). The two common primary sialic acids are N-acetyl-neuraminic acid (2-keto-5-acetamido-3,5-dideoxy-D-glycero-D-galactononulopyranos- 1-onic acid) (Neu5Ac), which is believed to be the biosynthetic precursor for all other members of the family and 2-keto-3-deoxy-D-glycero-D-galactonononic acid (2-keto-3-deoxynononic acid (Kdn). (Roseman, 1970; Rosenberg and Schengrund, 1976; Schauer, 1982) (Figure 2.4). The only difference is the substitution at the C-5 position. All other sialic acids are apparently metabolically derived from these two, with the exception of some bacterial molecules such as legionaminic acid. Neu5Ac is more common than Kdn in most vertebrate cell types (Varki and Schauer, 2009). Sia’s are found to be α2,3- or α2,6-linked to a ß-D-galactopyranosyl (Gal) residue, or α2,6-linked to a ß -D-N-acetylgalactosaminyl (GalNAc) residue or a ß -D-N-acetylglucosaminyl (GlcNAc) residue. Sialic acids are also found α2,8-linked to sialic acid residues in gangliosides and in polysialic acid (PSA) which is a linear α2,8-homopolymer observed on several glycoproteins.

2.7 Diversity in the sialic acids

Sialic acid is the metabolic precursor of a family of more than fifty 9-carbon acid sugars, in which structural diversity is generated by various substitutions at the 4, 5, 7, 8 and 9-carbon positions (Figure 2.4) (Varki, 1997). Nomenclature that permits easy abbreviation of the names of substituted sialic acids is now available (Schauer, 1991; Schauer *et al.*, 1995; Reuter and Gabius, 1996) and has found general acceptance

(the term "Sia" is used as a generic abbreviation for all family members). Further diversity in the presentation of these molecules is generated by several different linkages from the 2-carbon of sialic acids to a variety of underlying sugar chains (Schauer, 1991; Varki, 1992; Reuter and Gabius, 1996; Tsuji, 1996). Combinations of the different substitutions and the variety of linkages afford many ways in which sialic acids can present themselves. Further complexity arises from the fact that 0-acetyl esters can migrate along the side chain under physiological conditions (Schauer, 1991; Varki, 1992; Reuter and Gabius, 1996). This diversity is found in a cell type-specific and developmentally regulated manner, implying important roles in intercellular recognition phenomena.



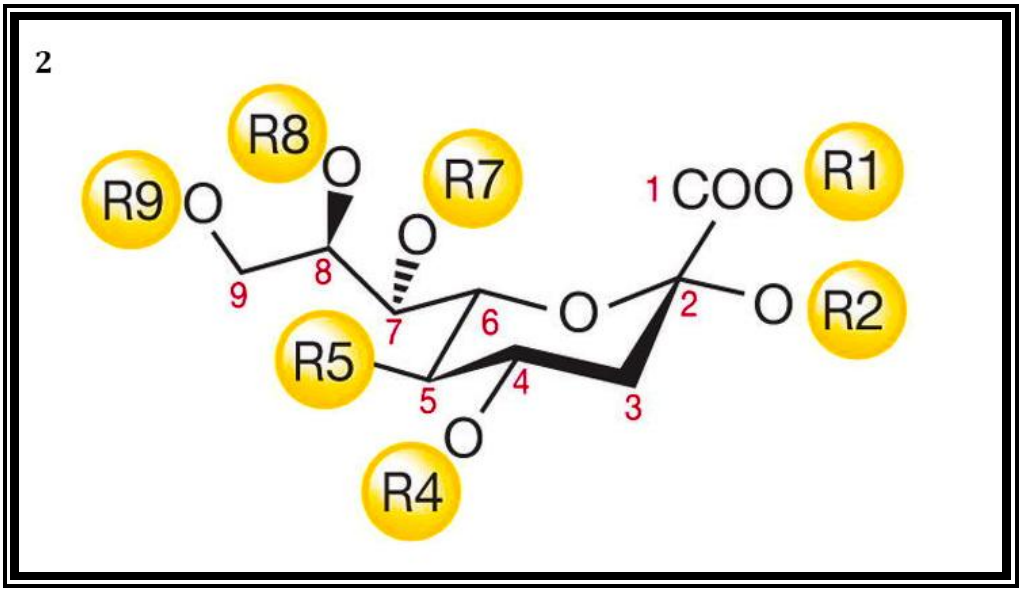


Figure 2.4: 1. Two common primary sialic acids shown are *N*-acetylneuraminic acid, (Neu5Ac); and 2-keto-3-deoxynononic acid, (Kdn). 2. The nine-carbon backbone common to all known Sias showing the diversity in the sialic acids.

2.8 Roles of Sialic acids

The high expression of Sias on outer cell membranes (e.g., more than 10 million molecules per human erythrocyte) on the interior of lysosomal membranes and on secreted glyco-proteins (such as blood proteins and mucins) suggests that they have roles in the stabilization of molecules and membranes, as well as in modulating interactions with the environment (Varki, 1992). Some functions arise from the relatively strong electronegative charge of Sias, for example, binding and transport of ions and drugs, stabilizing the conformation of proteins including enzymes, and enhancing the viscosity of mucins (Varki, 1992; Rahmann, 1992). Sias can also protect molecules and cells from attack by proteases or glycosidases, extending their lifetime and function. Furthermore, Sias can regulate the affinity of receptors and are reported to modulate processes involved in transmembrane signaling, fertilization, growth, and differentiation. In one system, apoptosis was reported to be inhibited by Sia *O*-acetylation. A recently described general property of Sias seems to be their free-radical scavenging antioxidative effect, which could be particularly significant on endothelia of blood vessels (Varki and Schauer, 2009).

Another prominent role of Sias is dualistic; they act either as masks or recognition sites. In the first case, they mask antigenic sites, receptors, and, most importantly, penultimate galactose residues. After Sia loss, molecules and cells can be bound, for example, by macrophages and hepatocytes, via Gal-recognizing receptors, and can even be taken up and degraded. This phenomenon has been most extensively studied with serum glycoproteins and blood cells (Varki and Schauer, 2009). Where addition of sialic acid

to the sub-terminal galactose impedes the binding of galactose-specific receptors of macrophages and hepatocytes hindering the degradation of those molecules (Schauer and Kamerling, 1997; Thotakura *et al*., 1994). On the other hand, Sias themselves can serve as ligands for a variety of microbial and animal lectins playing an important role in the specific recognition processes of many endogenous substances like cytokines and other hormones. Likewise, many pathogenic agents such as bacteria (e.g. *Escherichia coli* (Angstrom *et al*., 1994; Parkkinen *et al*., 1986) *Helicbacter pylori* (Hirmo *et al*., 1996), viruses (e.g. Influenza viruses (Rogers *et al*., 1986)), toxins (e.g. cholera-toxin (Schengrund and Ringler, 1989)) and protozoa (*Trypanosoma cruzi* (Schenkman *et al*., 1993)) bind to host cells via sialic-acid containing receptors. Siglecs (sialic acid binding immunoglobulin –type lectins) are proteins that bind sialic acids. There are 14 different mammalian siglecs, providing an array of different functions from regulating B-cell activation of the immune system to mediatiating cell-cell interactions via recognition of sialylated glycoconjugates (Crocker *et al*., 1991; Crocker *et al*., 1995; Powell *et al*., 1993; Powell and Varki, 1994).

Further confirming the pivotal role played by sialic acids, expression of some of the genes involved in the biochemistry of sialylated glycans are altered in cancer, injury and inflammation, resulting in changes in the glycan patterns. Sialic acids are also critical in the development of vertebrates and play a main role in human evolution (Schauer, 2004; Schauer, 2009). Today 60 genes are known to be directly involved in sialic acid biology and among them at least 10 show unique genetic changes in humans when compared with our closest evolutionary relatives (Varki, 2010). These modifications resulted in tissue specific changes of cell surface glycosylation that in turn caused adaptations in

various endogenous proteins that interact with these glycans, as well as in pathogen recognizing them. Chemical modification of Sias can strongly influence all of these properties, in particular ligand functions. For example, 9-O-acetylation or N-acetyl-hydroxylation of Neu5Ac can create new receptor functions or decrease the affinity of binding (Varki and Schauer, 2009).

2.9 Sialidase, Sialyltransferase and Tran-sialidase

2.9.1 Sialidases

Sialidases or neuraminidases (EC 3.2.1.18, systematic name: acetylneuraminyl hydrolase) are glycohydrolases widely distributed in nature, from viruses and microorganisms such as bacteria, protozoa and fungi, to vertebrates (Saito and Yu, 1995; Monti *et al*., 2010). They catalyze the removal of the acidic sugar sialic acid from a great variety of gangliosides and glycoproteins, generally termed glycoconjugates, which are mainly exposed on the cell surface or are secreted in the extracellular matrix (ECM) (Schauer and Kamerling, 1997). The particular chemical structure, the terminal position on oligosaccharide antennas and the negative charge of sialic acid residues confer special structural properties to glycoconjugates, accounting for their biological relevance as key regulators of molecular and cellular interactions (Schauer, 2009). Depending on their ability to act on α-(2→ 3)-, α -(2→ 6)-, α -(2→ 8)-, α -(2→ 9)- glycosidic linkages of terminal sialic acid residues, or on α -(2→ 8)-sialosyl linkages in oligo- and poly-sialic acids, sialidases are subdivided into exo- or endo-sialidases, respectively (Monti *et al*., 2010; Schauer, 2009). Since the first cloning of a mammalian sialidase in 1993 boosted the research on these enzymes (Miyagi *et al*., 1993), four distinct genes have been identified, encoding the lysosomal NEU1, the cytosolic NEU2, the plasma

membrane-associated NEU3 and the mithocondrial/ER associated NEU4 types.

Sialidases play a role in acquisition of sialic acid from the sialoconjugate sugar sialyl-lactose, which contains sialic acid linked to galactose via an α -2,3 or α -2,6 linkage, and furthermore to glucose via a α -1,4 linkage (Roy *et al*., 2010). This family of glycohydrolases which cleave the terminal sialic acid residue from sialoglycoproteins, and have been shown to play a role in virulence for a range of pathogens, including Streptococcus pneumoniae and Pseudomonas aeruginosa (Corfield, 1992; Reinholdt *et al*., 1990). Their actions include inducing chemokine release from epithelial cells (Kuroiwa *et al*., 2009), unmasking sialic acid-masked epitopes for adhesion (Corfield, 1992; Tong *et al*., 2000), promoting biofilm formation (Soong *et al*., 2006), and degrading host glycoproteins to obtain nutrients (Bradshaw *et al*., 1994; Byers *et al*., 1999; King *et al*., 2006; Severi *et al*., 2007).

It has also been shown that the *T. forsythia* NanH sialidase is important in interactions with human gingival epithelial cells (Honma *et al*., 2011) in a mechanism that may expose underlying glycosyl epitopes for adhesion and invasion. It is well documented that the sialidase of some pathogenic bacteria contribute to virulence, especially of those that reside on and/or invade mucosal surfaces. This is likely due to the abundance of sialic acid on the host glycoproteins in these tissues (Corfield, 1992). Human salivary glycoproteins are no exception, and contain various complex sugar substrates such as mucin and fetuin (Pigman & Gottschalk, 1966).

2.9.2 Sialyltransferase

Sialylation in nature is usually catalyzed by a family of enzymes named sialyltransferases. They catalyze the transfer of the sialic acid residue from cytidine 5-

monophosphate sialic acid (CMP-Sia) to an acceptor containing a Gal, GalNAc, or another sialic acid at the non-reducing terminus (Tsuji *et al*. 1996). The transfer of sialic acids to carbohydrate chains is performed by specific sialyltransferases in the cell (Taniguchi *et al*., 2002; Angata and Varki, 2002). Therefore, the sialyltransferases are considered to be key enzymes in the biosynthesis of sialylated glycoconjugates (Takeshi, 2010). These group of enzymes form α -2,3; α -2,6; α -2,8 or α -2,9 linkages between sialic acid and an appropriate acceptor molecule (Schauer and Kamerling, 1997). To date, many sialyltransferases, and the genes encoding them, have been isolated from various sources including mammalian, bacterial, and viral sources (Schauer, 2004; Sujino *et al*., 2000).

The biological function of the various sialyltransferases *in vivo* is not yet elucidated and modern molecular genetics; mouse gene knockouts in particular, have revealed extensive functional redundancy in sialyltransferase gene families. However, several reports have elucidated some roles *in vitro* such as cell surface sialylation is emerging as an important feature of cancer cell metastasis. Sialyltransferase expression has been reported to be altered in tumors and may account for the formation of sialylated tumor antigens. Sialyltransferases so far seem to have a wide impact on the biology of cancer and can be the target of innovative therapies (Dall'Olio' *et al*., 2014). Sialyltransferase have also been shown to regulate nervous system function in Drosophila (Repnikova *et al*., 2010).

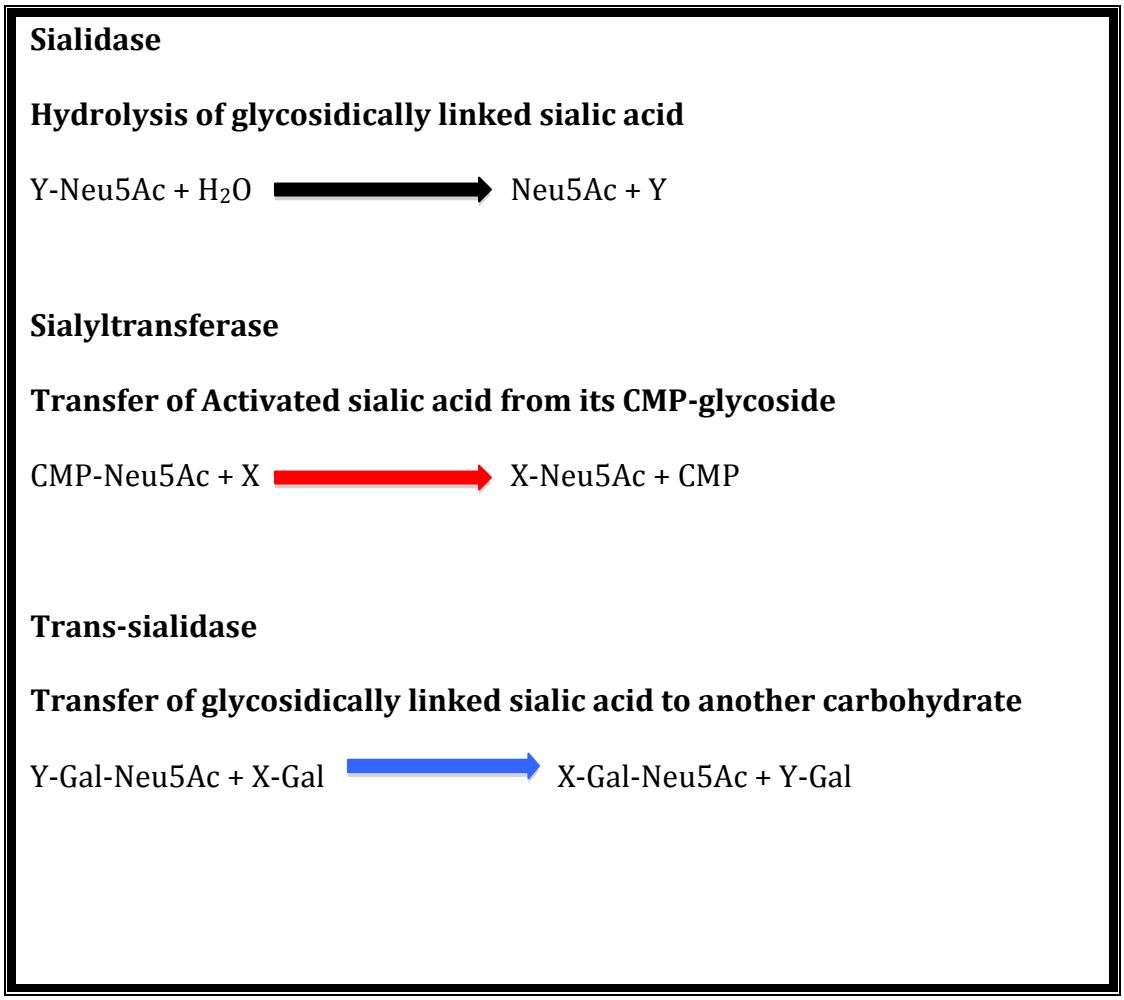


Figure 2.5: Comparison of sialidase, sialyltransferase and trans-sialidase

2.9.3 Trans-sialidases

Besides the sialidases and sialyltransferases mentioned above, a particular class of sialidases in some trypanosomal species have been discovered; these behave like normal sialidases if only water is present, but preferentially transfer sialyl residues (α2,3-carbohydrate linked sialic acids) from one glycan chain to the terminal galactose residue of another non-sialylated oligosaccharide or glycoconjugate (Figure 2.6A). Thus, they are well suited for glycan sialylation (Schauer and Kamerling, 2011). Unlike sialyltransferases, which require CMP-Neu5Ac as a monosaccharide donor, TS is able to transfer sialic acids from a variety of sialyl- α-galactose donor molecules (Tsuji *et al*., 1996).

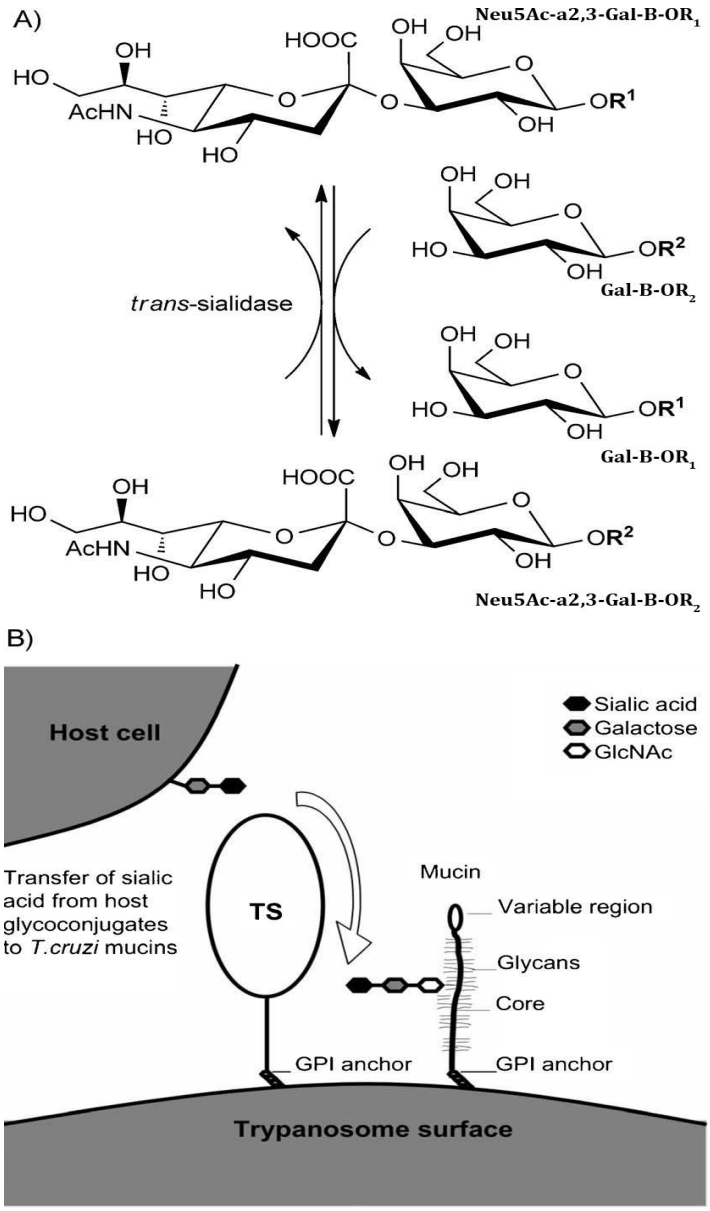


Figure 2.6: A) Reversible trans-glycosylation of (α2–3)-linked N-acetylneuraminic acid between Neu5Ac(α2–3)Gal-OR1 and Neu5Ac(α2–3)Gal-OR2, catalyzed by trypanosomal trans-sialidases. B) Trypanosomes do not synthesize sialic acids. However, some species express trans-sialidases that are used to transfer sialic acids from host cell glycoconjugates to terminal b-galactose residues of GPI-anchored glycoproteins on the pathogen surface, as shown here for mucins of *T. cruzi* (adapted from Jacobs *et al*., 2010).

2.10 Occurrence of Trans-sialidase in Trypanosome species

2.10.1 American Trypanosomes

Trans-sialidase was initially discovered in parasitic protozoan *Trypanosoma cruzi*, the causative agent of Chagas disease, which is an endemic parasitic infection in Latin America and is frequently associated with ventricular tachyarrhythmia and sudden death (Schenkman *et al*. 1991; Frasch 2000; Bern *et al*., 2008). In nature, sialylation can also be achieved by trans-sialidases, which catalyze the transfer of a sialic acid from one sialoside to an acceptor to form a new sialoside (Cheng *et al*., 2010). The first hint of the existence of an unusual sialic acid transfer reaction was given in 1983 when the sialic acids N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) detected in *Trypanosoma cruzi* was found that these sugar derivatives were not synthesized by the parasites themselves (Schauer *et al*., 1983; Pereira, 1983).

The *Trypanosoma cruzi* trans-sialidase is highly specific toward Neu5Ac α2,3Gal linkage and acts as a sialidase to produce free sialic acid in the absence of an acceptor (Cross and Takle 1993; Harrison *et al*. 2001). The parasite uses this surface bound enzyme to "steal" sialic acid from host to decorate its own surface (Figure 2.7) and the sialylation is proposed to be necessary for parasite's successful invasion of the host or protecting the parasite from host's immune recognition and defense (Colli, 1993). The parasites are transmitted through skin lesions from faeces-contaminated blood-sucking insects, mainly *Triatoma infestans, T. dimidiada,* and *Rhodnius prolixus* (the "kissing bug"). These species can transfer the protozoa between humans and from animals to man. Thus, Chagas disease can be considered as a zoonosis (Schauer and Kamerling, 2011). In the mammalian stage, *T. cruzi* mainly lives and multiplies inside the cells of

many tissues (Clayton, 2010; Nussbaum *et al*., 2010). The parasites are acquired as trypomastigotes from the insect and penetrate into the host's cells, where they multiply as amastygotes. After transformation into trypomastigotes and cell lysis, they enter the blood stream and are taken up by blood-sucking insects. In the insect's gut they become epimastigotes and multiply in the midgut. Trans-Sialidase is expressed in all stages, but with different enzymatic and biological activities (Telleria *et al*., 2010). Although drugs are available for the treatment of Chagas disease, new drugs with less toxicity are under investigation. One target of such medicaments is believed to be the trans-sialidase because it has been revealed to be a crucial pathogenic factor involved in spreading and survival of trypanosomes in the host (Schauer and Kamerling, 2011).

In order to understand the biochemical role of trans-sialidase in the life cycle of *T. cruzi*, the expression of this enzyme was studied in the insect vector and during the blood stage and intracellular life in the mammalian host. It was found that trans-sialidase seems to play a minor role in the insect's gut but is very important for replication and persistence in the host. Studies have questioned the role of trans-sialidase and sialic acids for the survival of *T. cruzi* in the insect (Takano-lee *et al*., 2002). The *T. cruzi* Silvio strain has two subpopulations, a smaller one that expresses trans-sialidase (TS^+) and a larger one without enzyme activity (TS^-). Surprisingly, when infecting *R. prolixus* with these subpopulations, the absence of trans-sialidase activity led to a higher parasite load in the insect. The addition of monoclonal anti-trans-sialidase antibody to TS^+ cells stimulated their multiplication, while the addition of trans-sialidase to TS^- trypanosomes reduced parasite numbers in the insect. These studies suggest a different biological role for TS^+ and TS^- subpopulations in the insect vector and vertebrate host. TS^- parasites might more

easily reproduce in the insect, whereas TS^+ trypanosomes are better suited to invasion of vertebrate cells (Takano-lee *et al*., 2002; Pereira *et al*., 1996). Thus, the polymorphism of *T. cruzi* might increase their fitness in different host environments. Further evidence that sialic acid is not essential for *T. cruzi* survival and development in the vector is suggested by the observation that the parasites grew well when feeding various insect species with a blood-free diet (Alvarenga and Brenar, 1978). In the last 20 years, many studies have examined the role of trans-sialidase as a strong virulence factor during the life and multiplication of *T. cruzi* in the mammalian host (Risso *et al*., 2004; Takano-lee *et al*., 2002) These showed that sialylation events catalysed by trans-sialidase on either parasite or host cells have many effects and finally enable *T. cruzi* to survive for a long time—often several decades—in their hosts (Schauer and Kamerling, 2011).

Chagas disease can run a rather chronic course. The trans-sialidase activity helps the parasites to better resist the host's innate and acquired immune systems, to enter cells, and even to manipulate the host's immune system and the biology of various other cell types; this enables the parasites' long residency in their hosts, and thus supplies a constant trypanosome reservoir for further distribution (Moore-lai and Rowland, 2004). The crucial role of trans-sialidase in the infection mechanism of *T. cruzi* has been well established since the finding that sialic acid deficient CHO (Lec2) cells are relatively resistant to trypanosome invasion (Schenkmann *et al*., 1993).

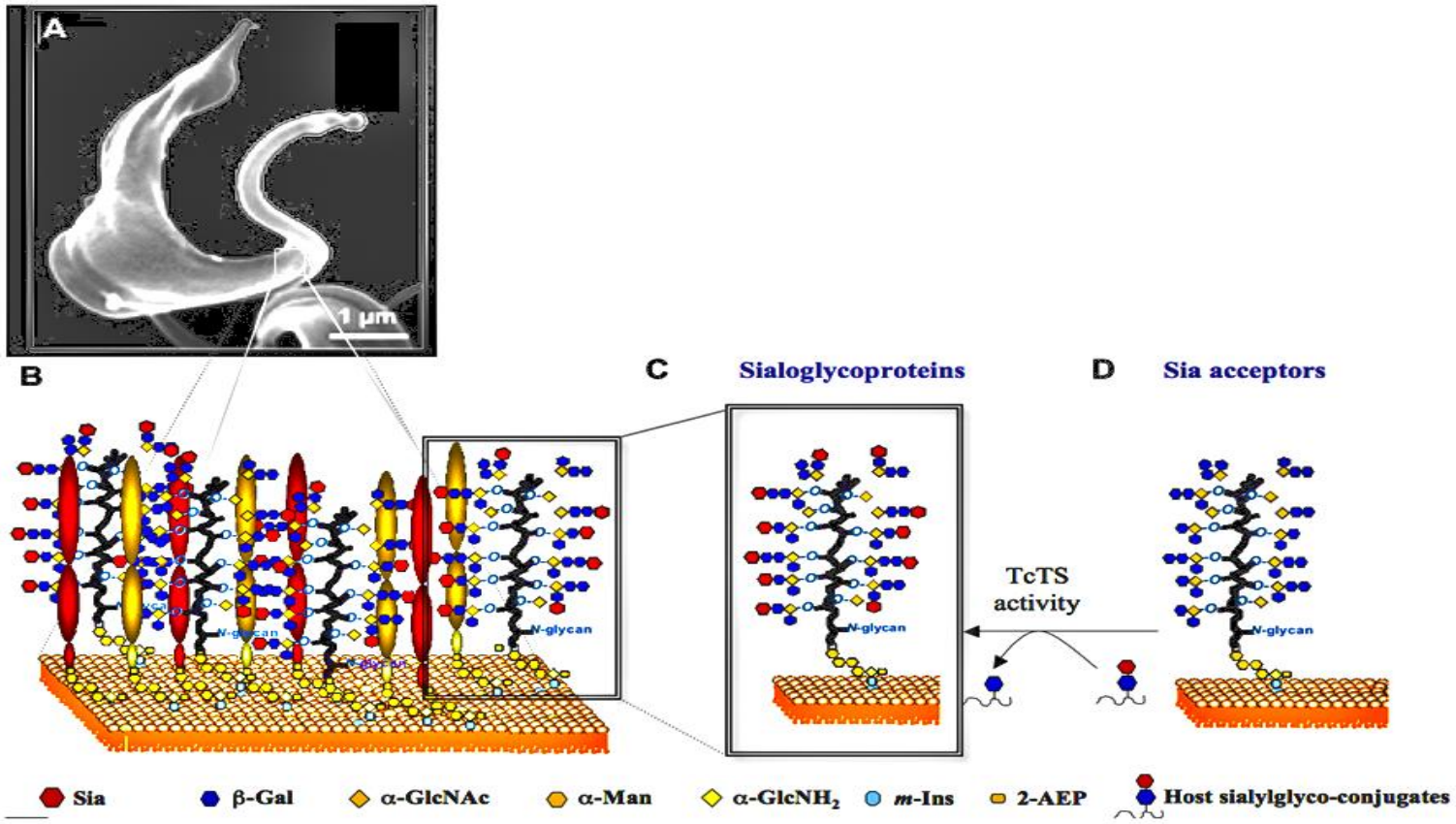
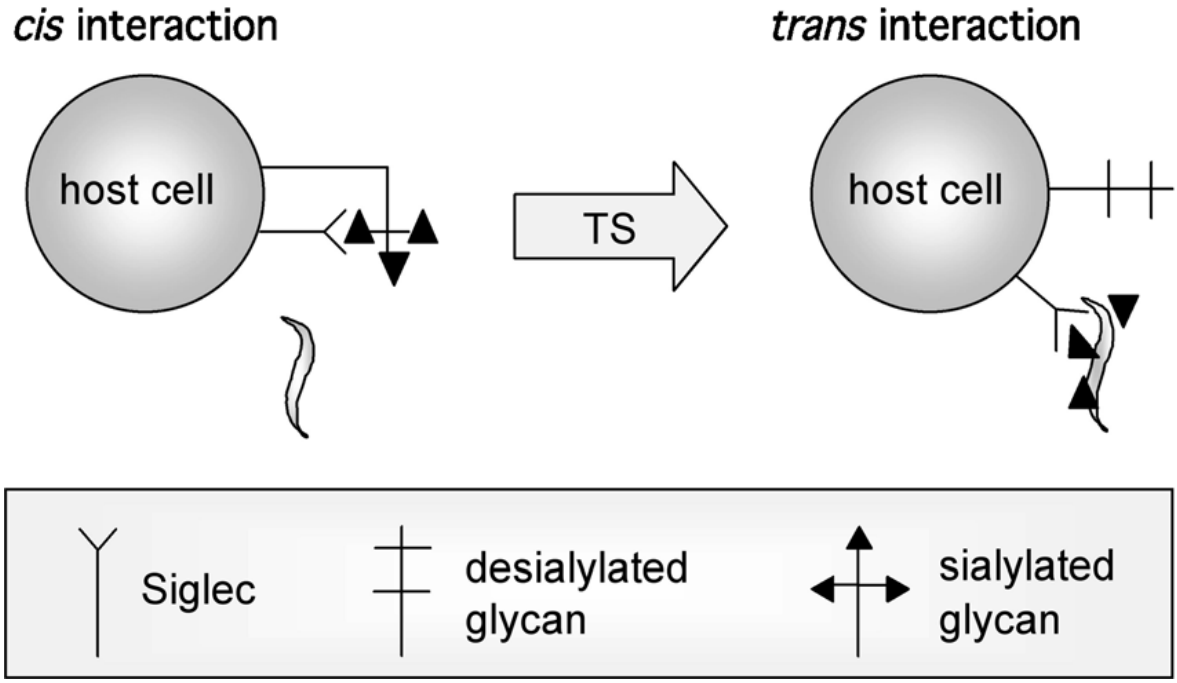


Figure 2.7. Representation of the TcTS sialoglycoproteins system on *T. cruzi* trypomastigotes surface**.** (A) An electron micrograph of a bloodstream form *T. cruzi* trypomastigote. (B) GPI-anchored TcTS sialoglycoproteins covering the trypomastigote surface. (C) Sia present on *T. cruzi* sialoglycoproteins is transfered from host sialoglycoconjugates to β-Gal*p* containing glycoproteins expressed on parasite surface (D) TcTS activity (Adapted from Freire-de-lima *et al*., 2012)

Active or inactive trans-sialidase can bind to mammalian cells, for example, heart cells or T lymphocytes, in a lectin-like manner (Todeschini *et al*., 2002; Alcntara-Neves *et al*., 2004). Of special importance in the biology of Chagas disease is the binding of trypanosomes by their mucin-bound sialic acid coat to siglecs (sialic acid-binding immunoglobulin-like lectins). In mice, the binding of sialoadhesin (siglec-1)-positive macrophages to sialylated ligands on *T. cruzi* cells might be important in the initial trypomastigote infection (Jacobs *et al.*, 2010; Monteiro *et al*., 2005). Siglec-E of mouse phagocytic and dendritic cells was found to be the target of sialic acid binding for the *T. cruzi* Tulahuen strain that expresses high trans-sialidase activity (Jacobs *et al*., 2010; Erdmann *et al*., 2009). This event suppresses secretion of the pro-inflammatory cytokine IL-12 and consequent T-cell activation. The molecular mechanism behind this phenomenon might be disruption of *cis*-interactions between siglecs and sialic acids on immune-competent host cells (Figure 2.8; Jacobs *et al.*, 2010). *T.cruzi* trans-sialidase activity mediates the transfer of the host cell's sialic acid to the parasite cell by its trans-sialidase activity. The resulting *trans*-interaction with the parasite can impair the immune response, similarly to the inhibition of the antibacterial function of neutrophils after the ligation to siglec-9 of sialylated glycans by group B Streptococcus (Carlin *et al*., 2009). Such sialic acid-mediated molecular mimicry seems to be the basic mechanism by which parasites sabotage host cytokine-secretion aimed at weakening the host's defense system and improving the parasite's chances of survival (Gao and Pereira, 2003).



Source: Jacobs *et al*., 2010

Figure 2.8: Representation of the interaction of siglecs with *T. cruzi* cells. Most siglecs are involved in cis interactions by binding to sialylated glycans on cell surfaces. Removal of sialic acid from the cell surface glycans by trypanosomal trans-sialidase leads to release of siglecs from their cis interactions. The trans-sialidase-mediated sialylation by *T. cruzi* parasites generates ligands for trans interactions.

2.10.2 African Trypanosomes

The trans-sialidases of African trypanosomes are strong virulence factors involved in both sleeping sickness and in a variety of animal diseases (Schauer and Kamerling, 2011). After the first isolation of trans-sialidase was reported from *T. cruzi* (Schenkmann *et al*., 1991) this was followed by a corresponding enzyme from cultured procyclic forms of the African trypanosome species *T. brucei* (Engstler *et al*., 1992) and *T. congolense* (Tiralongo *et al*., 2003) The enzymes are bound to cell membranes of the trypanosomes by glycolipid anchors (Figure 2.6B), therefore detergents are required for their solubilisation. When grown in cell culture, some of the enzyme is released and can be found in the medium. The molecular masses of the trans sialidases were estimated to be 160–200, 67, and 90 kDa (monomeric form) for the enzymes from *T. cruzi, T. brucei* and *T. congolense*, respectively. Regarding kinetic, structural and other properties, the trans-sialidases from the two continents show striking similarities. *T.congolense*, however, expresses various trans-sialidase forms that exhibit either high or low trans-sialidase activities when compared with their sialidase activities (Tiralongo *et al*., 2003). Expression of this enzyme also depends on the trypanosome strain, and this much influences its pathogenicity (Risso *et al*., 2004). The expression of trans-sialidases also differs between parasites living in mammalian (trypomastigotes) and insect (epimastigotes) hosts.

In contrast to *T. cruzi*, the trans-sialidases of some African trypanosome species are expressed only in the insect stage (Engstler *et al*., 1993; Ensgtler *et al*., 1995; Nussbaum *et al*., 2010; Brun *et al*., 2010). Trypanosomes from the blood meal develop in the midgut of the tsetse fly to the procyclic form, multiplies and expresses trans-sialidase. It

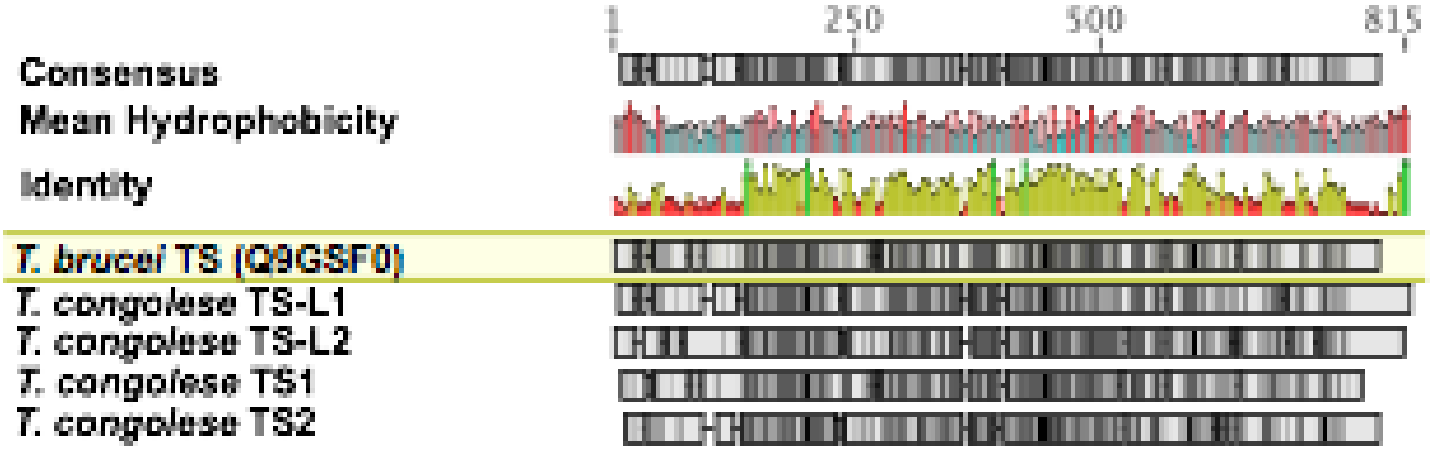
is interesting to note (and this points to its function) that this enzyme is only expressed in those trypanosomes that exhibit this type of developmental cycle in the vector, such as *T. brucei* group and *T. congolense* (Ensgtler *et al*., 1995). The other pathogenic (for animals only) African trypanosomes *T. equiperdum* and *T. evansi* do not express trans-sialidase activity. They are transferred between mammalian hosts and lack the midgut stage in the tsetse fly. Also, *T. vivax* lacks transformation in the fly's gut and correspondingly trans-sialidase activity. It expresses classical sialidase activity in the host's blood stream (Ensgtler *et al*., 1995; Esievo, 1979). This distribution of trans-sialidase activity leads to the assumption of a crucial role of this enzyme and of sialic acids for its life in the insect. Culturing trypanosomes in sialic acid containing medium revealed sialylation of their cell surface, but only for (sub-species) with an insect stage (Engstler *et al*., 1993; Engstler *et al*., 1995). The site of sialylation was found to be the glycolipid anchor of the developmentally regulated PARP (a procyclin - procyclic acidic repetitive protein) in *T. brucei* (Engstler *et al*., 1993).

It was estimated that there are five sialic acid residues in this membrane anchor, with a polylactosamine structure (Ferguson *et al*., 1993). Although trypanosomal trans-sialidases have a narrow specificity regarding the sialic acid accepting sugar and the linkage formed, they exhibit broad specificity concerning the sialic acid donor and acceptor glycans. Transfer of sialic acids from various oligosaccharides, glycoconjugates, for example, serum glycoproteins or PARP and, from cells like erythrocytes and even the trypanosomes themselves was demonstrated not only with solubilised enzyme but also with trans-sialidase still bound to procyclic trypanosomes in culture (Engstler *et al*., 1993; Engstler *et al*., 1995). It is not known whether trans-

sialidase is additionally required to sialylate molecules of the insect's gut, or whether sialic acids help the trypanosomes to attach to the fly's epithelia by lectins. It is more feasible, based on knowledge about the many fold functions of sialic acids (Schauer and Kamerling, 1997; Schauer, 1985; Angata and Varki, 2002; Schauer, 2009; Varki and Schauer, 2009; Schauer and Kamerling, 1997) that coating of the trypanosomes with sialic acids might protect them during their intestinal life from destruction by proteases and other digestive enzymes, by complement factors, immunoglobulins, trypanocidal lectins, and antimicrobial peptides (Engstler *et al*., 1993; Engstler *et al*., 1995; Ferguson *et al*., 1993). Some of these agents might be derived from the blood meal. Furthermore, it is conceivable that sialic acids are required for transformation of the procyclic trypanosomes into the epimastigotic and metacyclic forms in the fly's salivary gland (Schenkmann *et al.*, 1991; Engstler *et al*., 1993; Nussbaum *et al*., 2010; Brun *et al*., 2010). The latter form is infective and is transferred into the host's skin during the insect's bite. From the developing chancre the trypanosomes enter the blood stream and loose trans-sialidase activity. It is, however, not known whether trans-sialidase activity persists in the salivary gland or is required during the short stay of the trypanosome in inflamed skin after the bite. Strong evidence exists to support the theory that sialic acids are required for trypanosome survival in fly vectors (Nagamune *et al*., 2004) and it has been shown that disruption of the linkage of procyclin or trans-sialidase to the glycolipid anchor, or disturbance of GPI synthesis by gene knockout (KO) with cultured procyclic trypanosomes, resulted in less procyclin or trans-sialidase activity. These strongly reduced their survival in the fly's midgut. After transfection of GPI8KO cells, which lack GPI transamidase activity, with a truncated gene that expressed a soluble form of *T.*

brucei trans sialidase, partial restoration of the viability of the parasites was obtained, possibly a consequence of acquisition of sialic acids by the secreted trans-sialidase from the incubation medium. This confirms the hypothesis that an intact sialoglycocalyx coat is essential for the life cycle of African trypanosomes that require a procyclic insect stage (Schauer and Kamerling, 2011).

Trans-sialidases from *T.congolense* have been identified TconTS1 (Tiralongo *et al*., 2003), Tcon TS2, TconTS3, TconTS4 (Gbem *et al*., 2013) with the full length cloned and sequenced (Kower-Brandl *et al*., 2011), opening new perspectives for investigations on the biological role of these enzymes in the pursuit of a cure for Nagana and sleeping sickness. From ‘‘reads’’ of the WTSI (Wellcome Trust Sanger Institute) *T. congolense* genome-sequencing project (http://www.sanger.ac.uk), the full-length sequences coding TconTS1 and TconTS2 were assembled. Further BLAST hits with smallest sum probabilities (P(N)**,**1–10) were identified and arranged into contiguous sequences leading to further five genes with sequence similarities (Figure 2.9) Two of the putative gene products shared over 40% sequence identity with TconTS1 and TconTS2 and contained all the conserved amino acids required for transfer reactions (Buschiazzo *et al*., 2002; Amaya *et al*., 2004; Montagna *et al*., 2006). Consistent with the naming of TconTS1 and TconTS2 (Tiralongo *et al*., 2003), they were referred to as TconTS3 and TconTS4. The other three genes were distantly related with 20–30% amino acid identity (Figure 2.9) and lack several of the conserved amino acid residues. It was assumed that these set of genes are likely without sialidase or TS activity and were named TconTS-Like1, TconTS-Like2, and TconTS-Like3 (Gbem *et al*., 2013).



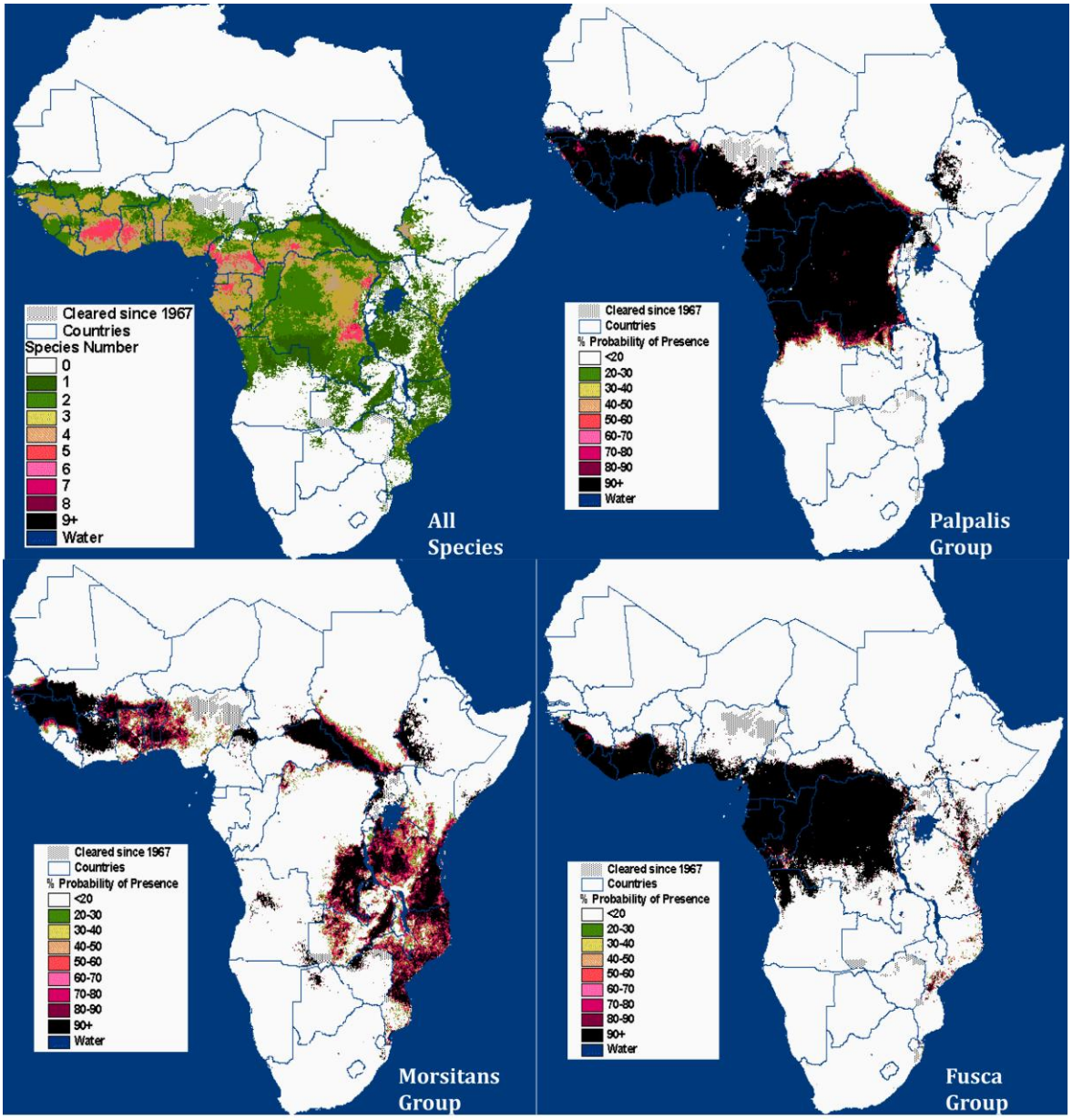
Trans-sialidase	TconTS2	TconTS3	TconTS4	TconTS-Like1	TconTS-Like2	TconTS-Like3
Tcon TS1	42.2%	43.6%	46.2%	21.1%	26.3%	29.8%
Tcon TS2	-	48.3%	42.8%	20.8%	26.2%	29.3%
Tcon TS3		-	48.9%	21.1%	25.1%	29.9%
Tcon TS4			-	21.3%	27.8%	29.8%

Source: Gbem *et al.*, 2013

Figure 2.9: *Trypanosoma congolense* trans-sialidase sequence similarities expressed as percentage of identical amino acids in pairwise alignments.

2.11 Tsetse Flies

Only tsetse flies are biological vectors of trypanosomes i.e. capable of cyclically transmitting African trypanosomes whilst other biting insects may transmit them mechanically (Desquesnes and Dia, 2003). There are 31 species and subspecies of tsetse flies (*Glossina, Glossinidae*) restricted to sub-Saharan Africa. Based on morphological and ecological considerations the genus has been divided in three groups or subgenera namely morsitans group (subgenus Glossina), palpalis group (subgenus Nemorhina) and the fusca group (subgenus Austenina). Tsetse flies are distributed discontinuously over 10 million square kilometres of sub-Saharan African land (14^0N and 29^0S), with each group of tsetse restricted to a relatively specific habitat. Flies belonging to the fusca group are mostly found in the forest or dense riparian forest while those of the palpalis group are found in riverine forest but also inhabit the rain forest. The morsitans group of flies are mostly restricted to the savannah woodlands (Leak, 1999). Figure 2.10, shows the distribution of tsetse flies in Africa with *G.morsitans* and *G.palpalis* being the most prevalent species in Nigeria (Figure 2.11). In general the fusca and palpalis groups occur in West and Central Africa while the morsitans group is widely distributed throughout the drier zones. In



Source: http://www.tsetse.org/FAQ/allspec.html

Figure 2.10. Distribution of Tsetse flies in Africa.



Source: http://www.raywilsonbirdphotography.co.uk

Figure 2.11. Male Savannah and female riverine tsetse flies.

Despite the high incidence of trypanosomiasis in mammals in sub-Saharan Africa, it is puzzling that relatively few tsetse captured in the wild show midgut trypanosome infections, with estimates ranging from 2% to 20% in different tsetse species and sample sites (Jamonneau *et al*., 2004; Auty *et al*., 2012). Susceptibility to *Trypanosoma* species midgut infections and the maturation index (MI) vary between tsetse species (Dyer *et al*., 2013). Some species (e.g., *Glossina pallidipes* Austen and *Glossina palpalis palpalis* R-D) are more refractory vectors than others (e.g., *Glossina morsitans morsitans* Westwood), as demonstrated under laboratory conditions (Welburn *et al*. 1989; Nayduch and Aksoy, 2007; Peacock *et al*., 2012). This relative difference in susceptibility is particularly interesting for *G.pallidipes* and *G. m. morsitans*, because the two species are sympatric relatives and primary vectors for *Trypanosoma brucei rhodesiense* (Nayduch and Aksoy, 2007). Also, *Glossina m. morsitans* has a relatively weak barrier to infection with *T. brucei* strain J10, with 11.3% of flies becoming infected at age 24–48 h, but with a MI below 20%. By contrast, *Glossina pallidipes* has a huge barrier to midgut establishment, with only 1.3% of flies developing midgut infections under the same conditions, but a MI as high as 88%. Similarly, *G.m.morsitans* have been shown to be more susceptible to *T.congolense* infection than *T.brucei* (Peacock *et al*., 2012).

Midgut susceptibility to *T. congolense* was maternally inherited, suggesting an extra chromosomal susceptibility factor. The refractory lines were more refractory to other trypanosome strains and species, including *Trypanosoma vivax*, which develops outside the midgut, suggesting a general effect of the factor on fly immunity (Moloo *et al*. 1998; Maudlin *et al*., 1986). The extra chromosomal susceptibility factor might be the genotype or density of rickettsia-like organisms (RLOs), later identified as *Sodalis glossinidius*

(Weiss and Aksoy, 2011)). In regions where Sodalis is variably present in the field, trypanosome-infected flies are more likely to harbor Sodalis than uninfected flies (Farikou *et al*., 2010). Consistently, selective elimination of Sodalis using streptozotocin (which did not kill the obligate tsetse symbiont *Wigglesworthia glossinidia*) led to almost 40% reduction in susceptibility to midgut infection in the progeny of treated flies (Dale and Welburn, 2001). Therefore, Sodalis appears not to be essential for trypanosome midgut infection, but increases the proportion of susceptible flies. How Sodalis influences vector competence remains to be elucidated (Dyer *et al*., 2013).

Van Hoof *et al.*, (1937), first observed the "teneral phenomenon" by studying the infection rates of *Trypanosoma brucei gambiense* in *G. fuscipes fuscipes*. In this study, it was noted that newly emerged flies that were fed a trypanosome-infected first bloodmeal had higher susceptibility to parasite infection than flies that were fed trypanosomes in subsequent bloodmeals. Wijers, (1958) further investigated this "first-day factor" with young *G. palpalis palpalis* emergents and demonstrated that flies were more permissive to *T. b. gambiense* midgut infections when fed trypanosomes in the first bloodmeal. This observation confirmed that susceptibility was dependent on the post-eclosion age of the fly upon ingestion of the infectious bloodmeal (Otieno *et al*., 1983; Weiss *et al*., 2013), density of maternally derived trypanolytic effector molecules present in the gut, and symbiont status during development (Weiss *et al*., 2013). Using *T. b. brucei*, it was demonstrated that a pronounced teneral phenomenon also existed in newly emerged *G. m. morsitans*. The highest number of salivary gland infections was achieved by feeding infected blood to flies aged <8 h post-emergence (hpe). Distelmans *et al*., 1982 also confirmed that a different trypanosome species, *T. congolense*, had higher infection rates

in teneral flies. In this large study consisting of 800 male and female *G. p. palpalis*, only flies aged less than 32 hpe developed mature proboscis infections. This phenotype was evident in both sexes, although to a lesser extent in females (Distelmans *et al*., 1982; Maudlin *et al*., 1990).

Midgut lectins are involved in tsetse refractoriness (vector competence) and agglutinate and kill trypanosomes in vitro (Welburn *et al*. 1989, Ingram and Molyneux, 1990). Lectins levels are low in teneral (newly eclosed unfed) flies, and they are inhibited by sugars produced from the chitinolytic activity of commensal bacteria (Welburn *et al.* 1993). However, first-blood feeding results in lectin production in amounts that exceed the symbiont effects. Thus, lectin production has been attributed to the **"**teneral phenomenon,**"** where, irrespective of species, teneral flies are more easily infected with trypanosomes (Nayduch and Aksoy, 2007).

2.12 Current and future approaches to Trypanosomiasis control

Besides the problems associated with the use of current trypanocides, most pharmaceutical industries have not eagerly pursued the development of effective trypanocides because it rarely seems profitable to them.

Besides being expensive, drug resistance and toxicity are problems associated with the classical trypanocides Pentamidine and Suramin (Anene *et al*., 2001). Melarsoprol is capable of crossing the blood brain barrier in the late-stage of sleeping sickness and therefore considered a precarious chemotherapeutic agent. However, due to its extremely toxic nature, an approximated 5% of cases die from drug toxicity alone (Aksoy, 2003). Difloromethylornithine (DMFO), an ornithine decarboxylase inhibitor, has been

considered the only alternative to this drug for late stage treatment. Futhermore, it is expensive and for reasons not known, *T.b.rhodesiense* displays natural resistance to the drug (Aksoy, 2003). Isometamidium, diminazene aceturate and homidium have demonstrated viability therefore have remained acceptable to veterinarians and farmers for over three decades. Every year, cattle in Africa are administered millions of doses of these trypanocides. Consequently, drug resistance has become apparent and may gravely shorten the use of these important compounds in the nearest future. Trypanocidal drugs notwithstanding remain the key approach in controlling animal trypanosomiasis in most African countries (Aksoy, 2003).

Vector control is the primary strategy aimed at controlling spread of the disease by minimizing contact with tsetse hence breaking the disease cycle since there is no available vaccine and low repertoire of effective and affordable drugs for treatment against African Trypanosomiasis (Aksoy, 2003). Insecticides and tsetse fly traps have been used as adjuncts and have been proven to be effective in controlling tsetse populations.

Another approach to vector control is the use of the Sterile Insect Technique (SIT), which is the first insect pest control method that employs genetics. This is an area-wide based method which has been successfully applied in controlling pests like New World screwworm fly in the USA, the Mediterranean fruit fly in Chile and Mexico and tsetse fly in the Island of Zanzibar. This was achieved by exposing male flies to low doses of radiation. High numbers of sterile male flies were released in infested areas over a period of two decades where they mate with wild females until the reproductive capacity of the target population was reduced. Consequently, the wild fly population died out (Vreysen *et al*., 2000).

Aksoy *et al*., 2008 recently described the vectorial and symbiotic biology of tsetse with emphasis on the current knowledge on tsetse symbiont genomics and functional biology, and tsetse's trypanosome transmission capability. Expanding knowledge on tsetse immunity effectors, on genomics of tsetse symbionts and on tsetse's parasite transmission biology stands to enhance the development and potential application of paratransgenesis as a new vector-control strategy.

The current methods available for the control of African trypanosomiasis have been shown to be effective where they have been vigorously applied but they do no more than limit the disease.

Advances in cell biology, immunology, genomics and molecular genetics have resulted in the evolvement of powerful technologies for laboratory study of the vector's biology to discover the vulnerable points in disease transmission. One of the most challenging applications of these developments is Transgenesis (is the process of introducing an exogenous gene — called a transgene — into a living organism so that the organism will exhibit the new trait which is subsequently transmitted to its progeny) is the most challenging applications of this advancement, which can result in the regulation of vectorial competence of insects (i.e. the ability of insects to transmit pathogens). Such genetically modified tsetse, which are incapable of transmitting parasites, can replace vulnerable wild tsetse.

It is of uttermost importance to meticulously assess the peril associated with discharge of such genetically modified organisms (GMOs) into the field before this approach can be put into practical use. Hence, an excellent expert system on the transmission biology of pathogens in their hosts cannot be over emphasized (Aksoy, 2003).

Eventhough developing vaccines against trypanosomiasis seems to be an almost impossible task primarily due to the ability of African trypanosomes to change their variable surface glycoproteins by antigenic variation. The question still stands, "Can this be done or is the trypanosome truly the ultimate immune destroyer and escape artist?" (La Greca, and Magez, 2011). Finding a suitable vaccine against trypanosomiasis seems to be the likely way out in combating this menace (La Greca, and Magez, 2011). Considering the fact that it is impossible to annihilate the whole parasite backlog of endemic regions, only a feasible vaccine will provide a resilient alternative impeding human casualties as well as far-flung economic losses due to livestock infections (Aksoy, 2003).

A futuristic approach to trypanosomiasis control is the DNA vaccine, which is the prime example of modern genetic vaccine (Fioretti *et al*., 2010). This primarily involves evoking the immune system against diseases through the use of naked plasmid DNA as a vaccine. Thus, putting forward practical benefits for substantial vaccine production that other forms of vaccines wouldn't manage easily. DNA vaccines have proven to be beneficial in cancer immunotherapy due to its potency in expeditious screening for antigens and in the design of specific construct against the tumor cells (Yannelli and Wroblewski, 2004; Haupt *et al*., 2002). The effectiveness and safety of DNA vaccines against HIV-1 infection in the first human clinical trial (MacGregor *et al*., 1998) also emphasized the safety and potentiality of this vaccine to induce appreciable immune, cellular and antibody responses (MacGregor *et al*., 1998; Mincheff *et al*., 2000; Le *et al.*, 2000).

DNA vaccines have also been demonstrated to be effective against protozoan parasites; *Leshmania major* and *Plasmodium yoclii* (Sedegah *et al*., 1994; Xu and Liew, 1995). Costa *et al*., 1998, reported that DNA vaccination could be feasible with other parasitic

infections as he demonstrated immunization of mice with a plasmid DNA containing the gene of Trans-sialidase gene reduced *T.cruzi* infection. Most recently, Silva *et al*., (2009), demonstrated that DNA vaccine coding for trans-sialidase gene from *T.b.brucei* is a promising target for treatment of animal trypanosomiasis as the vaccine was able to protect 60% of BALB-c mice challenged with the parasite.

Thus, these results have proven DNA vaccine as a plausible intervention strategy opening doors for new investigations for vaccine development against trypanosomiasis. However, it cannot be over emphasized that the roles these genes play in the developmental cycle of these parasites need to be investigated and elucidated, as it may be the key to achieving the desired success.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Reagents/Kits

All reagents used were of molecular biology and analytical grade.

3.1.1 Tsetse flies

Teneral male *Glossina morsitans sub morsitans* (about 8 hours old) were obtained from wild tsetse populations obtained from Nagana endemic foci in Nigeria and were maintained as colonies under standardized conditions at the Nigerian Institute for Trypanosomiasis and Oncocerchiasis research, NITOR as part of the PATTEC (Pan African Tsetse and trypanosomiasis Eradication Campaign) program. This line of tsetse has a high vectorial capacity (Van den Abbeele *et al*., 2001). The tsetse flies were maintained in a customized incubator (RUMED, Rubarth Apparate GmBH, Germany) which provided controlled conditions as obtained on the field: Temperature 25^0C, Humidity 77% and 12 hours of light and 12 hours of darkness for survival of the tsetse.

3.1.2 Trypanosomes

T.congolense was isolated from an infected cow at an abattoir in Kaduna State, Nigeria and was characterized in the DFG laboratory at the Center for Biotechnology Research and Training, Ahmadu Bello University, Zaria and passaged into albino rats. Cyclical transmission from tsetse flies to goats, serial passages in rats and cryostabilates was used to maintain the parasites.

3.1.3 Animals

The West African Dwarf breeds of goats used for the experiment were between 4-6 months of age. They were purchased from Ungwar-Dankali in Zaria Local Government area of Kaduna State, which is a tsetse free zone of northern Nigeria. The animals had no record of exposure to trypanosome infection. They were acclimatized and housed at the Center for Biotechnology Research and Training, Ahmadu Bello University, Zaria in clean fly-proof pens pre-treated with chlorphen-vinphos (Pfizona, Pfizer) against external parasites for three weeks and were dewormed with Albendazole (Pantex Holland B.V.). The animals were fed on groundnut hay, guinea corn, cowpea, maize chaff and sometimes-fresh grass/leaves and clean water was provided *ad libitum*.

3.2 METHODS

3.2.1 Infection of tsetse flies

Aliquots of 1 x 10^6 trypanosomes (*T.congolense*) were injected intraperitoneally into albino rats. The infection was monitored every other day by examining tail blood using a light microscope at a 40x magnification. Teneral flies were fed on the bellies of infected mice (on average of 20 flies on each rat) displaying levels of parasitemia > 1 x 10^9 parasites/ml (determined using the matching method of Herbert and Lumsden, 1976). Flies failing to feed were removed from the experiment while engorged flies were placed in individual cages. All the flies were maintained by feeding on freshly collected anti-coagulated (Sodium Citrate) goat blood through a silicone membrane, three days a week, until the end of the experiment.

3.2.2 Monitoring infection

The anal drops of each fly were analyzed at different post-infection times on days 4 through day 14. This biological fluid was examined by PCR for the detection of infections.

3.2.3 Infection of experimental animals

Twenty-one days after the infective feeding, flies confirmed to be positive for *Trypanosoma congolense* by PCR were fed once on a clean goat, and blood collected from the jugular vein was monitored by microscopic examination and PCR.

3.2.4 Dissection of flies

Twenty-four days after flies had been fed on experimental goats, the flies that were still alive were dissected according to the method described by Penchenier and Itard (1981). The time lag between infection and dissection is sufficient for the completion of the *T. congolense* developmental cycle in tsetse. Before dissection, flies were starved for 48 hours to reduce partially digested blood, and thus facilitate observation of the trypanosomes in the midgut. To avoid contamination, the dissection instruments were cleaned between organs and between each dissected fly with 70% ethanol followed by flaming.

3.2.5 Sample Preparation and Storage

Blood and tissue collected from animals and tsetse flies was stored in RNAlater (Sigma) i.e. 1000µL of RNAlater was added to 400µL of blood in 2mL cryotubes which were properly labeled, sealed and incubated at room temperature for 24 – 48 hours before they were stored at -20^0C and subsequently in liquid nitrogen for subsequent analysis.

3.3 Optimisation of PCR-based diagnosis of trypanosomes

Nested PCR allows a sensitive and reliable diagnosis of an infection with trypanosomes at all stages of the life cycle. Therefore, optimising the conditions for nested amplification of ITS-1 regions with two sets of primers which allow amplification of DNA fragments from several relevant *Trypanosoma* species, each with a typical size of the product (Adams *et.al.*, 2006).

3.3.1 DNA extraction (DNeasy Blood and Tissue kit, Qiagen)

Exactly 100µl anticoagulated blood/blood in RNALater was pipetted into 1.5ml or 2ml microcentrifuge tube. Proteinase K (20µl) was dispensed into the cocktail and adjusted to 220µl with PBS. Two hundred microlitres buffer AL (Lysis buffer) was added to the solution and mixed thoroughly by vortexing, and incubated at 56°C for 10 minutes. Two hundred microlitres ethanol (96–100%) was added to the sample, and mixed thoroughly by vortexing. The mixture was pipetted into the DNeasy Mini spin column placed in a 2ml collection tube and centrifuged at 6000x g (8000 rpm) for 1 minute using a refridgerated centrifuge (Eppendorf). The flow-through and collection tube were discarded. The DNeasy Mini spin column was placed into a new 2ml collection tube and 500µl buffer AW1 was added, and centrifuged for 1 minute at 6000x g (8000 rpm). The eluent and collection tube were discarded. The DNeasy Mini spin column was placed in a new 2ml collection tube, and 500µl buffer AW2 was added, and the tube was centrifuged for 3 minutes at 20,000x g (14,000 rpm) to dry the DNeasy membrane. The DNeasy Mini spin column was again placed in a clean 1.5ml microfuge tube, and 200µl buffer AE (Elution Buffer) was pipetted directly onto the DNeasy membrane. The tube was incubated at room temperature for 1 min, and then centrifuged for 1 min at 6000x g (8000 rpm) to elute. The eluent was quantified using a Nano drop spectrophotometer (ND 2000) and stored in a 2mL microfuge tube at -20^{0}C.

3.3.2 Primers

Generic primers were designed by alignment of 11 trypanosome DNA sequences from the NCBI database using Genious. Sequences used for alignment were *T. brucei* (AF306771,

XO5682), *T. congolense* savannah (U22315), *T. congolense* forest (U22319), *T. congolense* kilifi (U22317), T. (U22320), *T. simiae* Tsavo (U22318), *T. evansi* (D89527), *T. vivax* (U22316). Table 3.1 shows the sequences of the generic and specific primers. The specificity of the primers was verified using a BLAST search against the human and mouse genomes, representing possible contaminating mammalian DNA, and alignments against the ITS-1 region of Drosophila (no tsetse sequence available), and tsetse bacterial symbionts *Wigglesworthia glossinidia*, *Sodalis glossinidius* and *Wolbachia pipientis* to rule out non-specific matches to vector derived sequences (Adams *et al*., 2006).

Table 3.1: Tcon ITS-1 Generic and Specific Primers

ITS-1 Generic primers	
Name	Primer Sequence 5'-3'
OSK 1424	AAG CCA AGT CAT CCA TCG
OSK 1423	TAG AGG AAG CAA AAG
OSK 1426	TGC AAT TAT TGG TCG CGC
OSK 1425	CTT TGC TGC GTT CTT
ITS-1 *T.congolense* Specific primers	
Name	Primer Sequence 5'-3'
OSK 1426	TGC AAT TAT TGG TCG CGC
OSK 1449	TGT TGG TCG ACA CTG AGA
OSK 1447	TCG CGT GTC TCA CGT
OSK 1448	TCA AAG ATT GGG CAA TGT

3.3.3 Nested PCR

Two consecutive PCR reactions were carried out: a set of outer primers was used in the first round reaction, followed by inner primers in the second round. One microliter of the PCR product from the first round reaction was added in the second round reaction in a fresh PCR tube. The nested PCR was performed using a 25µL reaction volume containing 1µL (1ng/µL) template DNA, 25µM of each primer, 10mM dNTPs, 25mM 10x Dream Taq polymerase buffer, 5U/µL Dream Taq polymerase to a reaction volume of 25µL. Cycling conditions were as follows: 1 cycle at 95^0C for 300 seconds followed by 30 cycles at 95^0C for 60 seconds, 56^0C for 60 seconds, 72^0C for 30 seconds, and final extension 72^0C for 220 seconds. The products were resolved by electrophoresis (45 minutes to 1 hour at 100V) on 1.5 or 2% Agarose gels stained with G-Stain (Serva®) and bands were visualized using GelDoc-It^2 Imaging System (Analytik Jena AG Jena, Germany).

Another set of nested PCR was also performed using the Phusion Blood direct kit (Finzymes) that did not require DNA extraction. The reaction was done using a 20µL reaction volume containing 10µL Phusion buffer (10X), 1uL of each outer primer, 0.4µL of Phusion enzyme, 6.6µL of double distilled water and 1uL of blood that served as template in the first reaction. The cycling conditions were as follows: 1 cycle at 98^0C for 300 seconds followed by 35 cycles at 98^0C for 1 second, 57^0C for 5 seconds, 72^0C for 20 seconds and a final extension at 72^0C for 60 seconds. At the end of the reaction, the tubes were centrifuged at 1800 rpm for 3 minutes and 1µL of the supernatant was used as template in the second reaction where Dream Taq polymerase (Fermentas) was employed (as described in the previous paragraph).

Another set of nested PCR was performed on fresh blood, blood spotted on Whatman filter or tsetse fly droppings for quick detection of trypanosomes in the anal drop of one tsetse fly and in the blood of an infected animal. Anal drops of the flies or blood spotted on Whatman were recovered in 50 µL of sterile water or 50 µL of whole blood was left to incubate for 1hour at 56^0C. 50 µL of 5% Chelex 100 resin (Biorad, CA, USA) was dispensed into the mixture and incubated for 30 minutes at 95^0C. The solution was centrifuged at 13, 400 rpm for 2 minutes. 1µL of the supernatant was used to perform a nested PCR as described above.

3.3.4 Monitoring the developmental status of *Trypanosome congolense* in tsetse fly and Goat by means of nested PCR

Twenty teneral flies were fed on an infected rat (on average 10 flies) displaying parasitaemia of 1 x 10^9 parasites/ml (determined using the matching method, Herbert and Lumsden, 1976). Feeding was done at room temperature and relative humidity of 77%. Flies failing to feed were removed; only fully engorged flies were used in the experiment. All the flies were maintained by feeding on 3 uninfected goats (one goat/week), every other day for 21 days. Sixteen infected flies were fed on a fourth goat every other day for a week. Flies were dissected 32 days post infective feeding. The blood of the goat was screened daily by microscopy and PCR to detect the presence of parasites. The anal drops of the flies were analyzed at different post infection times by nested PCR. The anal drops were recovered from slides in 10µL of sterile water and incubated at 56^0C for 1hour. Followed by incubation at 95^0C for 30 minutes in 50µL of 5% Chelex 100 resin (Biorad, CA, U.S.A). The solution was centrifuged at 13400 rpm for 2 minutes. One microliter of

the supernatant was used for subsequent trypanosomal DNA amplification. Nested PCR was carried out as earlier described.

3.4 Reverse Transcriptase PCR based detection of TS in blood stream forms of *Trypanosoma congolense*

After the establishment of parasitaemia in an infected goat by PCR and microscopy, four batches of male teneral flies (*Glossina morsitans sub morsitans*) consisting of 10 flies per batch were allowed to feed on the *T.congolense* infected goat. Flies were maintained on blood from an uninfected goat using the membrane feeding technique.Twenty-one days later, the infected flies were allowed to feed on an uninfected goat three times a week after which all surviving flies were dissected and the midgut and proboscis prepared. Blood was collected daily by jugular venepuncture from day 0 to day 62. All samples, blood and fly tissues, were stored in RNAlater for RT-PCR. Daily microscopic screening of the blood was done to check for the presence of parasites. PCR was also performed daily to check the establishment of parasitemia in the goat.

3.4.1 Reverse Transcriptase PCR based detection of TS in Procyclic forms of *Trypanosoma congolense* (Single-fly-Goat experiment)

Again teneral flies were fed on an infected goat at peak parasitemia. Flies failing to feed were excluded from the experiment. Fully engorged flies were placed in individual cages and clean glass slides placed beneath the cages to collect fly droppings. Infected flies were detected by PCR analysis of the droppings at different post infection times using ITS-1 primers. Once parasites were established, the infected fly was fed on a clean goat once.

Flies were dissected 24 days post infection stored in RNAlater at -20^{0}C for subsequent analysis. Blood was collected from the goat via jugular venepuncture daily to check the onset of parasitemia and blood stored in RNAlater for subsequent analysis.

3.4.2 RNA Preparation and PCR Analysis

3.4.2.1 RNA Extraction from Blood

850 µL of Tripure isolation reagent (Roche) was dispensed into a microfuge tube containing 150 µL of blood. The solution was incubated at 25^{0}C for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Two hundred microliters of Chloroform was added and shaken vigorously for 15 seconds and incubated at 25^{0}C for 15 minutes. The tube was centrifuged at 12000-x g for 15 minutes at 4^{0}C. After centrifugation, the colourless upper phase, which contained the RNA, was transferred into a fresh microfuge tube. Five hundred microliters of Isopropanol was dispensed into the solution and inverted several times to mix thoroughly. The tube was incubated at 25^{0}C for 10 minutes to allow RNA precipitate. The tube was centrifuged at 12000-x g for 10 minutes at 4^{0}C. The supernatant was discarded and 1ml of 75% ethanol was added and the RNA pallet washed by vortexing. The tube was centrifuged at 7500-x g for 5 minutes at 4^{0}C. Excess ethanol was removed from the RNA pellet by air-drying. The pellet was resuspended in diethylpyrocarbonate (DEPC)- RNase -free water and incubated at 55^{0}C for 15 minutes to dissolve the pellet. RNA was quantified using a nanodrop (ND-1000 Spectrophotometer).

3.4.2.2 RNA Extraction from Tsetse tissue

An RNeasy micro-kit (Qiagen) was employed as follows: 1mg tissue stored in RNA*later* was disrupted and homoginized in 100µL of buffer RLT using 2.3 mm metal beating beads (MO-BIO) by vortexing for 15 minutes. The lysate was centrifuged at 13400 rpm for 3 minutes and the supernatant carefully transferred into a new microfuge tube by pipetting. One-volume of 70% ethanol was added and mixed by pipetting. The solution was transferred into an RNeasy MinElute spin column placed in 2mL collection tube and centrifuged at 10000 rpm and the flow through discarded. 350µL of buffer RW1 was dispensed into the column and centrifuged at 10000 rpm for 15s. 80µL of buffer RDD containing DNase I was dispensed directly into the column and incubated at room temperature for 15 minutes. Three hundred and fifty microliters of buffer RW1 was dispensed into the column and centrifuged at 10000rpm for 15s. The column was placed in a new collection tube and 500µL of buffer RPE was dispensed into the spin column and centrifuged. 500µL of 80% ethanol was dispensed to wash the spin column and the flow through discarded. The column was spun for 5minutes again at 14000rpm to get rid of residual buffer or contaminants. The column was placed in a new microfuge tube and 15 µL of RNase free water was dispensed directly to the centre of the spin column membrane and spun at 14000rpm for 1 minute. The eluate was quantified and used for subsequent RT-PCR and Real-Time PCR.

3.4.2.3 RNA Quantification

RNA was first quantified using the Nanodrop 2000c UV-Vis Spectrophotometer (Thermoscientific). Briefly, samples were lightly vortexed and centrifuged before checking

the concentration with the nanodrop. The pedestals were wiped clean with Kimwipe and 1.5μL of MilliQ water was placed on the pedestals to initialize the machine. The sample type RNA-40 was selected for RNA quantification. 1.5μL of elution buffer (buffer solution in which RNA was eluted in) was placed on the pedestals to blank the machine. Pedestals were again wiped thoroughly with Kimwipe. 1.5μL of RNA sample was dispensed and the concentration of RNA measured in ng/μL.

Also to further confirm the quality of RNA extracted, 500ng of sample was denatured using 5μL Formamide, 2μL Stain-G (Serva), 1μL loading dye and solution made up to 10μL with water. Tubes were kept on ice for 5 minutes and incubated at 70^0C for 6 minutes in a PCR machine. Tubes were place on ice for a minute and samples ran on Agarose gel at 100V for 1hour.

3.4.2.4 DNase 1 Digestion of Extracted RNA

Extracted RNA was subjected to DNase 1 (Fermentas) treatment to remove contaminating genomic DNA as follows:

To an RNase free-tube, 1 μg of RNA, 1 μL of 10X reaction buffer with $MgCl_2$, 1 μL (1 unit) of DNase I, RNase-free, 7 μL of DEPC-treated water was dispensed. The tube was incubated at 37^0C for 2 hours. The reaction was terminated by adding 1 μL of 50 mM EDTA and incubated at 65^0C for 10 minutes. This step was not necessary for fly tissue samples because a digestion step was included in the extraction protocol.

3.4.2.5 Reverse Transcriptase - PCR

cDNA was synthesised using the Revert Aid Premium First Strand cDNA Synthesis Kit (Fermentas) as follows:

The following were added into an RNase free-tube on ice in the indicated order, 1 pg – 5 µg of total RNA, 1 µL (100 pmol) of random hexamer primers, 1 µL of 10 mM dNTP Mix, the volume was made up to 15 µL with nuclease free water. This was followed by 4 µL of 5X RT Buffer and 1 µL of Revert Aid Premium Enzyme Mix in a final reaction volume of 20 µL. The solution was mixed gently, centrifuged and incubated for 10 minutes at 25^0C followed by 30 minutes at 50^0C. Heating at 85^0C for 5 minutes terminated the reaction.

3.4.2.6 Primer Design

TconTS primers for detection of TconTS mRNA expression were designed by alignment of *Trypanosoma congolense* TS sequences from the NCBI database using Genious software. Accession numbers of the TconTS genes were TconTS1 (HE583283), TconTS2 (HG531955), TconTS3 (HG531956), TconTS4 (HG531957) are shown on Table 3.2. The specificity of the primers was verified using a Primer BLAST search against the human, rat, mouse, Drosophila, Fruit fly, African dwarf goat genomes as well as tsetse bacterial symbionts *Wigglesworthia glossinidia*, *Sodalis glossinidius* and *Wolbachia pipientis* to rule out non-specific matches to vector derived sequences.

Table 3.2: Primer sequences for TconTS RT-PCR

Name	Primer Sequence 5'-3'
TS1	
OSK 1468	TAT TAC TTT ACC AAC AG
OSK 1436	GAC GAT CCC GTC GTG
OSK 1433	CCG TTG CTG GGG TTT
OSK 1434	ACC ACC TGG TGG CTC
TS2	
OSK 1437	GGC TTC AGG CTC TTA
OSK 1438	GTT TTC CAC GTC TTC
OSK 1439	ATG TGA ACA CTC GAG
OSK 1440	CTA TGA GAC TGC TGT
TS3	
OSK 1445	GTG GGA TGA CAA ACT
OSK 1446	GCA ATA GAA CTC CCT
OSK 1443	TAC TCA ACA GAT GAA
OSK 1444	GAG CAT CAC ACG CAT
TS4	
OSK 1447	CGT GGA AAA CTG AAG
OSK 1448	TAG TCC GGT TTC CAG
OSK 1449	CAT TGA AAA TGC TCG
OSK 1450	ATA CCG AGG TCC ACT

3.4.2.7 Nested PCR

Nested PCR was performed using a 25uL reaction volume containing 0.5µl of each primer (100 pmoles), 0.5µL of dNTP (10mM), 1 µL of $MgCl_2$ (25mM), 0.2µL of Dream Taq Polymerase (Fermentas), 2.5µL of Dream Taq buffer (10x) followed by 18.5 µL double distilled water. 1µL of cDNA was used as template in the reaction. The cycling conditions were as follows: 1 cycle at 95^0C for 300s followed by 35 cycles at 95^0C for 60 s, 54^0C for 60 s, 72^0C for 30 s and a final extension 72^0C for 210 s. Two consecutive PCR reactions were carried out; a set of outer primers (Table 3) was used in the first round reaction, followed by inner primers in the second round. 1 µl of the PCR product from the first round reaction was added to 25 µL of the second round reaction in a fresh PCR tube.

3.5 SDS-PAGE and Immunoblot based detection of TS in blood and fly tissue

3.5.1 Sample preparation

Fly tissue was homogenized by grinding in 100µL of 1x TNE lysis buffer for 20 minutes on ice. The lysate was centrifuged at 14000 rpm (4^0C) for 15 minutes. 1:1 volume of supernatant to 1x sample buffer containing 10mM dithiothreitol (DTT) was boiled.

3.5.2 SDS-PAGE

SDS-PAGE was performed according to Laemmli (Laemmli, 1970) in a Mini Protean II Cell (Bio-Rad, Munich, Germany) in the presence of a reducing agent (dithiothreitol). Polyacrylamide gels usually consist of 12% resolving and 4% stacking gel. As molecular weight markers, pre-stained SDS-PAGE standards from Fermentas (for immunoblotting) or SDS-PAGE Marker High Range from Sigma were applied (for staining). Proteins were

transferred onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) using a Mini-V 8–10 Blot Module (Life Technologies, Eggenstein, Germany) as described by the manufacturer.

3.5.3 Immunodetection

For immunodetection, blots were blocked for 1hour in Tris-Buffered Saline-Tween 20 TBST and 3% skimmed milk (blocking buffer), washed four times with TBST for 40 minutes, and then incubated for overnight at 4 °C (anti-*T.congolense* TS1 mAB, dilution1:1000). Following incubation, the blots were washed again four times with TBST buffer and then incubated for 2 hours at room temperature with horseradish peroxidase-conjugated anti-donkey or anti-mouse IgM (1:20000). After washing four times for 10 min with TBST buffer, bands were visualized using the Pierce ECL Western blotting substrate detection reagent kit (Fermentas) as described by the manufacturer.

3.6 Real Time PCR (qPCR) based TS quantification

3.6.1 Primers and Probes

In the qPCR assay, primers and probes for the detection of all TS isoforms were designed (Table 3.3). *T.congolense* TS sequences were selected from NCBI database (www.ncbi.nlm.nhi.gov). TconTS1 (HE583283), TconTS2 (HG531955), TconTS3 (HG531956), TconTS4 (HG531957). The sequences were aligned in the Genious Aligner and the primers and TaqMan probes designed with Primer Express software version 3.0. A Primer BLAST search of GenBank was performed on the primers and probes, the human genome, and other relevant species to ensure specificity. A simultaneous amplification of

the House keeping Genes (HKG) GAPDH genes of *T.congolense* (AJ620290.1), *Capra hircus* (XM_005680968.1), *Glossina morsitans morsitans* (DQ016434.1) were ran as internal controls to prevent detection of false positives. To check the effectiveness of the primers, the four primer pairs (Sigma-Aldrich, Germany) were tested for amplification of the appropriate amplicon length using 10pg of each TS plasmids in a total volume of 25 µL per reaction on a Mastercycler® Gradient (Eppendorf, Germany) using the TaqMan® Fast universal PCR Master Mix (Applied Biosystems). Thermocycling conditions consisted of an initial denaturation of 95^0C for 20s, 40 cycles of 95^0C for 1s and 60^0C for 20s. The PCR products were analysed by gel electophoresis on a 1.5% agarose gel stained with G-Stain (Serva, Germany) and bands were visualized using GelDoc-It^2 Imaging System (Analytik Jena AG Jena, Germany).

Table 3.3. Primers and Probes sequences for novel TconTS qPCR

	Primer/Probe Sequence	Length	Product Size	Sequence Identity
TconTS1				
Forward	5' CTGACGATGGAAAGTCATGG 3'	20	133	N/A
Reverse	5' ATCATACGGTAGCCCTGTCC 3'	20		
Probe	FAM 5'-TCCGAGGCTGCCCTCACTG-3' BHQ1	19		
TconTS2				
Forward	5' GCCATAACTGTGGAGGGAGT 3'	20	129	N/A
Reverse	5' AATCTGTCCAACAAGCCACA 3'	20		
Probe	FAM 5'-ATCGCGACCGAATGCGACTG-3' BHQ1	20		
TconTS3				
Forward	5'-TTCATCAAGTCGCACTCACA-3'	20	136	N/A
Reverse	5'-GGATGCCCAACAAAGAAGTT-3'	20		
Probe	FAM 5'-CGCCCGCAACCTTCGTATCC-3' BHQ1	20		
TconTS4				
Forward	5'-TCGCCGAAAGCAACTATATG-3'	20	136	N/A
Reverse	5'-AACCCGTCAGCAGCTCTTAT-3'	20		
Probe	FAM 5'-TTGTCCCTCAACCGGGAGGC-3' BHQ1	20		

3.6.2 RNA Extraction and Quantification

RNA extraction, RNA quantification, DNase1 treatment and cDNA synthesis of goat blood and fly samples were as described in an earlier section...

3.6.3 Preparation of Serial dilutions

A 5-point 10 fold serial standard curve for each TS isoform plasmid DNA was prepared by pipetting 18μL of Nuclease free water into five 0.2mL tubes labeled 1-5. A tube containing Plasmid DNA (100pg/μL) was briefly vortexed and centrifuged. 2μL of the stock solution was pipetted into tube 1. The tube was briefly vortexed and centrifuged. Using a fresh tip, 2μLwas pipetted from tube 1 to 2. The same process was repeated for all the tubes and isoforms of TS plamid DNA.

3.6.4 Real-time PCR Amplification

qPCR was performed with a Step-One Plus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The qPCR was performed in a 10μL volume containing 0.2 ng cDNA, 1μM of each primer, 0.1μM of each FAM-labelled probe, 2x TaqMan Fast Universal PCR Master mix (Applied Biosystems). The reaction mixes were loaded into a MicroAmp® Optical-96-Well reaction plates (Applied Biosystems) and covered with MicroAmp® Optical Adhesive film (Applied Biosystems). The qPCR cycle protocol started with the initial denaturation at 95^0C for 20s, 40 cycles of 95^0C for 1s and 60^0C for 20s. All qPCR amplifications were reproduced in triplicates including those for the standard curve.

3.6.5 Data Analysis

As soon as the run was over, the Step-One software (3.0v) automatically opened the data analysis window showing the amplification plot which is a plot of Log ΔRn vs C_T

where R_n is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye. ΔR_n is R_n minus the baseline value. The C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold. The software also generated standard curves which provided the following information about the PCR assay:

- Slope (S) of the line which is a measure of the assay's efficiency
- R-squared (R^2) which is a measure oft he assay performance and is the coefficient of correlation between data generated and the results obtained under ideal conditions.
- PCR/Amplification Efficiency (%) can be defined as the increase in amplicon per cycle. Efficiency = 10(-1/slope) – 1.

Using serial dilutions of the plasmid DNA of TS isoforms, the PCR efficiency and sensitivity in detecting and quantifying trypanosomal TS was analyzed. For the limit of detection serial 10-fold dilutions ranging from 10pg to 0.1fg were tested for each TS plasmid positive control. This covers 9.26 x 10^5 to 9.26 copies/ng of plasmid DNA respectively. (Number of copies/ng of plasmid DNA was determined using the URI Genomics and sequencing center calculator for determining the number of copies of a template on the following link: www.cels.uri.edu/gsc/cndna). The reaction was evaluated using the StepOne Software version 3.0 and regarded as positive if the amount of fluorescence exceeded a threshold value and followed a curve of a sigmoid shape.

CHAPTER FOUR

RESULTS

4.1 Extraction of DNA

The purity of the extracted DNA as assessed on a Nano drop spectrophotometer (ND 2000) was given as the ratio of the absorbance at 260/280 nm and 260/230 nm, which ranged between 1.76–1.82 and 2.0–2.1 respectively. Gel electrophoresis was also employed to further confirm the purity of DNA samples (Figure 4.1). As shown on the electrophoregraph, all samples (Lanes 1-14) had varying band intensities indicating the presence of DNA in all blood samples collected from animals infected with *Trypanosoma* species.

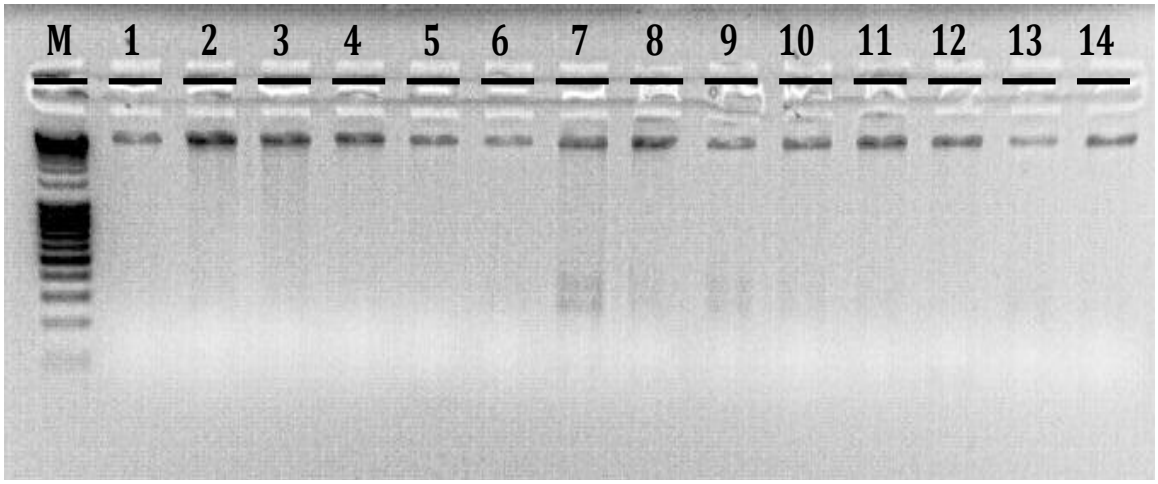


Figure 4.1: Electrophoresis gel (1.5% Agarose, stained with 0.5 μg/ml Ethidium Bromide) photographed under ultraviolet light showing from left to right, DNA extracted from blood samples collected from goats infected with *Trypanosoma* species.

M – Marker

Lanes 1 – 14 – gDNA from infected goat blood

4.2 Optimization of Polymerase Chain Reaction (PCR)- based diagnosis of *Trypanosoma* species

Generic primers were used to amplify the ITS-1 region of genomic DNA from *T.congolense* and other related trypanosome species each with a typical size product (Table 4.1), 640 bp in the case of *T.congolense savannah, w*hich is in agreement with previous studies (Adams *et al*., 2006; Desquesnes *et al*., 2001). Furthermore, primers were designed for species-specific amplification for confirmation and higher sensitivity.

Figure 4.2 shows the PCR amplification of ITS-1 region from genomic DNA (gDNA) isolated from *T.b.brucei* and *T.evansi* using a set of generic primers. The observed band sizes were approximately 400bp. Lanes 1, 2 and 3 represent amplicons from different amounts of *T.evansi* gDNA, 1ng, 0.1ng and 0.01pg respectively. While lanes 4, 5, 6, 7 represent amplicons from *T.brucei* gDNA starting from 0.1ng, 0.01ng, 0.001ng and 0.1pg respectively. No amplification was observed in the negative control.

To test the limit of detection of the generic primers used, a 10-fold serial dilution of 1ng trypanosomal DNA was done and used as template in a nested PCR. Amplicons of varying intensities were generated. A consistent decrease in intensity of the bands with decrease in amount of template was observed. The highest and lowest intensities represented as 1ng and 0.01fg respectively as shown in Figure 4.3. *T.brucei* and water were employed as positive and negative controls respectively. After optimisation and serial dilutions of DNA with both primers as little as 0.01fg of trypanosomal genomic DNA was detected reproducibly (Figure 4.3) and mixed infections could be clearly identified.

Table 4.1. Predicted species ITS-1 band sizes based on generic primers

ITS-1 band size (bp)	**Predicted species based on ITS-1**
640	*T.congolense* savannah
430	*T.brucei, T.evansi*
380	*T.simiea, T.grayi*
240	*T.godfreyi*
160	*T.vivax*

Adams *et al*., 2006

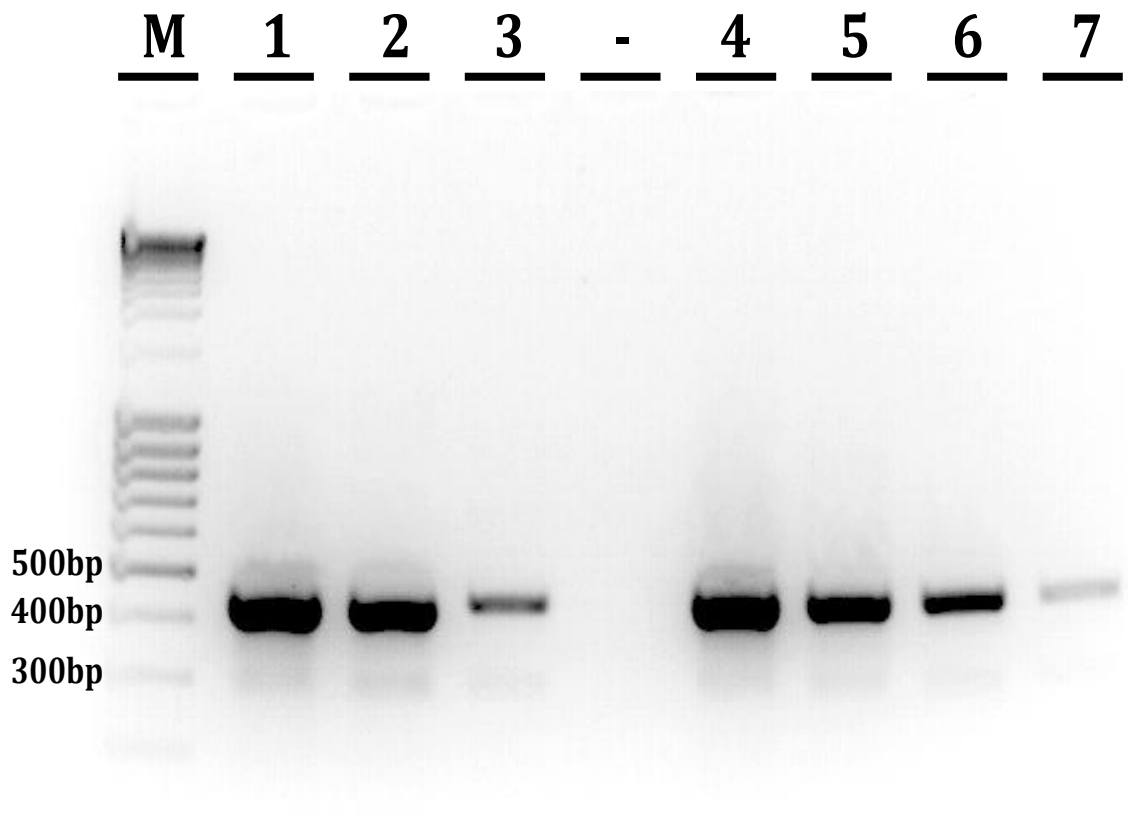


Figure 4.2: Amplification of ITS-1 region from *T.evansi and T.brucei* genomic DNA by nested PCR employing generic primers.

M: Marker, Lanes 1, 2, 3 – *T.evansi*

Lanes 4, 5, 6, 7 - *T.brucei*

- Negative control with water

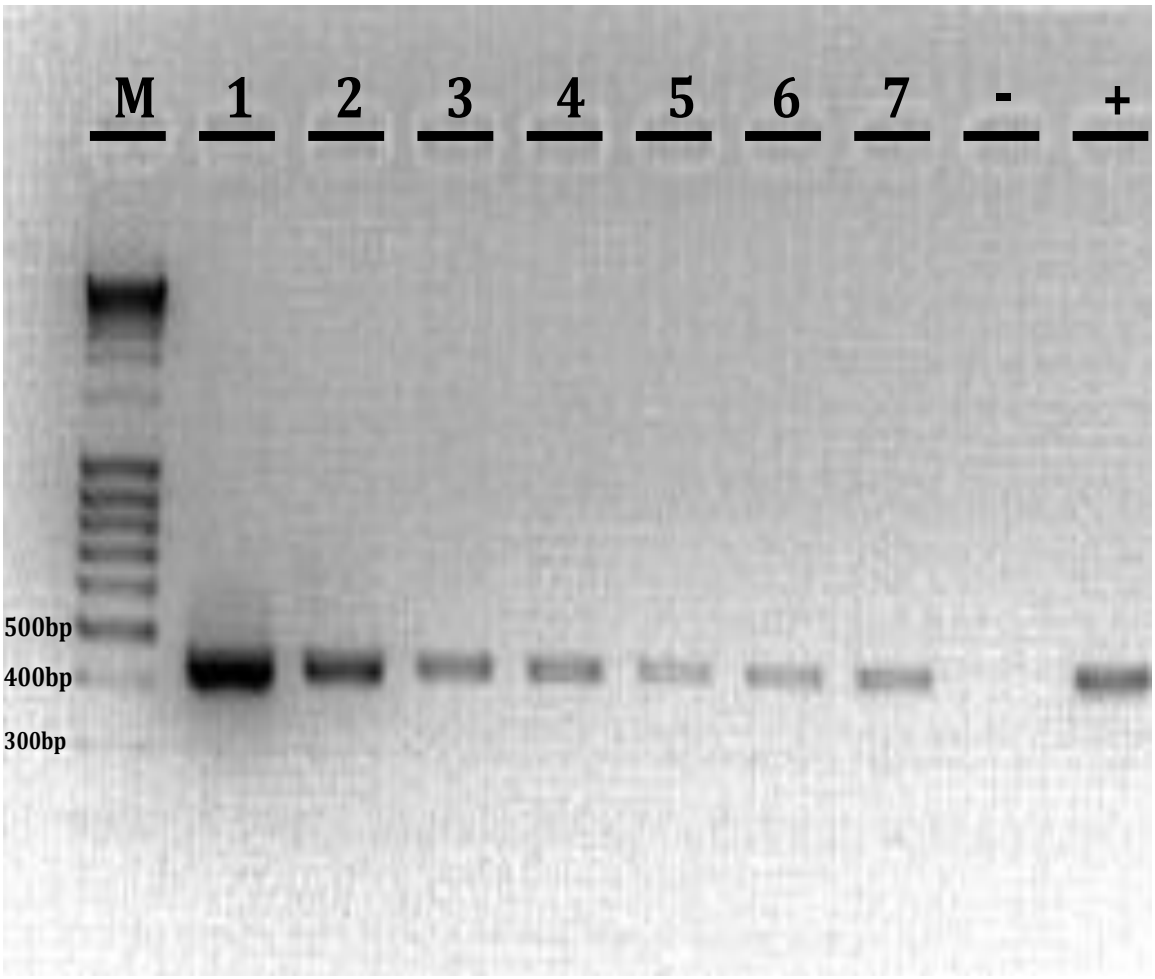


Figure 4.3: Amplification of ITS-1 region from *T.evansi* genomic DNA by nested PCR employing generic primers. M: Marker, Lanes 1, 2, 3, 4, 5, 6, & 7 - 1ng, 10pg, 0.1pg, 0.01pg, 1fg, 0.1fg, 0.01fg

- Negative control with water

+ Positive control with *T.brucei* gDNA

Once the limit of detection was certified, the PCR method was used on field samples. Figures 4.4a and 4.4b show amplification of ITS-1 region of the trypanosomal gDNA isolated from blood samples collected from livestock at Laduga Grazing Reserve, in Kachia Local Government Area, Kaduna state, Nigeria. Tsetse endemic focus. A total of 72 animals were screened and 33.3% were detected positive. Three *Trypanosoma* species were identified *T.b.brucei/T.evansi* (19.4%), *T.congolense* (12.5%) and *T.godfreyi* (1.4%). PCR also detected mixed (9.7%) and single (11.1%) infections.

The method was further adapted to be used with whole blood without parasite purification by using a high fidelity Phusion enzyme (Finzymes) and Dream Taq Polymerase (Fermentas) in the first and second reactions, respectively.

Figure 4.5 shows PCR detection and identification of various Trypanosome species in blood samples using a Phusion blood direct kit (without gDNA isolation). The PCR results showed Lanes 1 and 3 had a mixed infection of *T.congolense* kilifi and *T.brucei/T.evansi*. Lane 2 also had a mixed infection of T.congolense savannah, *T.brucei* and *T.vivax*. *T.congolense* specific primers were used to confirm the presence of *T.congolense* in lane 2 as well as the positive control (+).

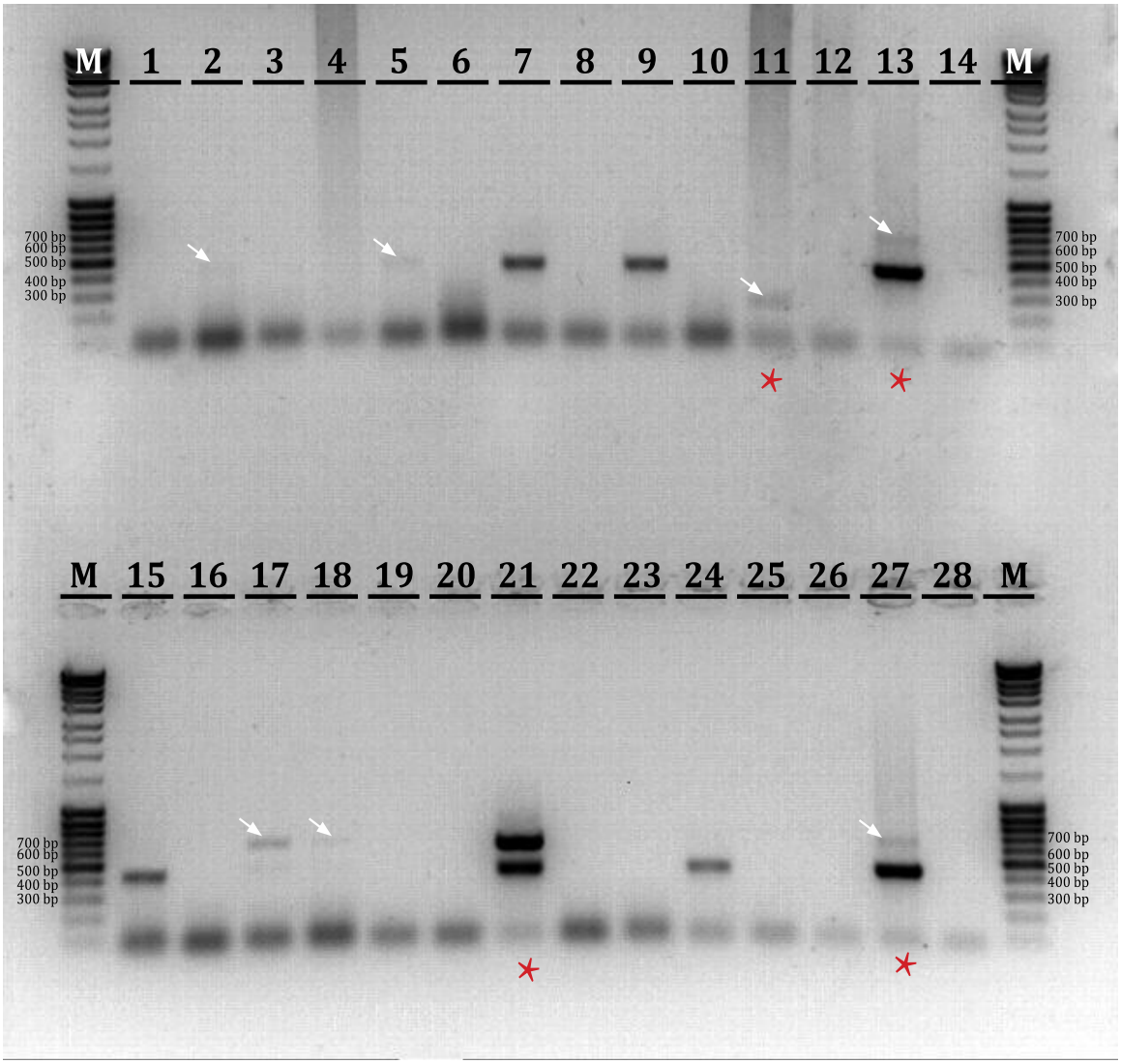


Figure 4.4a: Amplification of ITS-1 region from genomic DNA by nested PCR employing generic primers on blood samples collected from livestock at Laduga Grazing Reserve, Kachia Local Government Area, Kaduna State. A Tsetse endemic focus.

M: Marker, Lanes 2, 5, 7, 9, 11, 13, 15, 17, 18, 21, 24, 27 show *Trypanosoma* positive animals.

* - Indicates samples having mixed infections.

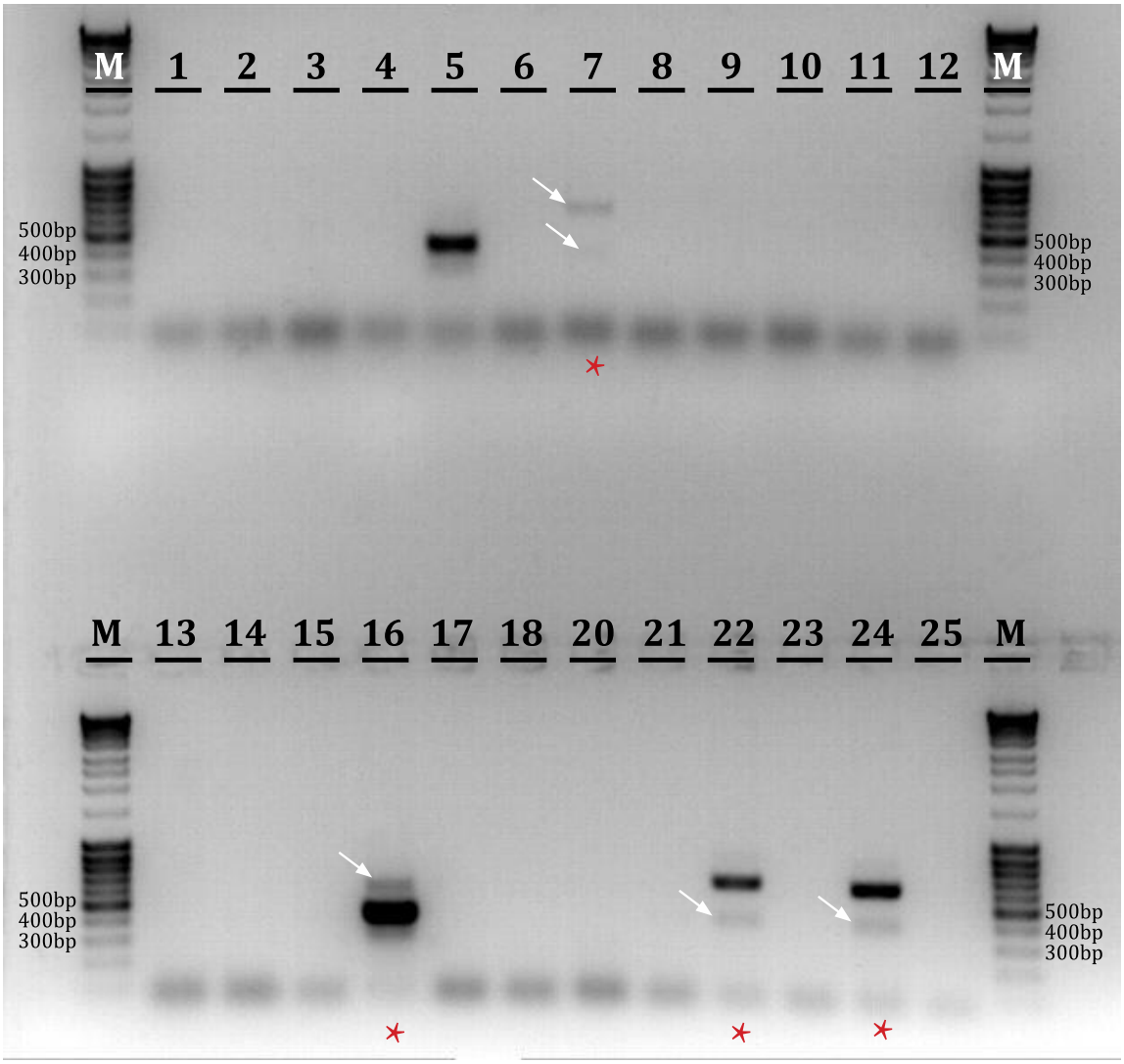


Figure 4.4b: Amplification of ITS-1 region from genomic DNA by nested PCR employing generic primers on blood samples collected from livestock at Laduga Grazing Reserve, in Kachia Local Government Area, Kaduna State. A tsetse endemic focus.

M: Marker, Lanes 5, 7, 16, 22, 24 show *Trypanosoma* positive animals.

* - Indicates samples having mixed infections.

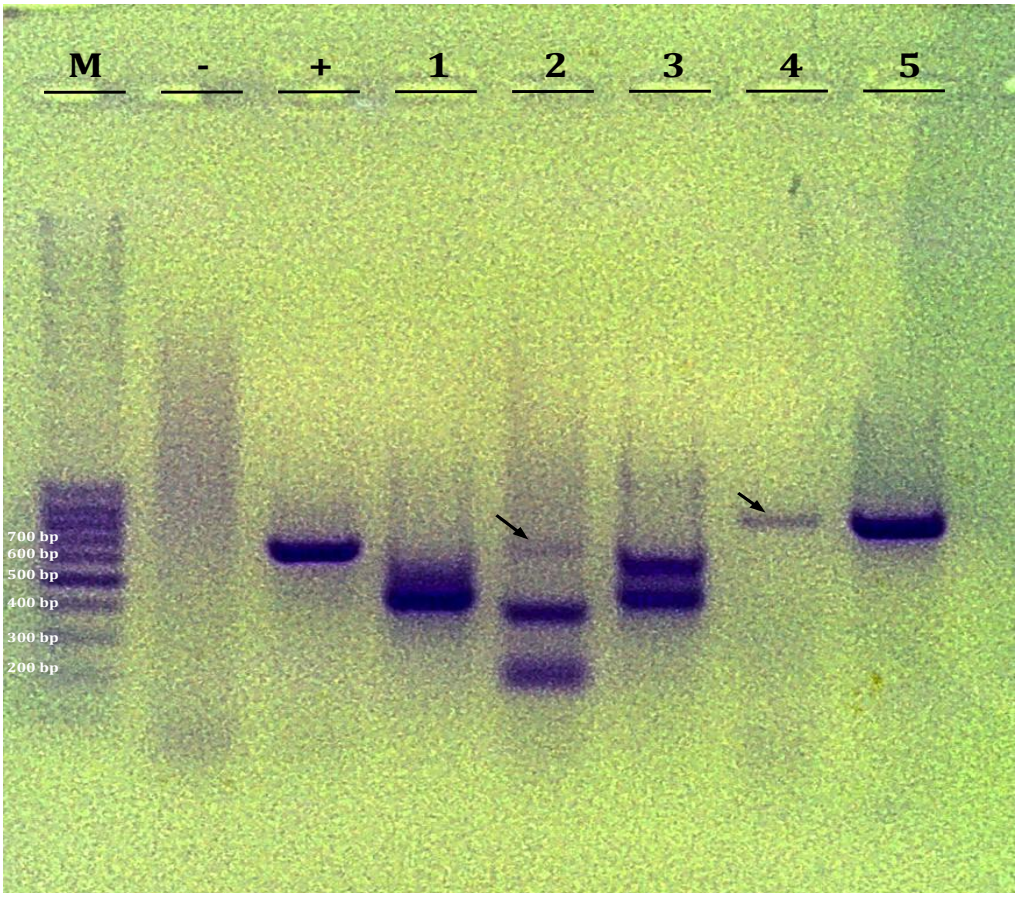


Figure 4.5: Nested PCR identification of *Trypanosoma* species from blood of infected animals by Phusion blood direct kit (Finzymes) using ITS-1 generic and *T.congolense* specific primers.

Lane 1 – Blood from mouse blood infected *T.evansi* using generic primers.

Lane 2 – Blood from cow blood infected with *T.congolense, T.brucei* and *T.vivax* using generic primers

Lane 3 – Blood from cow infected with *T.congolense* and *T.evansi* using generic primers

Lane 4 & 5 – Blood from mice infected with T.congolense using specific primers.

- & + - Negative and positive controls from uninfected and infected mice respectively

Figures 4.6 and 4.7 show successful amplification of ITS-1 region from blood of *T.congolense* infected mice using generic and specific primers respectively. The species-specific primers further confirmed the identification with band sizes of about 800bp. No amplification was observed in the negative controls as expected. Figure 4.8 shows PCR amplicons of different amounts of *T.congolense* genomic DNA, 5ng, 1ng and 10pg in lanes 1, 2 and 3 respectively.

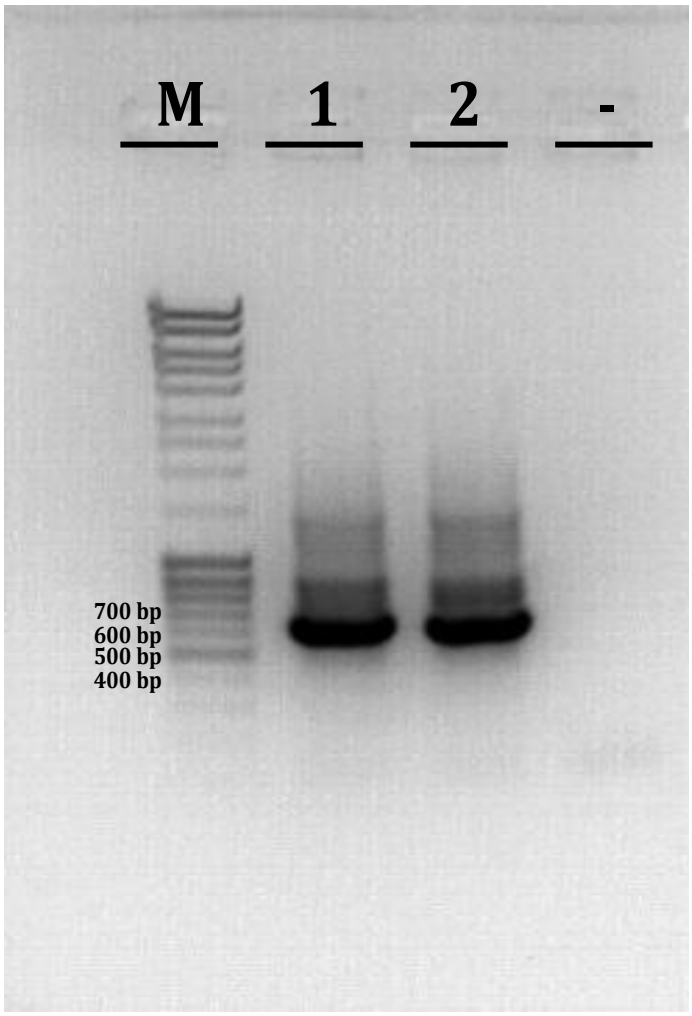


Figure 4.6: Nested PCR identification of *T.congolense* using ITS-1 generic primers. The PCR products were analysed by electrophoresis on a 1% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M- Marker

Lane 1 & 2 – DNA from blood of *T.congolense* infected mice

- Negative control with water

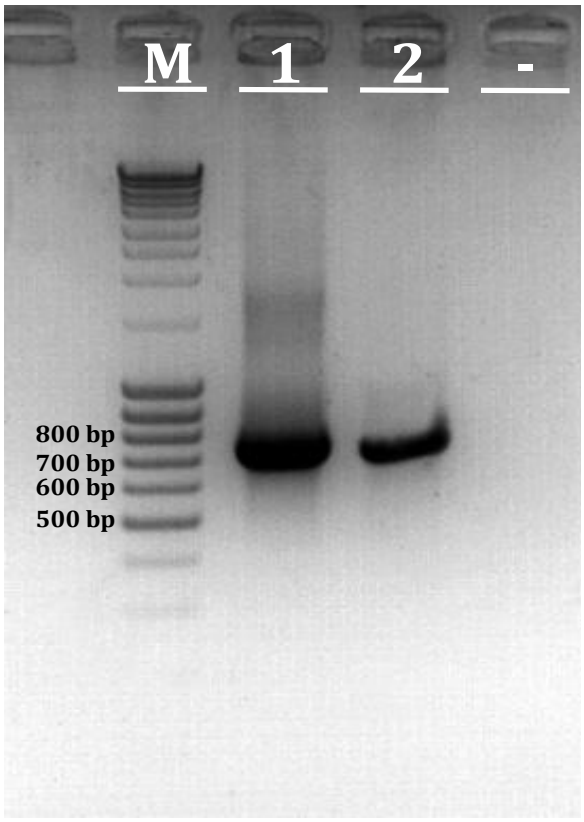


Figure 4.7: Nested PCR identification of *T.congolense* using ITS-1 *T.congolense* specific primers. The PCR products were analyzed by electrophoresis on a 1.5% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M- Marker

Lane 1 & 2 – DNA from blood of *T.congolense* infected mice, 1ng & 10pg respectively

- Negative control with water

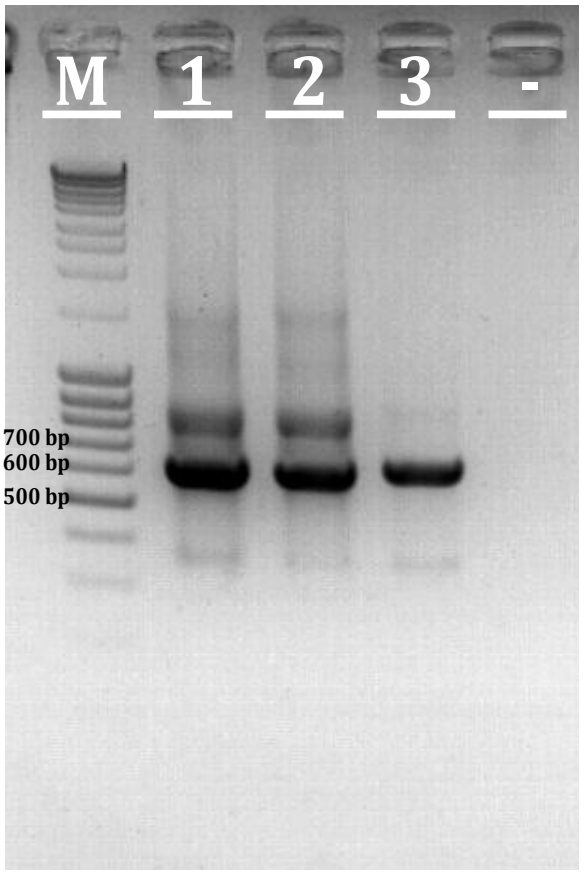


Figure 4.8: Agarose gel electrophoresis of PCR products obtained from nested PCR amplification of varying amounts genomic DNA of *T.congolense* infected mice. The PCR products were analyzed by electrophoresis on a 1.5% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany)

M: Marker,

Lanes 1, 2, 3 - 5ng, 1ng, 10pg respectively.

- Negative control with water.

Figure 4.9 shows a nested PCR amplification of ITS-1 region from genomic DNA of a *T.congolense* infected mouse while comparing amongst three methods; Qiagen extraction kit method, Chelex resin reagent method and Phusion blood direct kit. Of the three methods, Chelex resin reagent gave distinct bands without unwanted amplicons as observed with the other figures (Lanes 3 and 4).

The final method can be used with freshly collected blood or samples stored in RNAlater employing the generic or specific primer sets.

Figure 4.10 is a 3-D chart comparing the volumes of template obtained from DNeasy, Phusion and Chelex methods relative to the cost of procurement. Chelex clearly had the lowest cost of N15,500 when compared to DNeasy and Phusion methods which cost N56,000 and N21,000 respectively. However, the yield in terms of volume of template was highest for Chelex with 500,000µL. A five thousand fold and a hundred-fold decrease in volume of template was observed between Phusion (100µL) and DNeasy (5000µL) kits respectively.

Also, comparing between DNeasy, Phusion, and Chelex method on the cost of isolating the DNA template to amplification of the target gene (ITS-1), Chelex method again was clearly shown to be the most affordable of the three methods with a cost of #48,000 for 200 reactions. Representing a 1.9 and 1.6-fold increase in cost for DNeasy and Phusion kits respectively (Figure 4.11).

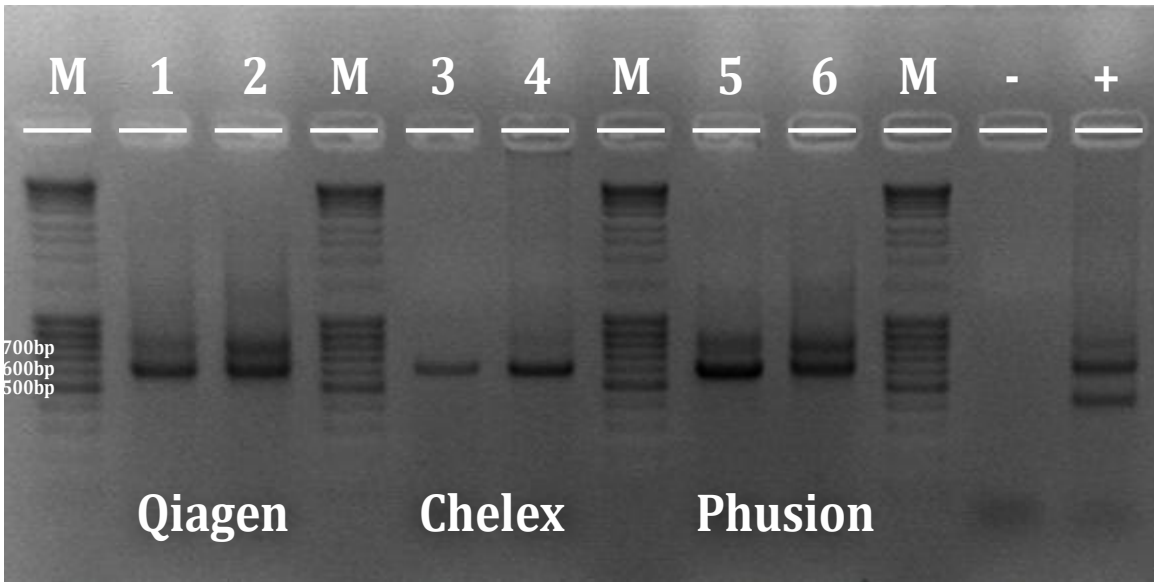


Figure 4.9: Nested PCR amplification of ITS-1 region from genomic DNA of a *T.congolense* infected mouse employing three different methods. The PCR products were analyzed by electrophoresis on a 1.5% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M – Marker

Lane 1 & 2 – *T.congolense* infected mouse blood DNA isolated using DNeasy blood and tissue kit (Qiagen).

Lane 3 & 4 - *T.congolense* infected mouse blood DNA isolated using Chelex Resin (Biorad).

Lane 5 & 6 – *T.congolense* infected mouse blood using Phusion Blood direct kit (Finzymes) without DNA isolation.

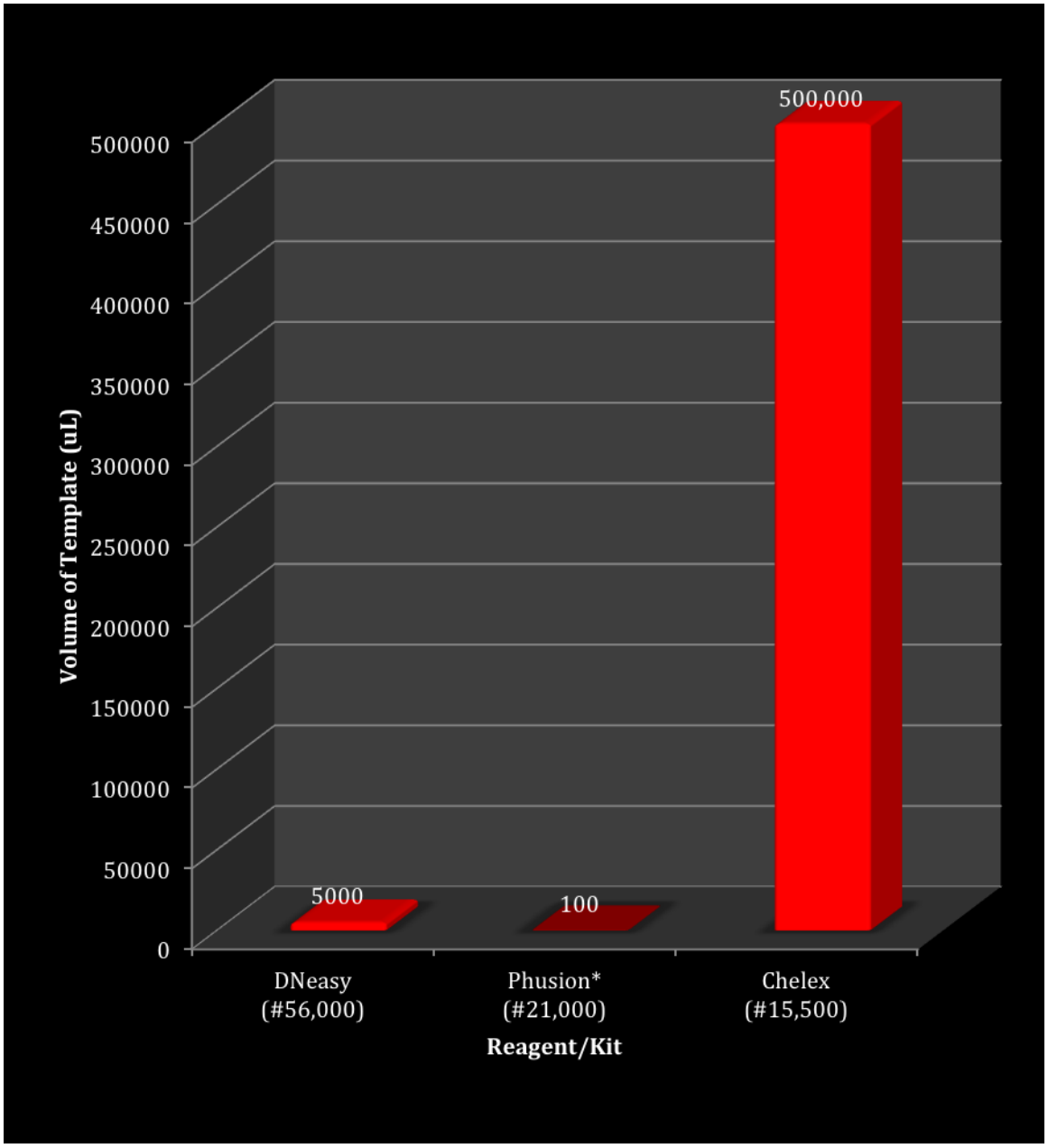


Figure 4.10: Comparing the volumes of template obtained from DNeasy, Phusion and Chelex methods relative to the cost of procurement.

* Indicates Phusion kit doesn't require DNA isolation.

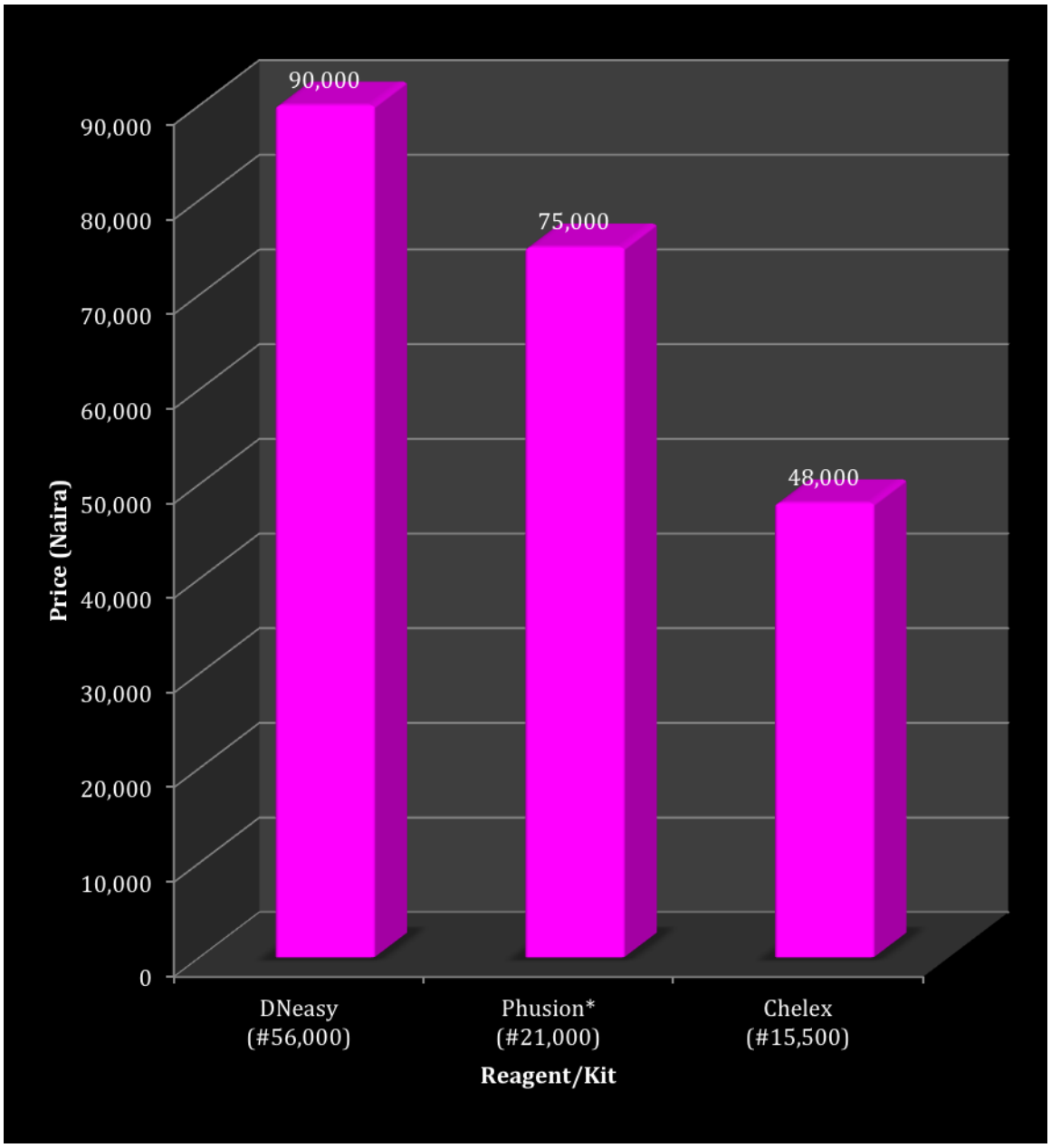


Figure 4.11: Comparison between DNeasy, Phusion, and Chelex method on the cost of isolation to amplification of a target gene.

* Indicates Phusion kit doesn't require DNA isolation.

4.3 Monitoring the developmental status of *Trypanosoma congolense* in tsetse fly and Goat by means of nested PCR

A total of twenty male teneral flies were fed on the donor rat. However, only 80% (16/20) had the infective blood meal and were retained for the experiment. The mortality rate was 12.5% (2/16). The proportion of infected flies wasn't determined since the samples were pulled together. The infection was monitored by nested PCR analysis of the anal droppings of the sixteen tsetse flies that had the infective blood meal at different time intervals. Anal drops of uninfected tsetse flies served as negative controls. Parasites were first detected by PCR in the anal drop of flies on day 8 post infective blood meal through day 18 post infective blood meal (Figure 4.13). Amplification attempts failed on anal droppings from uninfected control flies as expected. As shown in Figure 4.12, PCR was employed to monitor the course of the infection in the mammalian host (goat) at different time intervals. PCR detected the presence of parasites in the blood for the first on day 17 (14 days post infective feed on goat). However as shown on table 4.2, microscopy only detected parasites in the blood 14 days later (Day 31). Amplicons observed in lane D42 and D53 indicated successful amplification of parasite DNA at the mid-stage and chronic stages of the infection respectively. As expected, no amplification was observed in the negative control as well as lane D0 that represented blood collected for baseline data.

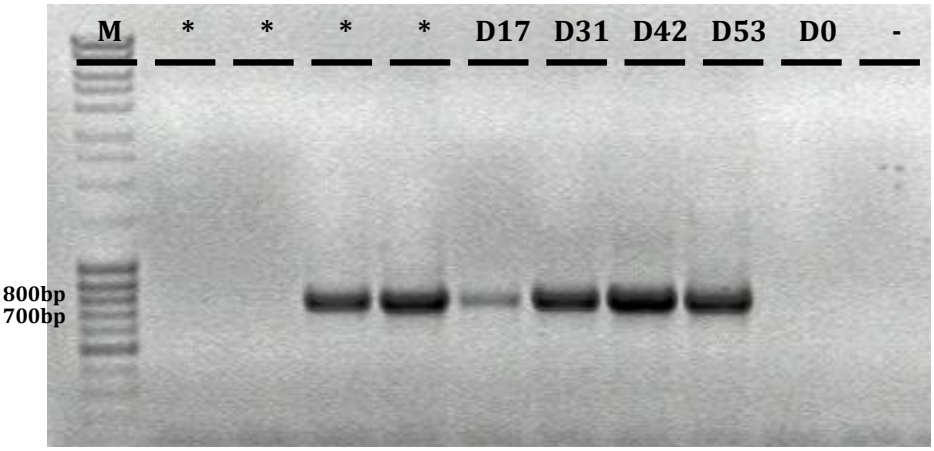


Figure 4.12: PCR identification of *T.congolense* in goat blood employing ITS-1 *T.congolense* generic primers using the Phusion blood direct kit. The PCR products were analysed by electrophoresis on a 1% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M- Marker

D17 – Day parasites were first detected by PCR

D31 - Day parasites were first detected by microscopy

D42 – Time point at the mid-stage of the infection

D53 - Time point at the chronic stage of the infection

D0 – Blood for baseline data

- Negative control with water

* Samples for a separate experiment which served as positive controls

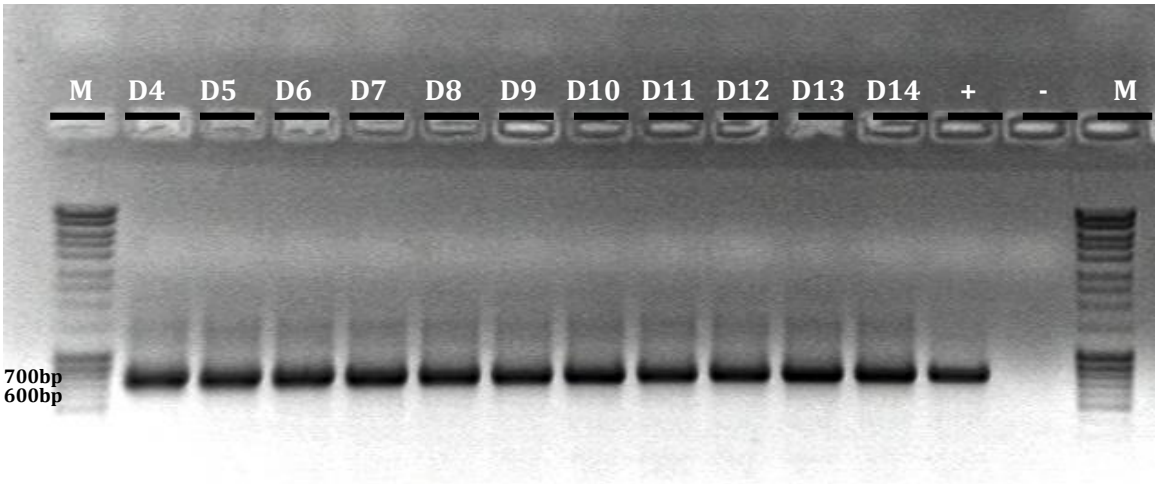


Figure 4.13: PCR identification of *T.congolense* in anal droppings of tsetse flies using the Chelex resin reagent method and employing *T.congolense* generic primers. The PCR products were analysed by electrophoresis on a 1.5% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M – Marker

D4 – D14 – Parasites were first detected in the anal drop of tsetse flies on Day 4 (D4) and was observed through Day 14 (D14).

+ Positive control with *T.congolense* gDNA

- Negative control with water

Table 4.2: Summary of the developmental status of *Trypanosoma congolense* in the host and TS expression in bloodstream forms.

Days	Pre/post fly infective feeding	Parasitemia (x 10^6/mL of blood)	RT-PCR Analysis	Remarks
0	20 Infected flies infective blood meal on clean goat	-	*	Blood was collected from the clean goat for baseline data
1		-		
2		-		
3	16 flies fed on clean goat	-		
4		-		
5		-	*	
6	Flies fed on goat	-		
7		-		
8		-		
9	Flies fed on goat	-	*	
10		-		
11		-		
12		-		14 flies were dissected
13		-		
14		-		
15		-	*	
16		-		
17		-	*	Pre-patent period (PCR)
18		-		
19		-	*	
20		-	*	1st TS expression detected by RT-PCR
21		-		
22		-	*	
23		-		
24		-		
25		-	*	
26		-	*	
27		-		
28		-		
29		-	*	
30		-		

31		0.251	**	Pre-patent period (Microscopy)
32		0.501	*	
33		1.000		
34		2.328	*	
35		1.333		
36		1.000		
37		1.995	*	
38		3.981		
39		7.943	*	
40		15.850		
41		23.715	**	
42		3.981		
43		1.000		
44		0.350		
45		None	*	
46		None		
47		0.314	*	
48		0.942	*	
49		0.251		
50		0.752	*	
51		0.501		
52		0.796	*	
53		1.991		
54		7.943	*	
55		3.981	*	
56		7.943	*	
57		11.888		
58		19.813	*	
59		23.775	*	
60		27.668	**	
61		31.620	*	
62				Goat Died

* Indicated days which RT-PCR analysis was done.

** Indicated different time intervals in which TS expression by RT-PCR analysis was presented.

In the single-fly-goat experiment, 100% (10/10) took the blood meal and no mortality was recorded throughout the experiment. The proportion of infected flies was 30% as PCR detected the presence of *T.congolense* in the anal drop of three out of 10 flies on day 5, 8 and 11-post infection respectively at the time of first analysis (Figure 4.14, 4.15, 4.16). An early detection of parasites by PCR in the anal droppings of one fly (denoted as fly 1) was observed on day 5 through day 14 (Figure 4.14). However, PCR first detected parasites on days 8 and 12 in flies 2 and 3 respectively (Figure 4.15 and 4.16). The other 7 flies remained uninfected throughout the duration of the experiment, as confirmed by the absence of amplification. A similar observation was made in the uninfected negative controls.

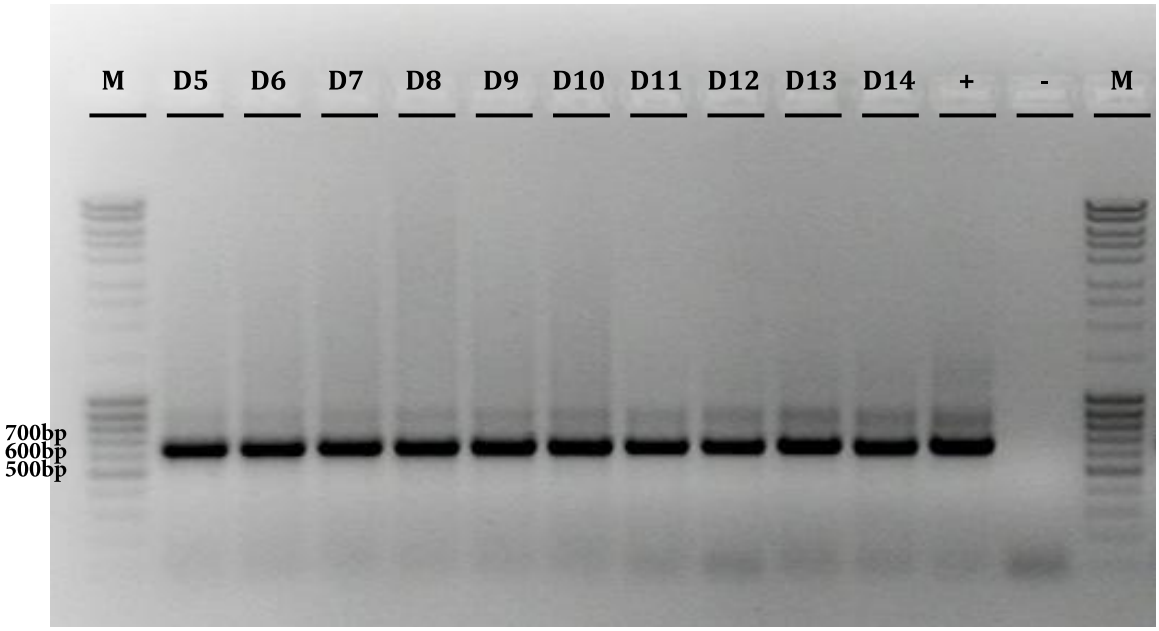


Figure 4.14: PCR identification of *T.congolense* in anal drop of fly 1 using ITS-1 *T.congolense* generic primers. The PCR products were analysed by electrophoresis on a 1.5% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M – Marker

D5 – D14 – Parasites were first detected in the anal drop of tsetse flies on Day 5 (D5) and was observed through Day 14 (D14).

+ Positive control with *T.congolense* gDNA

- Negative control with water

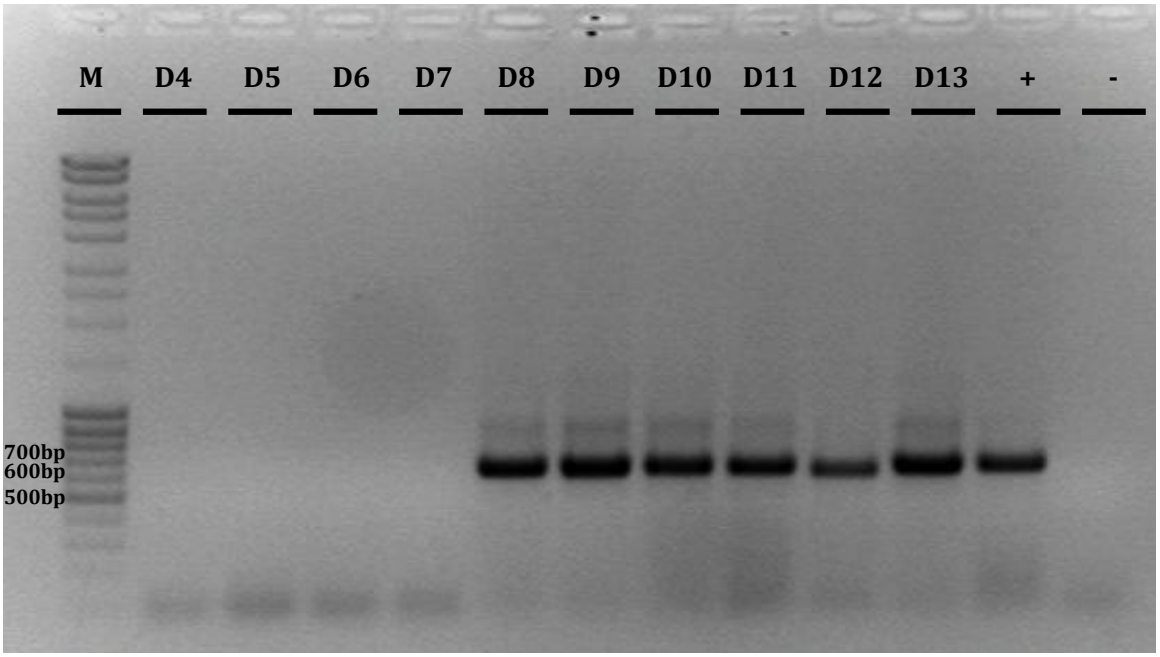


Figure 4.15: PCR identification of *T.congolense* in anal drop of fly 2 using ITS-1 *T.congolense* generic primers. The PCR products were analysed by electrophoresis on a 1% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M – Marker

D8 – D13 – Parasites were first detected in the anal drop of tsetse flies on Day 8 (D8) and was observed through Day 13 (D13).

+ Positive control with *T.congolense* gDNA

- Negative control with water

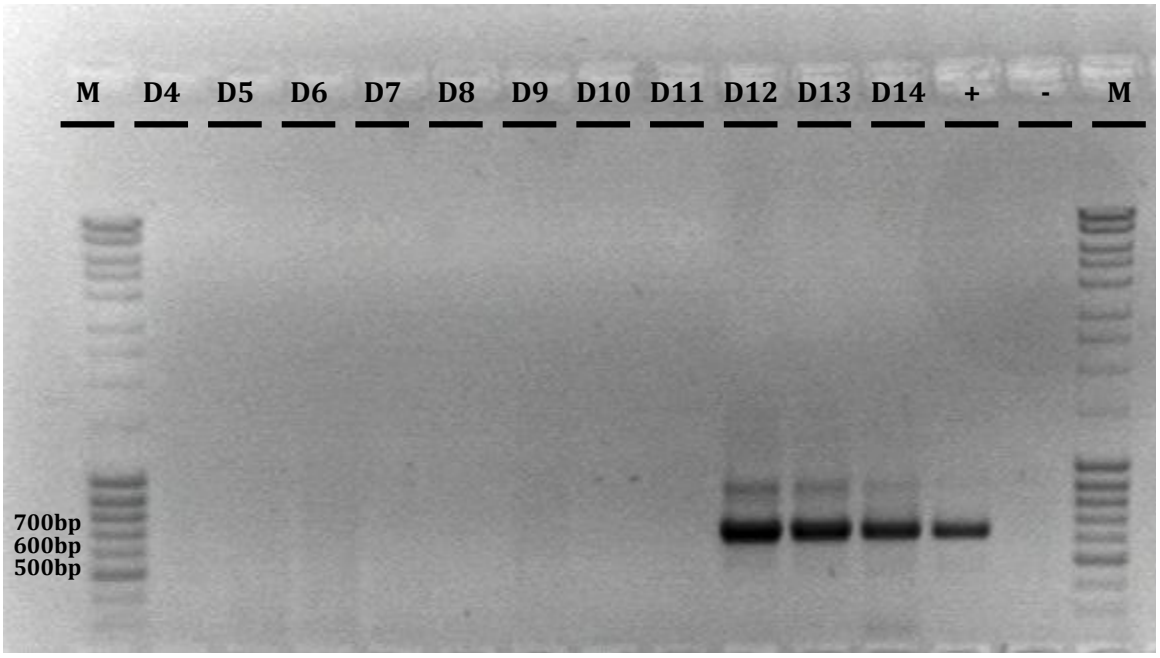


Figure 4.16: PCR identification of *T.congolense* in anal drop of fly 3 using ITS-1 *T.congolense* generic primers. The PCR products were analyzed by electrophoresis on a 1% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M – Marker

D12 – D14 – Parasites were first detected in the anal drop of tsetse flies on Day 12 (D12) and was observed through Day 14 (D14).

+ Positive control with *T.congolense* gDNA

- Negative control with water

4.4 Expression of *T.congolense* Trans-sialidase genes in mammalian host (Goat) and vector (Tsetse fly).

The ratio of the absorbance at 260 nm and 280 nm was used to assess the purity and amount of RNA after extraction on a Nano drop spectrophotometer (ND 2000) is shown on Table 4.3.

RNA was digested with DNase 1(Fermentas) to remove possible residual genomic DNA. The success of the digest was confirmed by performing the nested PCR reactions without reverse transcription of the RNA using ITS-1 generic primers (Figure 4.17 and 4.19). All DNase I treated RNA samples were negative in this experiment and were therefore used in the RT-PCR experiment.

Using TS specific primers by nested PCR, Figure 4.19 clearly showed the expression of TS genes in blood stream forms of *T.congolense* being detected at early, mid and chronic stages of the course the infection, 28, 38 and 58 days post infective feed on goat respectively (Table 4.2). To further authenticate amplification of TS genes, cDNA was spiked with genomic DNA, which served as a positive control while water was used as the negative control.

Table 4.3: The amount and purity of RNA assessed by ratio of the absorbance at 260/280 nm and 260/230 nm.

Sample	260/280	260/230	ng/μL
Goat blood	1.91	2.31	409.62
Fly Midgut	1.98	2.18	2289.03
Fly Proboscis	1.96	1.88	387.37
Fly Saliva	2.06	1.48	259.70

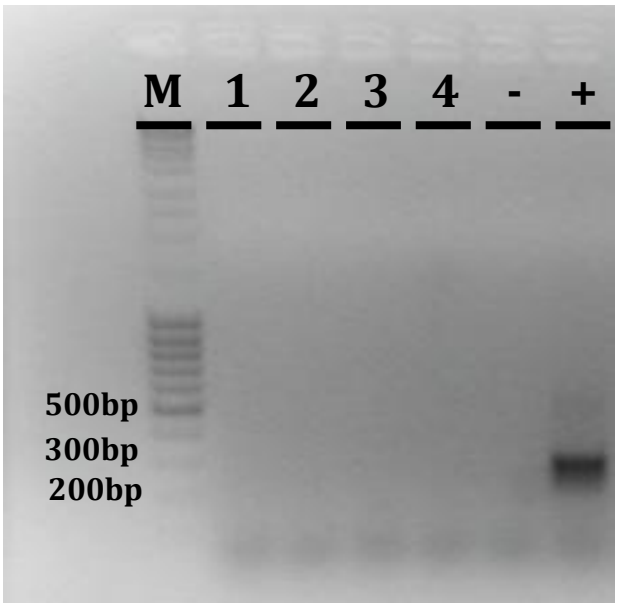


Figure 4.17: Amplification of ITS-1 region from DNase 1 treated RNA from *T.congolense* infected goat blood at different time points of the infection.

M – Marker

Lane 1 - DNase 1 treated RNA from goat blood for base line data

Lane 2 - DNase 1 treated RNA from day parasites were first detected by microscopy

Lane 3 - DNase 1 treated RNA from time point at the mid-stage of the infection

Lane 4 - DNase 1 treated RNA from time point at the chronic stage of the infection

- \- Negative control with water
- \+ Positive control

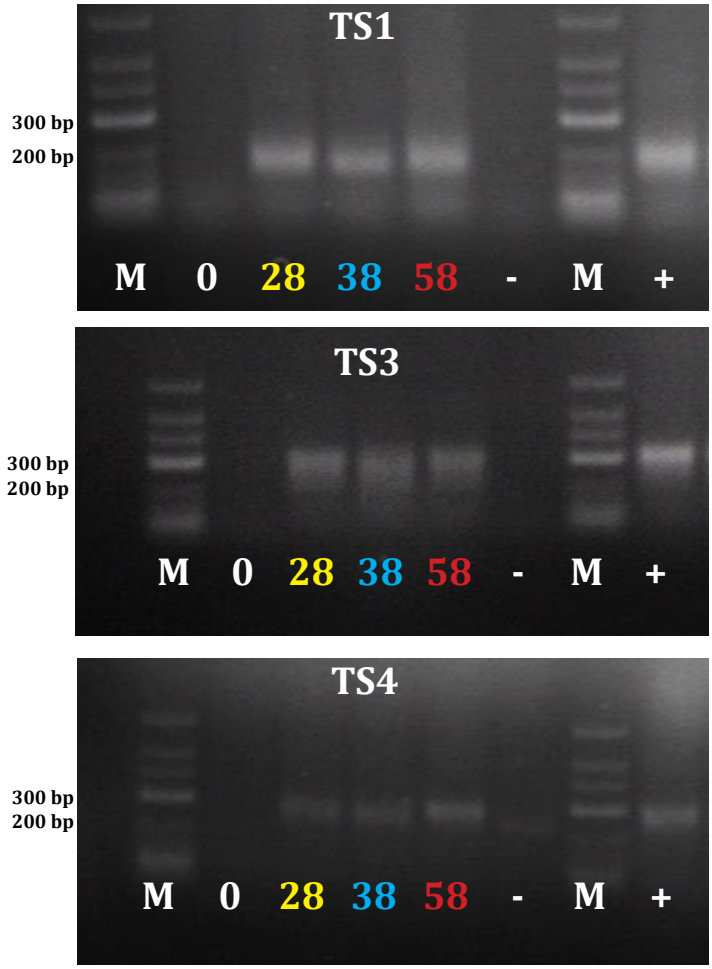


Figure 4.18: Amplification of Trans-sialidase (TS) genes with TS specific primers from cDNA of a *T.congolense* infected goat blood collected at different time points.

Lane 0 – Blood collected before onset of the experiment (for base line data)

Lane 28 – Blood collected when parasites were observed for the first time microscopically

Lane 38 – Blood collected ten days after parasites were first observed in the blood

Lane 58 – Blood collected at the chronic stage of the disease

- \- - Negative control with water
- \+ - cDNA synthesis with genomic DNA spiking

Figure 4.19 shows the absence of amplicons in the DNase 1 treated RNA samples from tsetse fly parts (midgut, proboscis and saliva) after nested PCR amplification using ITS-1 primers. However, an amplicon was observed in the positive control as expected.

Figures 4.20 and 4.21 also show the successful amplification of *T.congolense* TS genes in organs of an infected tsetse fly by PCR amplification using TS specific primers with specific product sizes for the different genes. There is a clearl indication of mRNA expression of TconTS1, TconTS2, TconTS3, TconTS4 in the midgut, proboscis and saliva of tsetse fly.

No amplification was present in the uninfected fly tissue (midgut and mouthparts) as shown in Figure 4.22.

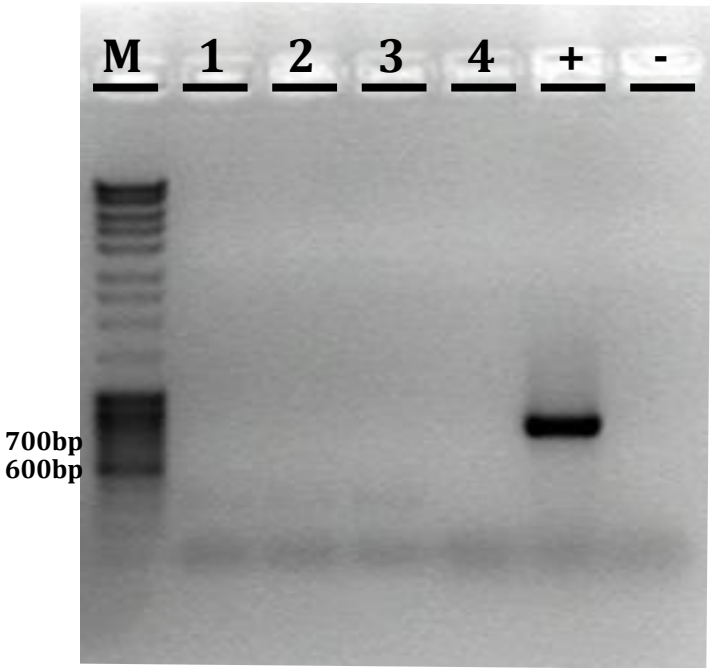


Figure 4.19: Amplification of ITS-1 region from DNase 1 treated RNA from organs a single *T.congolense* infected tsetse fly. The PCR products were analyzed by electrophoresis on a 1% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M – Marker

Lane 1 - DNase 1 treated RNA from midgut

Lane 2 - DNase 1 treated RNA from tsetse proboscis

Lane 3 - DNase 1 treated RNA from tsetse saliva

- \- Negative control with water
- \+ Positive control (*T.congolense* gDNA)

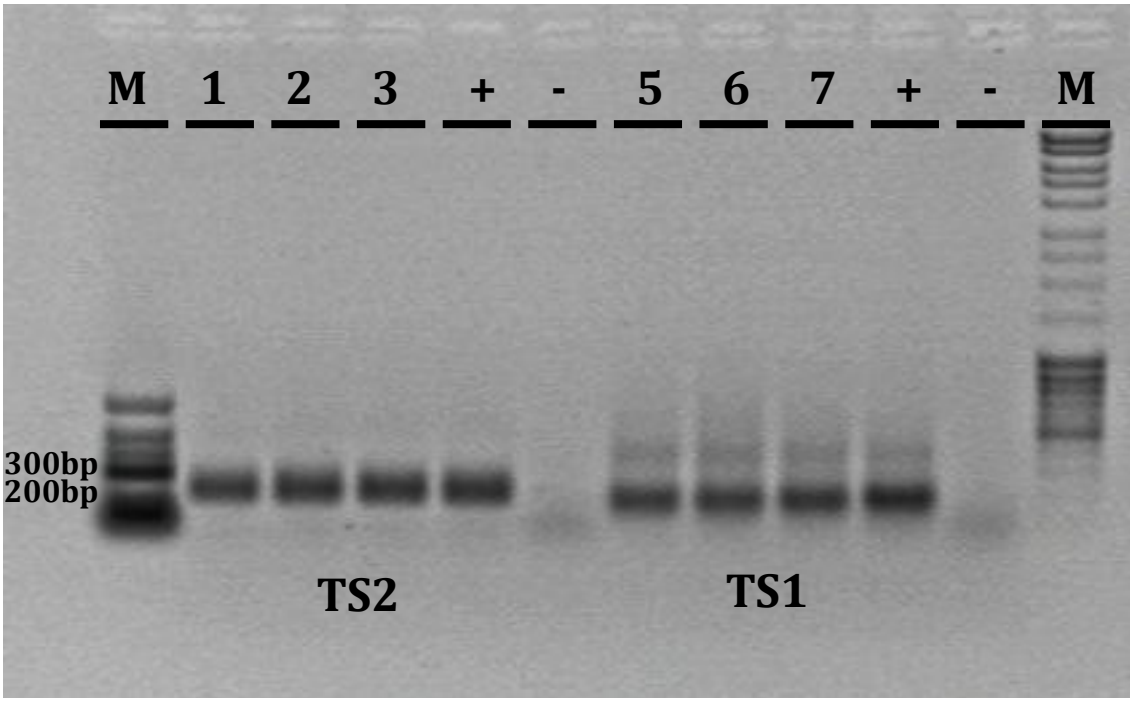


Figure 4.20: mRNA Expression of *T.congolense* TS genes in an infected tsetse fly by PCR amplification using TS specific primers. The PCR products were analyzed by electrophoresis on a 1.5% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M – Marker

Lane 1 - cDNA from tsetse midgut

Lane 2 - cDNA from tsetse proboscis

Lane 3 - cDNA from tsetse saliva

- \- Negative control with water
- \+ Positive control

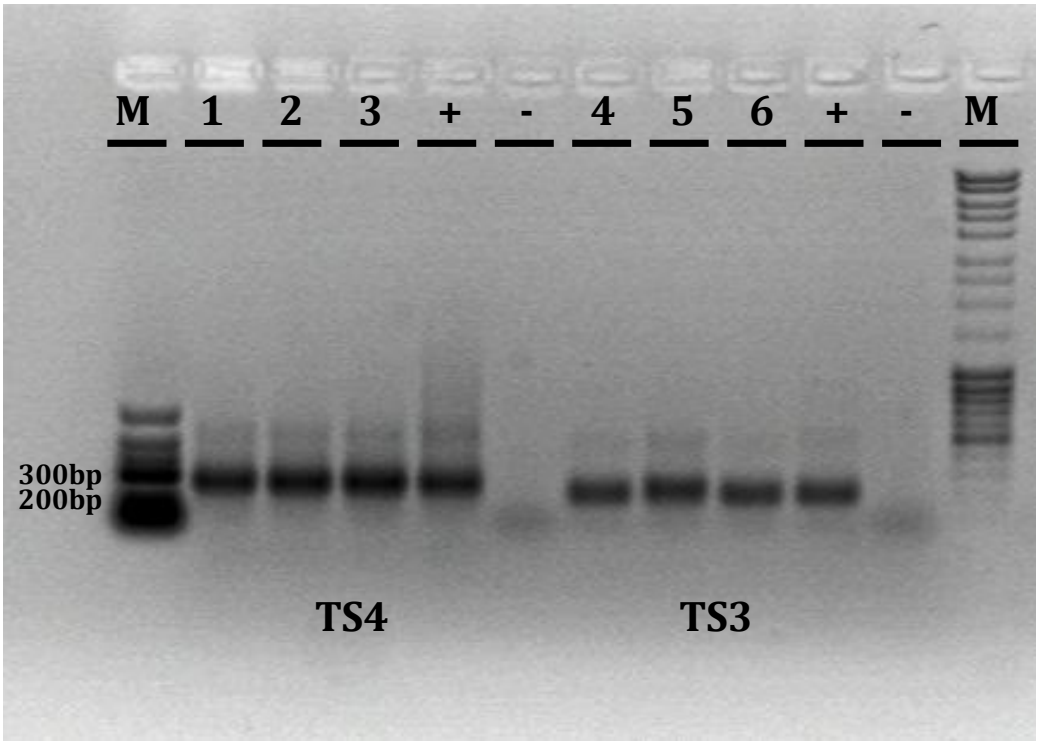


Figure 4.21: mRNA Expression of *T.congolense* TS genes in an infected tsetse fly by PCR amplification using TS specific primers. The PCR products were analyzed by electrophoresis on a 1.5% Agarose gel and stained with G-Stain (Serva Electrophoreis GmbH, Heidelberg, Germany).

M – Marker

Lane 1 - cDNA from tsetse midgut

Lane 2 - cDNA from tsetse proboscis

Lane 3 - cDNA from tsetse saliva

- \- Negative control with water
- \+ Positive control

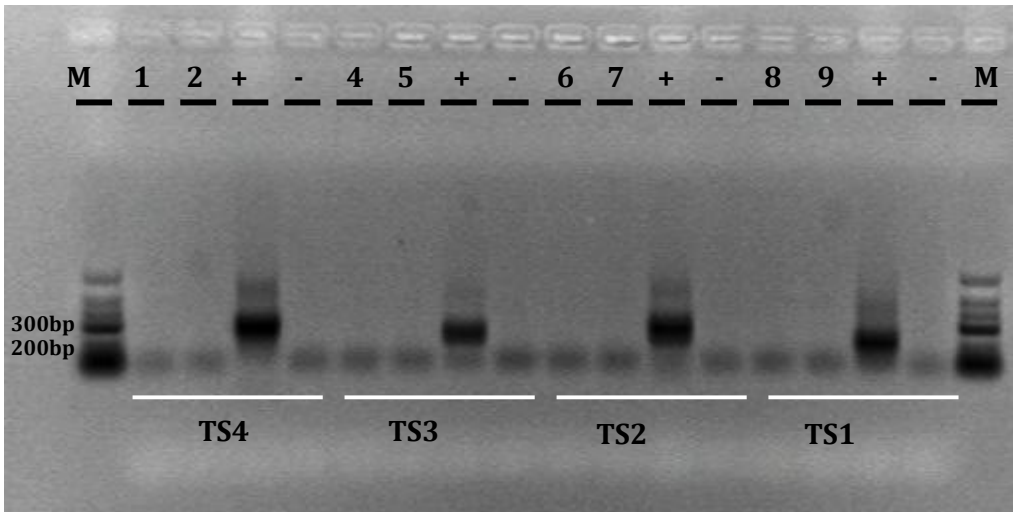


Figure 4.22: Amplification of TS genes from mouthpart and midgut of uninfected tsetse flies. The PCR products were analyzed by electrophoresis on a 1% Agarose gel and stained with G-Stain (Serva Electrophoresis GmbH, Heidelberg, Germany).

Lane 1, 4, 6, 8 – cDNA prepared from the mouthpart of an uninfected fly.

Lane 2, 5, 7, 9 – cDNA prepared from the midgut of an uninfected fly.

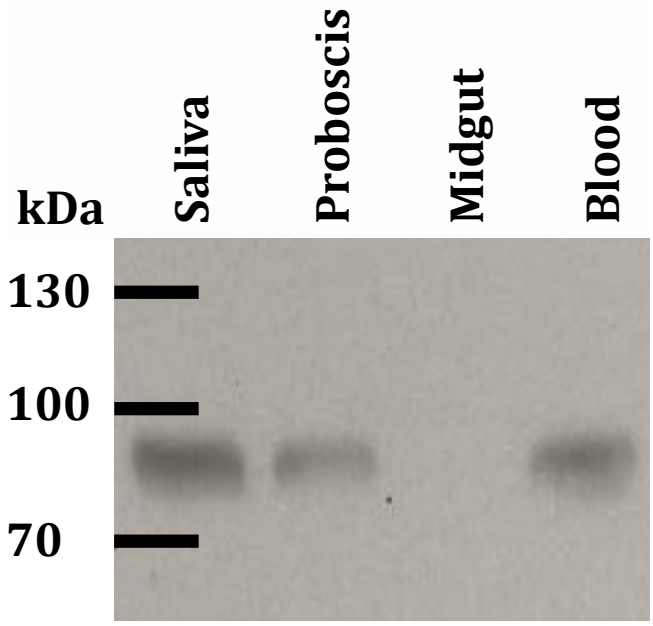
+ Positive control (cDNA from midgut of an infected fly)

- Negative control with water

4.5 *T.congolense* Trans-sialidase protein expression by immunoblotting with Anti-TconTS monoclonal antibody

Samples from saliva, proboscis, midgut and blood were analysed with anti-TconTS monoclonal antibody. As shown in Figure 4.23 (1), TconTS protein was detected from saliva and proboscis of infected flies and in the blood of infected goats. However, there was no detection of the protein in the midgut. The ~90 kDA TconTS protein detected concurred with the results of Tiralongo *et.al*., 2003 (Figure 4.23 (2).

1.



2.

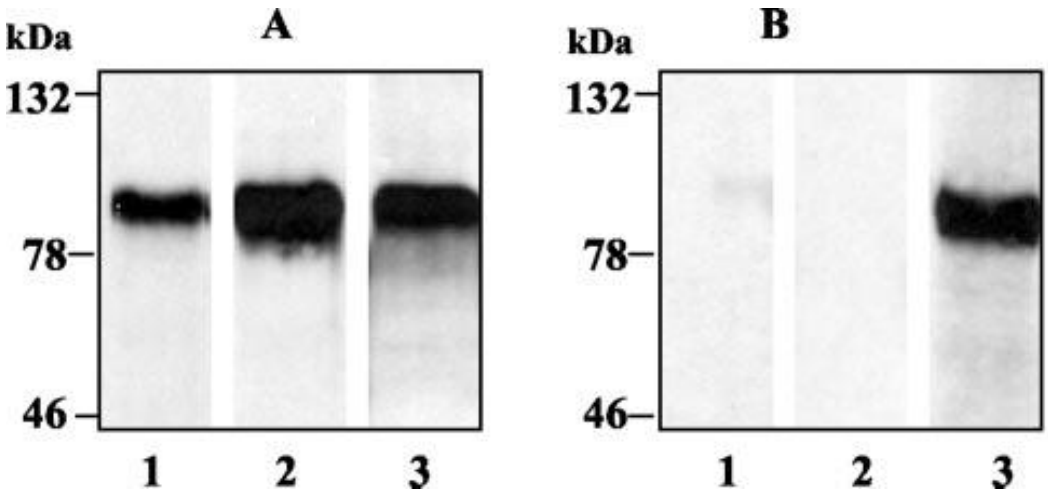


Figure 4.23: 1. Immunoblots of *T. congolense* TS forms with anti-*T. congolense* TS1 monoclonal antibody (mAb). Lane 1, Saliva from *T.congolense* infected tsetse fly. Lane 2, Proboscis of *T.congolense* infected tsetse fly. Lane 3, midgut from *T.congolense* infected tsetse fly. Lane 4, blood from *T.congolense* infected goat.

2. Immunoblots of *T. congolense* TS forms with anti-*T.congolense* TS mAb (mAb 7/23). (Tiralongo *et.al*., 2003).

4.6 Quantitative *T.congolense* Trans-sialidase gene expression by Real-Time PCR

Highly specific primers and probes targeting TS1, TS2, TS3 and TS4 mRNA genes were designed having appropriate melting temperatures and with no tendency to form secondary structures. When tested in a conventional PCR with TS1, TS2, TS3, TS4 plasmids, they generated short amplicons of the predicted sizes; 133bp, 129bp, 136bp and 136bp respectively, which is necessary in real-time PCR to achieve maximal efficiency (Table 3.3, Figure 4.24). A Primer BLAST search in GenBank revealed that the primers had no matches with *Capra hircus*, *Glossina morsitans morsitans* genome as well as genomes of other related species, which ensured specificity of primers in goat blood or tsetse samples.

First, the primers were tested with purified *T.congolense* TS plasmids to evaluate real-time PCR performance. Log-linear standard curves were obtained by plotting the C_T values of the assay versus the input Plasmid DNA amount (which is represented as gene copy number). With C_T values between 18.6 and 35.9, the real-time PCR detected *T.congolense* TS genes amounts with linear relationship about five orders of magnitude between 10 pg and 1 fg per 10μL reaction which covers 9.26×10^5 to 92.6 copies per ml respectively. By analyzing these serial dilutions, no signal could be obtained from samples containing ≤1fg of plasmid DNA. To increase the specificity of the Trans-sialidase assay, the primer annealing temperature was optimized at 61^0C, 64^0C, 60^0C, 60^0C for TS1, TS2, TS3, TS4 respectively. The assay performance of the primers was assessed in separate reactions. The TS1 assay efficiency was 90.1%, slope Y= -3.611 and RSq: 0.997, TS2 assay efficiency was 97.8%, slope Y= -3.375 and RSq: 0.997, TS3 assay efficiency was 96.8%, slope Y= -3.401 and RSq: 0.989, TS4 assay efficiency was 91.6%, slope Y= -3.541 and RSq: 0.999.

Figures 4.25, 4.28, 4.31 and 4.34 are amplification plots of purified TconTS1, TconTS2, TconTS3 and TconTS4 plasmids respectively which were assayed in triplicates generated after a 10-fold serial dilution of the plasmids which were used in 10µL PCR assays for the detection and absolute quantification of *Trypanosoma congolense* trans-sialidase genes in the different biological samples.

PCR efficiency plots of purified *T.congolense* TS1, TS2, TS3, and TS4 plasmid DNA by real-time PCR is shown in Figures 4.26, 4.29, 4.32 and 4.35 respectively. 10-fold serial dilutions of the different plasmids were used in 10-µL real-time PCR assays with TconTS1F, TconTS1R primers and TconTS1 probe with TaqMan as detector. Data points represent triplicate assays.

Standard curves of the different purified *T.congolense* TS plasmids for detection of TS genes by real-time PCR presented as plots of mean C_T versus the number of copies/ng of Plasmid DNA. PCR assay was performed in triplicates and data points represent mean values of three independent assays (Figure 4.27, 4.30, 4.33, 4.36).

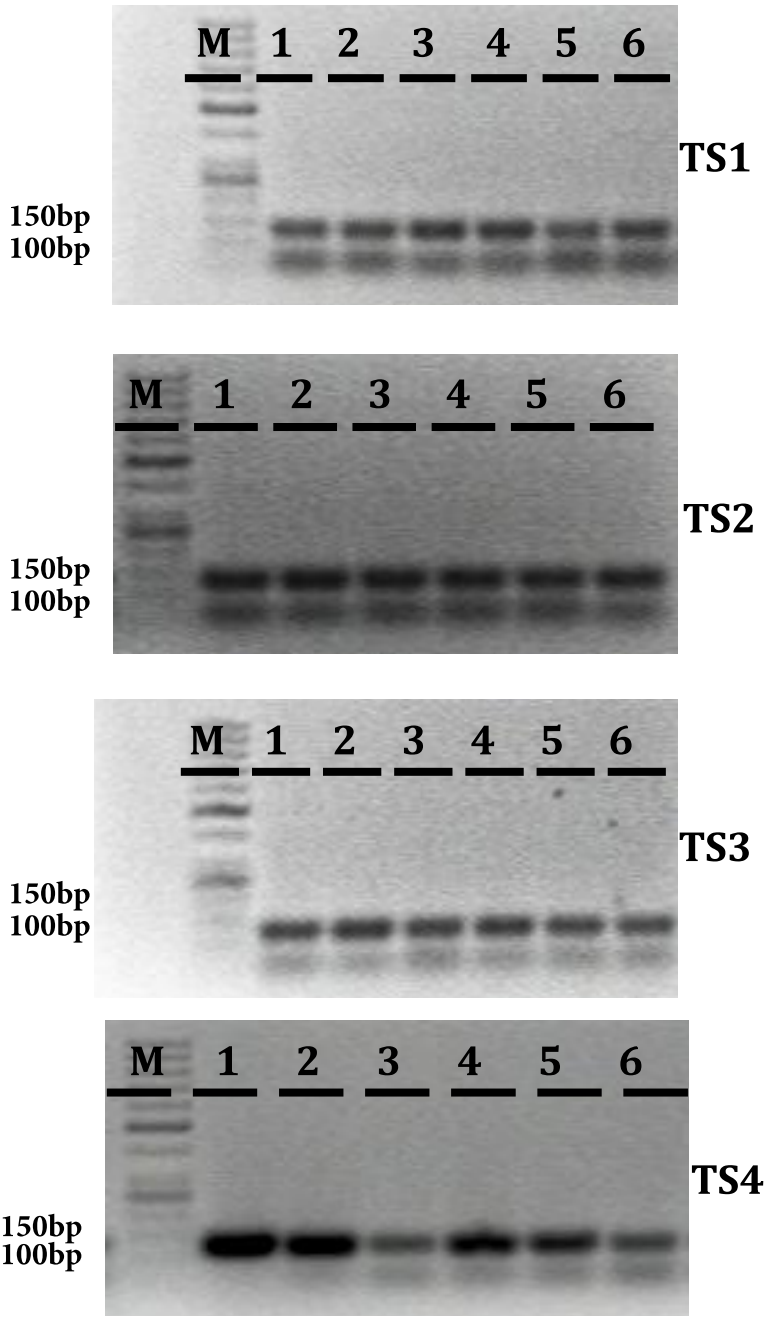


Figure 4.24: Amplification of TS plasmid DNA to check effectiveness of TS primers. The PCR products were analyzed by electrophoresis on a 1.5% Agarose gel against an O'GeneRuler ™ 50bp ladder (Fermentas) and stained with G-Stain (Serva Electrophoresis GmbH, Heidelberg, Germany).

- Lanes 1, 2, 3, 4, 5, and 6 represent 1ng of the different isoforms of TconTS plasmids

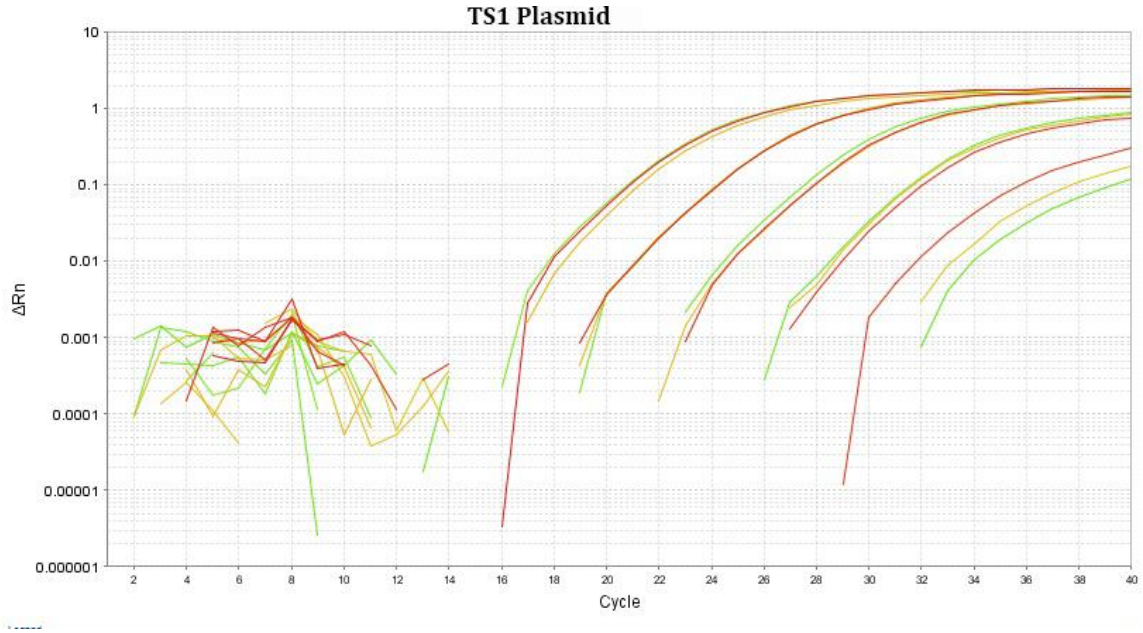


Figure 4.25: Amplification plot of log-fold dilutions of purified *T.congolense* TS1 plasmid DNA (10pg-0.1fg) which were assayed in triplicates using TaqMan Fast Universal PCR Master mix and a dual fluorescent probe and primers targeting the trans-sialidase gene. Assay was performed in a Step-One Plus Real-Time PCR System (Applied Biosystems) instrument according to the manufacturers recommended protocol.

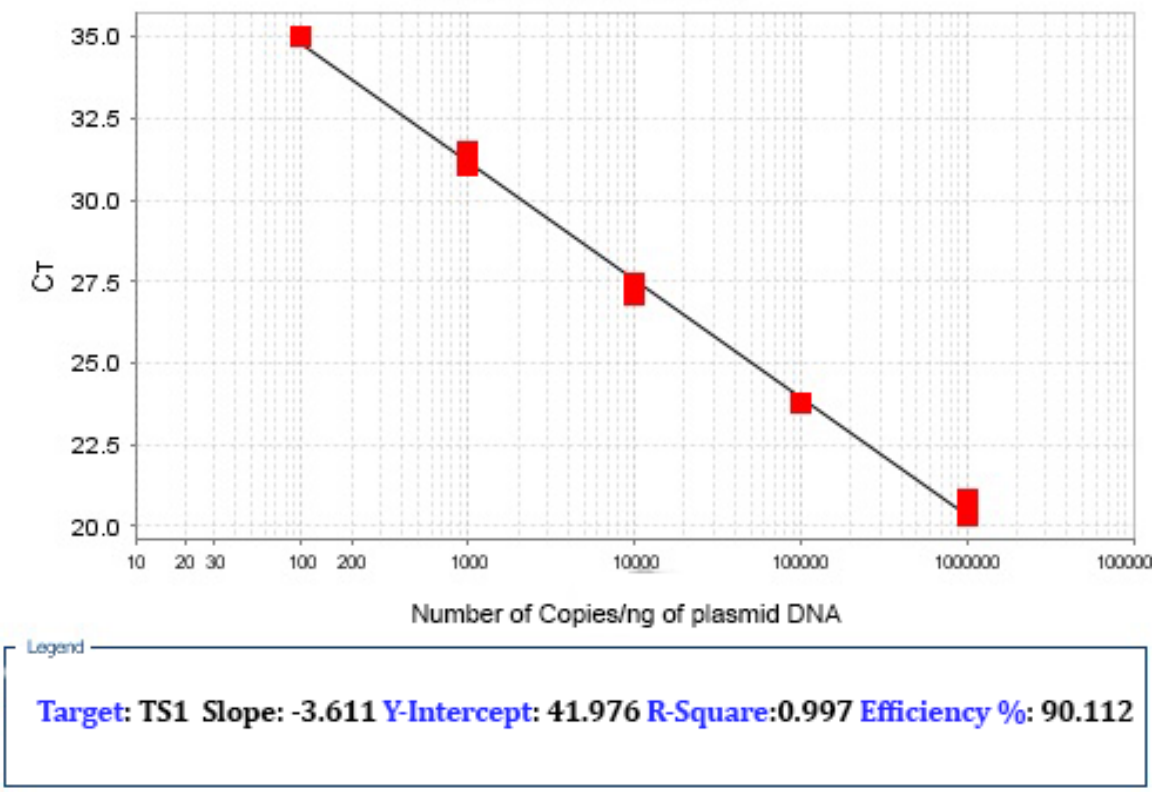


Figure 4.26: PCR efficiency plot of purified *T.congolense* TS1 plasmid DNA for detection of TS1 gene by real-time PCR. 10-fold serial dilutions of *T.congolense* TS1 plasmid DNA were used in 10-µL real-time PCR assays with TconTS1F, TconTS1R primers and TconTS1 probe with TaqMan as detector. Data points represent mean values of three independent assays. From the slope of the curve, the amplification efficiency of 90.112% was calculated.

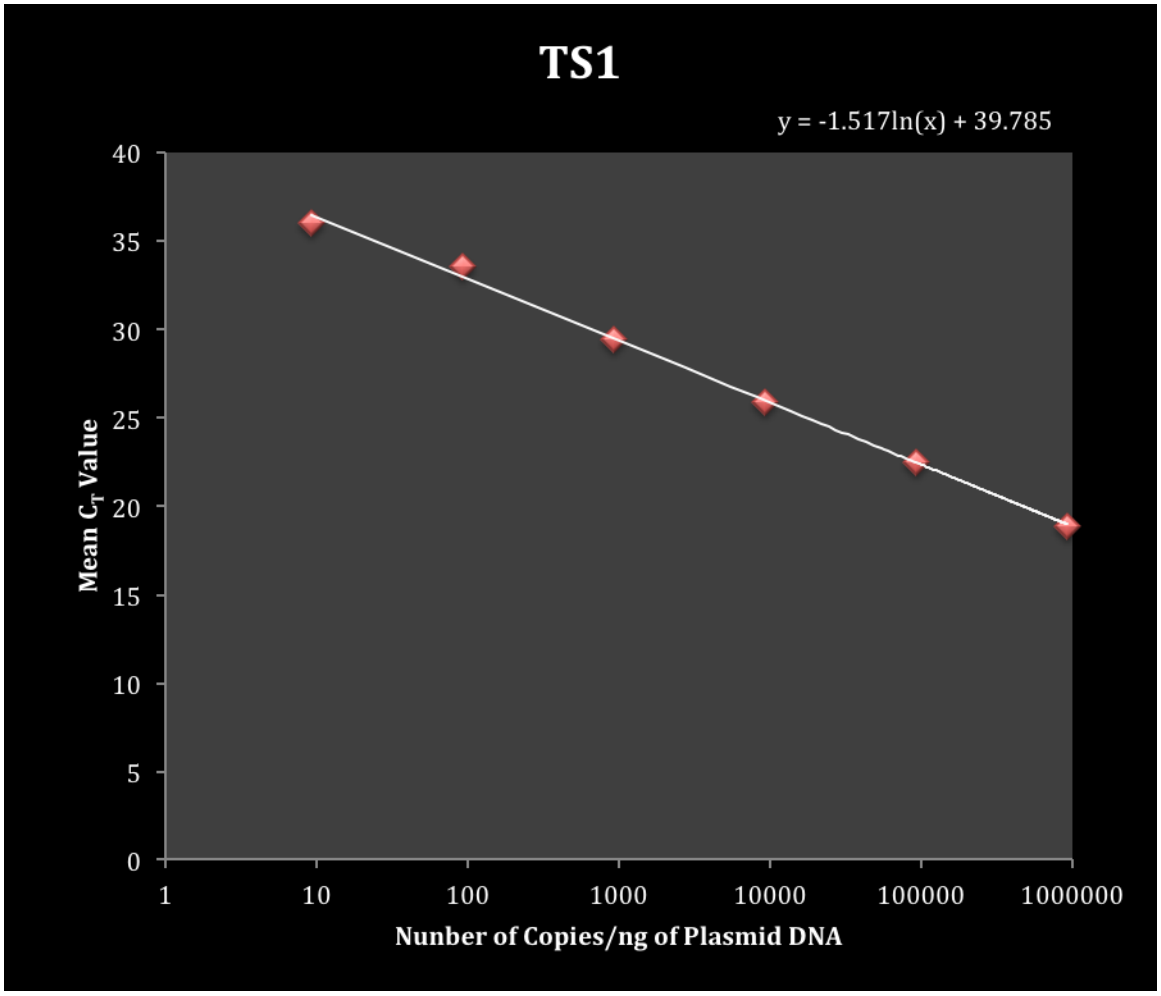


Figure 4.27: Standard curve of purified *T.congolense* TS1 plasmid DNA for detection of TS1 gene by real-time PCR presented as a plot of mean C_T versus the number of copies/ng of Plasmid DNA. PCR assay was performed in triplicates and data points represent mean values of three independent assays.

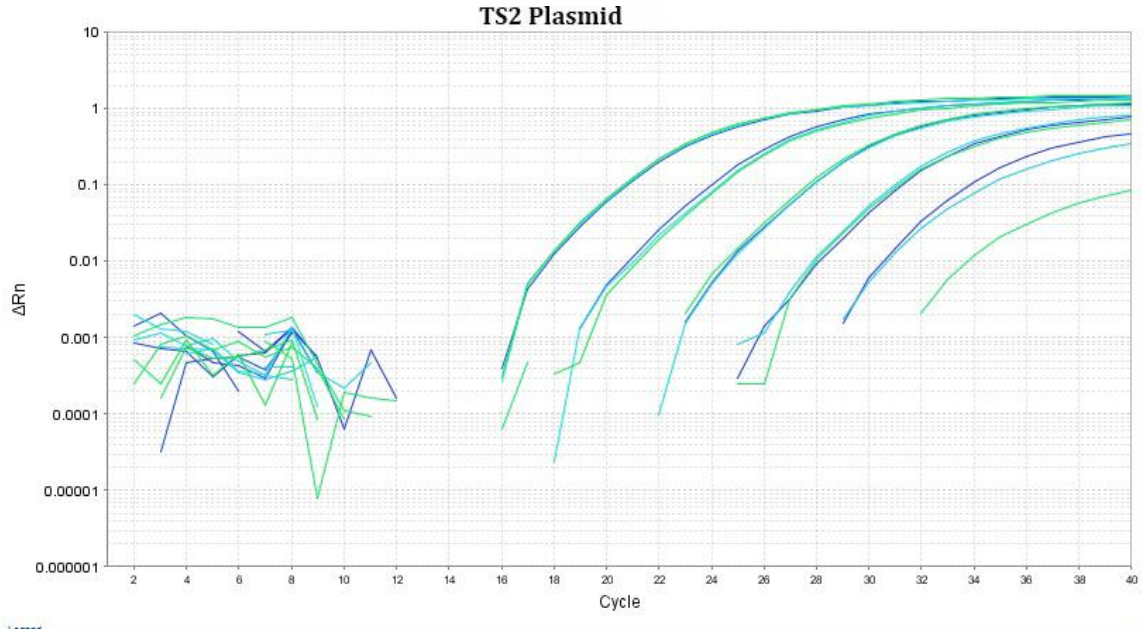


Figure 4.28. Amplification plot of log-fold dilutions of purified *T.congolense* TS2 plasmid DNA (10pg-0.1fg) which were assayed in triplicates using TaqMan Fast Universal PCR Master mix and a dual fluorescent probe and primers targeting the trans-sialidase gene. Assay was performed in a Step-One Plus Real-Time PCR System (Applied Biosystems) instrument according tot he manufacturers recommended protocol.

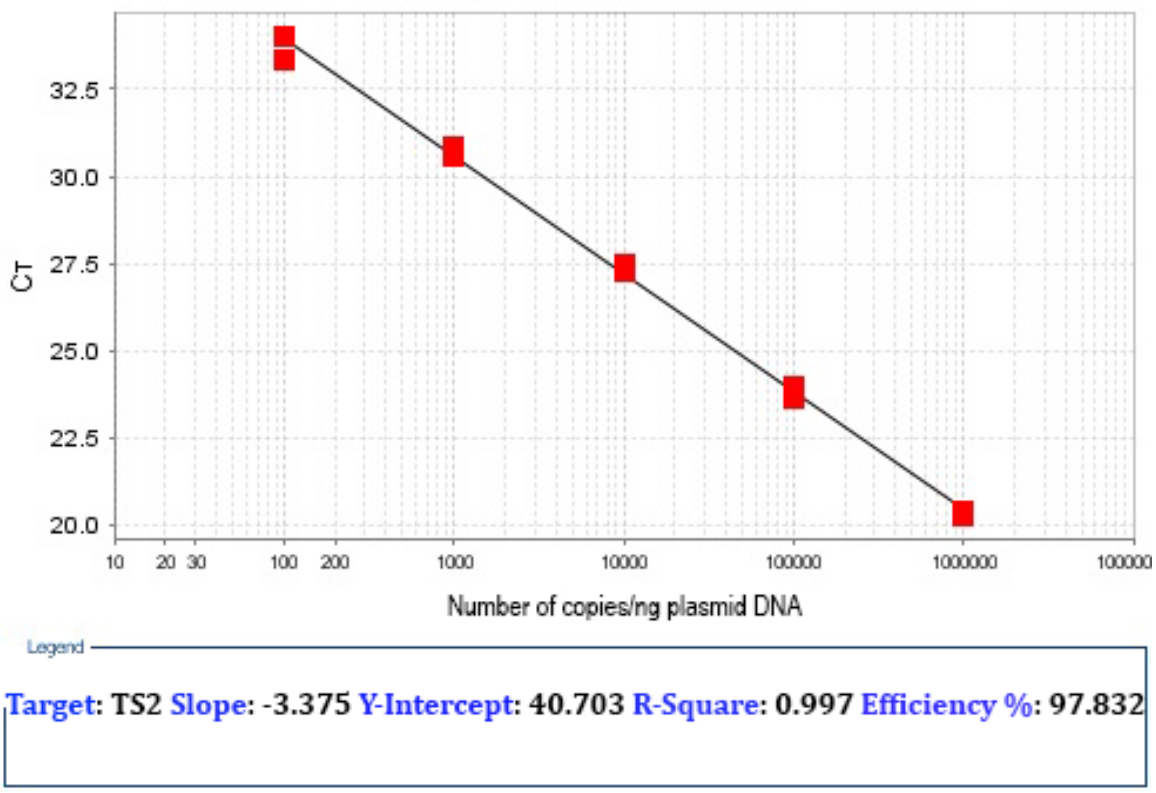


Figure 4.29: PCR efficiency plot of purified *T.congolense* TS2 plasmid DNA for detection of TS2 gene by real-time PCR. 10-fold serial dilutions of *T.congolense* TS2 plasmid DNA were used in 10-µL real-time PCR assays with TconTS2F, TconTS2R primers and TconTS2 probe with TaqMan as detector. Data points represent mean values of three independent assays. From the slope of the curve, the amplification efficiency of 97.832% was calculated.

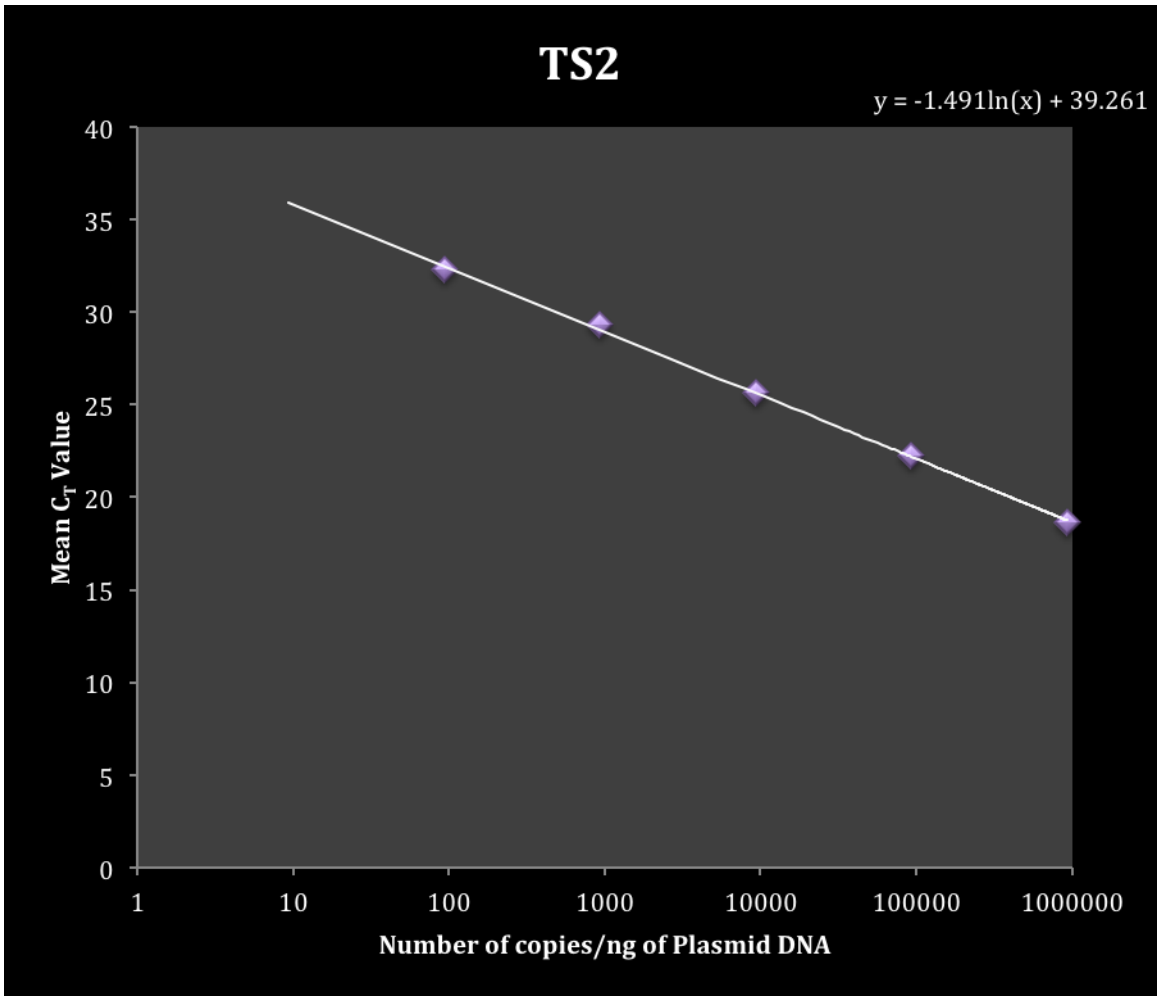


Figure 4.30: Standard curve of purified *T.congolense* TS2 plasmid DNA for detection of TS1 gene by real-time PCR presented as a plot of mean C_T versus the number of copies/ng of Plasmid DNA. PCR assay was performed in triplicates and data points represent mean values of three independent assays.

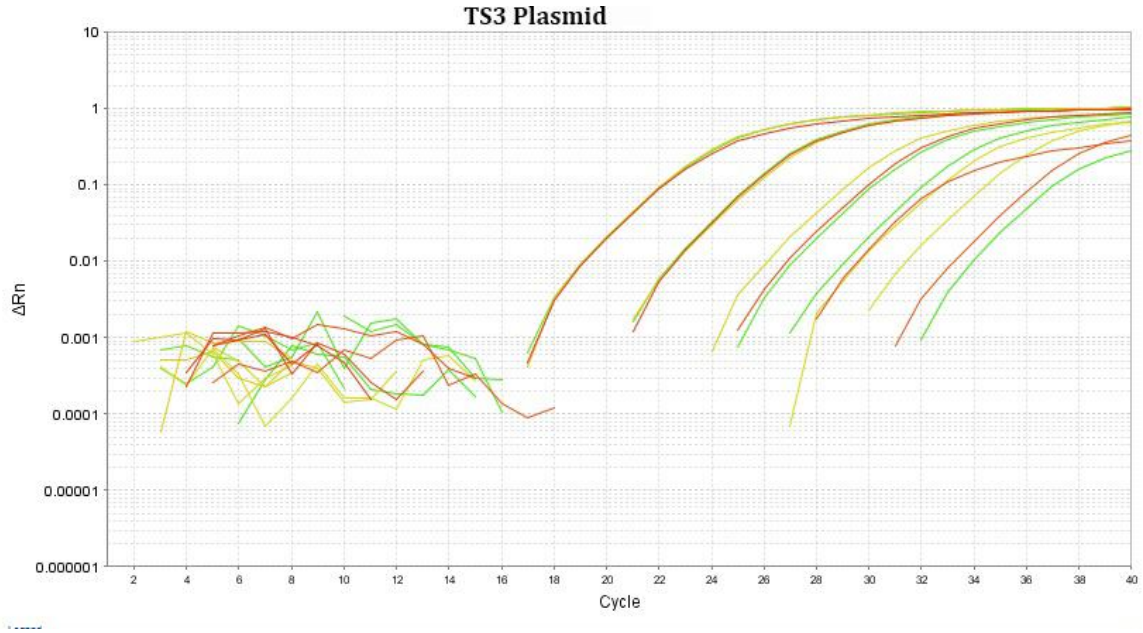


Figure 4.31: Amplification plot of log-fold dilutions of purified *T.congolense* TS3 plasmid DNA (10pg-0.1fg) which were assayed in triplicates using TaqMan Fast Universal PCR Master mix and a dual fluorescent probe and primers targeting the trans-sialidase gene. Assay was performed in a Step-One Plus Real-Time PCR System (Applied Biosystems) instrument according tot he manufacturers recommended protocol.

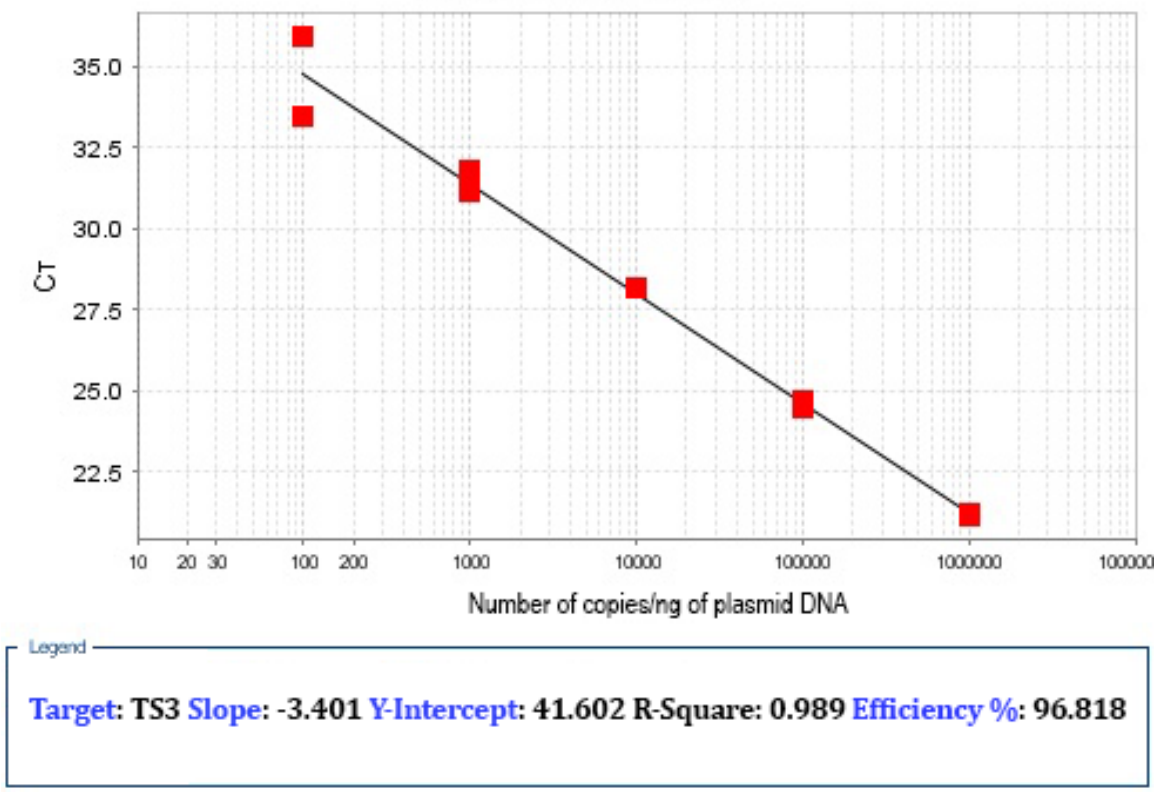


Figure 4.32: PCR efficiency plot of purified *T.congolense* TS3 plasmid DNA for detection of TS3 gene by real-time PCR. 10-fold serial dilutions of *T.congolense* TS3 plasmid DNA were used in 10-µL real-time PCR assays with TconTS3F, TconTS3R primers and TconTS3 probe with TaqMan as detector. Data points represent mean values of three independent assays. From the slope of the curve, the amplification efficiency of 96.818% was calculated.

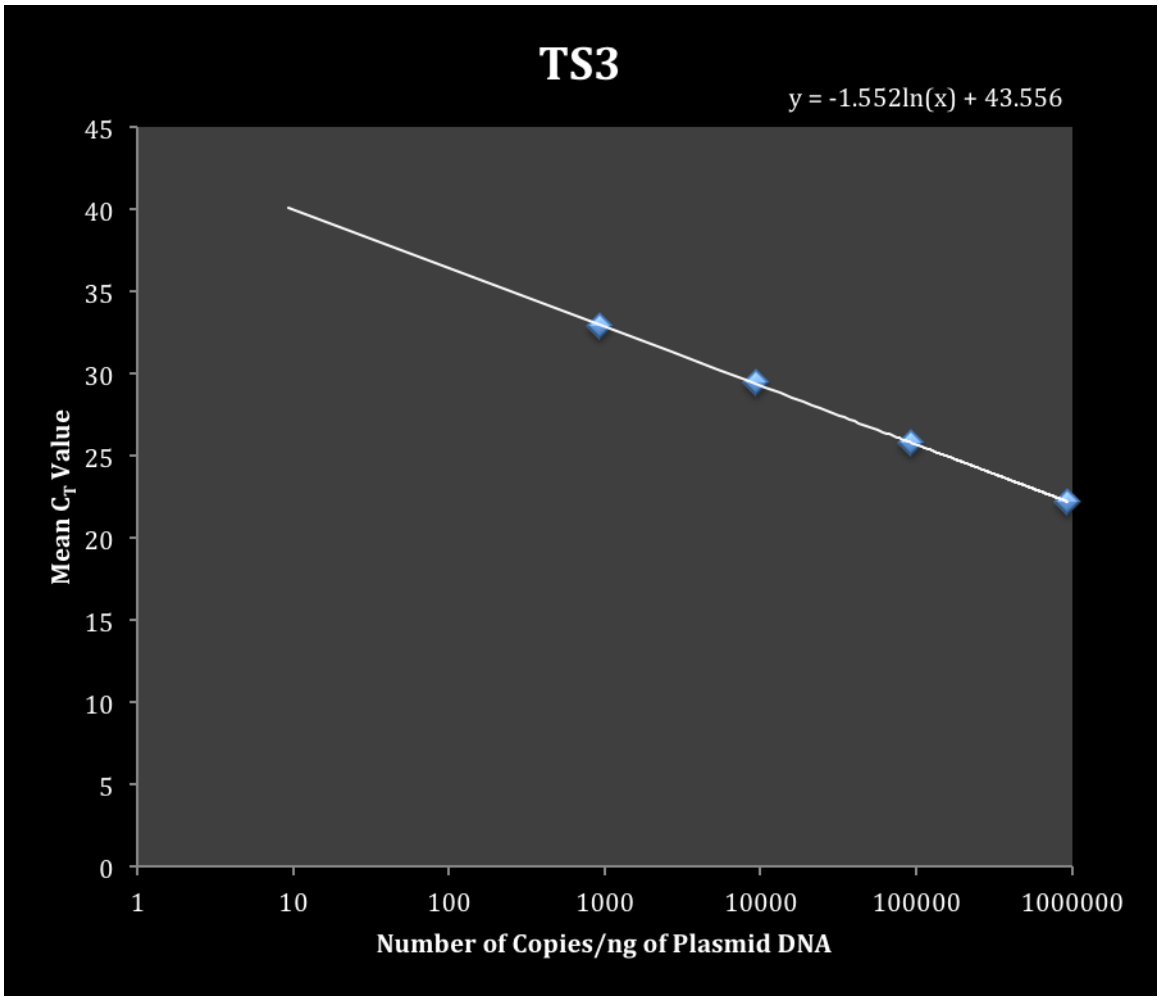


Figure 4.33: Standard curve of purified *T.congolense* TS3 plasmid DNA for detection of TS1 gene by real-time PCR presented as a plot of mean C_T versus the number of copies/ng of Plasmid DNA. PCR assay was performed in triplicates and data points represent mean values of three independent assays.

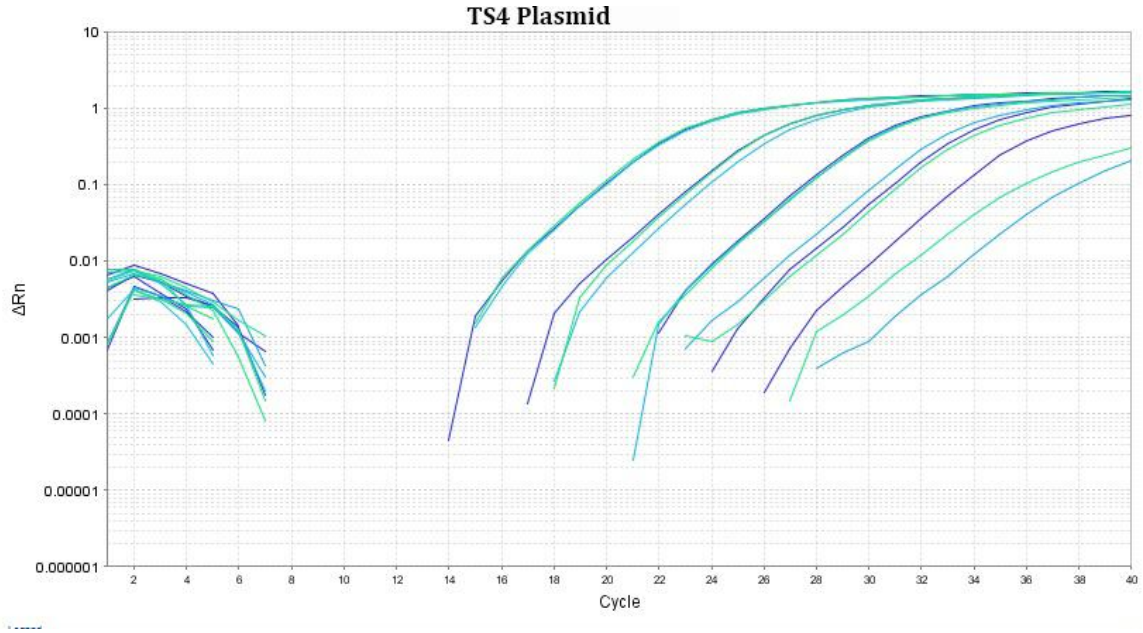


Figure 4.34: Amplification plot of log-fold dilutions of purified *T.congolense* TS4 plasmid DNA (10pg-0.1fg) which were assayed in triplicates using TaqMan Fast Universal PCR Master mix and a dual fluorescent probe and primers targeting the trans-sialidase gene. Assay was performed in a Step-One Plus Real-Time PCR System (Applied Biosystems) instrument according tot he manufacturers recommended protocol.

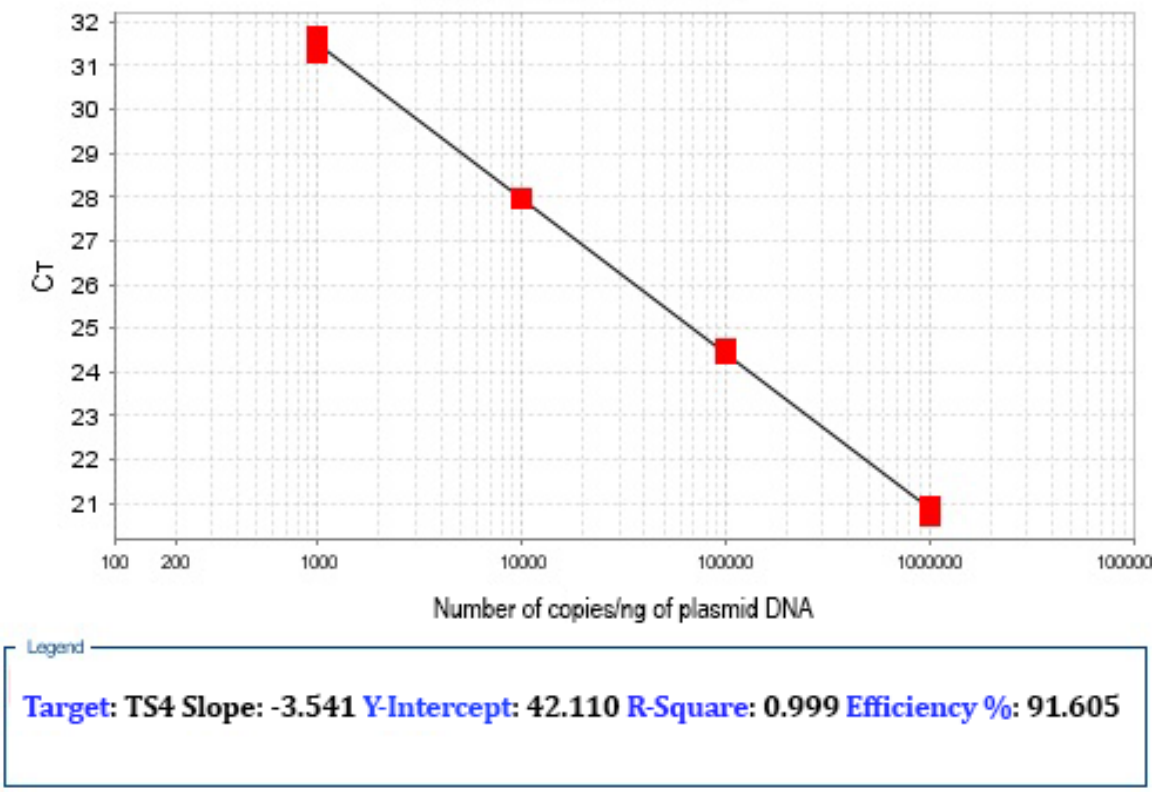


Figure 4.35: PCR efficiency plot of purified *T.congolense* TS4 plasmid DNA for detection of TS4 gene by real-time PCR. 10-fold serial dilutions of *T.congolense* TS4 plasmid DNA were used in 10-µL real-time PCR assays with TconTS4F, TconTS4R primers and TconTS4 probe with TaqMan as detector. C_T values were plotted against the amount of plasmid DNA in single assays. Data points represent mean values of three independent assays. From the slope of the curve, the amplification efficiency of 91.605% was calculated.

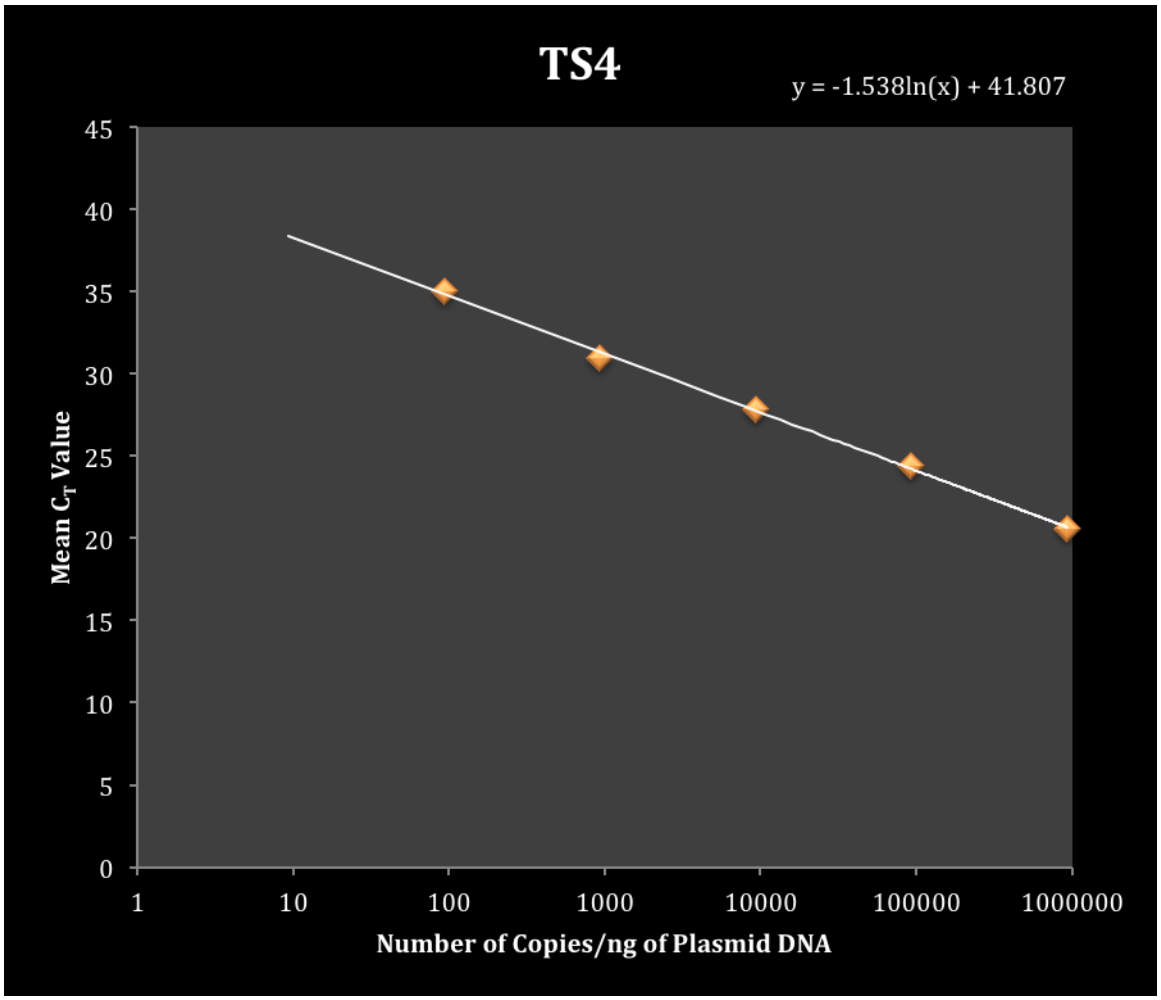


Figure 4.36: Standard curve of purified *T.congolense* TS4 plasmid DNA for detection of TS1 gene by real-time PCR presented as a plot of mean C_T versus the number of copies/ng of Plasmid DNA. PCR assay was performed in triplicates and data points represent mean values of three independent assays.

Next, the real-time PCR assay was evaluated with cDNA from goat blood and tsetse fly organ samples that contained various numbers and forms of *T.congolense.* Considering, the sensitivity of the real-time PCR assay, absolute care was taken in RNA and cDNA preparation. This was observed in the purity and amount shown in the spectral pattern assessed by absorbance ratio of approximately 2.0 and 2.2 at 260/280 nm and 260/230 nm respectively on a Nano drop spectrophotometer (ND 2000). Spectral patterns of all RNA samples also showed an absorbance peak at 260 nm, which further confirmed the purity of the RNA samples (Figure 4.37, 4.38, 4.39, 4.40).

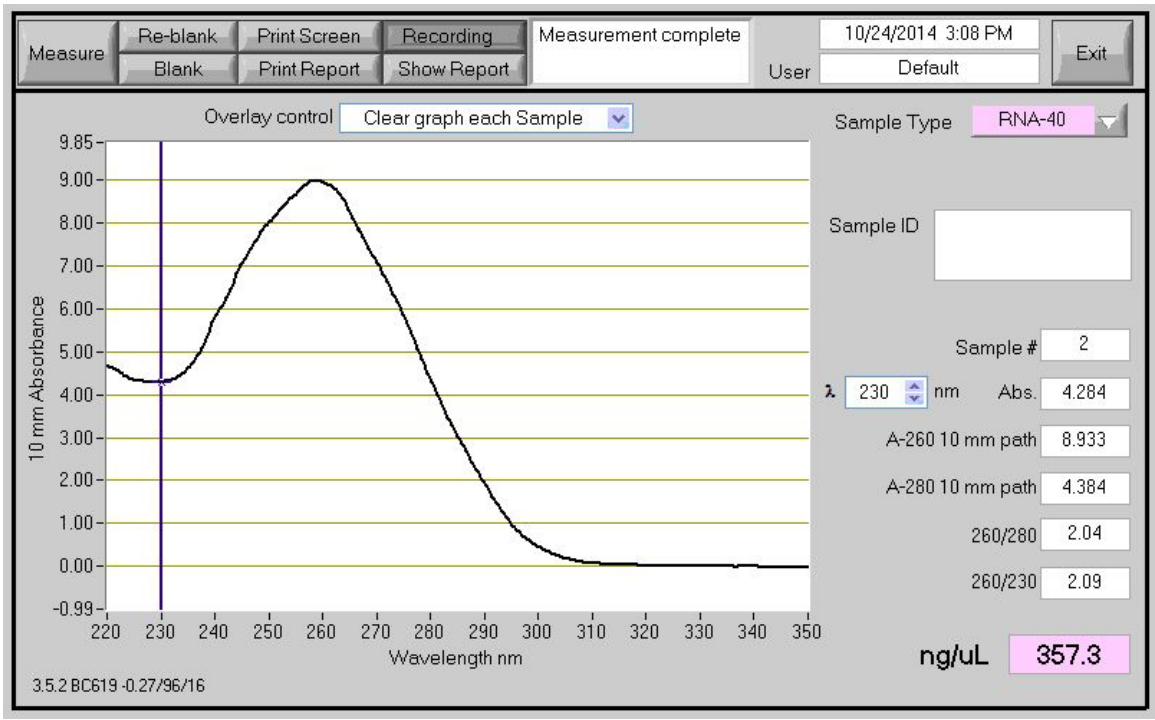


Figure 4.37: Spectral pattern of RNA from blood of a *T.congolense* infected goat. The purity of the samples was assessed by the absorbance ratios of 2.04 and 2.09 at 260/280nm and 260/230nm respectively with a yield of 357.3ng/μL.

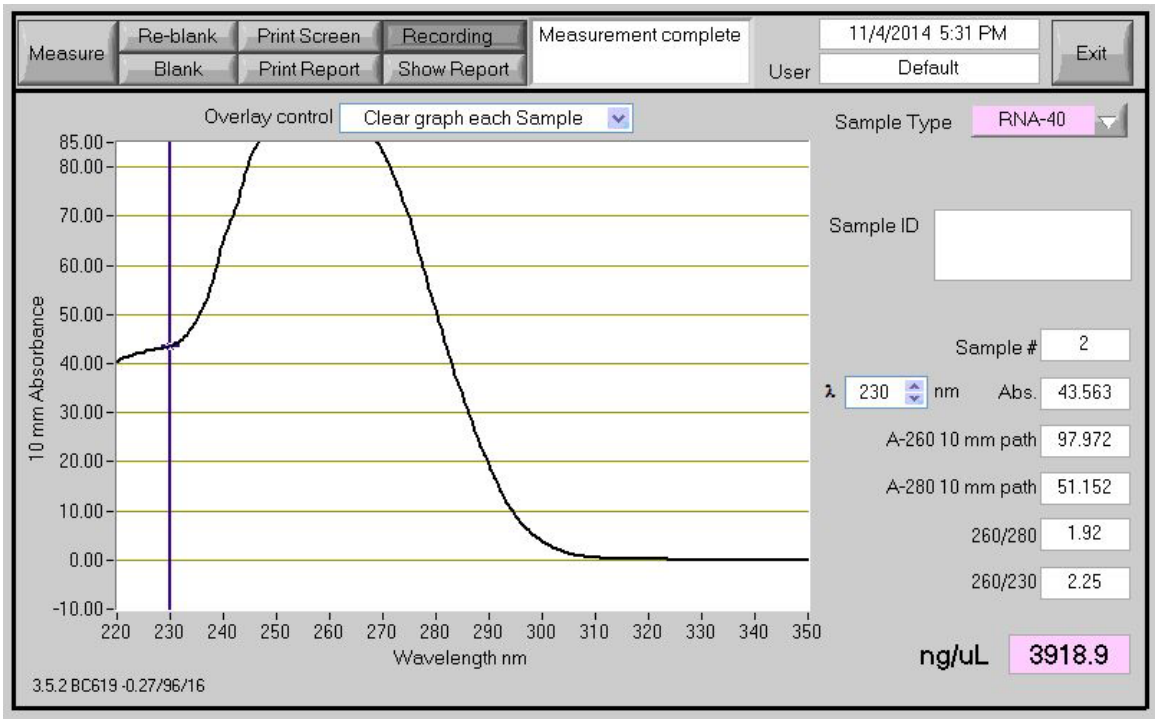


Figure 4.38: Spectral pattern of RNA from midgut of a *T.congolense* infected tsetse fly. The purity of the samples was assessed by the absorbance ratios of 1.92 and 2.25 at 260/280nm and 260/230nm respectively with a yield of 3918.9ng/μL.

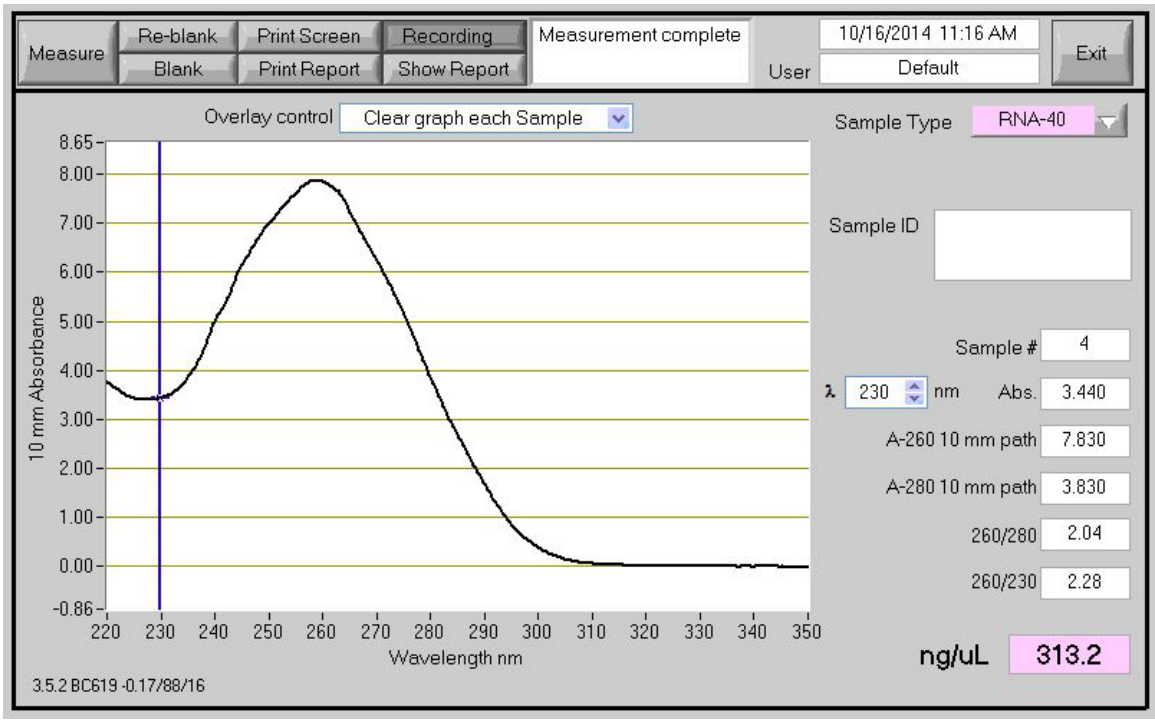


Figure 4.39: Spectral pattern of RNA from proboscis of a *T.congolense* infected tsetse fly. The purity of the samples was assessed by the absorbance ratios of 2.04 and 2.28 at 260/280nm and 260/230nm respectively with a yield of 313.2ng/μL.

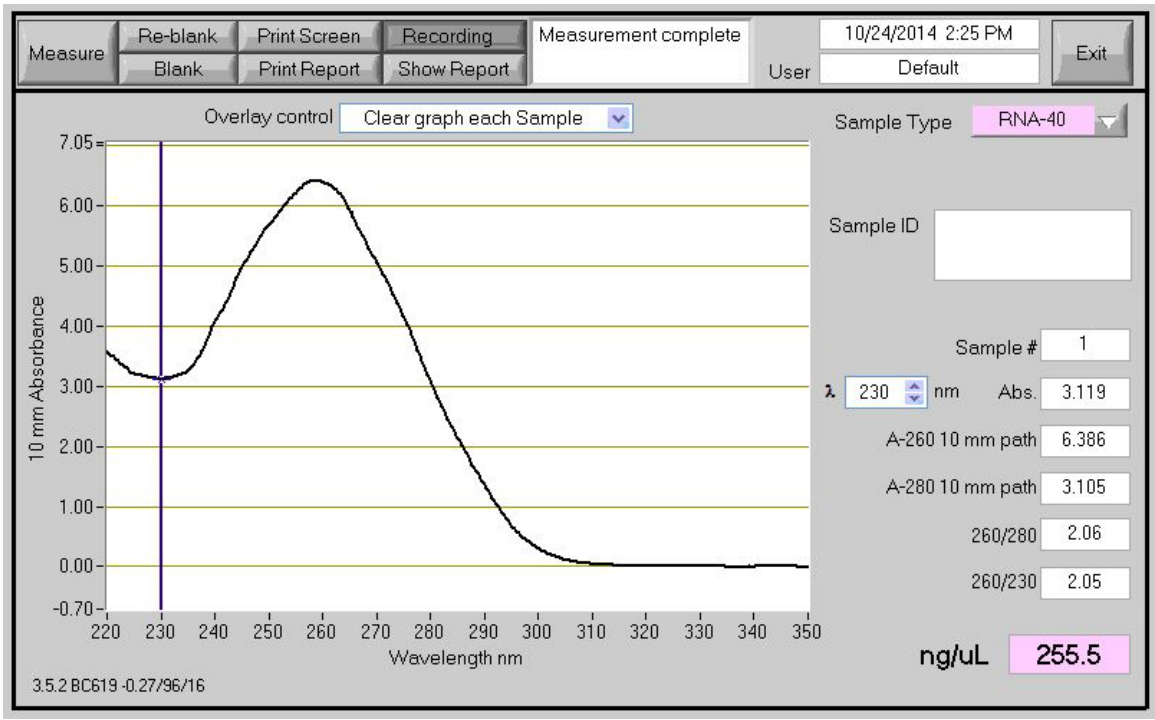


Figure 4.40: Spectral pattern of RNA from saliva of a *T.congolense* infected tsetse fly. The purity of the samples was assessed by the absorbance ratios of 2.06 and 2.05 at 260/280nm and 260/230nm respectively with a yield of 255.5ng/μL.

The identities of amplification curves of the log-fold dilutions of the cDNA (Goat blood, midgut, proboscis and saliva) as shown in Figures 4.41, 4.42, 4.43 and 4.44 confirms that the real-time PCR assay detected the presence of the TS isoforms in goat blood and tsetse fly samples, which resulted in the positive signals with mean C_T values within the linear range of the standard curves (Table 4.4).

TS4 was the most expressed in blood samples with a mean C_T value and copy number of 22.26 ± 0.03 and 330497.5 respectively (Figure 4.45) while TS1 was the most expressed in fly samples (Table 4.4). A steady stage-specific increase in the expression of all TS isoforms was observed in the procyclic forms but an approximated 3-fold decrease in the expression of TS2 and TS3 from 16,417 and 62,242.2 copies/ng plasmid DNA to 9139.6 and 19,916.2 copies/ng plasmid DNA respectively was observed when the metacyclic forms were transformed into blood stream forms (Table 4.4). However, the steady increase in TS1 and TS4 expression was maintained in the entire life cycle of *T.congolense* (Figure 4.45). TS2 on the other hand showed the lowest expression in all samples analyzed (Table 4.4).

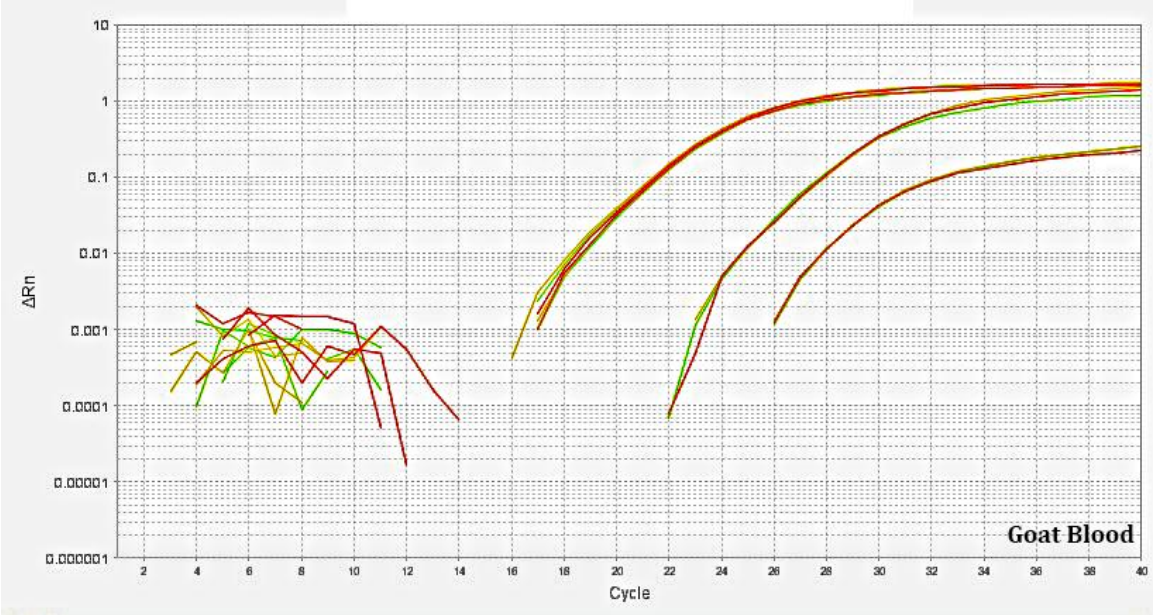


Figure 4.41: Real Time PCR amplification plot of *Trypanosoma congolense* Trans-sialidase cDNA from Goat blood which were assayed in triplicates using TaqMan Fast Universal PCR Master mix and a dual fluorescent probe and primers (TconTS Fand TconTSR) targeting the trans-sialidase gene. Assay was performed in a Step-One Plus Real-Time PCR System (Applied Biosystems) instrument according tot he manufacturers recommended protocol.

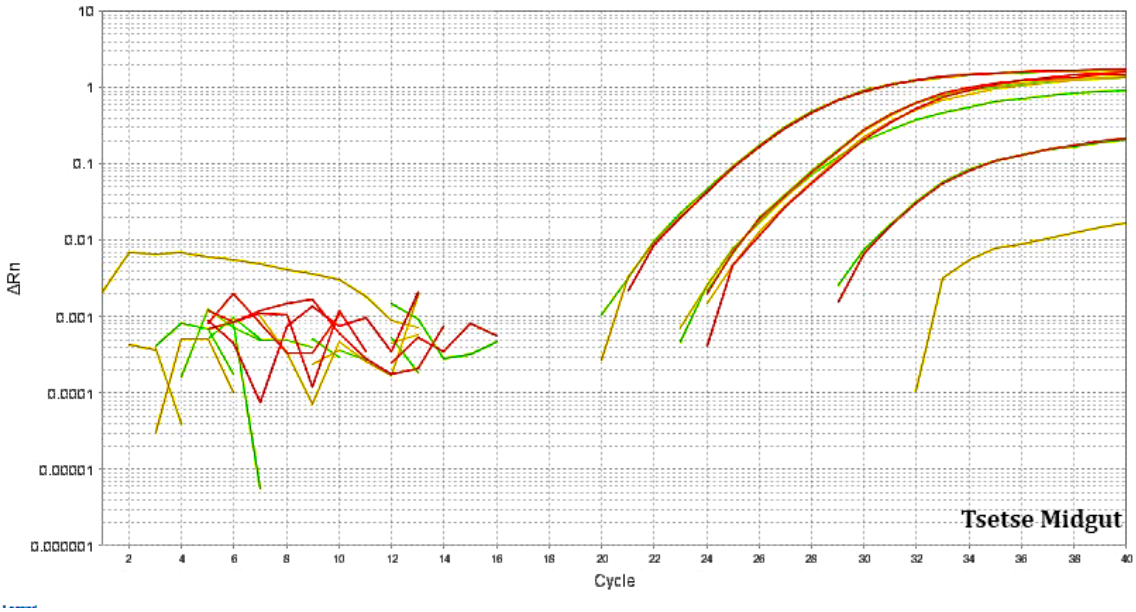


Figure 4.42: Real Time PCR amplification plot of *Trypanosoma congolense* Trans-sialidase cDNA from Tsetse fly midgut which were assayed in triplicates using TaqMan Fast Universal PCR Master mix and a dual fluorescent probe and primers (TconTS Fand TconTSR) targeting the trans-sialidase gene. Assay was performed in a Step-One Plus Real-Time PCR System (Applied Biosystems) instrument according tot he manufacturers recommended protocol.

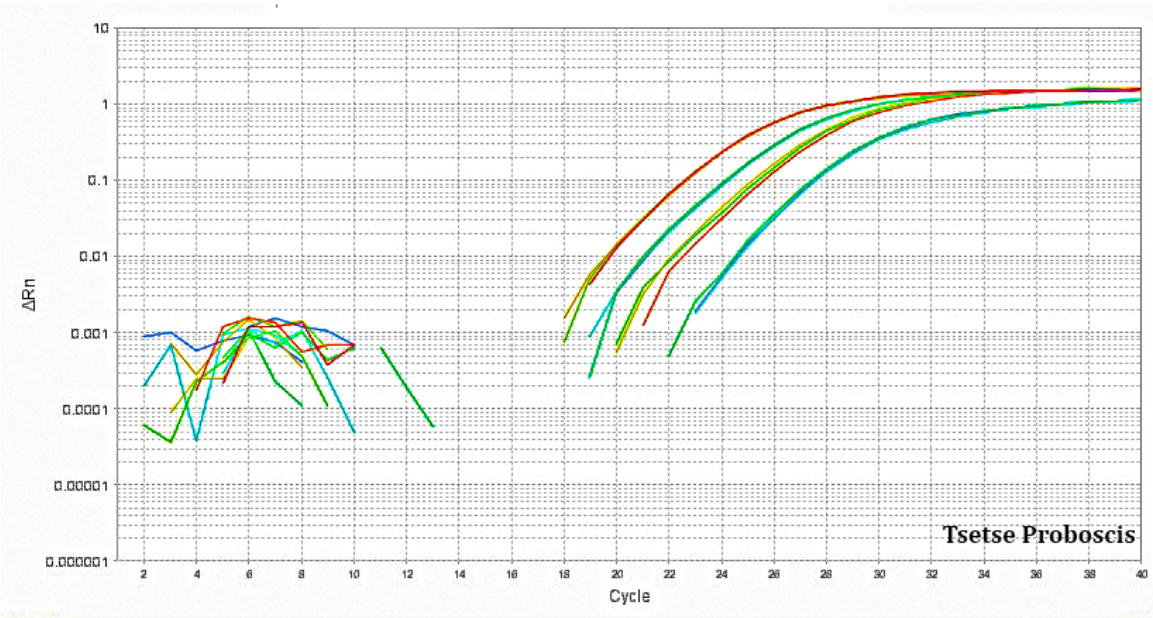


Figure 4.43: Real Time PCR amplification plot of *Trypanosoma congolense* Trans-sialidase cDNA from Tsetse fly proboscis which were assayed in triplicates using TaqMan Fast Universal PCR Master mix and a dual fluorescent probe and primers (TconTS Fand TconTSR) targeting the trans-sialidase gene. Assay was performed in a Step-One Plus Real-Time PCR System (Applied Biosystems) instrument according tot he manufacturers recommended protocol.

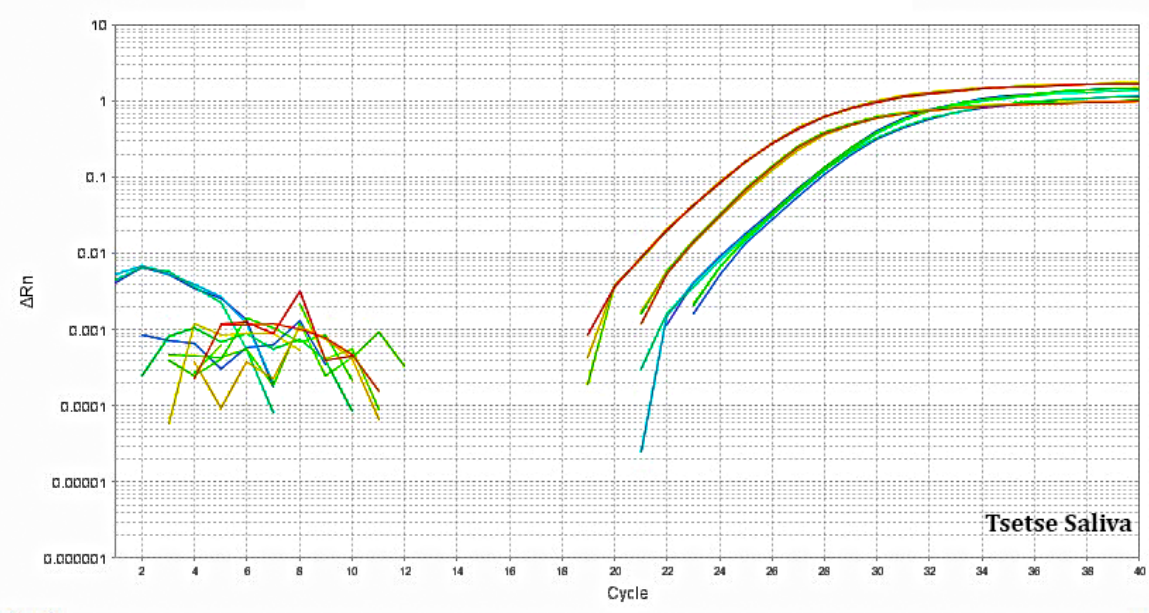


Figure 4.44: Real Time PCR amplification plot of *Trypanosoma congolense* Trans-sialidase cDNA from Tsetse fly saliva which were assayed in triplicates using TaqMan Fast Universal PCR Master mix and a dual fluorescent probe and primers (TconTS Fand TconTSR) targeting the trans-sialidase gene. Assay was performed in a Step-One Plus Real-Time PCR System (Applied Biosystems) instrument according tot he manufacturers recommended protocol.

Table 4.4: Real-time PCR data showing expression of Trans-sialidase with samples from goat and tsetse fly with confirmed *T.congolense* infection.

Sample	Parasite detection	Mean C_T±SD	Absolute cDNA levels (Copy Number/ng of plasmid DNA)
Goat Blood	PCR/Microscopy		
TS1		22.28±0.04	102527.7
TS2		25.66±0.03	9139.6
TS3		28.19±0.02	19916.2
TS4		22.26±0.03	330497.5
Fly Midgut	PCR		
TS1		26.62±0.03	5888.6
TS2		29.032±0.09	953.8
TS3		31.37±0.08	2577.0
TS4		29.67±0.05	2672.0
Fly Proboscis	PCR		
TS1		23.64±0.05	41890.5
TS2		26.26±0.06	6112.6
TS3		27.13±0.08	39596.9
TS4		26.82±0.07	17048.0
Fly Saliva			
TS1		22.89±0.03	68689.0
TS2		24.79±0.05	16417.0
TS3		26.37±0.06	64242.2
TS4		24.99±0.05	56044.5
House Keeping Genes			
GAPDH Goat		19.94±0.03	
GAPDH Fly		16.96±0.01	

*The Real-time PCR assay was carried out in triplicate and mean C_T ± SD are shown.

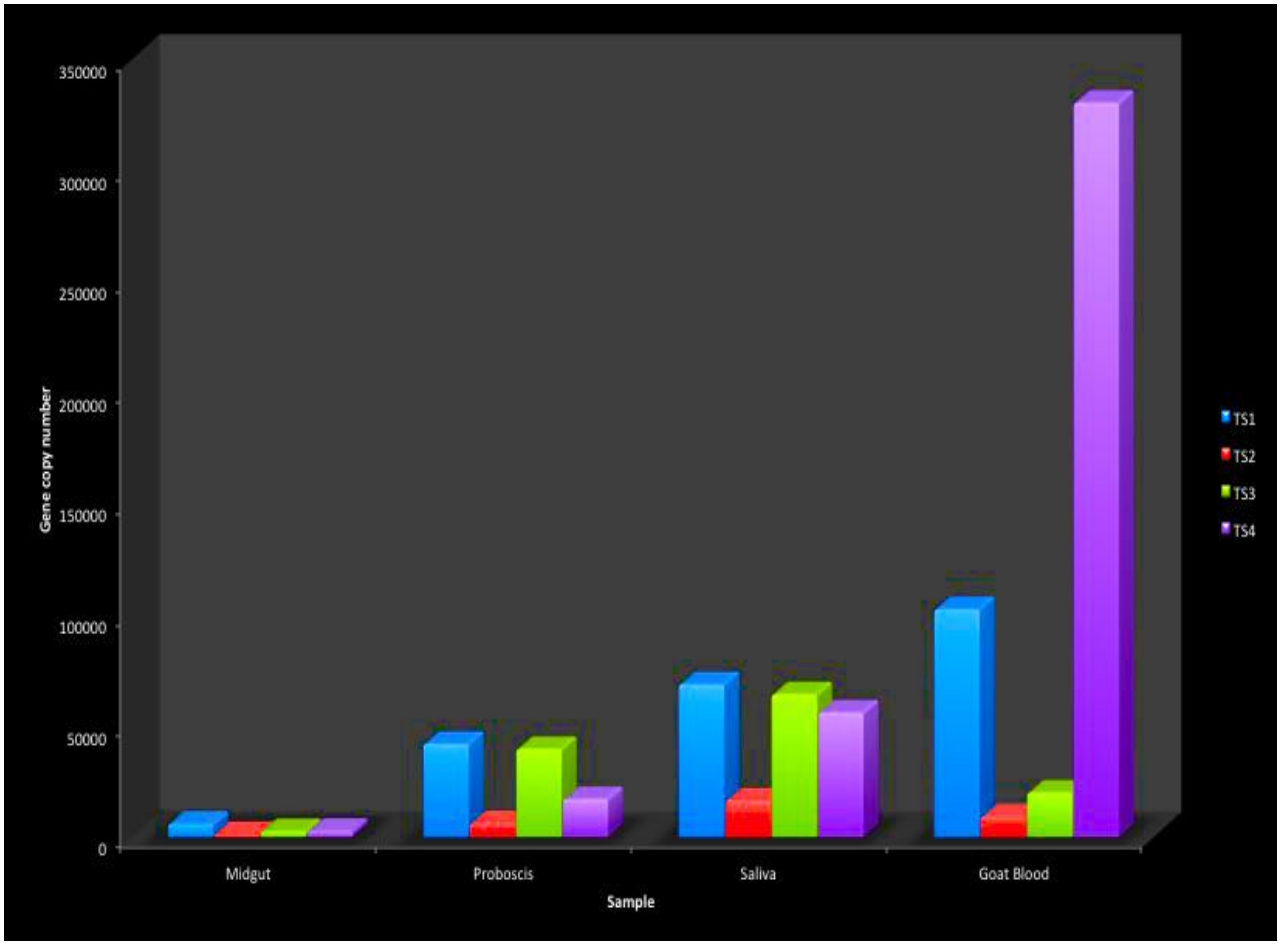


Figure 4.45: A 3-D clustered chart depicting mRNA expression of TS1, TS2, TS3 and TS4 genes in the entire life cycle of *Trypanosoma congolense* (Vector-host).

CHAPTER 5

DISCUSSION

All biological samples used in this experiment were stored in RNAlater® (Ambion) which is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissue to stabilize and protect the integrity of nucleic acids in unfrozen tissue samples, minimizing the need to immediately process or freeze tissue samples in liquid nitrogen. In the present work, this method was used on the field and laboratory, as samples were stored for a week at room temperature or a month at 4^0C or indefinitely at -20^0C or in liquid Nitrogen and the nucleic acid quantification and gel electrophoresis results confirmed clearly that the purity as well as nucleic acid integrity was not compromised. This makes them suitable for use in subsequent downstream applications i.e. PCR, qPCR, RTPCR, DNA sequencing and western blots. Thus, not only the diagnostic method, but also the procedure of sampjhjhle preparation is a driving factor for improved sensitivity in PCR assays (Silbermayr *et al.*, 2013).

The purity of the extracted DNA was evident in the spectral pattern as assessed by the ratio of the absorbance at 260/280 nm and 260/230 nm ranging between 1.76–1.82 and 2.0–2.1 respectively on a Nano drop spectrophotometer (ND 2000c) were within the acceptable range and further confirmed by Agarose gel electrophoresis. Efficient, effective nucleic acid extraction methods are of paramount importance in molecular biology more so the ability to extract pure DNA efficiently is critical yet often overlooked (Kelly *et.al.*, 2012). Sometimes, it is essential to know the exact concentration of a nucleic acid sample as it prevents unnecessary consumption, enhance amplification of difficult targets, and standardize downstream protocols for optimal performance as inaccurate quantification can

increase variability in downstream assays which leads to reduced result confidence (Haque, *et al.*, 2003).

This work is primarily aimed to test the hypothesis that trans-sialidase (TS) and TS-like proteins play a role in the infection cycle of *T. congolense* and related trypanosomes, particularly in the establishment of infection in the fly vector and the subsequent transmission to mammalian hosts. Accurate identification of trypanosome species is essential for understanding the epidemiology and pathogenesis of these parasites (Gibson, 2007). Therefore, it was necessary to detect the right *Trypanosoma congolense* specie to be used in the study. In the present work we developed a simple, sensitive, cost and time effective PCR-based approach for detecting tsetse-transmitted trypanosomes from some biological samples (blood, tsetse midgut, tsetse anal droppings). This was apt and deployed in monitoring the infection cycle of *T.congolense* between the vector (Tsetse fly) and the host (goat). Thus, becoming a means to an end.

Nested PCR allows a sensitive and reliable diagnosis of an infection with trypanosomes at all stages of the life cycle. This strategy is particularly effective and could be explored in conventional approach in monitoring infections. The project was started by optimising the conditions for nested amplification of ITS-1 regions with two sets of primers which allow amplification of DNA fragments from several relevant Trypanosoma species, each with a typical size of the product (Adams *et.al*., 2006). As such, the nested PCR was deployed for optimal detection using species-specific primers, which were designed for confirmation and higher sensitivity. After optimisation with both primers as little as 0.01fg of genomic trypanosomal DNA was detected reproducibly which is the highest sensitivity so far reported implying that very low level and mixed infections, particularly in farm animals

could be clearly identified. The method was further adapted to be used with whole blood without parasite purification by using direct Phusion enzyme (Finzymes) and Dream Taq Polymerase (Fermentas) in the first and second reactions, respectively. This contradicts with the report of Penchenier *et al*., (1996) which claims that direct amplification from blood samples is untenable. Furthermore, the results on the use of Chelex 100® in this study is novel as it has been previously reported that Chelex 100® could lead to incomplete purification of DNA leaving inhibiting factors in the PCR reaction mixture (Ravel *et al*., 2004; Becker *et al*., 2004). Sequel to the results obtained, when the electropherogram of these three methods were compared after amplification of *T.congolense* ITS-1 in blood samples stored in RNAlater showed absence of unspecific amplicons in the Chelex method, confirming higher specificity of the Chelex method over the other two methods. In principle, the Chelex method not only has the advantage of high specificity but is cost effective when compared to other methods as reported by Masake *et al*., (2002) and Cabrera *et al*., (2009) who used a PCR-ELISA/Hybridization format for detection of *Trypanosoma brucei* and *Trypanosoma vivax* but not for *Trypanosoma congolense* which is laborious and not cost-effective in terms of reagents. Also, PCR-RFLP developed by Geysen *et al*., (2003) used for diagnosis and characterization of "pan-trypanosomes" (Desquesnes *et al*., 2001) is relatively complex and a time-consuming procedure.

Another important advantage of this method is attributed to its use in the detection of immature infections in anal droppings of single tsetse flies at different post-infection times without having to dissect the flies, which was key in monitoring expression of Trans-sialidases in the life cycle of *T.congolense*. Understanding the complex interaction between tsetse flies and trypanosomes in a given geographical setting is epidemiologically relevant

and a key element for the establishment of rational and effective disease control measures (Morlais *et al*., 1998b). Determining whether a tsetse fly is infected by trypanosomes is extremely laborious, time consuming and required careful dissection by a skilled personnel. Adams *et al*., (2006) reported that their PCR method was unable to identify a high proportion (43.1%) of samples. This may have been due to the presence of inhibitors such as proteases or haem moieties from the fly midgut. Compared to existing methods of detecting *Trypanosoma* in *Glossina* (Solano *et al*., 2001; Ferreira *et al*., 2008), the Chelex method however gave an alternative approach to detection of trypanosomes in tsetse without necessarily dissecting them.

This final method was adopted and can be used with freshly collected blood, samples blotted on Whatman filter paper/FTA cards as well as wet/dry anal droppings of tsetse flies or samples stored in RNAlater employing both the generic and the specific primer sets and the same enzyme (Dream *Taq* polymerase). The detection limit of infections was clearly below that of conventional microscopy as parasites were detected much earlier by PCR. Besides its application in monitoring the trypanosomal life cycle, the PCR-based diagnosis was essential in identifying a suitable *T. congolense* strain for this project. Two examples for spin-offs are; a new collaboration with scientist from Cameroon investigating the presence of non-brucei *Trypanosoma* DNA in human blood and its application as reliable diagnosis of low level infected farm animals.

For many years, microscopy has been the standard available tool for the detection of parasites through inspection of blood smears (Healy and Ruebush, 1980; Tanowitz *et al*., 1992; Herwaldt, 1999; Duffy and Fried, 2005; Melrose *et al*., 2004; Costa-juniour *et al*., 2012), tissue specimens (Cobo *et al*., 2007; Ferguson *et al*., 1989; Sims and Talbot, 1989),

feces, lymph node aspirates (Chappius *et.al*., 2006; Ozensoy *et al*., 1998), bone marrow (Cruz *et al*., 2006), and even cerebrospinal fluid (Croft *et al*., 2006). This method however, is time-consuming, labour intensive, and proper diagnosis depends on qualified laboratory technicians. Although this is an inexpensive diagnostic test, it has very low sensitivity (Böse *et al*., 1995; Costa-juniour *et al*., 2012). In endemic regions, where resources are limited, this posses a problem as misdiagnosis can significantly impact patient (human or animal) care. Therefore, the need for improved or new parasitic diagnostic tools (Momar, 2009).

Diagnosis of *Trypanosoma* species in blood is difficult, either because of low parasitemias (especially in field samples) or due to the limits of parasitological techniques: centrifugation on mini-anion exchange columns (mAEC) needs fresh blood (Lanham and Godfrey, 1970), is delicate to perform and is almost forsaken for mass detection; haematocrit centrifugation technique (HTC) is much easier to achieve (Woo, 1971) but lacks in sensitivity (detection of one parasite for 75 uL of blood theoritically). Previous reports have compared the sensitivity of molecular-based assays for detection and identification (Wuyts *et al*., 1995; Penchenier *et al*., 1996; Morlais *et al*., 1998a; Mostafa *et al*., 1999; Koffi *et al*., 2006; Adams *et al*., 2006; Adams *et al*., 2008; Adams and Hamilton, 2008; Cabrera *et al*., 2009; Muhammad *et al*., 2010) of *Trypanosoma* species, which have been proven to be clearly below conventional microscopy and other parasitological techniques. The search for a suitable *T. congolense* strain turned out to take much more time than expected. Most of the strains collected from different sources in Nigeria, declared as *T. congolense* turned out to be different species diagnosed by PCR amplification of the ITS-1 region. For several of these the identities *T.evansi* was later

confirmed with specific primers and sequencing parts of the ITS-1. Finally, a *T. congolense* strain was obtained, which reproducibly caused parasitaemia in rats, goats and Tsetse flies (*G. morsitans submorsitans*). The strain was confirmed by PCR using generic as well as species-specific primers.

In developing countries, most *Trypanosoma* species that are used in trypanosomiasis research are obtained from the field. Microscopy, staining and haematocrit centrifugation techniques are the main methods used for detection and identification of trypanosomes in most laboratories and these methods have been shown to lack reliability and sensitivity. Various reports have shown that mixed trypanosome infections exist in the field in the vector as well as the host **(**Majiwa and Otieno, 1990; Masiga *et al*., 1996;Woolhouse *et al*., 1996; Morlais *et al*., 1998b; Lehane *et al*., 2000; Van den Bossche *et al*., 2004; Adams *et al*., 2006). Following the limitations of the current methods used in our laboratories for identifying trypanosoma species for research, it could be deduced that quite a number of research findings could be based on erroneous identities of parasites or even failure of current techniques to detect mixed infections. Unfortunately, the PCR Technology (PCR-ELISA, PCR-RLFP etc.), which brings the required sensitivity and specificity, is not affordable for laboratories in developing counties. Therefore, this optimized Chelex method which brings with it higher sensitivity, specificity and affordability would go a long way in alleviating this problem. Microscopy however remains relevant to detect motility and morphology of trypanosomes since PCR doesn't distinguish between dead and living trypanosomes. Research is ongoing to find a suitable method of separating mixed infections in order to obtain pure single trypanosome isolates which will be used in research in developing countries (Unpublished data, 2013).

Trypanosomes undergo a complex life cycle between the mammalian host and the insect vector. The mechanisms of refractoriness or susceptibility of tsetse flies to a trypanosome infection are not fully understood but parasite-related factors do play an important role in determining the transmissibility of the trypanosome (Akoda *et al*., 2008). In our earlier experiments, the 16 flies (over 60 hpe hours post-emergence) used in the experiment were all in a single cage which made it difficult to tell which fly was infected. Besides we were only trying to establish cyclical transmission from vector to host at the time. However, in line with earlier observations by Nantulya *et al*. (1978b), high transmissibility of *T. congolense* of 30%, which we observed from our single-fly experiment, could be associated with high parasitaemia since the flies were fed on a donor rat at peak of parasitemia. The findings of Akoda *et al*., (2008) observed that the proportion of infected flies was significantly higher in teneral tsetse flies that were not infected at the peak of parasitemia compared with those infected at peak parasitemia since the experimental setup allowed for a thorough analysis of variability between fly batches and variability between days on the rising, plateau or declining phase of the parasitaemia. An important note however, is that the teneral flies used in the single-fly experiment were about 8 hpe compared to 32 hpe flies reported by Akoda *et al*., (2008). This suggests a role in high transmissibility observed in the single fly experiment. Previous studies suggest that host age and microbiome status modulate the ability of insect disease vectors to transmit mammalian pathogens therefore associating susceptibility to infection with pathogenic trypanosomes more in teneral tsetse flies compared with their mature counterparts (Weiss *et al*., 2013; Haines, 2013; Weiss and Aksoy, 2011; Cirimotich *et al*., 2011; Wanj *et al*., 2009). Otieno *et al*. (1983), using *T.b.brucei*, demonstrated that a pronounced teneral

phenomenon also existed in newly emerged *G.m.morsitans*. The highest number of salivary gland infections was achieved by feeding infected blood to flies aged < 8 hpe. Distelmans *et al*., (1982) also confirmed that a different trypanosome species, *T. congolense*, had higher infection rates in teneral flies. In this large study consisting of 800 male and female *G. p.palpalis*, only flies aged less than 32 hpe developed mature proboscis infections. Starvation can also impact the vector competence of teneral (unfed) and mature (fed) flies (Kubi *et al*., 2006). *G. m. morsitans* were subjected to extreme starvation (>72 hpe) prior to infection with either *T. congolense* or *T. b. brucei*. Nutritional stress increased the percentage of flies with mature proboscis and salivary gland infections, respectively. The authors suggested that key immunological responses must be suppressed when starvation depletes fat body lipid reserves. Starvation has been shown to reduce the levels of the fat body- expressed host defense peptides (HDPs), attacin, and cecropin, and this may further contribute to the increased susceptibility to trypanosomes in nutritionally stressed flies (Akoda *et al*., 2009).

The roles of fat body immunity and presence of lectins in tsetse flies could be associated with the variation observed in the time of establishment of midgut infections. Lectins are a group of proteins, or glycoproteins with a range of properties, notably that of binding specific carbohydrates, causing agglutination. In tsetse, lectins play an important role in their defence against trypanosome infections (Welburn and Maudlin, 1999). A lectin specific for D-glucosamine (GlcN) and with lesser affinity for GlcNAc from the midgut of *G. m. morsitans* is capable of binding trypanosomes (Ibrahim *et al*., 1984; Ingram and Molyneux, 1988). Feeding lectin inhibitory sugars, either GlcN (Maudlin and Welburn 1987, 1988; Mihok *et al*., 1992, 1994; Peacock, 2006) or GlcNAc (Welburn *et al*., 1993,

1994; Peacock, 2006) increased infection rates in tsetse. However, supporting data for the direct role of lectins in the tsetse/trypanosome interaction is still limited since a trypanocidal lectin has not been purified yet from tsetse (Kubi *et al*., 2006). Tsetse fat body is loosely distributed throughout the hemocoel and is functionally related to both adipose tissue and the mammalian liver. This multifunctional tissue provides an energy reserve in the form of stored lipids and is a key controller of the innate immune system. It is essential for teneral flies to acquire several bloodmeals to build up enough energy (lipid stores) to develop protective immunity against pathogens (Haines, 2013). The identification of 68 tsetse fly genes with potential implication on its immune system in a tsetse genome discovery project (Lehane *et al*., 2003) has opened the way for more detailed investigation of tsetse responses to Trypanosoma infections (Kubi *et al*., 2006). Four HDPs have so far been characterized in tsetse: attacin, defensin, diptericin, and cecropin (Hao *et al*., 2001; Boulanger *et al*., 2002), but with the pending release of the tsetse genome, more are likely to be found. However, in teneral flies HDP expression does not increase even 5 days after trypanosomes are imbibed (Akoda *et al*., 2009). Tsetse attacin has been shown to provide resistance to trypanosome infection and also constrains trypanosome levels in flies if they do become infected (Hao *et al*., 2001). Nayduch and Aksoy (2007) observed elevated attacin levels in the fat body, midgut, and PV of different fly species known to be naturally less permissive to trypanosome infection, thus directly implicating attacin as a key molecule used to inhibit trypanosome establishment. To confirm the role in tsetse refractoriness, attacin knockdown by RNAi increased both midgut and salivary gland infections. This was the first time an HDP was directly linked to parasite transmission (Hu and Aksoy, 2006). The observed delay in

HDP activation by ingested trypanosomes may be exacerbated in teneral flies as a result of an immature immune system and minimal lipid resources present in the fat body. It is also possible that other immune effector molecules are underexpressed or inactive in teneral flies because of similar nutritional restrictions (Haines, 2013).

Research so far suggests the expression of trans-sialidases is not only restricted to trypanosomes but also to other prokaryotes, eukaryotes and humans as well (Schenkman *et al*., 1991; Medina-Acosta *et al*., 1994; Engstler *et al*., 1995; Mattosguaraldi *et al*., 1998; Tertov *et al*., 2001; Tiralongo *et al*., 2003; Schauer and Kamerling, 2011). Trans-sialidases have been investigated in detail for the mammalian and insect stages of *Trypanosoma cruzi* and have been shown to be expressed in all stages but with different enzymatic and biological activities (Schauer and Kamerling, 2011). Among the African trypanosomes, *T.b.brucei* and *T.congolense* have also been shown to express trans-sialidase activity at different developmental stages (Pontes De Carvalho *et al*., 1993; Engstler *et al*., 1993; Schauer and Kamerling, 2011). However, Engstler *et al*., (1995), reported that *T.congolense* trans-sialidase expression was restricted only to procyclic forms, as blood stream forms of the parasites do not reveal trans-sialidase activity.

Multiple forms of TS were found in *T.cruzi* (Cross and Takle, 1993; Egima *et al*., 1996; Frasch, 2000; Risso *et al*., 2004; Jäger *et al*., 2008). *T.congolense* however, expresses various trans-sialidase forms that exhibit high and low tran-ssialidase activities when compared to their sialidase activities (Tiralongo *et al*., 2003). Most recently, Koliwer-Brandl *et al*., (2011) and Gbem *et al*., (2013) reported on the biochemical characterization of trans-sialidase TS1 variants from *Trypanosoma congolense* and biochemical diversity in the *Trypanosoma congolense* trans-sialidase family respectively.

The expression pattern of trans-sialidases in the entire lifecycle (Vector-host) of *Trypanosoma congolense* (natural parasite) in its natural environment and host has been investigated. From the results, RT-PCR clearly shows that this enzyme is developmentally regulated and is expressed in the lifecycle (metacyclic trypomastigotes, epimastigotes, bloodstream trypomastigotes) of *T.congolense*. To authenticate these results, RNA samples which had a 260/280 and 230/260 ratio of ~2.0 and above were used to ensure purity. The DNase I treated RNA was amplified using ITS-1 primers and only samples, which turned out negative in this experiment were used in the RT-PCR as it was very likely to have DNA contamination of RNA during the extraction process. This served as a negative control to futher confirm purity of RNA used ruling out false positives. Non-infected goat samples served as negative controls to avoid amplifying false positives. Using RT-PCR, we could detect mRNA encoding TconTS1, TconTS2, TconTS3 and TconTS4 in saliva, proboscis and midgut samples from infected flies. Although this experiment does not distinguish between epimastigotes and metacyclic parasites in the proboscis, infective parasites in the saliva express mRNAs for all four enzymes, suggesting that these can play a role in this stage of the lifecycle or the transmission itself. While RT-PCR techniques have reached broad acceptance and offer advantages such as high throughput and sophisticated data mining, it is still important to appreciate the strengths and limitations of this technique. The issue of whether the data are biologically significant is being addressed in several ways: through biological replicate samples and confirmation of results with other analytical techniques. In order to confirm the RT-PCR results, samples from saliva, midgut and blood were again analysed with anti-TconTS1 monoclonal antibody (which cross reacts with TS 1 and 2), since the presence of mRNAs is an indication not a proof for

the presence of encoded proteins as it is often misleading to correlate transcript abundance and protein expression levels which can vary as much as 30 fold or more leading to grossly distorted analysis of a biological system (Haines *et al*., 2010). TconTS was detected in the saliva and proboscis of infected flies and blood of infected goats. The results obtained concur with those of Tiralongo *et al*., (2003) where the immunoblots of *T.congolense* TS forms with anti-*T.congolense* TS mAb detected a 90-kDa protein band following SDS-PAGE and this was proved using micro sequencing that the isolated 90-kDa protein was indeed a TS. They produced anti-*T.congolense* TS mAb, which could, because of its specificity, be a valuable diagnostic tool for distinguishing between procyclic forms of *T.brucei brucei* TS and *T.congolense* TS (Tiralongo *et al*., 2003). However, without monospecific antibodies, it is impossible to investigate the presence of TconTS3 and TconTS4 proteins or to distinguish between TconTS1 and TconTS2. The missing signal in the midgut sample could be due to degradation, since samples were not prepared specifically for this experiment.

To assess the significance and reproducibility of changes in gene expression it is important to use true biological replicates. Therefore, a novel TaqMan® assay was developed, a Real-time PCR technique that allows for accurate and sensitive monitoring and quantification of trans-sialidase genes expression in the course of *T.congolense* infection in the natural vector and host. The assays were applied using blood samples on infected goats and tissue samples from tsetse flies. Compared with conventional PCR assays, the major advantage of the Real-Time PCR technology is its wide detection range due to the determination of the C_T values at an early stage of the reaction (Becker *et al*., 2004). In other words, this method can be used to monitor amplicon formation throughout the PCR reaction providing

the ability to perform sensitive, accurate and reproducible measurements of specific DNA present in a sample (Bell and Ranford-Cartwright, 2002; Matsuu *et al*., 2005; Oyamada *et al*., 2005). Real-time PCR has enhanced the speed and scope for detection of Trypanosomes in blood samples (Becker *et al*., 2004). Thus improving epidemiological studies due to the fact that it can reliably measure different nucleic acid targets within a single reaction (Zerr *et al*., 2000; Kearns *et al*., 2001; Mackay *et al.*, 2002). Most recently, Silbermayr *et al.*, (2013) reported a novel Real-time PCR assay for the detection of African animal Trypanosomiasis species; *T.congolense*, *T.b.brucei* and *T.vivax* in trypanotolrant and trypanosusceptible cattle breeds. Detecting the level of parasitemia is relevant in monitoring TS gene expression.

The results on qPCR clearly show that TconTS1, TconTS2, TconTS3 and TconTS4 are expressed in the life cycle of *T.congolense*, which is developmentally regulated confirming the results obtained in the present study on RT-PCR and Western blot observations. Similarly, in the African Trypanosome *T.b.brucei* (Engstler *et al*., 1993; Pontes De Carvalho *et al*., 1993 Montagna *et al*., 2002) and the American trypanosome *T. cruzi* (Schauer and Kamerling, 2011), the pattern of regulated development is observed. The importance of transsialidases during the time of infection in the mammalian host by *T.congolense* is observed in the ~40-fold higher expression of this TconTS in the bloodstream forms relative to that in the procyclic forms of the midgut vector stage. This observation was also made in the 10 to 20-fold higher expression of this enzyme in trypomastigotes relative to epimastigotes in the vector stage of *T.cruzi* (Pereira *et al*., 1983; Rubin-de-Celis *et al*., 2006; Schauer and Kamerling, 2011). The high intensity of *T.congolense* ITS-1 detected in the anal droppings of the infected flies could be due to the

low expression of TconTS observed in the mid gut samples. However, as soon as the parasites leave the midgut to the mouthpart where they mature, a higher expression of the enzyme is observed. Studies have been reported that have questioned the role of trans-sialidase and sialic acids for the survival of *T.cruzi* in the insect where the absence of trans-sialidase activity in *R.prolixus* led to a higher parasite load in the insect (Takano-Lee *et al*., 2002). In contrast, the enzyme seems very important for replication and persistence in the host since it is believed to play an important role in the parasite's invasion of the host's cells (Schauer and Kamerling, 2011). The summation of these observations could imply that while maturing in the mouthpart of the fly, the enzyme expression/activity is restored to prepare it for transformation into infective metacyclic trypamastigotes and onward transmission into the blood of the mammalian host. Again we observed that TconTS1 was the most expressed within the vector. The finding, that under mild conditions GARP (Glutamic acid/Alanine Rich Proteins), which is the major surface glycoprotein procyclin, found in *T.congolense* (Bütikofer *et al*., 2002) is co-purified and co-immunoprecipitated with TconTS1 (Tiralongo *et al*., 2003) seems plausible for some reasons. First, GARP is the natural substrate of *T.congolense* TS. Second, protein complexes and protein-protein interactions involving glutamic acid-rich proteins (Korschen *et al*., 1999; Poetsch *et al*., 2001) have the possibility of mediating or facilitating the formation of oligomers of TconTS1 is supported by the finding that the interaction between the cGMP gated channel and peripherin-2 proteins of the rod photoreceptor outer segment of vertebrates are mediated by a glutamic acid-rich protein (Poetsch *et al*., 2001). The fact that only TconTS1 is co-purified and co-immunoprecipitated with GARP may be due to the possibility that between TconTS1 and TconTS2 forms only TconTS1 can interact with GARP as reported

by Tiralongo *et al*., 2003. However, there is no scientific evidence showing the interaction between TconTS3 and TconTS4 with GARP.

As shown in the results obtained, the TS expression was not evenly spread across the entire life cycle of *T.congolense*. TS4 expression was highest in blood samples, followed by TS1 and TS3. However, TS2 was the least expressed of the four isoforms in the procyclic as well as blood stream forms of the parasites. Gbem *et al.,* (2013) reported the enzymatic properties of the four *T.congolense* TS genes, which were compared using fetuin as a model for blood glycoprotein donor substrate. TS2 was observed to have the lowest K_M for fetuin of 299µM followed by TconTS1 (395µM as reported by Tiralongo *et al*., 2003), TconTS3 (1104µM) and TconTS4 (1806µM) having the highest K_M value. This shows a disparity between the changes in mRNA abundance and enzyme activities of TconTS genes. This data suggests that there could possibly be different underlying regulatory mechanisms, which may be connected with the expression of these enzymes and this may not be unrelated to the fact that the experiment was conducted in the natural host and vector.

Differences in protein stability, post-translational modifications, and regulation of enzyme activity are other factors that complicate interpretation of the data. Presumably, different mechanisms of regulation of gene expression play a role here and could be the subject of detailed studies involving these genes. These data underscore the complexity of the problem of predicting enzyme activities from mRNA data and stress the need for incorporating external data in order to validate expression-profiling hypotheses (Glanemann *et al*., 2003). Even so, this interpretation is merely an inference. Discepancies between the gene expression, protein expression and enzymatic

activity have been reported (Miyamoto *et al*., 2001; Glanemann et al., 2003). Therefore, it is also wrong to assume that responses at the mRNA level reflect the response at the protein level or at the level of active enzyme, i.e. that there is a static correlation between levels of mRNA, protein, and active enzyme. Recently, researchers have begun to examine the relationship between transcriptome and proteome on a large scale (Gmuender *et al*. 2001; Yoshida *et al*. 2001). However, few studies have quantitatively examined the correlation between message and protein abundance (Glanemann *et al*., 2003).

CHAPTER 6

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

This work was aimed at testing the hypothesis that trans-sialidase proteins play a role in the infection cycle of *T.congolense* particularly in the establishment of infection in the fly vector and subsequent transmission to the mammalian host. Therefore, well-controlled laboratory experiments with the evaluation of the situation in the field through molecular and biochemical approaches have been deployed to investigate the expression pattern of trans-sialidase genes in the infection cycle of *Trypanosoma congolense.*

Three generic systems have been developed from this work to detect and identify trypanosome infections from a variety of biological samples ranging from tsetse tissue to blood based upon PCR analysis. We have demonstrated the benefits of these systems including cost that these systems possess. These methods have shown high levels of congruency when looking at individual or mixed infections, thus showing the systems to be reliable and the results reproducible. Therefore, the final method adapted is a very simple, sensitive, specific and affordable PCR-based diagnostic method that detects and identifies *Trypanosoma* species in the fly vector as well as the mammalian host, which are of relevance in the African trypanosomiasis research. These identification systems have enabled us to carefully study the establishment of the life cycle of *T.congolense* between the fly and host.

Tracking the establishment of the parasites proved to be a powerful approach to identifying at what stage(s) TS mRNA are being expressed. By RT-PCR, we were able to detect and

monitor the stage-specific TS mRNA expression of *T.congolense* with the results showing that TS mRNA are expressed in the entire life cycle of the parasite.

Since the presence of mRNAs is an indication not a proof for the presence of encoded proteins we used Immunoblot analysis, employing anti-*T.congolense* mAb to detect a single protein of ~90 kDa as *T.congolense* TS. This has now become a valuable diagnostic tool for detecting TconTS at the different stages of the parasite in the fly vector as well as the mammalian host and also for distinguishing between *T.brucei.brucei* TS and *T.congolense* TS (Tiralongo *et al*., 2003).

Real-time PCR was finally employed to quantify these genes. They are actually expressed since the previous methods were merely qualitative. The qPCR results, confirmed our findings that TconTS genes are expressed in the entire life cycle of the parasite. However, the expression is modulated depending on the stage of the infection.

6.2 CONCLUSION

Based on the results obtained, it was confirmed that contrary to previous reports that trans-sialidases are only expressed in procyclic forms of *T.congolense*, TconTS1, TconTS2, TconTS3 and TconTS4 are expressed in the entire life cycle of the parasites in their natural host/vector and are developmentally regulated. The results of this study on the expression pattern of trans-sialidase genes in *T.congolense* will contribute significantly towards understanding the mechanism and roles of these enzymes in the pathogenesis of Animal African Trypanosomiasis.

6.3 RECOMMENDATION

Up until now, no crystal structure of TS in African Trypanosomes exists. To guide this process, it is important to understand the underlying mechanism responsible for it's unusual sialyltransferase activity. Crystallographic study of the enzyme structure needs to be done which will provide a framework for the structure-based design of novel inhibitors, as this enzyme is a potential target for drug design.

Considering the fact that trypanosomiasis has reached epidemic magnitude in some countries especially in Africa, it's of uttermost importance to scrutinize other control methods not just the disease, but also its transmission stage in the vector as these TS inhibitors can serve in combating trypanosomes both in the host as well as the vector.

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