MIDGUT EP PROTEIN GENE EXPRESSION IN *GLOSSINA MORSITANS MORSITANS* INFECTED WITH *TRYPANOSOMA CONGOLENSE*

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P13SCBC8007

A DISSERTATION SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA NIGERIA

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN BIOTECHNOLOGY

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF LIFE SCIENCES
AHMADU BELLO UNIVERSITY,
ZARIA, NIGERIA

NOVEMBER, 2018

DECLARATION

I hereby declare that the work in this dissertation entitled "MIDGUT EP PROTEIN GENE EXPRESSION IN *GLOSSINA MORSITANS MORSITANS* INFECTED WITH *TRYPANOSOMA CONGOLENSE*" was performed by me in the Department of Biochemistry under the supervision of Professor I. A. UMAR and Professor Olayinka O. OKUBANJO. The information herein derived from literature has been duely acknowledged in the text and list of references provided. No part of this dissertation has been previously presented for another degree or diploma at any university to the best of my knowledge.

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CERTIFICATION

This dissertation entitled **"MIDGUT EP PROTEIN GENE EXPRESSION IN *GLOSSINA MORSITANS MORSITANS* INFECTED WITH *TRYPANOSOMA CONGOLENSE"*** by GIMBA HASSAN DONGBA, meets the regulations governing the award of the degree of Masters of Science Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This work is dedicated to Almighty God

AKNOWLEDGEMENTS

I must first of all give thanks to my creator for his love, mercy, and protection to me. I give him Glory for provision and devine health throughout my period of study in Zaria.

My endless gratitude to my project supervisors, Prof.I. A. Umar and Prof.O.O. Okubanjo for their limitless effort and fatherly suggestions, constructive critism, corrections, close supervision and inspiration during the course of this work. It was your assistance, in all ramification that made this work a success.

I also appreciate the African Center of Excellence and Neglected Tropical Disease, for funding my research work.

A very special thanks also goes to my entire family for their immense love and relentless support all the way.

My deep appreciation goes to my Head of Department, Prof. M.N. Shuaibu and the entire academic and non academic staff of the Department of Biochemistry for their individual and collective support.

I will especially appreciate Dr Ibrahim Auwal for dedicating his time in going through my work and corrections and constructive critism.

I will also like to acknowledge Mr AminuYusuf Bashir for assisting me with materials relevant to my research work. My profound gratitude also goes to Mr stephen S. Shaida the acting Head, Department of Vector and Parasitology, Nigerian Institute for Trypanosomiasis and Onchocerciasis NITOR Kaduna for his support and encouragement and also for his assistance with the laboratory teneral flies for my research work,

My sincere appreciation also goesto Dr (Mrs) AliceJonah and Mr Emmanuel Enoh and the entire staff of insectary (NITOR) Kaduna, for their assistance during my research work.

Lastly I will like to appreciate the following people for their assistance both academically and morally Dr Peter Dede, Head of Pan Africa Tsetse Trypanosome Eradication and Control

(PATTEC) NITOR, Mallam Usman Baba, Dr Simon Ayo Yila and my best friend Mr Daniel Danladi Gaiya for their encouragement and advice during the course of this research work, God will continue to bless and protect you all, amen.

ABSTRACT

Trypanosoma congolense, is a protozoan parasite that causes animal trypanosomosis also known as *'Nagana'* in cattle. It is transmitted by the vector tsetse fly in which it undergoes several cyclical differentiation and development. The vector expresses a protein called Glutamic acid proline (EP) protein that plays an immunity role as an antagonist to the parasite establishment in the midgut. The present work was aimed to study the expression of the glutamic acid proline (EP) protein gene during the parasite establishment in the fly midgut at day 3, 10 and 24 post infections. One hundred teneral *Glossina morsitans morsitans* flies were fed on *Trypanosoma congolense* infected rat's blood, and only fifty four fully engorged flies were selected for the experiment. DNA was extracted from the anal dropping (s) of each of the fifty four flies for polymerase chain reaction to determined if the flies were infected. Twenty non-infected flies served as control. The flies were dissected and midguts collected at day 3, 10 and 24 post-infection. Reverse transcriptase Polymerase chain reaction (RT-PCR) followed by Quantitative polymerase chain reaction (qPCR) were performed on the midguts samples to determine the expression of Glutamic acid Proline (EP) protein. The results obtained, showed fly mortality of 29% (14/54), and percentage infection of 20% (8/40) by PCR. The qPCR result reveals that EP protein transcript was significantly ($p < 0.05$) more expressed at day 3 with fold change of 43.02 , as compared to day 10 and 24 having fold change of 1.22 and 1.27 respectively. Overall, the results have shown that the expression of EP protein gene in *Glossina morsitans morsitans*is a time dependent process, and could also be influenced by the species of the fly/trypanosomes interaction.

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LIST OF ABBREVIATIONS

AAT: Animal African Trypanosomiasis

AMP: Antimicrobial Peptides

BSF: Blood Stream Form

cDNA: Complementary DNA

Cp value: Crossing Point Value

DNA: Deoxyribonucleic Acid

EP: Glutamic Acid ProlineELISA: Enzymes Link Immunosorbent Assay

HAT: Human African Trypanosomiasis

MI: Maturation Index

NITOR: Nigerian Institute for Trypanosomiasis

PGRPs: Peptidoglycan Recognition Proteins

PC: Procyclic Cell

PCV: Packed Cell Volume

PCR: Polymerase Chain Reaction

qPCR: Quantitative polymerase chain reaction.

ROS: Reactive Oxygen Species

RNA: Ribonucleic Acid

CHAPTER ONE

1.0 INTRODUCTION

African trypanosomes are insect-borne parasites that cause sleeping sickness in humans and '*Nagana*' in cattle. Although, exact number of deaths from Human African Trypanosomiasis (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 of the United Nations (FAO, 2014) 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 Gross Domestic Products (GDP) may be as much as US$4.75 billion (FAO, 2014).

Sleeping sickness is fatal if untreated (Holmes, 2013). In addition to their importance in public health, at least seven other species of African trypanosomes cause severe disease in livestock known as African Animal Trypanosomiasis (AAT) or *'nagana'*. *Trypanosoma vivax* and *Trypanosoma congolense* are the major pathogens of cattle and other ruminants, while*Trypanosoma simiae, Trypanosoma godfreyi,* and *Trypanosoma suis* causes high mortality in domestic pigs (Van Den Abbeele and Rotureau, 2013).

Male and female tsetse flies are obligatory blood feeders, able to carry and transmit trypanosomes during their entire life span (Van Den Abbeele and Rotureau, 2013). Tsetse flies are in general refractory to parasite transmission, although little is known about the molecular basis for refractoriness. In laboratory infections, transmission rates vary between 1 and 20%, depending on the fly species and parasite strain (Moloo *et al.*, 1998). Approximately 72 h following ingestion of the infected blood meal, a process of attrition leads to the complete elimination of the infection in a high proportion of flies, and there is

also indirect evidence to suggest that trypanosomes may be killed by an innate immune response in the fly (Croft *et al.*, 1982). However parasites are established in the gut during a successful infection, thus enabling the parasite to undergo successive rounds of differentiation, proliferation, and migration in different locations in the fly, resulting in a final infective stage that is ptransmitted to a new mammalian host during each tsetse fly blood-meal (Jan and Rotureau, 2013).

Once acquired through an infected blood meal, the blood stream form (BSF) parasites encounter a number of physical and immunological barriers in the gut (Roditi and Lehane,2008), such as the peritrophic matrix (Weiss *et al.*, 2013) and a number of host immune molecules, including reactive oxygen species (ROS), Antimicrobial Peptides (AMPs), Peptidoglycan Recognition Proteins (PGRPs), tsetse EP(Glutamic acid proline) Protein which restrict the establishment of successful infections (Weiss *et al.*, 2013). In susceptible flies BSF parasites differentiate in the midgut into Procyclic Cells(PC), which are characterized by an invariant surface coat made of procyclin proteins, (Stebeck and Pearson,1994).

Previously, Lehane *et al.*, (2003) reported a number of selected genes in the fly midgut (including genes related to fly immunity) that were significantly expressed in response to trypanosome infection, during their establishment in the fly gut, these include Serpin 1, 3 and 4, chitin binding protein, Sphinginase, Thioredoxin peroxidase2 Nitric oxide synthase3, C1- Tetrahydrofolate Synthase, Transferrin and Glossina tubulin. Tsetse EP (glutamic acid-proline)protein acts as a powerful antagonist in the establishment of parasite in the fly midgut, suggesting tsetse EP protein may be a major determinant of vector competence in all *Glossina* species (Haines *et al.*, 2010)

1.1 Statement of Research Problem

Several efforts to eradicate tsetse fly populations have proven difficult (Oluwafemi *et al.*, 2007, Holmes, 2013). The flies reinvade the previously reclaimed land areas. Many studies have addressed the biology of trypanosomes in the mammalian host, but there is sparse information on the vector parasite interaction (Morrison *et al.*,2009). As several control methods of the disease are failing, there is a need for alternative sustainable solution, such as knowledge on the molecular interaction between the tsetse and the parasite are lacking, which could be used as a strong potential for diminishing or even disrupting vector competence, hence breaking transmission process to improve the control of sleeping sickness.

1.2 Justification

Many factors partake in determining the success or failure of the infection and maturation process (Aksoy, 2003), with fly immunity factors of particular importance (Hao et al.,2001). Tsetse EP (glutamic acid-proline) protein acts as a powerful antagonist in the establishment of parasite in the fly midgut (Haines et al., 2010).

A clear test of hypothesis on the gene expression pattern of the Glutamic acid Proline(EP) protein at three different post infection time points during the parasites establishment in the tsetse fly gut could give insight into how the Glutamic acid Proline(EP) protein gene is expressed in the infected midguts at the 3 post infection time points

1.3 Aim

To determine the expression ofGlutamic acid Proline(EP) protein gene during the parasite establishment in the tsetse fly midgut at three post infection time points.

1.4 Specific Objectives

i. To determine the infection status of *Glossina morsitans morsitans*, at different post Infection time points using polymerase chain reaction (PCR).

ii. To determine the expression pattern ofGlutamic acid Proline(EP) protein geneinGlossina morsitansmorsitans infected midgut at three different post infection timepoints Using qPCR.

iii. To compare and quantify the expression level of EP protein gene at three time points of the Infection process using quantitative PCR.

CHAPTER TWO

LITERATURE REVIEW

2.1 Trypanosomiasis

Trypanosomiasis is group of devastating vector-borne diseases (Matetovici *et al.*, 2016), cause by protozoan parasites of the genus*Trypanosoma* that is cyclically transmitted by the bite of infected tsetse fly of the genus*Glossina*(Stervading, 2008).Trypanosomiasis affect both human and animals leading to death, great economic and agricultural loss, especially in Africa (Baral, 2010).

Trypanosomiasis is divided into two main type, they are African trypanosomiasis or sleeping sickness and American trypanosomiasis or Chagas diseases

2.2 African Trypanosomiasis:

African trypanosomiasis is an infectious disease of humans and animals of similar etiology and epidemiology. The etiological agents of the disease are unicellular flagellated eukaryotic protozoan parasites of the genus *Trypanosoma* that live and multiply extracellularly in blood and tissue fluids of their mammalian hosts, and are transmitted by the bite of infected tsetse flies (*Glossina spp.*), which initially causes fever, headache, and joint pain in humans (Dietmar,2008). Winter bottom's sign, a swelling of the lymph nodes characteristic of early trypanosome infection, has long been recognized in association with African trypanosome infection. As the disease progresses, parasites enter the braincausing neurological symptoms, such as confusion, disturbed sleep patterns, extreme lethargy (hence, "sleeping sickness"), and coma. If left untreated, the disease is invariably fatal.

Throughout history, African trypanosomiasis has severely stagnate the economic and cultural development of the entire continent (Dietmar, 2008).

African trypanosomiasis is divided into two, they are human African trypanosomiasis or sleeping sickness (HAT) and the animal African trypanosomiasis or 'nagana' (AAT;).

2.2.1 Human African Trypanosomiasis or Sleeping Sickness (HAT):

Human African trypanosomiasis (HAT) is a neglected disease that impacts 70 million people living in 1.55 million km^2 ofsub-SaharanAfrica (Simarro*et al*., 2012). About 500000 cases have been reported since the beginning of twentieth century to date (Headrick, 2014). Two species of African trypanosomes are responsible for Human African Trypanosomiasis (HAT), also known as "sleeping sickness". The term sleeping sickness describes the deregulation of the sleep-wake cycle and the intra-sleep cycle, which is observed in the late stage of Human African trypanosomiasis (Buguet *et al*., 2005). Human African trypanosomiasis caused by *Trypanosoma brucei gambiense* in Central/West Africa, is responsible for the chronic form of sleeping sickness and *Trypanosoma brucei rhodesiense* in East/Southern Africa, gives rise to rapid and acute development of sleeping sickness.Human African trypanosomiasisnormally affects people living in tsetse fly endemic communities and it is transmittedthrough the bite of infected tsetse fly during their daily outdoor activities(Aksoy*et al*., 2017). Unless treated, Human African trypanosomiasis is fatal and patients can die within weeks or months of the infection, whereas the West African form of Human African trypanosomiasis is more chronic that can last for several years (Baral, 2010). Both protozoan species are morphologically indistinguishable, but have drastically different epidemiological features. Both forms of the sleeping sickness affect the central nervous system. The *Trypanosoma brucei gambiense* transmission cycle is mostly from human to human with an occasional involvement of an animal reservoir.

The *Trypanosoma brucei rhodesiense* transmission cycle mainly involves wild and domestic animals, but intensified human-to-human transmission may occur during epidemic (Jan*and* Rutoreau, 2013). There are two distinct stages during the course of sleeping sickness. The first or early stage of the disease, also known as the haemolymphatic phase, is defined by the restriction of the trypanosomes to the blood and lymph system, The symptoms of this stage are fever, headaches, joint pains and itching. The second or late stage of the disease, also known as the neurological phase, is characterized by the presence of the parasites in the cerebrospinal fluid (WHO 2006).In general, when the typical signs of the sleeping sickness occur, it result to confusion, disturbed sleep pattern, sensory disturbances, extreme lethargy, poor condition and coma. If left untreated, patients die within months when infected with *Trypanosoma brucei rhodesiense*or within years when infected with *Trypanosoma brucei gambiense*. Wild and domestic animals may play a major role as parasite reservoirs for human infections with trypanosomes (Njiokou *et al*., 2006, Simo *et al*., 2006).

2.2.2.1Animal African Trypanosomiasis (AAT):

Animal african trypanosomosis (AAT), '*nagana'or 'surra'* is a disease caused by *Trypanosoma congolense*, *Trypanosoma vivax* or *Trypanosoma brucei spp* 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), resulting in major economic loss in the affected country (Alsan, 2015). 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 include fever, restlessness, emaciation, hair loss and discharge from the eyes, edema, anemia and paralysis. As the illness progresses the animals weaken more and eventually become unfit for work, hence the name of the disease "N'gana" which is a Zulu word that means "powerless/useless" (Dietmar, 2008).Animal African Trypanosomiasis limits agricultural and economic development on the African continent despite the availability of prophylactic and curative drugs. Moreover, it is of serious concern to see drug effectiveness threatened by an increasing resistance in animal trypanosomes (Delespaux *et al.,* 2008).The economic losses attributed to Animal African *trypanosomiasis (*AAT) include, decreased meat and milk production as a result of mortality, morbidity and infertility. It also impede agricultural and crop productivity due to reduced animal draught power and manure, the true economic losses could be much higher (Budd, 1999). The main trypanosome species causing trypanosomosis in East Africa include *Trypanosoma congolense*, *Trypanosoma vivax*, *Trypanosoma brucei* and *Trypanosoma evansi.* All except *Trypanosoma evansi* that affects camels are cyclically transmitted by tsetse flies. In East Africa the most important tsetse flies are *Glossina pallidipes, Glossina longipennis, Glossina swynnertoni, Glossina brevipalpis* and *Glossima morsitans*. Biting flies and insects include Stomoxys, Tabanus and tick,whichmechanically transmittrypnosomes(Dwinger and Hall, 2000).

2.2.2.2 Etiology

Trypanosomes are unicellular organisms belonging to Phylum Protozoa, class Mastigophora, order Kinetoplastida, family Trypanosomatidae and genus *Trypanosoma* (Uilenberg, 1998).Trypanosome species belong to three different subgenera: Trypanozoon (*Trypanosoma brucei, Trypanosoma brucei rhodesiense, Trypanosoma brucei gambiense, Trypanosoma evansi*, *Trypanosoma equiperdum*), Dutonella (*Trypanosomavivax*,

Trypanosoma uniforme) and Nanomonas (*Trypanosomacongolense, Trypanosoma simiae, Trypanosoma godfreyi*) (Antoine-Moussiaux *et al.*, 2009). The *Trypanosoma brucei* group of trypanosomes mostly invade tissues (humoral) whereas,*Trypanosoma congolense* and to a lesser extent *Trypanosoma*

vivax predominantly restrict themselves to the blood circulation (Igbokwe, 1994).

Trypanosomes transmitted by tsetse in Africa cause diseases of importance to the health of both humans and livestock. Of the livestock trypanosomes, *Trypanosoma congolense* (Nannomonas) is probably the most prevalent and widespread pathogenic trypanosome In tropical Africa, being found in ruminants, pigs, dogs and other domestic animals throughout the tsetse belt.

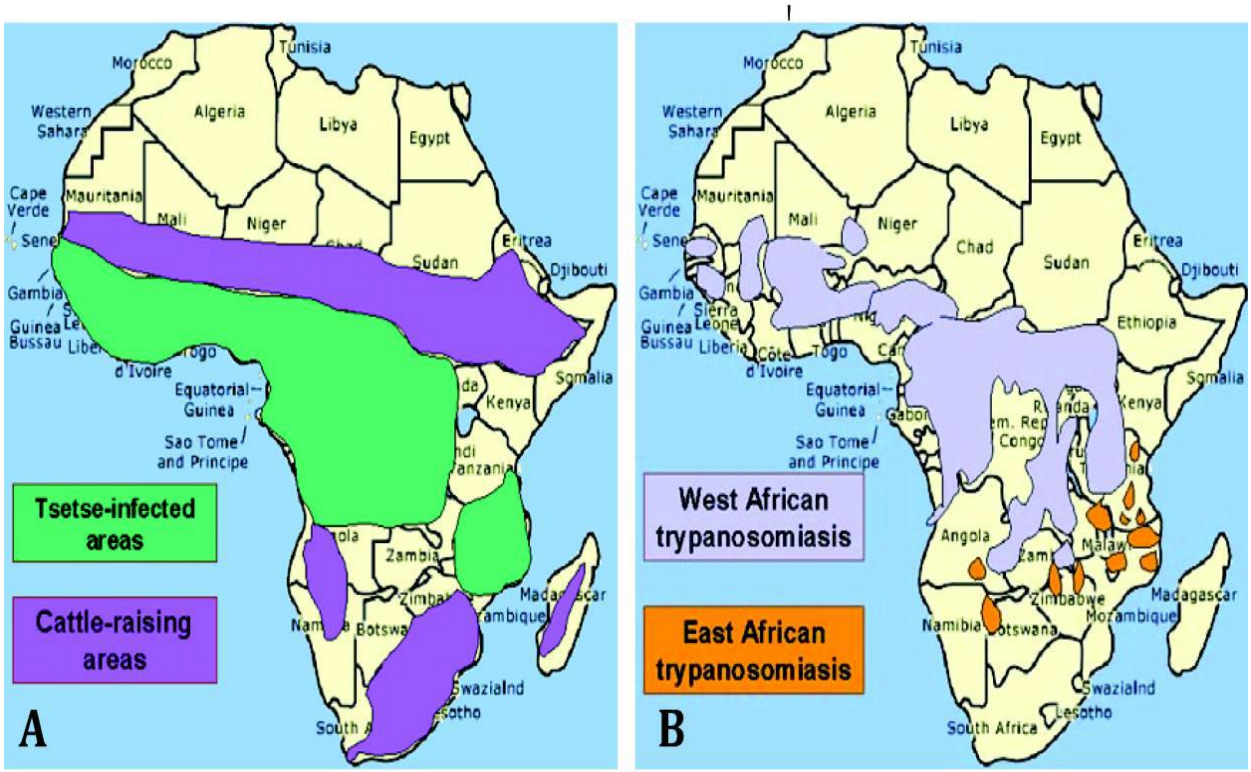
2.2.2.3 Host Range

Atleast seven species of African trypanosomes cause severe disease in livestock known as Animal African Trypanosomiasis (AAT) or '*nagana*'. *Trypanosoma vivax* and *Trypanosoma congolense* being the major pathogens of cattle and other ruminants, while *Trypanosoma simiae,Trypanosomagodfreyi,* and*Trypanosoma suis* causes high mortality in domestic pigs (Delespaux *et al.*, 2008). More than 30 species of wild animals can be infected with the pathogenic trypanosomes, and many of these remain carriers of the organisms. Ruminant are widely known to be active reservoirs of the trypanosomes and wild lions are carrier of trypanosomes.

2.2.2.4 Geographical Distribution

The major vectors responsible for the transmission of African Animal Trypanosomiasis and Human African Trypanosomiasis in the 37 sub-Saharan African countries between latitudes 14^0N and 29^0Sare Tsetse flies (**Figure 2.1**).Over 11 million $km^2$of Africa (about 37% of the

continent) is tsetse infected, putting millions of humans and livestock at risk of contracting trypanosomosis(Namangala, 2011). There are about 30 species and subspecies



(Adapted from: Chechet, 2015).

Figure 2. 1: Map of Africa, Showing A. Tsetse infested areas affected by Nagana (green), Cattle raising areas (purple) . B. Distribution of West (*Trypanosoma brucei gambiense* (lilacblue)and East African (*Trypanosoma brucei rhodesiense)* Trypanosomiasis (orange)

of Tsetse flies (Schofield,2008; Despommier *et al.*, 2005), and are classified into(i) savannah, (ii) riverine and (iii) forest groups(Geiger *et al.*, 2005). Thesavannah group includes*Glossinamorsitans, Glossina swynnertoni, Glossina longipalpis, Glossina pallidipes*and*Glossina austeni*(Swynnerton,1936).

Base onmorpholology and generalstructure, tsetse flies can also be grouped into (i) morsitans,(ii)palpalis and (iii) fuscagroup(Eloy and Lucheis,2009; Ugochukwu,2008)).This group,which is found at 900–1800 meter above sea level, is a vector of both African Animal Trypanosomiasis and Human African Trypanosomiasisin East and Southern Africa(Hargrove *et al.*, 2012).The presence of wildlife is the key factor controlling its distribution.(Kaare *et al.*, 2007).The riverine group, which prefersthe shaded habitat, riverine and lakes, includes*Glossina palpalis, Glossina fuscipes, Glossina martinii, Glossina quanzensis, Glossinacaliginea, Glossina pallicera and Glossina*tachinoides are mainly found in west and central Africa, transmitting*Trypanosoma brucei gambiense*, responsible for about 90% of all Human African Trypanosomiasis cases in sub-Saharan Africa(Katunguka *et al.*,1996). Their distribution depend on the forest vegetation and environmental climate, most species in this group inhabit the moist evergreen forests (Jordan, 1986)

2.2.2.5 Classification of Trypanosomes

Trypanosomes are classified into three different subgenera, such as Trypanozoon, Dutonella and Nanomonas. As represented in **table 2.1**

Table 2. 1:Classification of Trypanosomes

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*	

(Chechet, 2015)

2.2.3 Transmission

The three main species of tsetse flies responsible for transmission of trypanosomes are the *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 favors the high, dense forest areas.

2.2.3.1Pathogenesis of Trypanosomosis

Upon the bite of the mammalian host by a trypanosome infected tsetse fly, the parasites multiply locally in the skin and elicit a local host response that manifests itself as an indurate skin lesion called chancre. Eventually, the parasites enter the blood circulation via lymph vessels and can survive in the blood circulation throughout the infection (Akol and Murray, 1986) and remain continually exposed to the host's immune system. *Trypanosoma brucei* has the ability to penetrate the walls of capillaries, invade interstitial tissues, but always remain extracellular. *Trypanosoma congolense* is an extracellular, intravascular blood parasite that is unable to leave the circulation. It has a tendency to bind to walls of capillaries and small vessels (Banks, 1980).

African trypanosomes have evolved very sophisticated evasion mechanisms to survive in chronically infected hosts. The evasion mechanisms include antigenic variations, excessive activation of the complement system leading to persistent hypocomplementemia, down regulation of nitric oxide production, polyclonal B-lymphocyte activation, and marked immune suppression (Hudson *et al*., 1976). While the most common feature of Clinical signs of acute Trypanosomosisinclude anemia, weight loss, abortion, roughness of the hair, enlargement of peripheral lymph nodes, fever and reduced milk production, extravascular localization of the parasite includes meningoencephalitis, splenomegaly, and cachexia occur. The incubation period for *Trypanosoma congolense* varies from 4 to 24

days; for *Trypanosomavivax* from 4 to 40 days; and for *Trypanosomabrucei brucei,* from 5 to 10 days.

2.2.3.2 Diagnosis of Trypanosomiasis

Trypanosoma infection can be diagnosed by clinical, parasitological, immunological and molecular methods.

Parasitological diagnosis includes wet blood film (thick or thin) and buffy-coat techniques. Serological techniques such as rapid agglutination tests, immuno-fluorescence assays and enzyme linked immunosorbant assay (ELISA) can be used for antibody and antigen detection. The Polymerase Chain Reaction (PCR) is a molecular technique which theoretically is very sensitive and specific (Eisler *et al.*, 2004).

Thick or thin blood films are widely used for diagnosis. Blood can either be examined directly for parasitemia, or fixed and Giemsa-stained to identify the trypanosome specie. These methods are not sensitive enough to detect low levels of parasitemia (Eisler *et al.*, 2004). A more effective and reliable method of detecting parasitemia is to concentrate the parasites. The hematocrit centrifugation technique is used to concentrate parasites in capillary tubes containing blood samples (Woo, 1970). The advantage of this technique is a higher concentration of parasites that are easier to detect. In addition the method gives the possibility of measuring packed cell volume (PCV) at the same time (Stein, 2011).

2.2.3.3 Control and Eradication of Trypanosomiasis

2.2.3.4 Vector Control

Two main strategies are generally used for controlling tsetse flies. One strategy aims at killing the flies as they emerge, before they manage to deposit a larva in the ground. This approach has been carried out using insecticide with persistent residual properties like dieldrin or synthetic pyrethroids. These are applied at the resting sites of tsetse flies. Also

aerial application of non persistent insecticide such as endosulfan has been done (Vale, 1993).

The second strategy aims at reduction of Tsetse fly population over a long period. This is usually carried out by attracting tsetse flies to lethal baits. The baits may either be artificial devices, such as traps or insecticides treated targets baited with synthetic host odours, or natural baits such as cattle treated with insecticides. The low reproductive rate of tsetse means that a deployment of low density of targets, for example: 4 targets per kilometer of evenly spaced artificial baits can eradicate tsetse populations within two years (Vale, 1993) Similarly, spraying cattle with insecticides or acaricides has controlled tsetse populations successfully in Zimbabwe (Thompson *et al.,* 1991).

2.2.3.5 Use of Trypanocidal Drugs

Trypanocidal drugs remain the only widely available control method for Trypanosomosis.As decribed by (kalule, 2010), the compounds listed below are the only available therapeutic drugs for trypanosomal infection

a. Diminazene aceturate (Berenil®, Hoechest®, Veriben®, Sanofi, and various (other generic formulations).

b. Homidium bromide (Ethidium®, Laprovet), Homidiumchloride (Novidium®, Merial).

c. Isometamedium chroride Samorin®/Trypamidium®, Merial: Veridum®. Sanofi).

There are two main strategies for controlling bovine trypanosomosis when using trypanocidal drugs, one aimed at treatment of existing trypanosome infections, in which case they are termed as chemotherapeutic drugs. And the other aimed at preventing cattle at risk of contracting infection, in which case they are termed chemoprophylactic drugs (Kalule, 2010). Some of the drugs can be used for either purpose, although dose rates and

routes of administration may be adjusted for the particular circumstances. Isometamedium chloride is the most widely used drug. It is the most efficacious chemoprophylactic drug, but also has good chemotherapeutic activity (Leach *et al.,* 1980). Homidium salts are used mainly for chemotherapy, but also do have some prophylactic activity. Meanwhile diminazine acceturate is the most widely used chemotherapeutic agent, but has almost no prophylactic activity (Leach and Roberts, 1981).

The continues used of trypanocidal drugs among the poor can result to danger of drug resistance, thereby rendering trypanosomiasis control strategy useless (Geerts *et al.,* 2001). This concern should be taken most seriously, because of the slow livelihood development and marketing of new trypanocides in the near future (Geerts *et al.,* 2001)

2.2.3.6 Vaccination

Despite several ongoing research on the development of vaccines against trypanosomes, no vaccine has so far been developed (Authie *et al.,* 2001). The one area where there has been some progress is the development of an anti-disease vaccine. More efforts have been directed towards preventing the pathogenic effects of the parasite rather than infection itself (Authie *et al.,* 2001).

2.2.3.7 Trypanotolerance

Trypanotolerant animals are not refractory to trypanosome infections but limit proliferation of trypanosomes and reduce susceptibility to the effect of the disease (Bakhiet *et al*., 1996), they can survive, produce and reproduce better than susceptible animals under tsetse-*Trypanosomosis* challenge. These definitions are very wide and can possibly contain factors ranging from coat color of the animals that does, or does not, attract the tsetse fly. The use of trypanotolerant breeds is one possible and potentially cost effective solution in the control of *Trypanosomosis* (Stein, 2011).

The Taurine, N'Dama and West African Short horn cattle breeds of West and Central Africa are known to possess a significant degree of innate resistance to *Trypanosomosis, and* several other important infectious diseases (Murray *et al.*, 1990). The Sheko breed classified as a humpless Shorthorn is the only known breed of taurine type in eastern Africa which exhibit trypanotolerance (Rege, 1999). The breed is found in the Bench-Maji zone of Southern Region in the south-western parts of Ethiopia (Stein, 2011).

Trypanotolerance can therefore most easily be found in Taurine cattle breeds, whereas indicus breeds more often are trypano-susceptible (Murray *et al.*, 2004). The breeds are tolerant to both *Trypanosoma vivax* and *Trypanosoma congolense*, with a higher degree of resistance to *Trypanosoma vivax* (Murray *et al.*, 1982).

Trypanotolerance has also been investigated and found in other species of livestock such as small ruminants, pigs, horses and donkeys (Murray *et al.*, 2004). For instance, the Djallonke sheep and dwarf goats in West Africa and the Red Maasai sheep in East Africa have shown trypanotolerant characteristics (Murray *et al.*, 1982). Furthermore, people and some old-world primates are resistant to infection with the trypanosome species that causes nagana, i.e,*Trypanosomabrucei brucei, Trypanosomavivax* and *Trypanosoma congolense* (Black *et al.*, 2001).

2.3 *Trypanosoma congolense*

Trypanosomes transmitted by tsetse fly in Africa cause diseases of importance to the health of both humans and livestock. Of the livestock trypanosomes, *Trypanosoma (Nannomonas) congolense* is probably the most prevalent and widespread pathogenic trypanosome in tropical Africa, being found in ruminants, pigs, dogs and other domestic animals throughout the tsetse belt (Stephen, 1986).In the mammalian bloodstream *Trypanosoma congolense* is a small trypanosome, shorter in length than *Trypanosoma 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 develops in the proboscis for *Trypanosoma congolense* and in the salivary glands for *Trypanosoma 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 (Hoare, 1972).

Although*Trypanosoma congolense*generally considered to be monomorphic (Godfrey, 1960), similar pleomormism has been described in some strain and has been associated with parasite transmissibity (Nantulya *et al.,* 1978). In the fly,*Trypanosoma congolense*differentiates into elongated proliferative forms in the remains of the blood meal, which passes through the peritrophic matrix into the ectoperitrophic space before migrating interiorly 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 where they proliferate and subsequently develop into infective metacyclics (Peacock *et al.*, 2012).

2.3.1 Life Cycle of *Trypanosoma congolense*

The life cycle includes several phases alternating between the intermediate invertebrate and in the mammalian hosts (Stein, 2011) as shown in **Figure 2.2**.Transmission by insects may be cyclical by tsetse flies, *Glossina* species, or mechanical by other biting flies. But apart from transmitting trypanosomes cyclically, tsetse flies can also act as mechanical vectors.

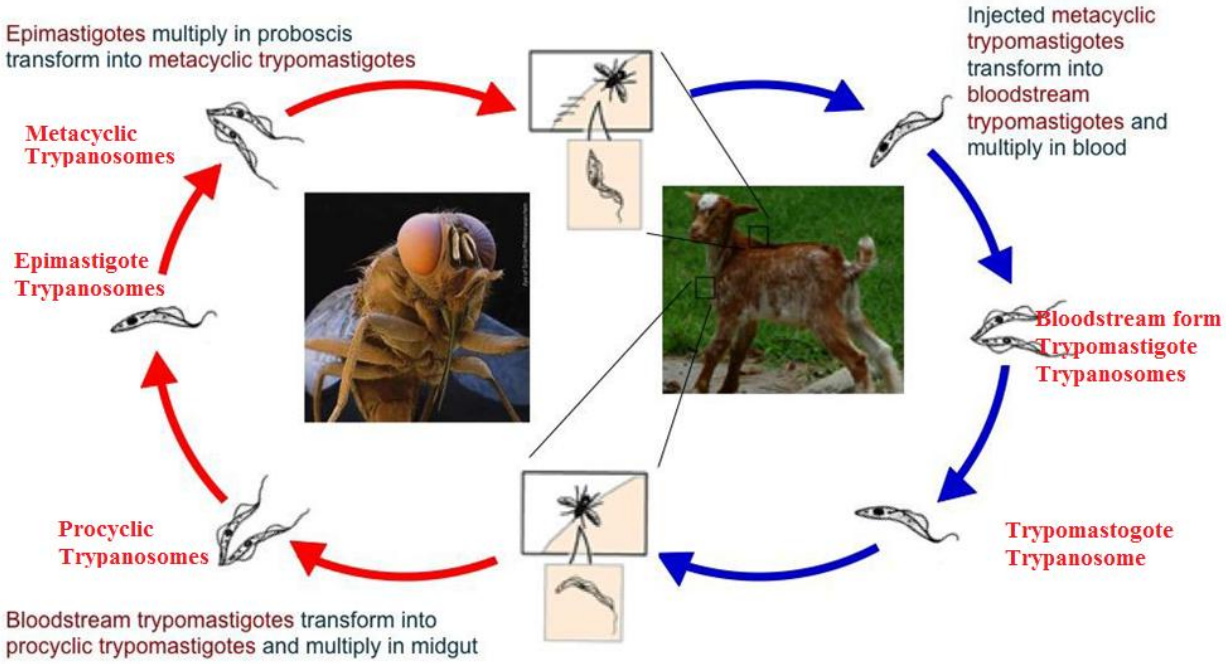


Figure 2.2: Life cycle of Trypanosomes (Adapted and modified from:Chechet, 2015**)**

In principle, any species may occasionally be transmitted congenitally (Uilenberg, 1998). The tsetse-transmitted African trypanosomes cause diseases of importance to the health of both Humans and livestock. The life cycles of*Trypanosoma congolense* and*Trypanosoma brucei*weredescribed in detail in the last century (Hoare, 1972). For *Trypanosoma* brucei, our understanding of the developmentalcycle in the fly has been steadily augmented by information emerging from molecular and cell biology analyses,e.g. differentiation of bloodstream to procyclic forms (Matthewsand Gull, 1997, Fenn and Matthews, 2007), major surface glycoproteins of developmental Stages(Urwyler*et al.*, 2007) cell cycle control (Sharma*et al.*, 2007)genetic exchangeand meiosis (Peacock *et al.*, 2011). Much less is known about the life cycle of*Trypanosomacongolense*, but evidence to date suggests thatmany aspects of the developmental pathway in tsetse fly aresimilar to that of its close relative,*Trypanosomabrucei(*Utz *et al.*,2006).

Ingestion of the bloodstream form trypanosomes by the heamatophagous tsetsefly, lead to a considerable change in morphology as well as their metabolism, into long slender forms called epimastigotes, multiply and finally give rise to the infective metacyclic trypanosomes(Peacock *et al.*, 2012). The metacyclic trypanosomes occur either in the biting mouthparts for *Trypanosoma congolense* and in the salivary glands for*Trypanosoma brucei*(Peacock *et al.*, 2012).Once infective metacyclic trypanosomes are present, the fly remains infective for the remainder of its life. A new host will be infected with the parasite when the fly takes another blood meal (Stein, 2011). During the act of feeding the fly penetrates the skin with its proboscis. Due to rupture of small blood vessels, a pool of blood is formed in the tissues and the fly injects saliva to prevent coagulation. Infection of the host takes place at this stage, with infective metacyclic trypanosomes (Uilenberg, 1998).

2.4 The Vector *Glosssina Spp*

2.4.1 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. Tsetse fly is divided into three group or subgenera based on morphological and ecological considerations, namely Morsitans group (subgenus *Glossina*), *Palpalis* group (subgenus *Nemorhina*) and the *Fusca* group (subgenus Austenina). Tsetse flies are distributed over 10 million square kilometres of sub-Saharan African land (14^0N and 29 ^{0}S), 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 is mostly restricted to the savannah woodlands (Leak, 1999). The map on **Figure 2.3**shows the distribution of tsetse flies in Africa with *Glossina morsitans* and *Glossina palpalis* being the most prevalent species in Nigeria. In general the Fusca and Palpalis groups occur in West and Central Africa while the Morsitans group **PlateI**is widely distributed throughout the drier zone.

Despite the high prevalence 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 samplesites (Auty *et al*., 2012). Tsetse fly susceptibility to *Trypanosoma* 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*) are more

refractory vectors than others ((e.g.,*Glossina morsitans morsitans* Westwood), as demonstrated under

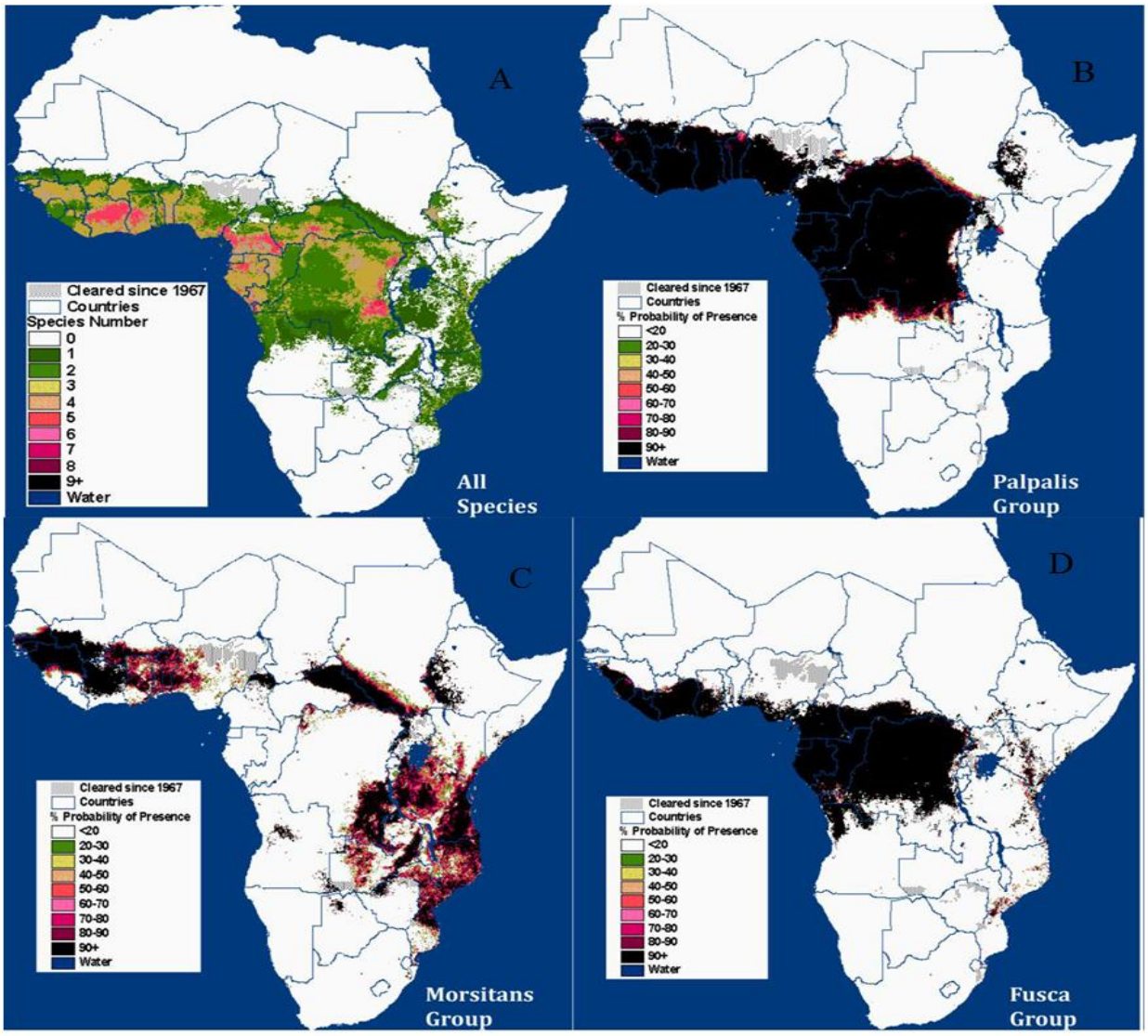


Figure 2.3: Distribution of Tsetse Flies species and Groups in Africa

(Source: Adapted and modified from Chechet, 2015)

A = Map of Africa showing the distribution of all Tsetse flies species

B = Map of Africa showing regions occupied only by Palpalis group of Tsetse flies

C = Map of Africa showing regions occupied only by Morsitans group of Tsetse flies

D = Map of Africa showing regions occupied only by Fusca group of Tsetse flies



PlateI: photograph of *Glossina morsitans morsitans*

laboratory conditions (Peacock*et al.*, 2012). This relative difference in susceptibility is particularly interesting for *Glossina pallidipes* and *Glossina morsitans morsitans*, because the two species are sympatric relatives and primary vectors for *Trypanosoma brucei rhodesiense* (Nayduch and Aksoy, 2007). Also, *Glossina morsitans morsitans* has a relatively weak barrier to infection with *Trypanosoma brucei* strain J10, with 11.3% of flies becoming infected at age 24–48 hour, 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, *Glossina morsitans morsitans* have been shown to be more susceptible to *Trypanosoma congolense* infection than *Trypanosoma brucei* (Peacock *et al.*, 2012).

Susceptibility to midgut*Trypanosoma 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).

Studies conducted by Van Hoof *et al.*, (1937), on the teneral phenomenon and infection rates of *Trypanosoma brucei gambiense* in *Glossinafuscipes fuscipes*, shows that newly emerged flies fed with a trypanosome-infected first blood meal had higher susceptibility to parasite infection than flies fed withtrypanosomes on subsequent blood meals. Further investigations by Wijers (1958), on this first-day factor, using newly emerged *G*lossina *palpalis palpalis*, demonstrate that flies were more permissive to *Trypanosoma brucei gambiense* midgut infections, when fed with trypanosomes in the first blood meal. This observation confirmed that susceptibility was dependent on the post-eclosion age of the fly upon ingestion of the infectious blood meal (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 *Trypanosoma brucei. brucei*, it was demonstrated that a pronounced teneral phenomenon also existed in newly emerged *Glossina morsitans morsitans*. The highest number of salivary gland infections was achieved by feeding infected blood to flies aged <8 h post-emergence (hpe). Studies on infecteion rate by Distelmans *et al.*, 1982 also confirmed that, a different trypanosome species, *T*rypanosoma*congolense*, had higher infection rates in teneral flies. In a study demonstrated by (Maudlin *et al.*, 1990), using 800 male and female *Glossina palpalis palpalis*, showed that 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. Midgut lectins are involved in Tsetse refractoriness (vector competence) and agglutinate and kill trypanosomes in vitro (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, lectin production in teneral flies is activated by first blood meal, resulting to amounts greater than that of the symbiont

effects. Thus, lectin production has been associated to the **"**teneral phenomenon,**"** where, irrespective of species, teneral flies are more susceptible to trypanosomes infection (Nayduch and Aksoy, 2007).

2.4.2 EP (Glutamic acid proline) protein

EP is a one letter notation for amino acids glutamic acid and proline which are negatively charge and non polar aliphatic amino acid respectively, these proteins are found in the tsetse fly midgut, which is a dipeptides repeats that plays an immunity role as an antagonist in the establishment of trypanosome in the Tsetse midgut (Haines *et al.*, 2010).The precise mechanism by which tsetse EP protein influences the refractorial capacity of the midgutremains to be elucidated(Haines *et al.*, 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents/Kits

All reagent and kits used were of molecular biology grade, they include:

Ethidium bromide Gibcobrl (Life technologies U.S.A).

Hotstart PCR premix (Bioneer U.S.A).

Molecular Ladder (Bioneer U.S.A).

2x Green star qPCR mix (Bioneer U.S.A).

Trizol (invitrogen)

Chelex 100 resin (Biorad U.S.A)

Light cycler PCR machine (Roche Germany)

3.1.2 Tsetse flies

Exactly 120 Teneral male and female *Glossina morsitans morsitans* (less than 32 hours old) were obtained from the insectary unit, at the Nigerian Institute for Trypanosomiasis and Oncocerchiasis research, (NITOR) Kaduna. This line of tsetse has a high vectoral capacity (Van den Abbeele *et al*., 2001). The tsetse flies were maintained in the insectary (NITOR Kaduna) at temperature of 25^0C, relative Humidity of 87% and 12 hours of light and 12 hours of darkness for survival of the tsetse (Chechet, 2015).

3.1.3 Trypanosome

Trypanosoma congolense was obtained from an infected albino rat at Biochemistry Department Ahmadu Bello University Zaria, Nigeria, and passaged into albino rats. In orderto maintain the parasites, they were sub-passaged into other albino rats.

3.1.4 Animals

A total number of 12 albino rats weighing between 120- 140 grammes were used for the experiment. They were purchased from NITOR; they were acclimatized and housed in a plastic cage at the experimental animal house NITOR, Kaduna. The animals were fed on chicken grower marsh (Vital Feeds, Jos) and drinking water was provided *ad libitum*.

3.2 METHODS

3.2.1 Preparation of Trypanosome Aliquot and Infective Feeding of teneral Flies

Exactly 1 ml of blood was collected from infected rat and diluted in 1ml of phosphate buffer saline, the parasitaemia of the diluted blood was checked using light microscope at a 400x magnification, as described by Herbert and Lumsden, 1976.

Exactly 0.5ml and 0.2ml 0f of the diluted blood containing 10^6 trypanosomes (*Trypanosoma congolense*) were injected intraperitoneally into five albino rats. These rats were monitored every day for parasitaemia by examining their tail blood using light microscopy at 400x magnification until their parasitamia reaches 10^9 Trypanosomes/ml. A total of 100teneral flies (less than 32 hours old) were fed on the abdomen of the infected rats (on average of 20 flies per rats) and only fully engorged flies were selected for the experiment (Chechet, 2015). The infective feeding was repeated three times, using different set of teneral flies of same species, and each set grouped as A,B andC, and another 20

different flies were fed on uninfected rats and used as control.Each fly in a group was placed in individual plastic cage, and were maintained by feeding on freshly collected rat's blood added to sodium citrate anticoagulant, using silicon membrane feeding system, three days a week until day 24 (Chechet, 2015).

3.2.2 Monitoring Infection

Collection of anal droppingsfrom each fly was done by dissolving it in 50µl phosphate buffer saline (PBS), and DNA extraction was performed on individual fly's anal droppings using chelex 100 resin DNA extraction kits according to method describe by (Chechet, 2015), as follows:

Exactly 50µl of PBS pipetted, was used to dissolve the anal droppings of the flies in each plastic container where each fly was kept. The solution was then transferred into a sterile Eppendorff tube and incubated at 56^0C for 1hour, followed by addition of 50µL of 5% Chelex 100 resin, andincubated at 95^0C for 30 minutes. The entire mixture was centrifuged at 14000rpm for 2 minutes and the supernatant was transferred into a new microfuge tube which is the DNA. Two microliter (2µL) of the extracted DNA was amplifedto detect the presence of infections from each infected fly at different post-infection times on day 3, 10 and day 24.

3.2.3 Dissection of Flies

Flies dissection was carried out at the three different post infection time point (day 3, 10 and 24), according to the method of Penchenier and Itard, (1981) in order to collect the guts. Before dissection, flies were starved for 48 hours to reduce partially digested blood. The dissecting instruments were cleaned and sterilized, between each dissected fly with Sodium Hypochloride (NaOCl) followed by flaming in order to avoid contamination.

3.2.4 Sample Preparation and Storage

Dissected midguts collected from tsetse flies was stored in RNA-later (Sigma) which was properly labeled in each tube, sealed and stored at -20 ^{0}C for later use.

3.2.5 DNA Extraction from *Trypanosoma congolense* Infected Blood, Using PhenolChloroform Method

Exactly 0.5 ml of blood from rat infected with *Trypanosoma Congolese* was collected and used for DNA extraction. Genomic DNA was extracted from the infected blood using phenol chloroform method, as follows:250µL of the infected blood was transferred into sterile eppendorff tube, 400µL of lysis buffer was added to the blood, followed by addition of 10µL of proteinase K, after which it was mixed by vortexing. The mixture was incubated at 55^{0}C for 1 hour (the sample was vortex every 20 minutes interval). After the incubation, 400µL of phenol chloroform was added, vortexed and spun at 13000 rpm for 10 minutes. The supernatant was transferred into a new tube and 400µL of chloroform was added vortexed and spun at 13000rpm for 5 minutes, and the supernatant was collected in a new tube followed by the addition of sodium acetate and 100% ethanol (40µL sodium acetate and 1000µL of ethanol). The tube was inverted and incubated overnight at -20^{0}C, after which it was centrifuged at 14000rpm for 10 minutes followed by addition of 70% ethanol (400µL) and spun at 14000 rpm for 5 minutes, air dried and the DNA was eluted with 50µL of double distilled water.

3.2.6 Primers Used For PCR

The *Trypanosoma congolense* species specific primers sequence used for PCR amplification of trypanosome DNA was obtained from previous work of Moser *et al.,(*1989). as shown in **Table 3.1**

Table 3.1: PCR Primer Sequence for Species specific *Trypanosoma congolense* (Savannah)

Specificity	code	primer sequence	amplicon size
Trypanosoma congolense (Savanna)	TCN Forward	TCGAGCGAGAACGGGCACTTTGCGA	341bp
	TCN Reverse	ATTAGGGACAAACAAATCCCGCACA	Moser *et al*.,(1989).

3.2.7Chelex DNA Extraction From flies Anal Droppings

Anal droppings was collected from individual fly, by dissolving with 50µL PBS from transparent disposable cups where each fly was kept, the desolved anal droppings was transferred into sterile eppendorf tubes, then incubated at 56^0C for 1hour, followed by addition 50µL of 5% Chelex 100 resin, and further incubated at 95^0C for 30 minutes in. The solution was centrifuged at 14000 rpm for 2 minutes and the supernatant (DNA) was transferred into a new microfuge tube. Two microliter(2µL) of the supernatant was used for PCR to detect infection status of each fly (Chechet, 2015).

3.2.8 PCR Identification of *Trypanosoma congolense* from Infected Blood

The PCR of the genomic DNA extracted from *Trypanosoma congolense*infected rat bloodwas performed in a 25µL reaction in order to identify the parasite used for the research work,

Asfollows: the following was added into the PCR tube,2µL template DNA, 1µL of each primer, 12.5µL of premix to a reaction volume of 25µL, was mixed by vortexing and transferred into the PCR machine. Cycling conditions was as follows: 1 cycle at 94 ^{0}C for 5 minutes followed by 35 cycles at 94 ^{0}C for 30 seconds, 65 ^{0}C for 30 seconds, 72 ^{0}C for 30 seconds, and final extension 72^0C for 5 minutes The products was resolved by electrophoresis (35 minutes at 100V) on 1.5% Agarose gel stained with Ethidium bromide and bands were visualized using GelDoc- Imaging System (BIORAD) Segrate (Millan) Italy.

3.2.9PCR Detection of Infection fromDNA of Infected Fly's anal droppings

PCR was performed on the extracted DNA of infected fly's anal droppings, to checked for the presence of infection in each fly, in a 20µL reaction volume using hot start PCR premix, as follows: 1µL each of primer, 2µL of template DNA and 16µL of water making up 20µLwere dispensed into hotstart PCR tube,the tube was vortex and spun. And were set on the thermal cycler PCR machine and run. The PCR condition was as follows: 1 cycle at 94^0C for 5 minutes followed by 35 cycles at 94 ^{0}C for 30 seconds, 65 ^{0}C for 30 seconds, 72 ^{0}C for 30 seconds, and final extension 72^0C for 5 minutes The products were resolved by electrophoresis (35 minutes at 100V) on 1.5% Agarose gel stained with Ethidium bromide and bands were visualized using GelDoc- Imaging System (millan) Italy.

3.2.10Midgut RNA Extraction and Quantification

An RNA column extraction kits (DNA LAND scientific total RNA extraction miniprep) was employed as described according to the manufacturer instructions, as follows: pooled infected gut tissue stored in RNA*later was* transferred into a new tube containing 200µL of solution compose of β Mercaptoethanol and RX buffer. The tissue was homogenized, and the lysate was centrifuged at 14000rpm for 5 minutes at 3^0C.Exactly 150µL of the supernatant was transferred into a new microfuge tube using micropipette, followed by addition of 150µl of 70% ethanol and mixed; the entire mixture was transferred into RNA miniprep binding column placed in 2ml collection tube and centrifuged for 1 minute at 14000rpm and the flow through discarded. Exactly 500µL of WF buffer was dispensed into the column and centrifuge at 14000rpm for 30s, the flow through was discarded again.700µL of WS buffer was dispensed into the column (100% ethanol + WS buffer) and spun at 14000rpm for 1 minute and the flow through was discarded. The empty column was

spun at 14000 rpm for 3 minutes, and the RNA binding column was transfer into a new RNAse free microfuge tube, following addition of 30µL of DEPC water and allowed to stand for 2 minute.

The extracted RNA was quantified using UV – spectrophotometer RNA/DNA calculator (gene quant) at absorbance at 260nm (A_{260}). Briefly, 5µl of the extracted RNA was dispensed into the cuvette and the concentration and purity of the RNA was taken using the UV spectrophotometer RNA/DNA calculator.

3.2.11 Reverse Transcriptase PCR or cDNA Synthesis

Principle: using the polymerase chain reaction, template RNA is transcribed to a more stable cDNA.This requires the enzyme reverse transcriptase, primers and dNTPs.

From the results of Midgut RNA extraction and quantification obtained, 0.5µg of cDNA was synthesized using RT-PCR premix (Bioneer U.S.A), as follows:

Exactly the amount of total RNA calculated in the 4 samples needed to synthesize 0.5µg of cDNA were added in the RT-PCR premix tube followed by 1µL Primers each, both forward and reverse, the remaining was nuclease free water, making the total volume to be 20µL. The solution was mixed gently in the reaction tubes, and incubated at 42^0C for 1 hour, and was terminated at 94^0C for 5 minutes.

3.2.12 RT-PCR Amplification of Glutamic acid Proline(EP) protein and Tubulin Transcripts

Conventional PCR of cDNA was performed in a light cycler PCR machine, in a 20 µL reaction volume containing 2 µL of cDNA, 16µL of distilled water and 1µL of each primer.

The reaction mixtures were loaded into the hot start PCR premix tube, capped vortex and centrifuged for 30 seconds. Cycling conditions are as follows:

1 cycle at 94^0C for 5 min followed by 30 cycles at 94^0c for 30 sec, 57^0C for 30 sec, 70^0C for 30 sec and final extension 70^0C for 5 minutes .

3.2.13 Primers used for qRT-PCR

Primers for qRT-PCR were obtained from previous work of Attardo*et al.*, 2006. As shown in **Table 3.2**

3.2.14 Real-time PCR (qRT-PCR)

Principle: Real-time PCR is carried out in a thermocycler with the capacity to illuminate beam of light and detect fluorescence. Involves the use of an intercalating dye whose fluorescence increases as more copies of gene is generated through cycles of polymerase chain reaction until the fluorescence is strong enough to be detected (threshold frequency).

qRT-PCR was performed with a light cycler real time PCR system. The qPCR was performed in a 25 µL reaction volume containing 1 µL of 0.5 µg cDNA, 12.5 µL of qPCR 2x greenstar premix (Bioneer USA), 1 µL each of primers and the remaining made up to 25 µL with ddH_2O. The reaction mixtures were loaded into the capillaries, capped and centrifuged for 30 seconds. The qRT-PCR cycle protocol started with 1 cycle at 95^0C for 15 min. Forty five cycles, 95^0c for 15 sec, 57^0C for 15 sec and 70^0C for 15 sec. All qPCR amplifications were reproduced in triplicates including those for the Tubulin housekeeping gene. Amplification plot was obtained for each quantitative PCR run, which was used to obtainedthe crossing point values (Cp), and use for data processing and analysisusing the relative quantification method described by Livark and Schmittgen., (2001).

Table 32: qRT-PCR Primer Sequences for EP Protein and Tubulin housekeeping gene.

Gene symbol	Gene name		Gene size (bp)
GMOY003306	EP protein	Forward GCT-GAA-GTT-GGG-AAG-ACT-GC	108
		Reverse AGC-TTG-CTC-GAA-AGC-TTG-AT	
GMOY000148	*Glossina* tubulin	Forward CCA-TTC-CCA-CGT-CTT-CAC-TT	149
		Reverse GAC-CAT-GAC-GTG-GAT-CAC-AG	

CHAPTER FOUR

RESULTS

4.1 Molecular Identification of Trypanosome by PCR

Trypanosoma congolense species-specific primer was used to amplify the trypanosome genomic DNA, and the expected product size was obtained when analyzed on the gel (341bp) for*Trypanosoma congolense* savannah (**Plate II**).

4.2 PCR Detection of Infection from Infected Tsetse Flies

From the three different sets of teneral flies of same species and age (less than 32 hours old)used for the post infective feeding (grouped as A, B and C), only 54% (54/100) were fully engorged with the infected blood meal. The mortality rate was 29.5% (14/54), and the percentage of flies that had midgut infection after PCR amplification of DNA extracted from each infected fly anal droppings at the three day post infection time point was 20% (8/40), PCR was ran along with negative control (water). The PCR obtained, detect infection in 2 flies at 3 days post infection time point, in 2 flies at 10 days post infection time point and in 1 fly at 24 days post infection time points, in group A (**Plate III**). For group B flies, PCR detected infection in 1 fly at day 10 and 1 fly at day 24 post infection time points (**PlateIV**). And for group C flies, PCR only detected infection in 1 fly at day 10 post infection time point (**PlateV**). No amplification was seen in the negative control as expected.

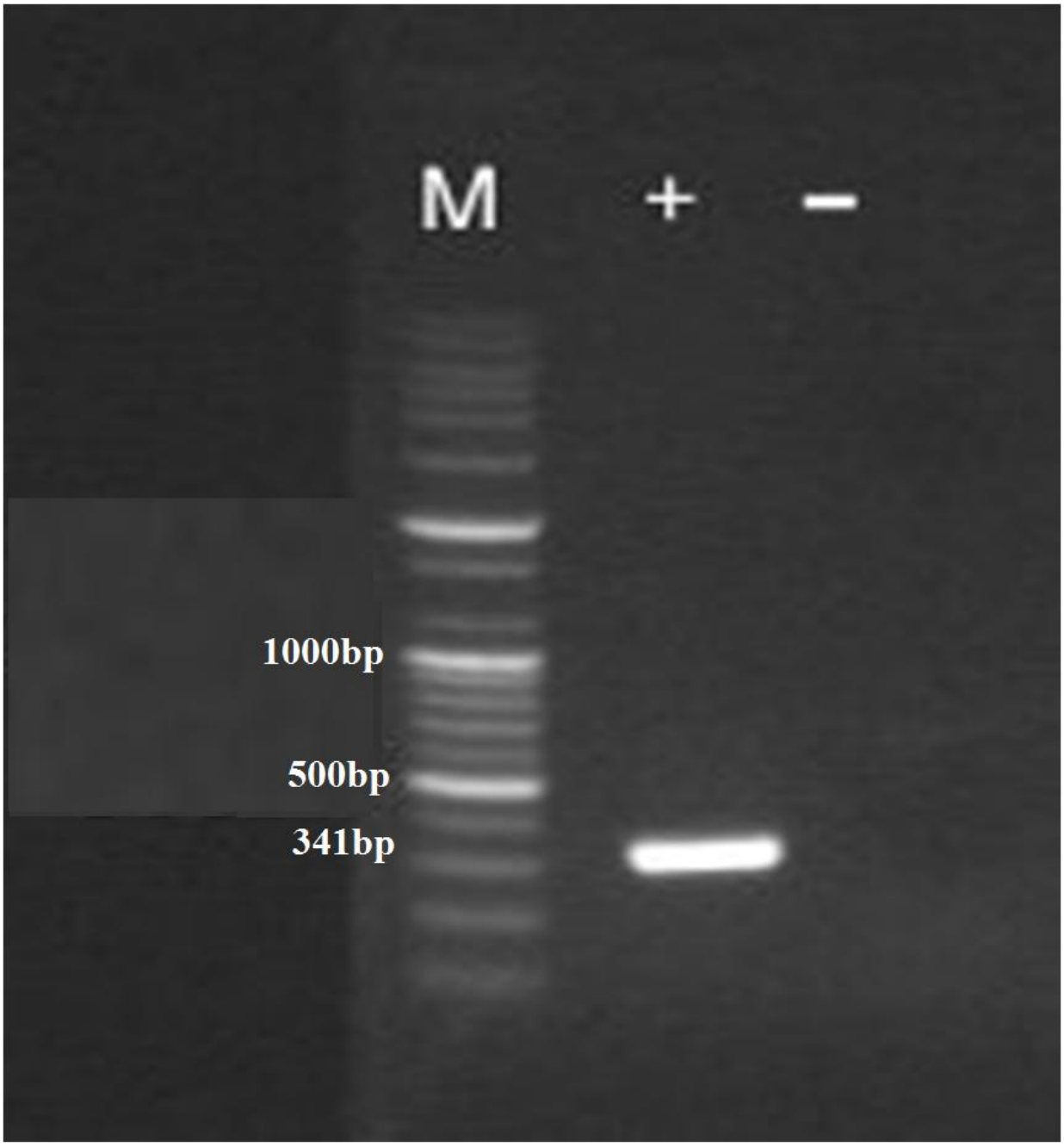
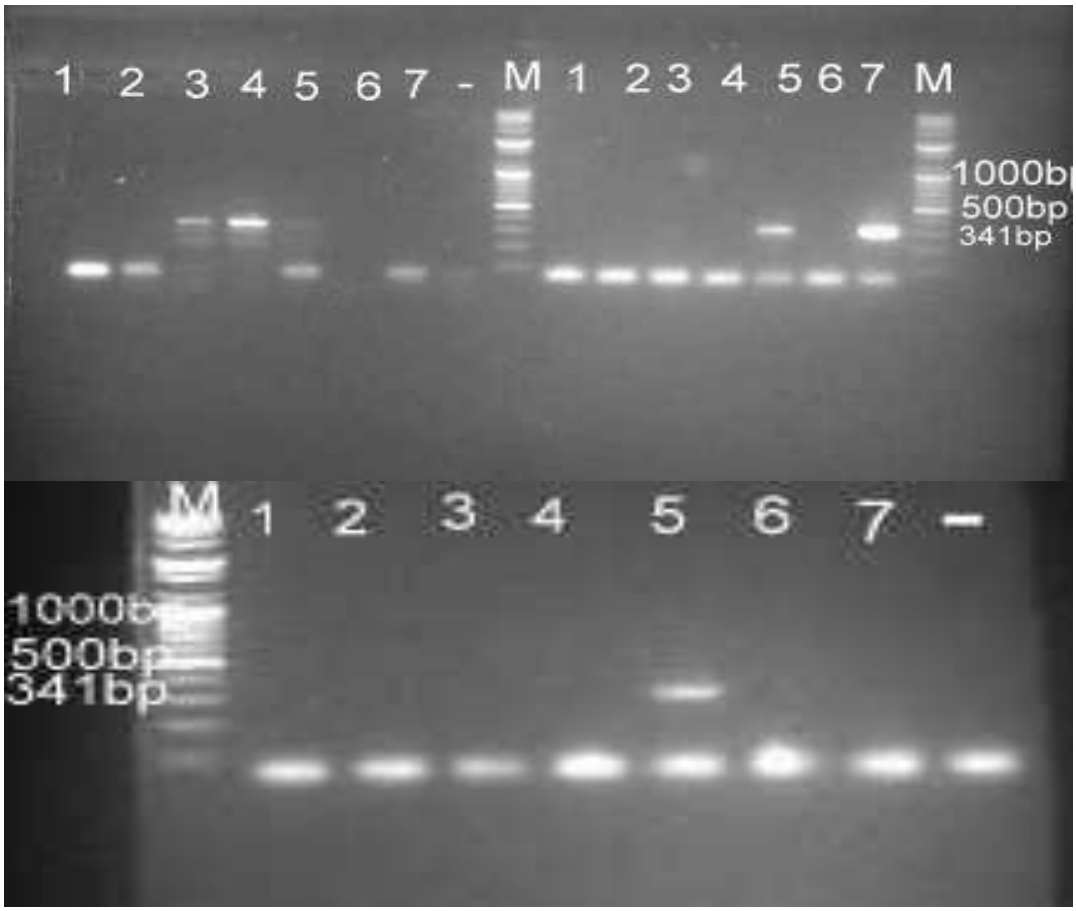


Plate II: Electropherograph of Genomic DNA ExtractedFrom *Trypanosoma congolense*, Amplified by PCR using Species Specific Primers.

Lane1: 100 bpmolecular ladder

Lane 2: positive sample (infected *Trypanosoma congolense* blood)

Lane 3: negative control.



PlateIII: Electropherograph ShowingAmplification of *Trypanosoma congolense*GenomicDNAExtracted DNA from Infected Tsetse Fly's anal droppings by PCR, using SpeciesSpecific primers, in Group A.

M= marker

- = Negative control

Top left, Lane 1-7 represent individual fly's anal droppings in day 3 post infection time points.

Top right, Lane 1-7represent individual fly's anal droppings in day 10 post infection time points.

Bottom, Lane 1-7 represent individual fly's anal droppings in day 24 post infection time points.

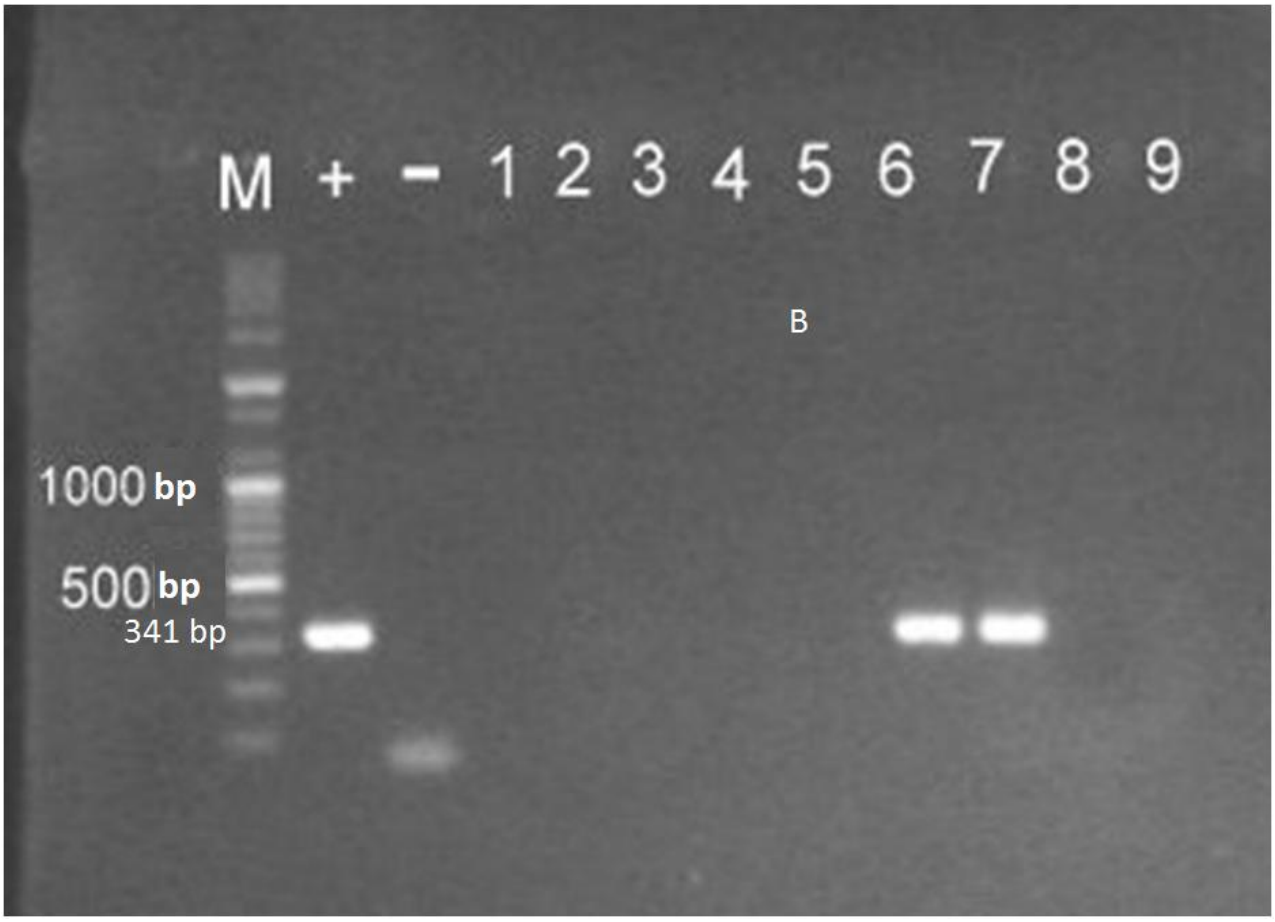


Plate IV: Electropherograph Showing Amplification of *Trypanosoma congolense* Genomic DNAExtracted DNA From Infected Tsetse fly's anal droppings by PCR, usingSpecies specific primers, in Group B .

Lane 1-3 represent individual flies anal droppings in day 3 post infection time points.

Lane 4-6 represents individual fly's anal droppings in day 10 post infection time points.

Lane 7-9 represents individual fly's anal droppings in day 24 post infection time points.

M= Marker

+ = positive control (infected blood)

- = negative control

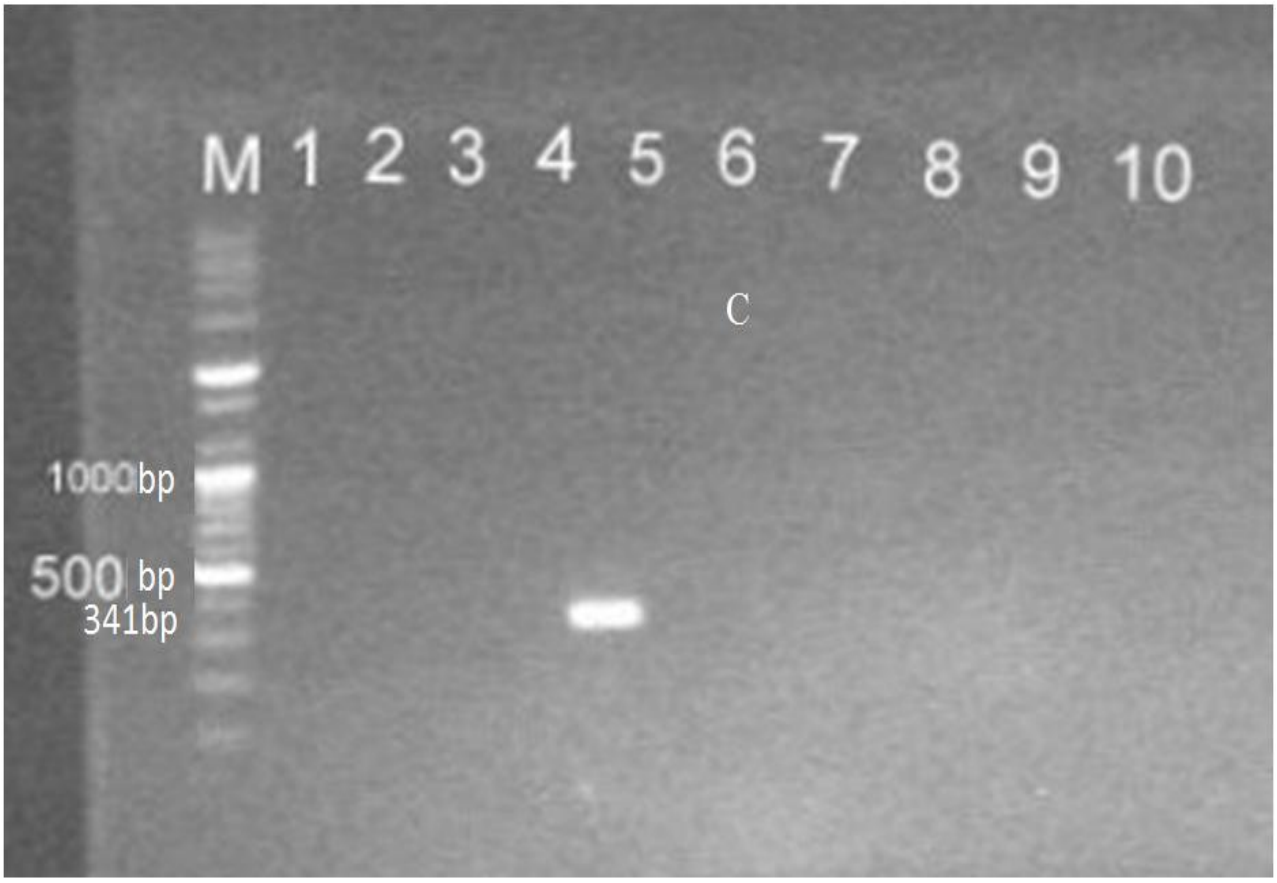


Plate V: Electropherograph Showing Amplification of *Trypanosoma congolense* Genomic DNAExtracted DNA From Infected Tsetse fly's anal droppings by PCR, using Speciesspecific primers, in group C.

Lane 1-3 represent individual flies anal droppings day 3 post infection time point.

Lane 4-6 represent individual flies anal droppings day 10 post infection time point.

Lane 7-10 represent individual flies anal droppings day 24 post infection time point.

M = Marker

4.3 Trypanosomal Infection Rate In Glossina Morsitans morsitans Detected by PCR and Mortality Rate

A total number of 54 fully engorged flies was used for the experiment, but only 40 flies survived during the experiment and the remaining 14 flies died. However, PCRdetect infection in only 8 flies in the three groups, as shown in **Table 4.1**

4.4 Quantification Of Midgut Extracted RNA

The extracted RNA from infected flys midgut, was quantified using UV – spectrophotometer RNA/DNA calculator (gene quant) at absorbance at 260nm (A_{260}), their concentration and purity in the four samples were determined. These was used to synthesized 0.50 µg of cDNA in the four samples as shown in **Table 4.2**

4.5 RT-PCR of Glutamic acid Proline (Ep) Protein and Tubulin gene

The cDNA synthesized from the extracted RNA of pooled infected flies and uninfected (control) flies midgut, at three different post infections time points that were amplified using the EP Protein and Tubulin housekeeping genes primers to test the effectiveness of the primers and to confirm their products size,the result obtained gave the expected band size for the Glutamic acid proline (Ep) protein and Tubulin housekeeping gene in the 4 samples, which are 108bp and 149bp, respectively**(Plate VI** and **Plate VII).**

Table 4.1Trypanosome Infection Rate In *Glossina morsitans morsitans* Detected By PCR, And Mortality Rate.

TOTAL ENGORGED FLIES	GROUPS	POSTINFECTION DAYS	INFECTION RATE	TOTAL MORTALITY
30	A	3 DAYS	2/7	9/30
		10 DAYS	2/7	
		24 DAYS	1/7	
12	B	3 DAYS	0/3	3/12
		10 DAYS	1/3	
		24 DAYS	1/3	
12	C	3 DAYS	0/3	2/12
		10 DAYS	1/3	
		24 DAYS	0/4	

Table 4.2: Midgut Extracted RNA Quantification Table

SAMPLE NO.	RNA CONCENTRATION (ng/µl)	PURITY	Volume of RNA required toSynthesise 0.5µg Cdna
1 control guts	89	1.9	5.6µl
2 Day 3 flies gut	183	2.1	2.7µl
3 day 10 flies gut	138	2.1	3.6µl
4 day 24 flies gut	83	2.0	6.0µl

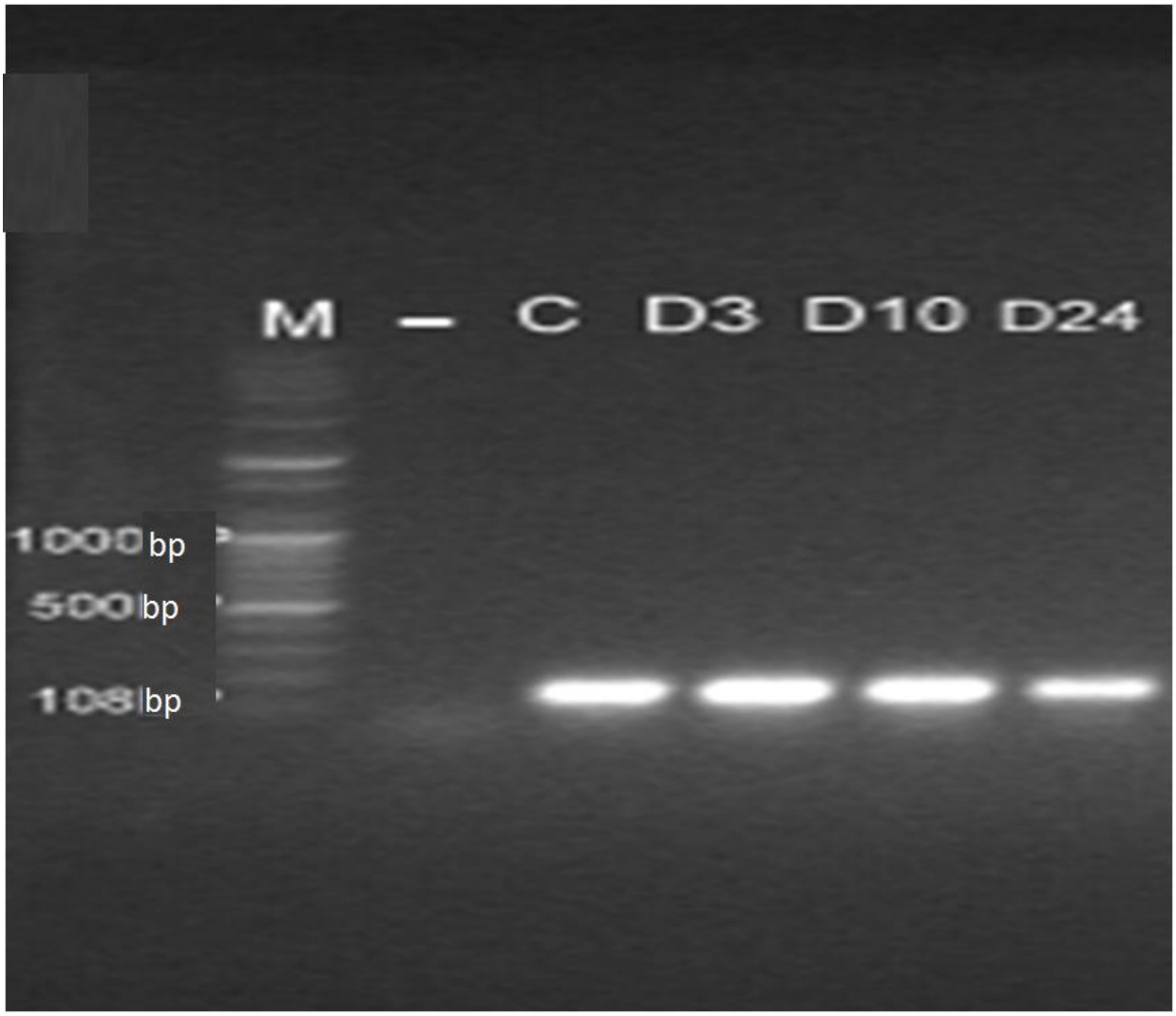


Plate VII: Electropherograph Showing Transcript profiles of midgut Amplified using tsetse EP primers

M = Marker

- =negative control (Water)

C = Control (uninfected midgut)
D3 = Pooled infected midgut for day 3 post infection timepoints
D10 = Pooled infected midgut for day 10 post infection timepoints
D24 = Pooled infected midgut for day 24 post infection timepoints

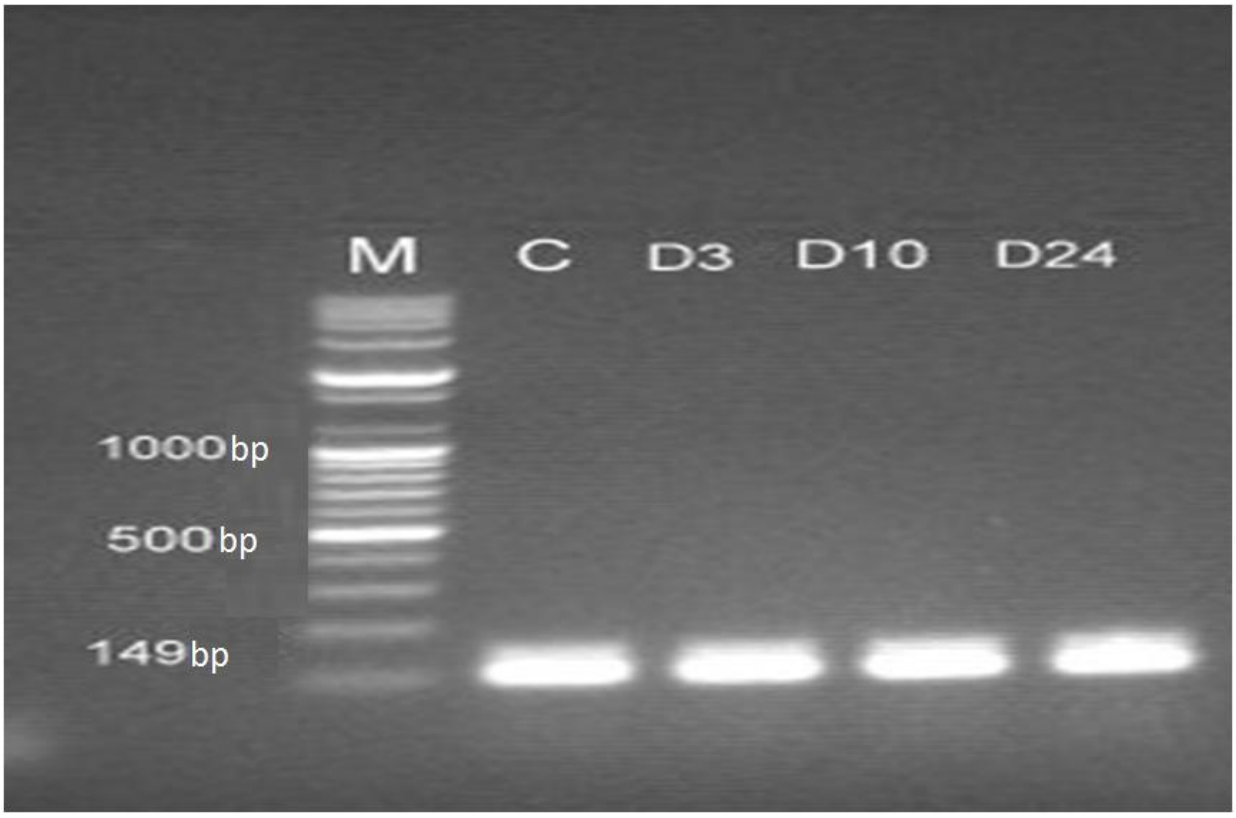


Plate VIII: Electropherograph Showing Transcript profiles of midgut Amplified using Tubulin (control) primer

M = Marker

C = Control (uninfected midgut)

D3 = Pooled infected midgut for day 3 post infection timepoints
D10 = Pooled infected midgut for day 10 post infection timepoints
D24 = Pooled infected midgut for day 24 post infection timepoints

4.6 qPCR Data Processing and Analysis

qPCR analysis of all the 4 samples (day 3, 10 and 24 pooled infected flies midgut and control mid gut) were ran in triplicate for the Ep protein and TubulinHousekeeping gene respectively, and the result obtained from the amplification curve (Cp values) graph, was used to calculate the fold change (expression) using the method describe by Livark and Schimitgen (2001). The analyzedresultwas used to plot a graph of fold change (expression)of Ep protein against post infection timepoints (days)in order to compare their expression levels at the 3 different post infection time points, as shown in**Table 4.3** , **Figure 4.1**and **Figure 4.2** respectively .

Table 4.3 Result of qPCR Data analysis

Sample	Gene		ΔCp	ΔΔCp	$2^{-\Delta\Delta Cp}$(Fold change) EP protein Expression
	Ep	**Tubulin**			
Control	41	6.816			
	41	6.541			
	41	7.253			
Mean	41	6.87	34.13	0	1
D3 P.I	36.23	11.51			
	34.93	7.662			
	41	6.878			
Mean	37.387	8.683	28.703	-5.427	43.022
D10 P.I	41	8.284			
	41	6.551			
	41	6.633			
Mean	41	7.156	33.844	-0.286	1.219
D24 P.I	41	6.404			
	41	6.591			
	41	8.66			
Mean	41	7.218	33.782	-0.3483	1.273

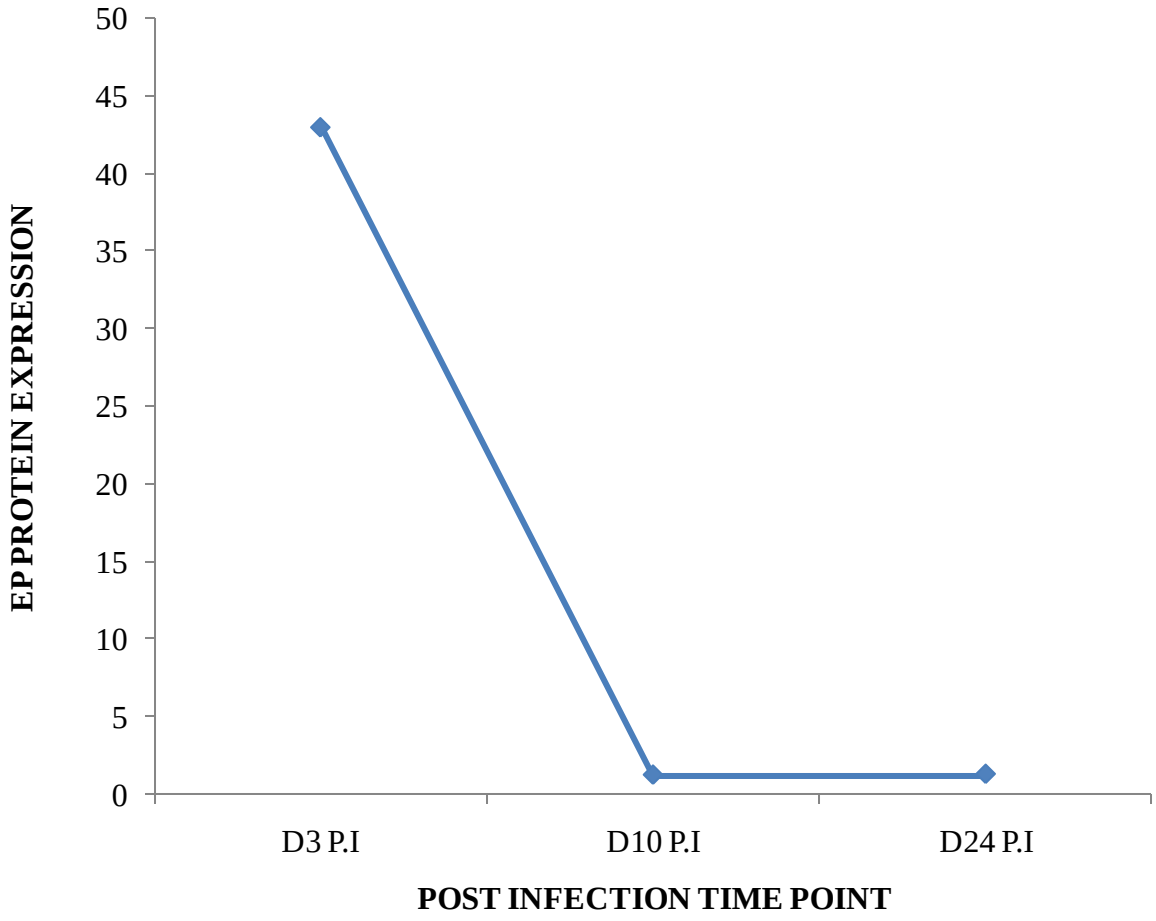


Figure 4.1: EP Protein Expression against Post Infection Time Point

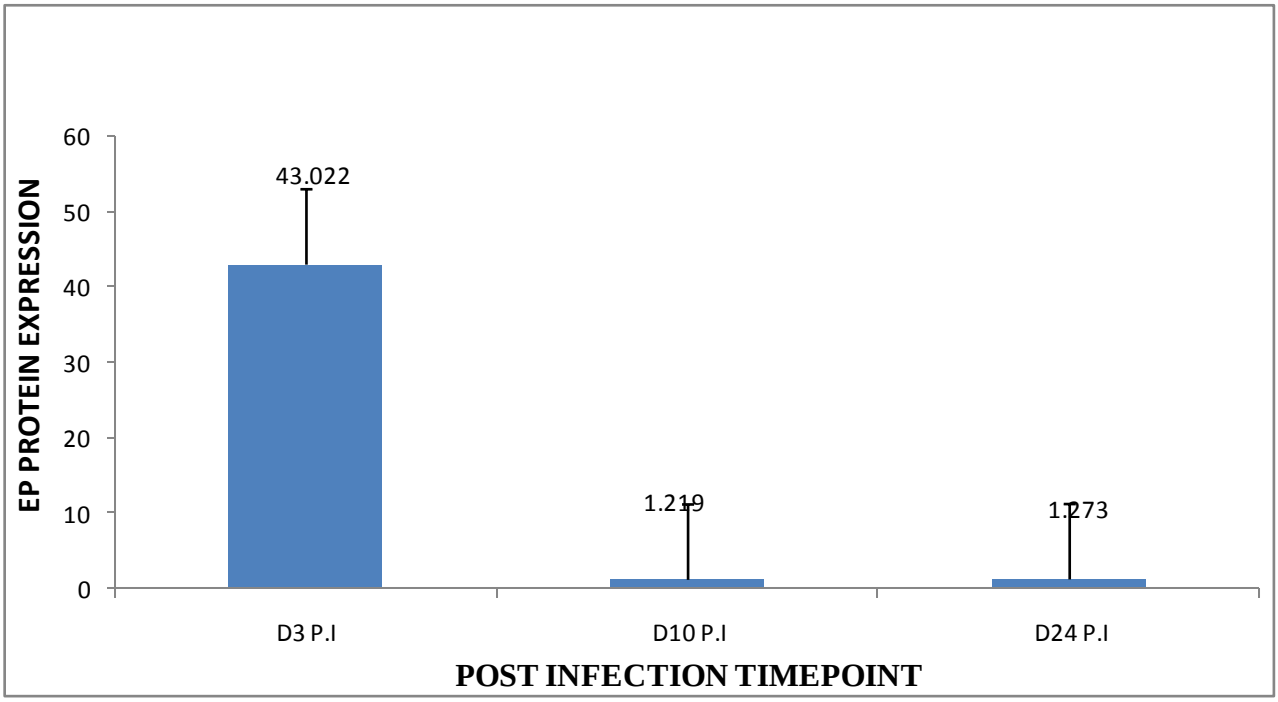


Figure 4.2: EP protein Expression against PostInfection Timepoint

CHAPTER FIVE

5.0 DISCUSSION

The aim of this experiment is to study the expression of midgut EP protein gene, in *Glossina MorsitansMorsitan* infected with *Trypanosoma congolense,* during early day (3 and 10) and late(day 24) establishment of the trypanosomes in the midgut.

To achieved this aim, it was very important to identify the parasite used for the experiment, using PCR.The PCR result obtained, confirmed that the parasite to be used was *Trypanosoma congolense* Savanah, as expected plate II. This was used for the infective feeding of the teneral flies.

PCR IS a molecular technique used as diagnostic tools for detection of infection due to it specificity and sensitivity (Elsier *et al.*, 2004).

The PCR technique was further used to detect the presence of Trypanosomes infection in the flies that Fed on the infected blood meal, at the three different post infection time point (day 3, 10 and 24) in the three different groups (A,B and C) respectively, using DNA extractedfromeach infected fly anal droppings. The result obtained in this study, shows that greater percentage (80%) of flies that had the infective blood meal were refractory to the infection, with only 20% having midgut infection, as recorded in the three groups (A,B and C) as shown in plate III, IV and V).

Studies demonstrated by (Lehane *et al.*, 2003, 2008), have shown that in ideal laboratory condition, 40%or more flies that feed on trypanosome infected blood meal are refractory to the parasite, depending on the fly species and parasite strain. However, (Haines *et al,*, 2010) also demonstrate that typically, 90%of flies infections fail, most during initial

establishment of the parasite in the fly midgut. Even under ideal artificial infection condition in the laboratory, frequently only 15- 20% of Tsetse flies fed on a trypanosome infected blood meal become infected (Ravel *et al.*, 2006). Another studies demonstrated by craft *et al.*, 1982. Shows that approximately 72 hour following ingestion of the infected blood meal, a process of attrition leads to the complete elimination of the infection in a high proportion of the flies, with only few percentage becoming infected.

In this study, qPCR was performed in order to determine the expression of the EP protein,

during the early (day 3 and 10) and late (day24) establishment. The result obtained showed that, EP protein was significantly unregulated in day 3 infected midgut, which could be as a resultof the Tsetse midgut immune response triggered by the parasite entry to the midgut during the early establishment in order to protect the midgut from being infected. Haines et al,, (2010) demonstrated that EP protein was strongly upregulated following fly challenge with gramme negativebacterial, thereby inducing increase expression of the Tsetse EP protein as well as in response to trypanosome infection, and thus EP protein is probably an immune molecule. Report by Illiasou *et al*. (2014), showed that, EP protein transcript level was downregulated at any stage of trypanosome bruicei gambiense infection. However this study detected expression of EP protein in day 3 infected fly gut, which contradict the findings of Illiasou et al. 2014, the tsetse immune response involvement may depend on the tsetse/trypanosome species interaction (Illiasou *et al.*, 2014). Akoda st al. (2009), also demonstrated that starvation can result to significant reduction in non induce baseline immune gene expression, but only after a longer starvation, (4 days for newly emerge flies, 7 days for older flies). In this study flies were starved for 2 days prior to dissection, to remove any blood meal in the fly gut, and the result obtained showed that EP protein was

significantly downregulated at day 10 and 24 post infection time point which could explain the variation in the expression of the EP protein transcript between day 3 infected midgut as compared to day 10 and 24 infected gut.

Tsetse Ep protein levels are however, dramatically reduce after day 3 of starvation Haines et al., 2010)

From the result of expression of Ep protein gene obtained, we can say that the expression of this gene is a function of time, and also the period of trypanosome in the midgut.

CHAPTER SIX

SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 Summary

i. PCR was able to detectinfection in anal drops of flies as early as day 3 post infection which is contrary to the infection cycle of 4-24 days, hence may be immature infection

ii.Expression of tsetse Ep protein is a time dependent factor and can be influence by the strain of parasite/vector interaction

iii. Tsetse Ep protein was significantly upregulated at day 3 post infection time point as compared to day 10 and 24 time point, hence may be as a result of the tsetse EP immune response triggered by the trypanosomes on entering the midgut, which is the first point of trypanosome establishment

6.2 Conclusion

The result obtained from these studies, was able to confirmed that EP protein transcript was significantly expressed in day 3 infected fly's midguts, but its expression for day 10 and 24 infected flies midgut, was significantly reduce as compared to that of day 3 infected flies midgut, which clearly gives an insight on the expression of the EP protein gene during early establishment and late establishment of the parasite, suggesting that the expression of this gene is a time dependent process and could also be due to some factors such as species and strain of tsetse/trypanosome interaction.

6.3 Recommendation

Further research interest should be focus on the biology of the tsetse-trypanosome interaction, especially at the initial stage of establishment in the midgut and targeting other immune proteins in the midgut, which could help to provide a way of breaking or

disrupting the developmental transformation of the parasite in the vector, therefore preventing cyclical transmission of these parasites from the tsetse flies to healthy animals during the tsetse blood feeding event.

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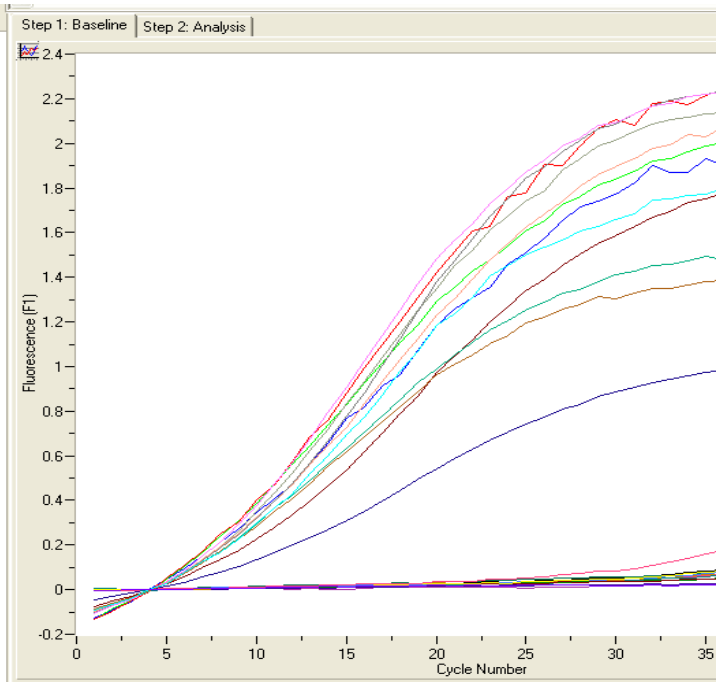
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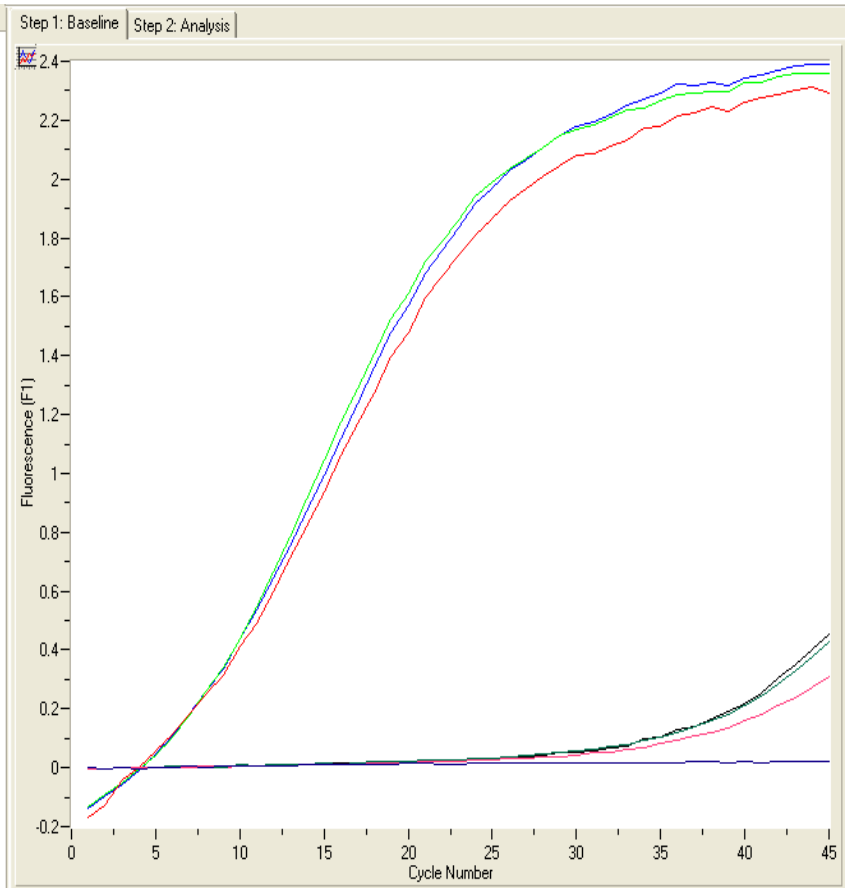
APPENDIX

P...	Name	Calculate...	Cros...
1	TC1	0.000E+00	6.816
2	TC2	0.000E+00	6.541
3	TC3	0.000E+00	7.253
4	EC1	<0.000E+00	>41.00
5	EC2	<0.000E+00	>41.00
6	EC3	<0.000E+00	>41.00
7	TD3A	0.000E+00	11.51
8	TD3B	0.000E+00	7.662
9	TD3C	0.000E+00	6.878
10	ED3A	0.000E+00	36.23
11	ED3B	0.000E+00	34.93
12	ED3C	<0.000E+00	>41.00
13	TD10A	0.000E+00	11.28
14	TD10B	0.000E+00	7.456
15	TD10C	0.000E+00	8.005
16	ED10A		
18	ED10B		
19	ED10C		
20	TD24A	0.000E+00	6.404
21	TD24B	0.000E+00	6.591
22	TD24C	0.000E+00	8.660
23	ED24A	<0.000E+00	>41.00
24	ED24B	<0.000E+00	>41.00
25	ED24C	<0.000E+00	>41.00
26	-VE		



qPCR Amplification Curve of Tubulin and EP protein cDNA from Tsetse Fly Midgut For day 3 and 24 Post infection Time point

Na...	Calculate...	Cros...
T10A	0.000E+00	8.284
T10B	0.000E+00	6.551
T10C	0.000E+00	6.633
E10A	<0.000E+00	>41.00
E10B	<0.000E+00	>41.00
E10C	<0.000E+00	>41.00
-ve		



qPCR Amplification Curve of Tubulin and EP protein cDNA from Tsetse Fly Midgut For day 10 Post infection Time point