STUDIES ON THE EXPRESSION OF EP PROCYCLIN – LIKE GENE IN CULTURED *Trypanosoma congolense.*

BY

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ZARIA, NIGERIA

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BY

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF MASTER OF SCIENCE DEGREE IN BIOTECHNOLOGY

DEPARTMENT OF BIOCHEMISTRY,
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SEPTEMBER, 2018

DECLARATION

I hereby declare that the work in this dissertation "**Studies on The Expression of EP Procyclin – like gene in cultured *Trypanosoma congolense***" was performed by me in the Department of Biochemistry, under the supervision of Prof. Y.K.E Ibrahim and Dr. I. A. Aimola. The information derived from literature has been duly acknowledged in the text and a list of references provided. No part of this work has been presented for another degree or diploma at any institution.

YUSUF BLESSING BECKY	_____	_____
P13SCBC8052	Signature	Date

CERTIFICATION

This dissertation titled "**Studies on The Expression of EP Procyclin – like gene in cultured*Trypanosoma congolense***" meets the regulation governing the award of the degree of Masters in Biotechnology of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This research work is dedicated to my dear husband, Mr. Taita Iliya, for your relentless love.

ACKNOWLEDGEMENT

My deep appreciation is first to God Almighty with whose Mercy, Grace, Provision, and Strength I am able to come this far.

I am grateful to my project supervisors, Prof. Y.K.E Ibrahim and Dr. I.A Aimola for their tireless effort and fatherly suggestions, corrections, close supervision and push during the course of this work. It was your assistance, in all ramifications that made this work a success.

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ABSTRACT

Trypanosama brucei is known to express EP procyclins in the Tsetse fly midgut. These proteins protect the parasite from digestive enzymes in the fly's mid-gut and are not known to be expressed in the blood stream forms of Trypanosomes. *T. congolense* was obtained from the blood of an infected laboratory mouse. Trypanosomes were adapted to culture conditions in RPMI 1640 culture medium supplemented with FBS, glucose, β-Mercaptoethanol and cysteine. The parasites were monitored at 6, 12, 24 and 48hours respectively and we observed that the *in vitro* culture conditions supported the survival of *T. congolense* and the parasites retained their animal infectivity potential after 48 hours of culture. Gene expression level of the cultured parasites was assayed, to determine the presence of *EP-procyclin* in the parasites. Primers designed from the conserved regions of *T. brucei EP-procyclin* gene to identify the *EP-procyclin* gene in blood stream form of *T. congolense*. *EP-procyclin* gene expression was monitored at 0, 24 and 48 hours of *T. congolense* culture. The results reveal that cultured blood stream form of *T. congolense* expressed *EP-procyclin-like* gene and that the optimum time to maintain a culture of bloodstream forms of *T. congolense* is between 12 and 24 hours.

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ABBREVIATIONS

AAT – Animal African Trypanosomiasis

BSF – BloodStream Forms

cDNA – Complementary DNA

C_P/C_T Value – Crossingpoint/ Crossing threshold value

DEPC – DiethylPyrocarbonate

DNA – Deoxyribonucleic acid

EMF – Epimastigote Forms

EP – Glutamic acid and Proline repeat

E-value – Expected value

FBS – fetal Bovine Serum

GPEET – Glycine-Proline-Glutamicacid-Glutamic acid-Threonine

GPI – Glycosylphosphtidyl Inositol

HAT – Human African Trypanosomiasis

MCF – Metacylic Forms

ORFs – Open Reading Frames

PARP – Procyclic acidic repetitive proteins

PCR – Polymerase Chain Reaction

PF – Procyclic Form

PV – Proventriculus

qRT-PCR – Quantitative real time PCR

RPMI – Roswell Park Memorial institute

RT-PCR – Reverse Transcriptase PCR

VSG – Variant Surface Glycoprotein

CHAPTER ONE

1.0 INTRODUCTION

Trypanosomiasis is a disease caused by the protozoan of the order; *Kinetoplastida*. The disease which is classified into two; the Human African Trypanosomiasis (HAT) and the African Animal Trypanosomiasis (AAT), is cyclically transmitted by biting flies of the *Glossina* genus (tsetse flies).(Haines, *et al.*, 2011). In animals, symptoms of the disease include fever, listlessness, emaciation, hair loss, and discharge from the eyes, oedema, anaemia, and paralysis (Steverding, 2008). As the illness persists, the animal becomes listless, it lags behind the herd, loses interest in its surroundings, its ears and tail hang limply and generally becomes unfit for work, hence the name "N'gana" which is the Zulu word for 'depressed spirit'.

Trypanosomiasis is caused by several species of trypanosomes in both humans and domestic animals. These disease-causing trypanosomes include *Trypanosoma congolense*, *T. vivax, T. brucei*, *T. evansi*, and*T. cruzi.*

Trypanosomes are flagellate protozoans that inhabit the blood plasma, the lymph and various tissues of theirhosts. They belong to the genus *Trypanosoma,* in the family*Trypanosomatidae,* and the order Kinetoplastida. (Desquesnes, 2004). They are responsible for several neglected tropical diseases, in man and animals such as cattle, horses, goats, etc.

The transmitting vector in trypanosomiasis is the tsetse fly.The vector is 6–15 mm in length.Tsetse flies are bloodsucking flies of the genus *Glossina*. These flies are found in tropical Africa between latitude 15° N and 29° S, and are important vectors of African trypanosomiasis in both humans and animals.

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 *T. congolense*is a small trypanosome, shorter in length than *T. brucei*and without a conspicuous undulating membrane. In the tsetse fly vector, both species develop and multiply in the midgut initially, before onward migration to the mouthparts.

When bloodstream form (BSF) trypanosomes are taken up by a tsetse fly during its blood meal, they enter the insect's midgut and a certain population differentiates to procyclic forms (PF) that are adapted to life in the midgut (Vickman, 1985). This adaptation is done by the remodeling of the parasite's surface and loss of the variant surface glycoproteins (VSG) coat. The surface of the parasites is then covered instead by other proteins termed procyclins (Roditti, *et al.*, 1989) (also known as procyclic acidic repetitive proteins). In *T. brucei,* procyclic forms of this coat consists of Glutamic acid and Proline (EP) and Glycine-Proline-Glutamic acid-Glutamic acid-Threonine (GPEET) repeat forms of procyclins (Roditi, *et al.*, 1998). EP and GPEET are the first letter representations of the amino acids they stand for.

The EP procyclins are a group of closely related proteins that are encoded by six or seven genes and contain extensive tandem repeat units of glutamic acid and proline (E and P, respectively, in the single-letter amino acid code). (Keonig, *et al.*, 1989; Mowatt,*et al.*, 1989).

Experiments have shown that EP is important for survival of trypanosomes in the insect host, possibly by mediating parasite-fly interactions (Ruepp,*et al.*, 1997). Its presence has also been discovered in the midgut of the insect host and its sequence similarity to the sequence of that in the parasite is remarkable (Haines, *et al.*, 2010) hence, the thought thatit confers resistance

against infection in the fly while at the same time ensuring the trypanosomes are able to further differentiate into the epimastigote forms and move to the salivary gland.

1.1 STATEMENT OF RESEARCH PROBLEM

African animal trypanosomiasis is an important disease of domestic livestock in sub-Saharan Africa, caused by the protozoan trypanosomes. Trypanosomes kills more than 3 million cattle annually, with *T. congolense*as a major contributor. The annual losses from trypanosomiasis in cattle amount to more than US $4.5 billion. Direct costs due to AAT involve decreased livestock productivity (mortality, fertility, milk yield, ability to work as traction animals),and expenditure on controlling the disease (Shaw, 2004) which are estimated to amount to between US$ 1-1.2 billion each year. Indirect impact of AAT on agriculture in sub-Saharan Africa far outweighs the direct cost. Although drugs for treatment of African Animal Trypanosomiasis are readily available; e.g., isometamidium chloride, homidium (bromide and chloride) and diminazene aceturate, cases of drugs resistance have been reported.

Resistance to immune responses due to the VSG and procyclic EP coats, have been shown to be very critical to the survival of the trypanosomes in both mammalian and insect hosts(Roditti, *et al.*, 1989; Ruepp, *et al.*, 1997). EP protein is crucial to the survival of the parasites in the fly, and their ability to protect them against fly's immune response facilitates transmission from vector to host. In addition, these changing surface coats enables the trypanosomes to evade immune responses of the host and eventually cause disease(Haines, *et al.*, 2010).

Previous studies have demonstrated the presence of EP- *procyclin* in some species like the *T. brucei*. The expression pattern of this protein in *T. congolense* has not yet been fully elucidated/ demonstrated. Detection of the presence and understanding the expression pattern of surface

proteins, particularly the EP protein in *T. congolense* can provide a template for the development of new strategies and treatment regimen in the control and management of trypanosomiasis in animals.

1.2 JUSTIFICATION

Drug and vaccine development is a promising approach for the prevention and treatment of many diseases. EP protein helps in survival and differentiation of parasites in the insect host (Haines *et al*., 2010). EP presence has been demonstrated in *T. brucei* and in the procyclic forms of the parasite.Targeting it may break the transmission cycle of the parasite, help in understanding the pathogenicity of the parasite, and add to new knowledge in the transition process of trypanosomes.Overall, a break in transmission chain of trypanosomiasis will improve livestock, increase agricultural output and nutritional quality and health of the people and upliftment of the African community.

Identification of this protein and its expression in other species (especially *T. congolense*)and also the cultured bloodstream forms, will be an interesting finding and target for vaccine development against the trypanosomiasis disease due to the regulatory role of *EP protein* in the parasites.

1.3 AIM AND OBJECTIVES

1.3.1 General Aim

To determine the expression of EP- procyclin gene in cultured Bloodstream Forms of *Trypanosoma congolense*, parasites.

1.3.2 Specific Objectives:

1. To identify EP-procyclin gene in *T. congolense* from available trypanosomal genome sequence.
2. To amplify EP - procyclin gene from cultured bloodstream forms of *T. congolense*.
3. To determine the expression level of EP-procyclin in cultured bloodstream forms of *T. congolense*.

1.4 HYPOTHESIS

1.4.1 Null Hypothesis

Ho_1 - the EP-procyclin gene is not expressed in *Trypanosoma congolense*.

Ho_2 - in vitro cultivation has no effect on the parasites and therefore the EP-procyclin gene is not expressed.

1.4.2 Alternate Hypothesis

HA_1 – EP procyclin gene is expressed in *T. congolense*.

HA_2 – in vitro culture of*T. congolense* affects its ability to express the EP-procyclingene

1.5 LIMITATION AND CONSTRAINT

The work was limited by the fact that it set out to identify the EP- procyclin transcript and not the protein in the bloodstream forms of *T. Congolense*.

` **CHAPTER TWO**

2.0 LITERATURE REVIEW

2.1 TRYPANOSOMIASIS

Trypanosomiasis is a disease caused by protozoan Trypanosomes. It is a disease which affects both humans (African and American human trypanosomiasis) and animals. In Africa, the disease is classified into two; the Human African Trypanosomiasis (HAT) and the African Animal Trypanosomiasis (AAT), and is cyclically transmitted by biting flies like of the *Glossina* genus (tsetse flies) (Haines, *et al.*, 2011).

Trypanosomiasis is an endemic disease particularly in the African continent, affecting 36 countries in western and eastern Africa, with about 60 million people at risk and 500,000 reported cases; and approximately 40 million cattle threatened, with 3million of these cattle being killedannually (Maudlin, 2006).

Tsetse-transmitted trypanosomiasis is usually an acute or chronic disease that causes intermittent fever with general symptoms like anaemia, oedema, lacrimation, enlarged lymph nodes,hair loss, and discharge from the eyes, paralysis, abortion, decreased fertility, loss of appetite and weight, leading to early death in acute forms or to digestive and/or nervous signs with emaciation and eventually death in chronic forms. (Steverding, 2008). As the illness persists in the AAT, the infected animal becomes unfit for work; hence the name N'gana which is the Zulu word for 'depressed spirit'.In humans, it causes what is popularly known as the 'sleeping sicknesss' with symptoms that occur in two stages. The first stage, known as the **hemolymphatic phase**, is characterized by fever, headaches, joint pains, and itching. Fever is intermittent, with attacks lasting from a day to a week. Invasion of the circulatory and lymphatic systems by the parasites

is associated with severe swelling of the lymph nodes, to tremendous sizes (Lundkvist, *et al.*,2004).

The second stage, called the **neurological phase**, begins when the parasite invades the central nervous system by passing through the blood–brain barrier. Disruption of the sleep cycle is a primary symptom of this stage (how the disease got the name 'sleeping sickness'). Infected individuals experience a disorganized and fragmented 24-hour rhythm of the sleep-wake cycle, resulting in daytime sleep episodes and nighttime periods of wakefulness(Lundkvist, *et al.*,2004).Other neurological symptoms associated with AAT include confusion, tremor, general muscle weakness, hemiparesis and paralysis of a limb. Individuals may also exhibit psychiatric symptoms such as irritability, psychotic reactions, aggressive behavior, or apathy (Brun, *et al.*, 2010).Without treatment, the disease is invariably fatal, with progressive mental deterioration leading to coma, systemic organ failure, and death. Damage caused in the neurological phase is irreversible.(Brun, *et al.*, 2010).

IMPACT OF TRYPANOSOMIASIS

Trypanosomiasis, both of humans and of livestock, is one of the most important factors restricting economic development in Africa today. Economic impact of this disease can be classified into two major categories:Qualitative Impact and Quantitative Impact.

The qualitative impact of trypanosomiasis consists of direct and indirect losses. Direct costs involve decreased livestock productivity (mortality, fertility, meat and milk yields, and ability to work as traction animals. The direct cost also includes the detection, treatment of infected animals, fly control and research (Finelle, 1974; Shaw, *et al*,2013). The indirect impact of trypanosomiasis mostly lies on crop production; through the availability and cost of animals'

traction power (Omotainse, *et al*,2004; Swallow,*et al.*, 2000). The indirect losses remain an important factor due to severe immunosuppression produced by the disease.

Quantitative assessment of the economic loss due to African Animal Trypanosomiasis (AAT) costs Africa US$5 billion a year and at least $30 million to control cattle trypanosomiasis in terms of curative and prophylactic treatments (Swallow, 1998)every year. Direct losses due to trypanosomiasis are estimated to range between US$ 1-1.2 billion each year. The total loss for the tsetse-infested lands in terms of agricultural Gross Domestic Product is US$ 4.75 billion per year (Simarro,*et al.*, 2011). According to Onyiah (1997), the Nigerian Institute for Trypanosomiasis Research (NITR) estimated the economic loss due to cattle trypanosomiasis in six states to be N837.20 million annually.

EPIDEMIOLOGY OF AFICAN TRYPANOSOMIASIS

The distribution of African trypanosomiasis is completely linked to the spread of its vector, the tsetse fly. The risk factors for the transmission of this disease are therefore determined by increase in the possibility with which humans/animals come into contact with a tsetse fly. Due to the tsetse fly's climatic restrictions, the disease is restricted to between 14^0 North and 29^0 South south on the African continent (Krafsur, 2009).

Countries where the disease is currently epidemic includes: Angola, Democratic Republic of the Congo, Uganda and Sudan. Countries where the disease is highly endemic are Cameroon, Congo, Cote d'Ivoire, Central African Republic, Guinea, Mozambique, Tanzania, and Chad, while low endemic countries are: Benin, Burkina-Faso, Gabon, Ghana, Equatorial Guinea, Kenya, Mali, Nigeria, Togo, and Zambia. The disease is a threat to more than 60 million people throughout Africa and approximately 40 million cattle are threatened, with 3million of these cattle being killed by trypanosomiasis yearly. Epidemiologists estimated that

between 300,000 to 500,000 cases of these threatened category actually come down with the disease during the same time period.

There have been three major epidemics in Africa in the last Century. One between 1896 and 1906 in Uganda and the Congo Basin. Another one occurred in 1920 involving several African countries, while the third one started in 1970 and before 2010 it has caused around 9,000 deaths, (Lozano, 2012).

2.2 TSETSE FLY AND TRYPANOSOMIASIS TRANSMISSION

Tsetse flies are bloodsucking flies of the genus *Glossina*. *Glossina* species (e.g. *Glossina mortisans and Glossina palpalis*)are 6–15 mm in length, and can be distinguished from other biting flies by their forward-pointing mouthparts (proboscis).They are the vectors of African trypanosomes, which cause sleeping sickness in humans and nagana in animals. These flies are exclusive to Africa in a distribution that stretches between 14^0 North and 29^0 South of the equator (Krafsur, 2009). The restrictedness of tsetse to Africa explains the presence of *T. brucei, T. vivax* and *T. congolense*which cause the disease within sub- Sahara Africa (Steverding, 2008). Tsetse flies inhabit 8.7 millionkm^2 of sub-Saharan Africa known as the "tsetse belt". This area represents approximately a third of Africa.In the areas where tsetse are prevalent, agricultural output is not optimal because of the risk of AAT. Every year approximately 40 million cattle are threatened and 3million are killed by trypanosomiasis, with a total gross domestic product asmuch as $4.5 billion per annum (Maudlin, 2006).

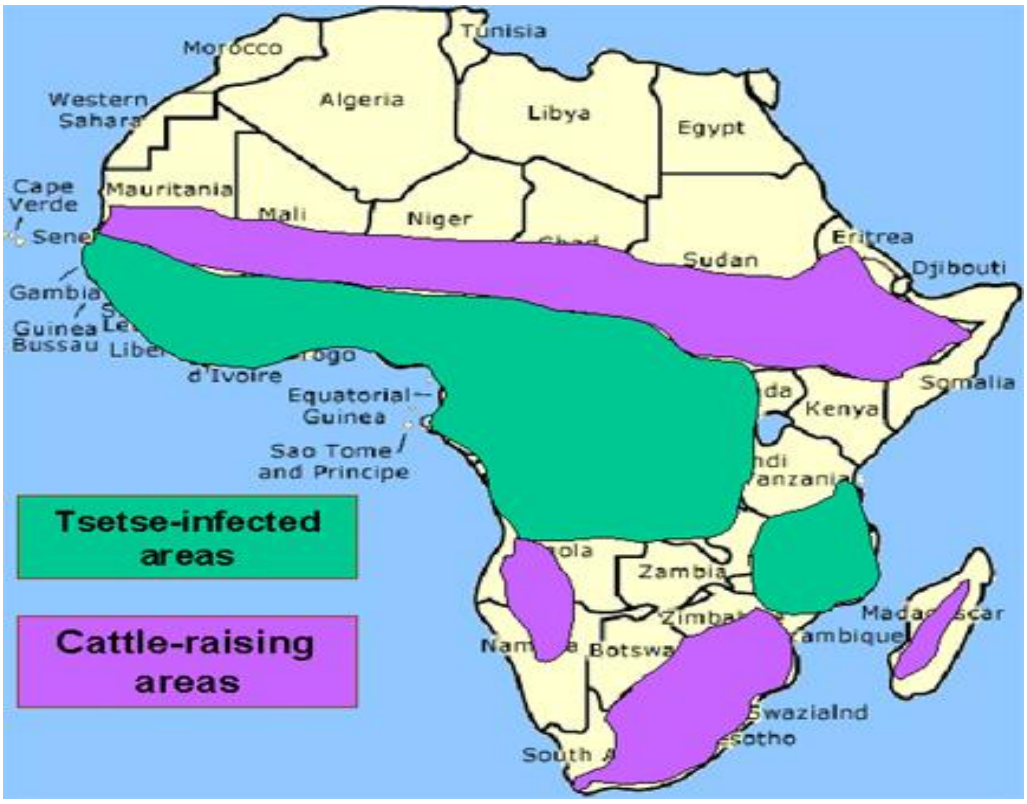


Figure 2.1: Map of Africa showing endemic areas for tsetse flies and trypanosomiasis.

Source:https://www.semanticscholar.org/paper/Human-African-trypanosomiasis-in-endemic-and-Blum-Neumayr/b09db5e9c902122cf652fe1dfdf00e4854ce3ccf

LIFE CYCLE OF TSETSE FLY

The female tsetse fly does not lay eggs but produces larvae, one at a time. The larva develops in the uterus over a period of 10 days and is then deposited fully grown on moist soil or sand in shaded places. It buries itself immediately and turns into a pupa. The fly emerges 22–60 days later, depending on the temperature. The flies pass most of their time at rest in shaded places in forested areas. Preferred locations are the lower woody parts of vegetation. They search for food only for very short periods during the day. Common risk areas where people are likely to be bitten by tsetse flies are: on forest trails;near water collection points in forests;in vegetations close to bathing and water collection sites along the banks of rivers.

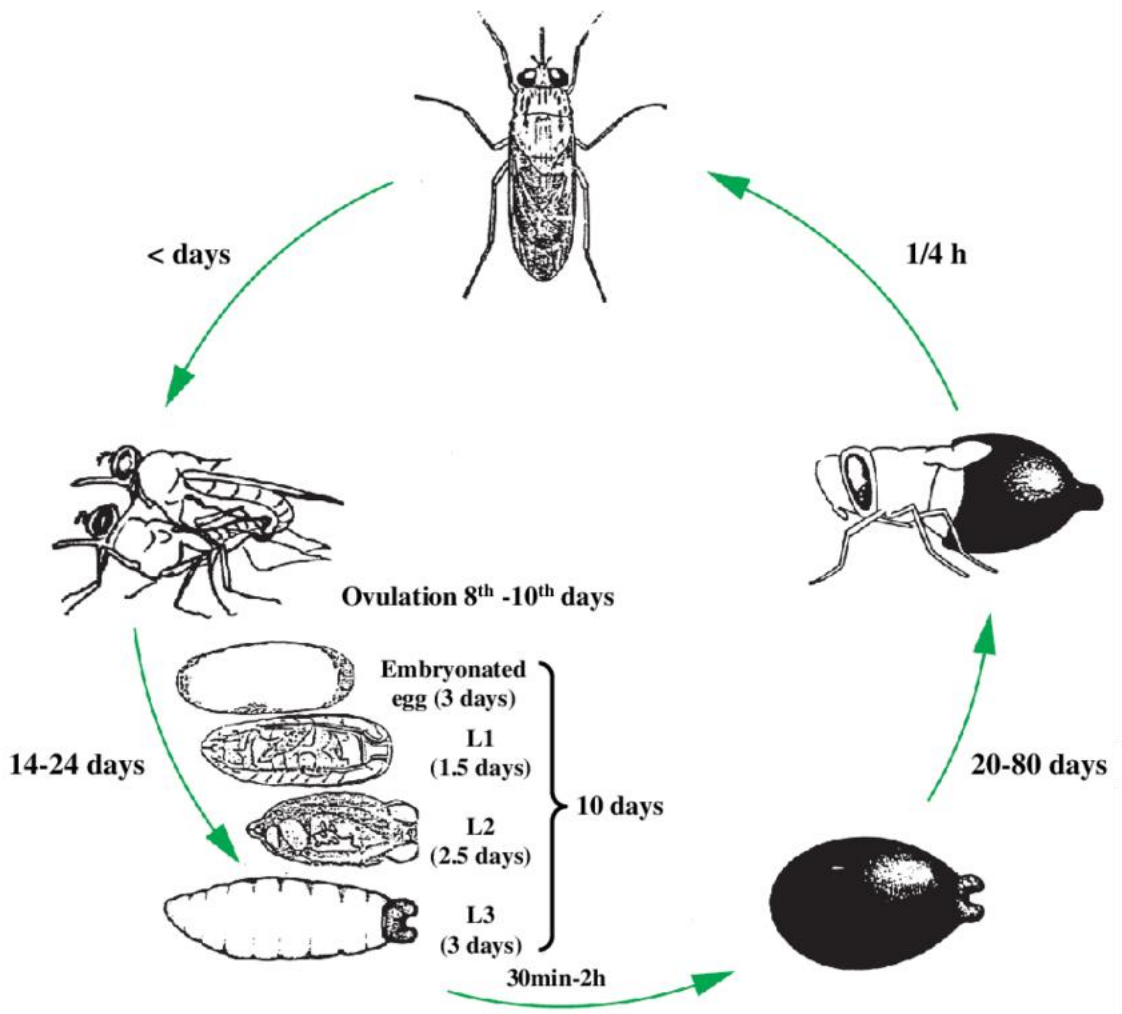


Figure 2.2: Life cycle of *Glossina palpilis* (tsetse fly).

Source:https://www.researchgate.net/figure/3-Reproductive-cycle-of-a-tsetse-fly-adapted-from-Cuisance-1989-When-the-tsetse_fig3_294263312

2.3 TRYPANOSOMES

Trypanosomes are flagellate protozoans that inhabit the blood plasma, the lymph and various tissues of theirhosts. They belong to the genus *Trypanosoma,* in the Family*Trypanosomatidae,* and the order Kinetoplastida (Desquesnes, 2004). This group of organisms infects millions of humans and animals; and is divided into two genera:Leishmania and the digenetic flagellates, Trypanosoma. They are responsible for several neglected tropical diseases, in man and animals such as cattle, horses, goats, etc. (Desquesnes, 2004). Of the trypanosoma genera,only two subspecies belonging to the *T. brucei* species cause significant human diseases (sleeping sickness) on the African continent. These are: *T. b. gambiense* and *T. b. rhodesiense*.

T.brucei, T. b. rhodesiense, and*T.b. gambiense* are morphologically indistinguishable. *T. rhodesiense*causes illness in both humans and animals, is known to be responsible for sleeping sickness,is associated with acute disease in humans and occurs in Southern and Southeastern Africa. *T. gambiense*on the other hand is thought to be principally a parasite of man,is found in West and CentralAfrica and causes the chronic form of the disease in humans whichoften takes months or years to develop into a severe illness(Desquesnes, 2004).

Another human infective Trypanosoma, *T. cruzi*, which is the etiological agent of Chagas' disease is responsible for the American trypanosomiasis, a condition that afflicts millions of people in central and southern America and kills about 15, 000 yearly (Carrabarin-Lima,*et al.*, 2013).*T. cruzi* has been studied more extensively than its African counterparts (Dc-Rubin and Schenkman, 2012).

A subspecies of *T. brucei* -*T. brucei brucei*(which is seen in figure 2.2),*Trypanosoma congolense* and *Trypanosoma vivax* are not pathogenic to humans but infects solely ruminants and other animals (Stephen, 1986). These three parasites, together with *T. b. rhodesiense* are the major

causative agents of the Animal African Trypanosomiasis (AAT), widely known as nagana. *T. evansi* is another causative agent of the animal trypanosomiasis known as "surra". But in contrast to the other animal trypanosomes, this specie has different vectors and is mechanically transmitted by flies of the genera *Tabanus* and *Stomoxys*. Its wider occurrence is attributed to the total adaptation to mechanical mode of transmission (Stephen, 1986) and to the transport of cattle from Africa in the 19th century (Jones and Davila, 2001).

Traditionally, trypanosomes have been classified on the basis of site of development in the tsetse vector. This has given rise to four subgenera comprising:

i. Trypanozoon e.g. *T. brucei* with midgut and salivary glands as sites of development,

ii. Nannomonas e.g. *Trypanosoma congolense* with midgut and proboscis as sites for development

iii. Duttonella e.g. *T. vivax*, where members develop in the proboscis and cibarium,

iv. Pcynomonas where members develop in the midgut, salivary glands and the proboscis. (Checklay,*et al.*, 2007).



Figure 2.3: *T. brucei gambiense*.

Source:http://www.jotscroll.com/forums/11/posts/206/trypanosoma-brucei-gambiense-life-cycle-of-trypanosoma-rhodesiense.html

2.3.1 *Trypanosoma congolense*

Trypanosoma congolense are small species of about 8–25μm, with the undulating membrane not obvious. Its Kinetoplast is medium sized and terminal, often laterally positioned. Although *T. congolense* is considered to be monomorphous, a degree of morphological variation is sometimes observed. A number of morphotypes have been described (Desquesne,*et al.*, 2012) from the slender to the stumpiest:

I. hyperleptomorph (rodhaini-form, very long and slender, with a free flagellum),
II. leptomorph (simiae-form, slender, with a free flagellum),
III. isomorph (congolense-form, short, without a free flagellum),
IV. pachymorph (montgomeryi-form, short and stout; without a free flagellum), and hyperpachymorph ('hyper-montgomeryi-form', short and very stout; without a free flagellum).

Within *T. congolense*, different types or subgroups exist (savannah, forest, kilifi or Kenya coast) that have different pathogenicity (Bengaly,*et al.*, 2002). These types can only be distinguished using PCR.

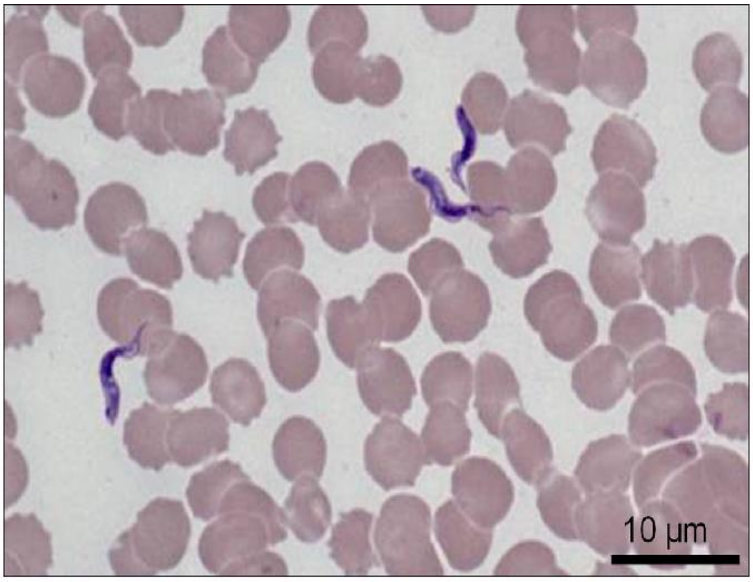


Figure 2.4: *Trypanosoma congolense*

Source:*http://www.fao.org/docrep/006/x0413e/X0413E02.htm*

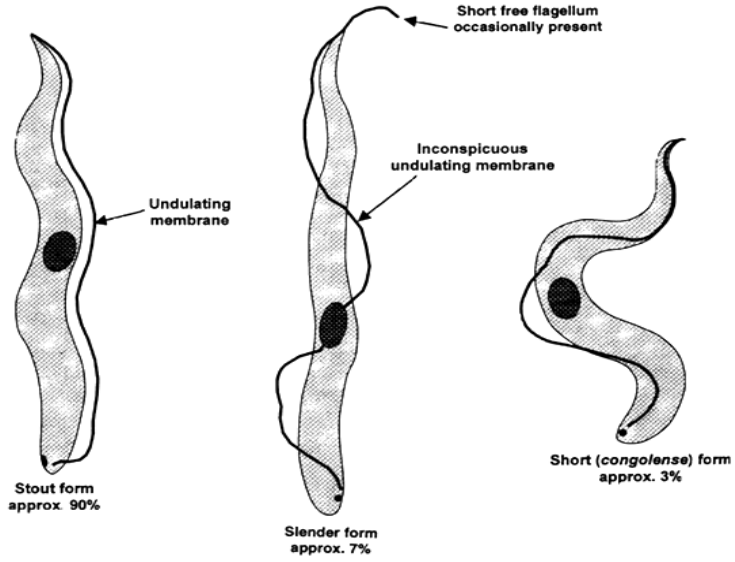


Figure 2.5: *T. congolense* as seen in a stained blood smear.

Source: https://www.semanticscholar.org/paper/Refractory-hypoglycaemiain-a-dog-infectedwithDeschampsDesquesnes/ffed2d80c408e1e92cdf0f3ec2d6108438f1a634

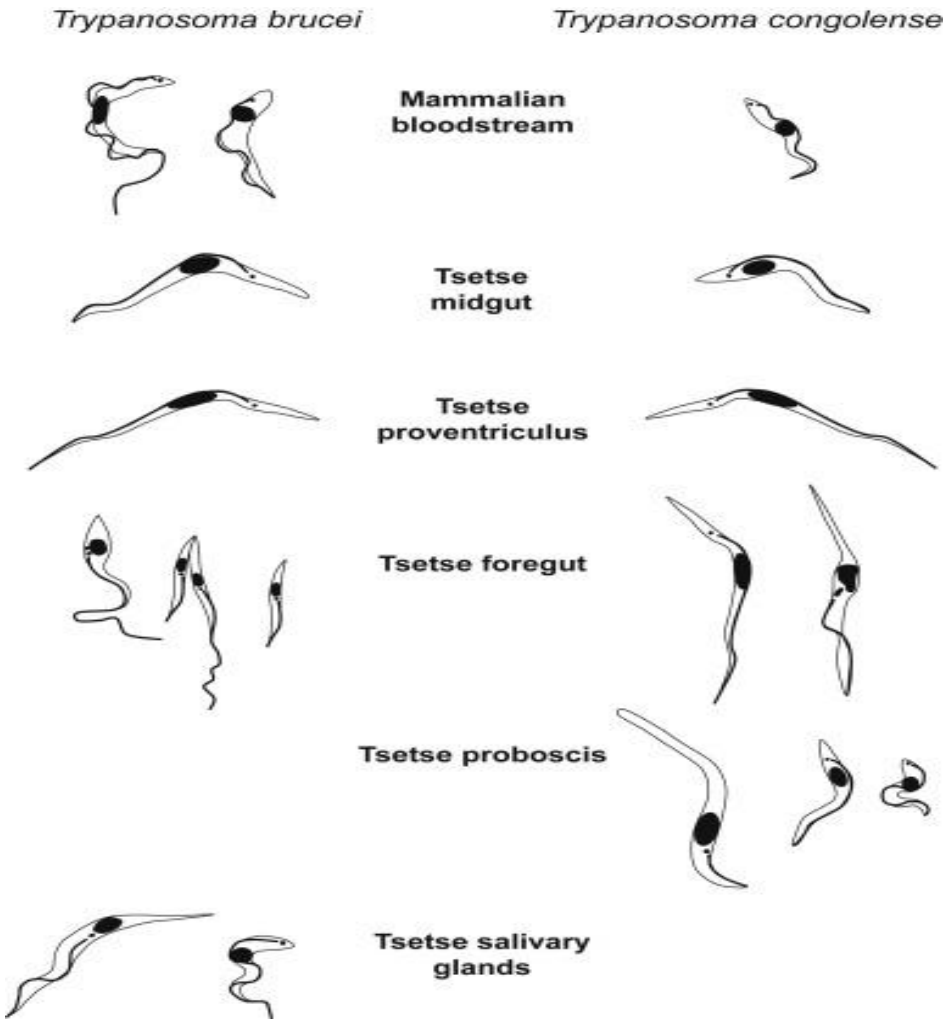


Figure 2.6: Difference between *T. brucei* and *T. congolense*.

Source:https://www.sciencedirect.com/science/article/pii/S0065308X17300441

2.3.2 LIFE CYCLE OF *Trypanosoma congolense*

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 *T. congolense* is a small trypanosome, shorter in length than *T. brucei*.

The life cycles of *T. brucei*have been well documented(Vickerman, 1985). For *T. brucei*, understanding of the developmental cycle in the fly has been steadily improved by information emerging from molecular and cell biology analyses, while on the other hand, not much is known about the life cycle of *T. congolense*. However evidence to date suggests that many aspects of the developmental pathway of *T. congolense* in tsetse are similar to that of its close relation, *T.brucei*(Vickerman, 1985).

Pathogenic African trypanosomes, including *T. congolense*, have a complex developmental cycle that involves both mammalian hosts and tsetse vectors(Tuner, 1985). When BSF trypanosomes (which rely on glycolysis and substrate level phosphorylation for their energy needs) are taken up by a tsetse during its blood meal, they enter the insect midgut and a certain differentiates to procyclic forms (PF) that are adapted to life in the midgut (Vickerman, 1985). Differentiation from BSF to PF is characterized by a switch to proline oxidation and oxidative phosphorylation for energy production. In the tsetse fly vector, the trypanosomes develop and multiply in the midgut initially, before onward migration to the mouthparts, where infective metacyclics develop in the proboscis for *T. congolense* and in the salivary glands for *T. brucei*. (Vickerman, 1985).

Once established in the fly, a small subset of the PF migrate from the midgut and begin to differentiate, becoming non-motile epimastigote forms (EMF) that adhere to the fly's salivaryglands (for *T. brucei*) or to the fly's proboscis and mouth parts (for *T. congolense*)

(Sharma *et al*., 2008). Finally, the dividing, adherent EMF differentiates into non-dividing, non-adherent, VSG-expressing and infectious metacyclic forms (MCF) and are injected into the host's bloodstream when the tsetse fly takes a blood meal. The parasites multiply at the site of the bite for 5–9 days after which they invade the bloodstream and lymphatic system, becoming BSF and thus completing their life cycle(Sharma *et al*., 2008).

Thus, both *T. brucei* and *T. congolense* undergo cyclical transmission and interact with both tsetse vectors and mammalian hosts. These interactions influence parasite differentiation, survival, maturation and infectivity and are thus critical for parasite transmission and perpetuation of disease. Several molecules involved in these processes,particularly VSGs in BSF(Sharma *et al*., 2008)have been well studied. In the last ten years, molecules of PF that are involved in tsetse–trypanosome interactions have received more attention (Roditti and Lehane, 2008). One interesting aspect of cyclical transmission is the extreme constraints placed on trypanosomes in the tsetse vectors and intransmission to mammals (Oberle *et al*., 2010), although the molecular mechanisms involved still remain vague.

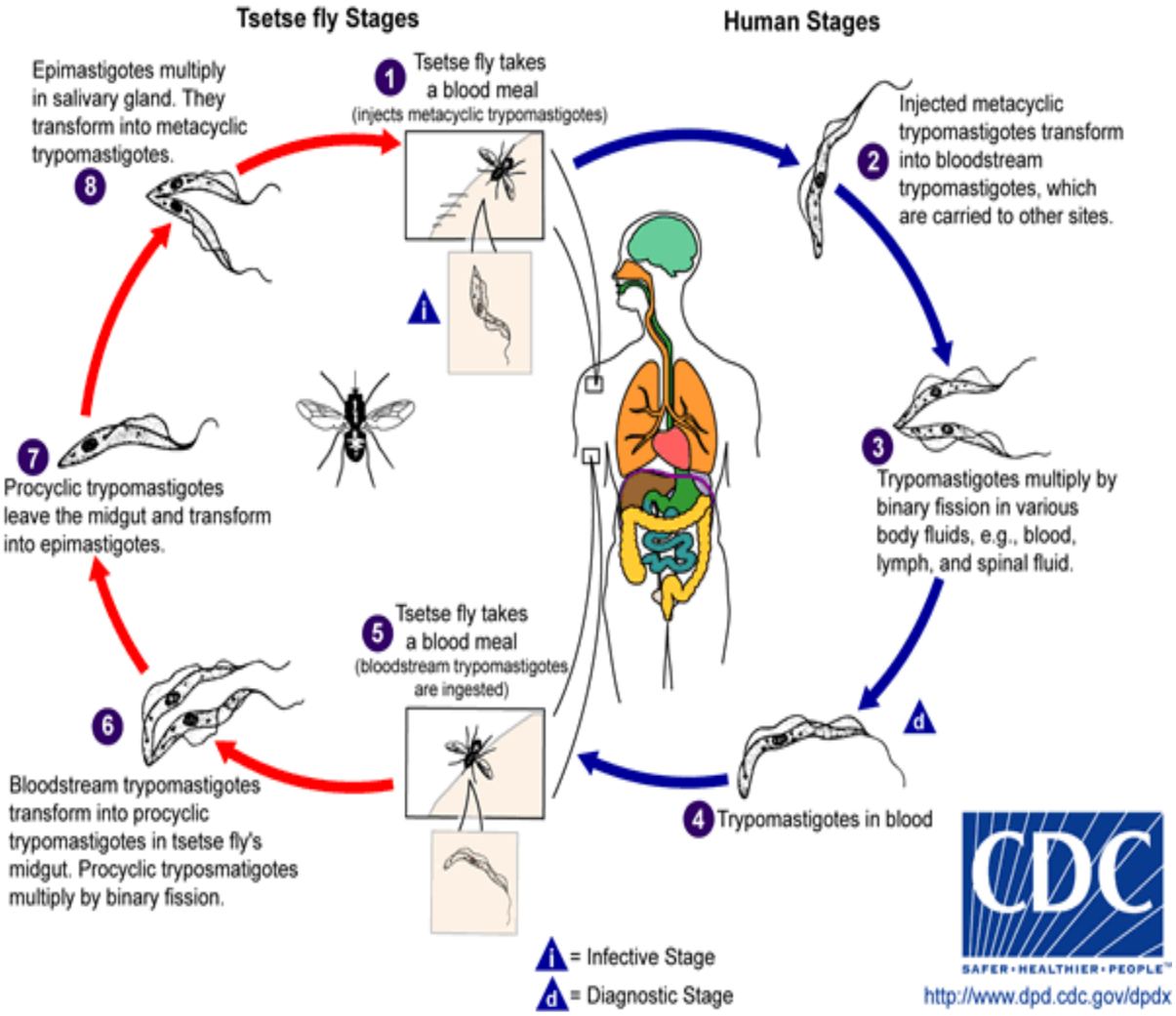


Figure 2.7: life cycle of trypanosoma congolense.

Adapted from CDC page on trypanosomiasis.
Source: http:/www.dpd.cdc.gov/dpdx

2.4 SURFACE PROTEINS AND EVASION STRATEGIES OF TRYPANOSOMES

2.4.1 VARIANT SURFACE GLYCOPROTEIN (VSG)

In the mammalian host, bloodstream forms (BSF) are covered with a dense coat consisting of approximately 10^7 copies per cell of a variant surface glycoprotein (VSG), which is arranged as a dense monolayer of homodimers on the parasite surface. This VSG is involved in antigenic variation, allowing the trypanosome population to avoid elimination by the host's immune system (Tuner, 1985; Rudenko, *et al.*, 1998).

VSG is a dominant protein in trypanosomes, accounting for about 10% of the total amount of protein. It is constitutively synthesized at a high rate.VSG is produced as a protein containing an N-terminal signal sequence and a hydrophobic C-terminal domain, which is post-translationally replaced by a Glycosyl Phosphotidyl Inositol (GPI) membrane anchor. This membrane anchor exchange is thought to be a single-step reaction performed by a transamidase (Udenfriend and Kodukula,1995).

The VSG is replaced by procyclins, a collection of GPI-anchored glycoproteins (Pearson, 2001) encoded by a small number of genes and hence limited potential for variation. (Butikofer *et al.*, 1997; Acosta-Serrano *et al.*, 1999; Utz *et al.*, 2006).

2.4.2 GLYCOSYLPHOSPHATIDYLINOSITOL ANCHOR TRANSAMIDASE (GPIT)

GPI anchoring is essential for mammalian development and many specific cellular functions (Takeda *et al.*, 1993; Kawagoe *et al.*, 1996; Tarutani *et al.*, 1997; Nozaki *et al.*, 1999) but not for cell survival itself. The requirement of GPI in protozoan parasites depends on the species and stage of life cycle (Hilley *et al.*, 2000). For example, the bloodstream form of*Trypanosoma brucei* requires GPI; in contrast the GPI-deficient procyclic form (insect stage) of this parasite is

viable but has decreased infectivity toward its vector, tsetse fly (Nagamune *et al.*, 2000; Lillico *et al.*, 2003).

Many eukaryotic cell surface proteins are tethered to the plasma membrane via glycosylphosphatidylinositol (GPI) (Udenfriend and Kodukula, 1995). The GPI moiety is synthesized in the endoplasmic reticulum (ER) by the sequential addition of sugars and ethanolamine phosphate to phosphatidylinositol (Udenfriend and Kodukula, 1995; Kinoshita and Inoue, 2000). In addition to the amino-terminal signal peptide required for entry into the endoplasmic reticulum, precursor proteins destined to be GPI-anchored have carboxyl-terminal GPI attachment signals that direct GPI modification. GPI transamidase mediates cleavage of the GPI attachment signal peptide and all together transfer a preassembled GPI to newly exposed carboxyl terminus of precursor proteins. During this transamidation reaction, GPI transamidase forms a carbonyl intermediate with substrate proteins (Udenfriend and Kodukula, 1995).

2.4.3 EP PROTEIN

At each stage of proliferation and differentiation of transmission, the survival and successful replication of *T. congolense* improve their chances of continuing the life cycle, (Reupp, *et al*., 1997). *Trypanosoma brucei* and *T. congolense* undergo a complex cycle of development in the tsetse which begins almost immediately after ingestion of an infected bloodmeal, when BSF differentiate to the procyclic forms in the fly midgut lumen. For the first three days following infection all flies contain trypanosomes. Between days 4 and 5, trypanosome infections are eliminated from most flies through a process of self-cure. The identified factors that influence vector competence include the age of the fly, the number of bloodmeals taken and the activation of fly immune processes, with both antimicrobial peptidesand lectins implicated in parasite-vector interactions(Hao*et al.*, 2001).

Metacyclic forms and bloodstream forms are covered by a coat of variant surface glycoprotein (VSG) that protects the parasite from the host's immune system (Barry and McCulloch, 2001). Differentiation of the bloodstream to the early procyclic form after ingestion by a tsetse fly involves shedding of the VSG coat and its replacement by a new invariant surface glycoproteins coat termed **procyclins** (also known as procyclic acidic repetitive proteins - PARP), over a period of two to three days(Roditti, *et al.,* 1989). In *T. brucei,* this procyclin coat was identified by Jackson *et al* (1993) using cDNA expression, as the **EP procyclin** and another protein initially called **procyclic stage surface antigen**, but now known as"**procyclic form surface glycoprotein**". This new stage-specific surface antigen had the features of a typical trans-membrane glycoprotein but with an unusual cytoplasmic extension composed of a proline – rich tandem repeat, hence given the name GPEET (Roditi,*et al.,* 1998). Later on, a similar molecule (to GPEET) was found to be expressed in *T.congolense* and that it was also up-regulated in the EMF of the parasite(Urwyler, *et al.,* 2005).

The EP procyclins are a group of closely related proteins that are encoded by six or seven genes and contain extensive tandem repeat units of glutamic acid and proline (E and P, respectively, in the single-letter amino acid code), while GPEET procyclin is encoded by two identical genes and contains internal pentapeptide (GPEET) repeats (Mowatt, 1989; Koenig,*et al.,* 1989). The structures of the EP and GPEET GPI moieties are indistinguishable from each other and consist of a GPI core structure that is substituted by heterogeneous sialylated polylactosamine-containing side chains (Ferguson,*et al.,* 1993; Treumann,*et al.,* 1997). This heavy glycosylation, together with the proposed extended peptide conformation of EP and GPEET, may account for their unusual migration on SDS-PAGE. There are four different isoforms of procyclins that arenamed according to internal repeats, GPEET—which containspentapeptide repeats and is

highly phosphorylated; and EP1, EP2 and EP3, all of which contain glutamic acid – prolinedipeptide repeats. EP1 and EP3 are modified byN-linked carbohydrates whereas EP2 has no such glycosylationsite (Roditti and Clayton, 1999). Using qualitative and quantitative RT-PCR to analyse the procyclin mRNAs expressed by trypanosomes in the tsetse midgut and salivary glands at different time points after infection it was discovered by Urwyler and others, that the coding regions of the three EP isoforms (EP1, EP2 and EP3) were extremely similar, but also that they had 39 untranslated regions (UTRs) with unique sequences.

Soon after differentiation, the parasites express different isoforms of the procyclic form surface coat, initially EP and GPEET procyclin (early procyclic forms) and then only two isoforms of EP procyclin (late procyclic forms). According to Urwyler *et al.*(2005), the study showed that the coding regions of EP1 and EP3 could not be distinguished by hybridization because their open reading frames (ORFs) differ by only three nucleotides outside the repetitive region.

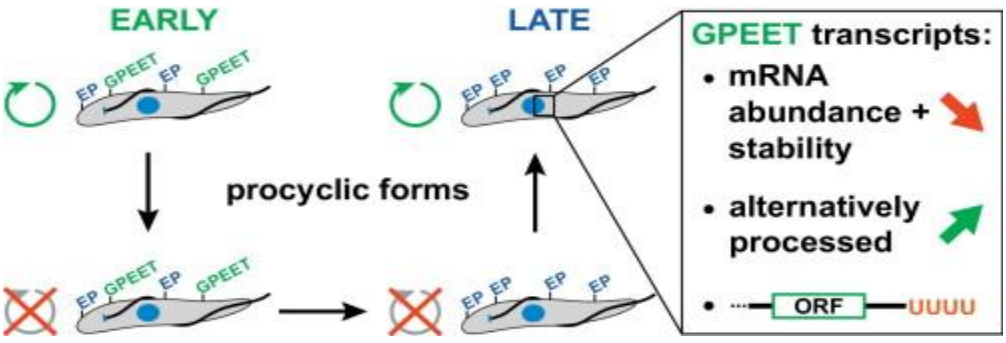


Figure 2.8: diagramatic expresion of EP and GPEET expresion and repression

Source: http://ars.els-cdn.com/content/image/1-s2.0-S0166685113001357-fx1.jpg

a. Tsetse Midgut EP Procyclin

Experiments have shown that EP is important for survival of trypanosomes in the insect host, possibly by mediating parasite-fly interactions (Ruepp,*et al.*, 1997). Its presence has also been discovered in the midgut of the insect host and hence is said to confer resistance against infection in the fly while at the same time ensuring the trypanosomes are able to further differentiate into the epimastigote forms and move to the salivary gland. Atpresent, the roles of the procyclins in parasite development are unclear(Haines *et al.*,2010). The GPEET and EP amino acid repeat domains of the proteins, are resistant totsetse proteases (Haines *et al.*,2010) and thus may protect trypanosomes from digestive enzymes in the fly midgut. Procyclins might playa role in directing the parasite through the digestive tract.This is particularly so as the GPEET procyclin is repressed (Vassella, *et al.*, 2001).The same order of events, i.e. expression of GPEET by early procyclic forms and its replacement with EP1 and/or EP3 in established procyclic forms also occurs during tsetse fly infections (Acosta-Serrano, *et al*., 2001).

Research has shown that the full-length tsetse EP protein contains 320 amino acids, including a signal peptide, and a run of 59 (EP) repeats. Immuno-blotting with a monoclonal antibody against (EP) repeats reveals expression in the gut, but not salivary glands, of female and male flies, whether or not fed (Haines *et al.*,2010).

It was also discovered by dissection of the tsetse midguts into distinct structuralregions, that tsetse EP transcripts were detected in allsections tested. However, lower levels were consistently observedin the proventriculus (PV) (Haines, *et al.*, 2010). Given the presence of tsetse EP transcript in the PV it was then concluded that tsetse EP protein was either not produced in PV, was rapidly turned over in that organ or was rapidly translocated from there into the anterior gut. Similarly, tsetse EP transcript was detected in salivary glands from teneral and fed flies

(Chandra,*et al.*, 2004) but tsetse EP protein was only weakly detected by immunoblotting suggesting it may be rapidly translocated to midgut.

From research also, it has been seen that when tsetse EP was knockdown in flies, there was statistically significant increase in susceptibility to *T. brucei brucei* establishment in the midgut (Haines, *et al.*, 2005). To determine if this phenomenon was present in other tsetse species and with other species of trypanosomes, it was investigated in *G. p. palpalis*. There were statistically significant increases in *T. b. brucei* establishment in the midgut of tsetse EP knockdown of *G. p. palpalis.T. congolense* also established higher midgut infections in EP knockdown flies (Haines, *et al.*, 2005). This suggests that the increase of vector competence to midgut inhabiting trypanosomes in tsetse EP knockdown flies ispossibly a genus-wide phenomenon in Glossina.

Tsetse EP protein levels decreased with fly starvation as discovered (Haines, *et al.*, 2005). After 3 days of starvation, there was a cleardecline in tsetse EP protein levels evident with fatbody atrophy apparent in these flies when viewed with adissection microscope. Tsetse EP protein levels increased again inflies 24 hours after feeding. Although there is no data to show if the starvation- induceddecrease in tsetse EP protein is specific or part of a generallowering of protein levels in the midgut in response to starvation.In addition, it has been demonstrated that tsetse EP protein is strongly up regulatedfollowing immune stimulation with E. coli (Haines, *et al.*, 2005) providing goodevidence that it is part of the immune response system.

From all these evidences, it is seen that the EP protein is very essential to both the fly and the parasites particularly the *T. brucei* specie.

2.5 *IN VITRO* CULTIVATION OF TRYPANOSOMES

Cultivation or culture refers to the growth of tissues or cells in a suitable selected culture medium.

A culture medium is defined as a nutrient material that is either solid or liquid, that is used to promote and help cells to grow and reproduce outside their natural environment.

In vitro (meaning: *in the glass*) studies are performed with microorganisms, cells, or biological molecules outside their normal biological habitat, and done in lab-ware such as test tubes, flasks, Petri dishes, and microtiter plates. Studies conducted using components of an organism that have been isolated from their typical biological surroundings allows for a more exhaustive and convenient analysis; however, results obtained from *in vitro* experiments may not entirely or accurately predict the effects on a whole organism (Artursson and Luthman, 2001).

Examples of *in vitro* studies include:

1. The isolation, growth and identification of cells
2. Polymerase chain reaction used selective replication of specific DNA and RNA sequences in the test tube.
3. Protein purification .
4. *In vitro* fertilization (used to allow spermatozoa to fertilize eggs in a culture dish before implanting the resulting embryo or embryos into the uterus of the prospective mother).
5. *In vitro* diagnostics .
6. *In vitro* drug testing by characterizing specific adsorption, distribution, metabolism, and excretion processes of drugs inside a living organism (Gargas, *et al.*, 1989).

2.5.1 ADVANTAGES OF IN VITRO CULTURE

There are several advantages that are garnered from *In vitro* studies. These include:

i. **Simplicity-** Living organisms are extremely complex functional systems that are made up of many tens of thousands of genes, protein molecules, RNA molecules, small organic compounds, etc. (Albert, 2008), that interact with each other in a complex manner. This complexity makes it difficult to identify the individual interactions between components and to explore their basic biological functions. *In vitro* work simplifies the system under study, so the investigator can focus on a smaller number of components per time (Vignais, *et al.*, 2010).

ii. **Species specificity -** Another advantage of *in vitro* methods is that human cells can be studied without "extrapolation" from an experimental animal's cellular response.

iii. **Convenience and automation-** *In vitro* methods can be scaled down and automated, yielding high-throughput screening methods for testing molecules in pharmacology or toxicology (Quignot and **Hamon, 2002).**

2.5.2 DISADVANTAGES

The primary disadvantage of *in vitro* experimental studies is that it may be challenging to extrapolate from the results of *in vitro* work back to the biology of the intact organism. Investigators doing *in vitro* work must be careful to avoid over-interpretation of their results, which can lead to inaccurate conclusions about biological systems (Rothman, 2002).

Trypanosomes have been cultured over time, with various degrees of success, with each specie requiring different culture conditions or a variation of previously established protocols. For example, Miralles, *et al.*, (2002), used six different trypanosomatids isolated from different geographical areas from South America (Peru and Brazil) and different vectors and reservoir

hosts to culture in vitro in five different media:Grace'ss medium (Sigma), RPMI-1640 (Gibco), SDM-79, MTL and TC-199 (Gibco) Grace's medium supplemented with 10% inactivated bovine foetal serum. These media were tested to identify the most adequate for isolates' growth. Grace's medium gave the highest growth density.

Several cultures of *T. brucei* have also been developed with several modifications. In earlier studies, bloodstream forms of *Trypanosoma brucei*were only grown *in vitro* when maintained by a feeder layer of mammalian fibroblasts, with HEPES-buffered Minimum Essential Medium (MEM), with Earle's salts, and supplemented with 15% inactivated rabbit serum (Brun, *et al.*, 1981). Although cultures of bloodstream forms from mammalian hosts and from cryopreserved stabilates transformed within 6 h to bloodstream forms, were infective for mice, and could also be cyclically transmitted through tsetse flies (Brun, *et al.*, 1981), there was the need to improve on the cumbersome nature of the feeder layer experiment. As science progressed, the feeder layer requirement was eliminated by including L-cysteine at regular intervals and appropriate concentrations (optimum cysteine concentration depending on cell density) (Duszenko, *et al.*, 1985). But it was then discovered that at concentrations greater than 24 mg/liter, cysteine became acutely toxic to trypanosome concentrations, hence the need to also handle that problem. Toxicity was stopped by addition of pyruvate or catalase, which neutralized hydrogen peroxide(H2O2) produced by cysteine autoxidation. The presence of a reducing agent (2-mercaptoethanol or thioglycerol) was later found to be essential for growth (Baltz,*et al*, 1985), as this supplement to the media is a free radical scavenger that maintains the Sulphydrin group of the L-Cysteine, and prevents it from oxidising to Cystine which is also toxic to trypanosomes.

Therefore, the standard medium for culture of *T. brucei* consisted of Hepes -buffered minimum essential medium (MEM), with Earle's salts supplemented with M 2-mercaptoethanol, M

pyruvate and 10% inactivated serum either from rabbit (Baltz,*et al*, 1985). Even though some other media like the RPMI-1640 were used to successfully grow these parasites, as carried out by Hirumi, *et al.*, (1997)

Bloodstream forms of *Trypanosoma congolense* were also able to be cultivated *in vitro* using HMI-93 medium (modified from Iscove's modified Dulbecco's MEM). The modification was done by supplementing the medium with bathocuproine sulphonate, L-cysteine, 2-mercaptoethanol, Sodium pyruvate, Thymidine, heat-inactivated young goat serum and 5% (v/v) Serum (Hirumi and Hirumi, 1991). Cultures were maintained by replacing the medium every 24 h and the shortest population doubling time of 9 Hrs, was achieved. The bloodstream forms propagated in this system were morphologically similar to those seen in infected mouse blood, they were covered with a surface coat as examined by electron microscopy and they were infective to mice (Hirumi and Hirumi, 1991).

2.6 MANAGEMENT OF TRYPANOSOMIASIS

Attempts to manage African trypanosomiasis have a long history, dating from the colonial period when the Europeans were concerned with epidemics of the human disease and the chronic loss of livestock hindering both transport and agriculture (Jordan, 1986). Their attempts were successful to an extent, based largely on active detection and treatment of sleeping sickness cases, combined with major programs to control tsetse by bush-clearance, wild game culling (to reduce the parasite reservoirs and host availability for tsetse), and insecticidal spraying of tsetse resting sites. An important initiative by the World Health Organization (WHO) led to steady improvements in the supply of drugs to treat Sleeping Sickness, was able to offer increasing support for national medical services involved in active detection and treatment. In both Africa

and in Latin America there had been a series of control projects following administratively-defined boundaries (rather than covering biologically-relevant areas) that had been technically successful until being halted before a sustainable end-point had been reached. In parallel, a number of externally-financed projects to control tsetse were set up in African countries of interest to external donor organisations (e.g. the Regional Tsetse and Trypanosomiasis Control Programme of Zimbabwe, Zambia, Mozambique and Malawi and the various FITCA projects (Farming in Tsetse Controlled Areas) supported by the European Commission (Jordan, 1986). Such projects were generally successful whilst in operation, but generally proved unsustainable once the formal project reached its endpoint. But for the African animal trypanosomiases, that contribute so much to poverty, underdevelopment, and food insecurity, tsetse elimination is now seen as the primary approach for the long-term. And in both continents, accumulating experience is showing that large-scale elimination of vector populations is feasible, and more likely to be sustainable in the face of changing circumstances and priorities.

Generally, there are two basic approaches to combating trypanosomiasis: tsetse control methods and treatment of the disease after been diagnosed.

2.6.1 METHODS OF TSETSE CONTROL

2.6.1.1Trypanotolerance control-Trypanotolerant animals are animals that do not get infected as often, and when infected, they survive, produce and reproduce better than susceptible animals under tsetse-*Trypanosomosis* challenge.Trypanotolerant and semi-trypanotolerant cattle (e.g. Taurine, N'Dama and West African Short horn cattle breeds)in West Africa account for about 10 million (in 1983) of the 45 million head of cattle living in and in close proximity to tsetse-infested areas. Although these cattle are not immune, they do possess a degree of tolerance that

allows them to remain productive while being infected. 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).

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).

2.6.1.2 Vector control or eradication- These vector-control techniques include:

a. Ground Spraying

This is the use of persistent insecticide applied from the ground. The technique is based on the knowledge that tsetse flies spend a greater part of their life resting in cool, shady places provided by trees, holes, etc. and directing a persistent insecticide at these sites should achieve a good control measure. In Zimbabwe, the insecticide of choice for this purpose has been the chlorinated hydrocarbon DDT, used as a 75% wettable powder which is applied to essential habitat of tsetse flies.

b. Aerial Spraying

Basically, aerial spraying is the sequential ultra-low volume (ULV) aerial application of an insecticide. The application of low dosages of endosulphan and deltamethrin make aerial spraying highly specific for tsetse, with little effect on non-target organisms.

Work conducted in Zimbabwe, and described by Allsopp (1984) demonstrated that aerial spraying is a feasible and rapid technique for dealing with emergency situation, as well as treating large areas with less manpower than required in ground spraying operations over comparable areas. However theoperation proved that the technique is poorly effective in hilly terrain and cannot guarantee eradication. While aerial spraying can achieve good control, the operation requires a high level of technical sophistication, strict timing of repeat sprays and areas sprayed are open to reinvasion and therefore require protection. (Allsopp, 1984).

c. Odor- Baited and Insecticide Treated Targets

Various trials undertaken in Zimbabwe have shown that tsetse flies can be controlled by attracting them to visual targets that are baited with odor attractants and coated with insecticide. The results from these trials suggested that targets could be used for large scale campaigns and they offer several advantages over conventional methods of control. (Allsopp, 1984).

2.6.1.3 The Sterile Insect Technique for Control of Tsetse Flies

The sterile insect technique is the use of mutagenic compounds to sterilize insects. These are then introduced into the field to mate with other tsetse flies. The principle of the Sterile Insect Technique (SIT) is that fertile insects are incapable of producing normal offspring after mating with a sexually sterile partner (Takken and Weiss, 1978). Insects are sterilized by treating them with mutagenic agents (e.g. gamma rays, chemostenlants). In spite of their genetic anomaly, sterile flies usually display the same behaviour as their wild counterparts, this of course, is a requirement for the success of the SIT.

A more recent technique is being researched, whereby a system has been developed to rear the flies successfully on guinea pigs by the Seibersdorf Entomology Laboratory (2004 annual report) (http://www-naweb.iaea.org/nafa/ipc/public/entomology-report-04.pdf).This research cancels the requirement for living hosts, by replacing it with an artificial an artificial membrane which simulates the skin. Flies pierce these membranes and suck up blood from an underlying blood-pool.

2.6.1.4 Integrated approaches to combating tsetse-trypanosomiasis

The development of these various strategies are still met with several challenges, which varies from cost, to maintenance of these programs for a longer period of time. As pointed out by Keatin, *et al.* (1997), there is no single solution that will be effective for all production systems, ecological zones, or regional or national markets. These observations support the analysis by Holmes (1997) that more integrated strategies need to be developed, which must be based on quality information, not only on the abundance of tsetse flies and the infection rates in the flies and the host livestock, but also on how the livestock owners view the extent of the problem.

2.6.2 DISEASE MANAGEMENT: DIAGNOSIS

Disease management is made in the following steps:

1. Screening for potential infection. This involves using serological tests and checking for clinical signs - especially swollen cervical lymph nodes.
2. Diagnosing by establishing whether the parasite is present in body fluids.
3. Staging to determine the state of disease progression. This entails examining the cerebrospinal fluid obtained by lumbar puncture.

4. Drug administration to treat the disease, particularly at the acute stage and early chronic stage. Drugs commonly used for treatment of trypanosomiasis include: suramine, pentamidine, melasoprol, benzindazole, eflornithine, nifurtimox, etc.

2.6.3 THE PROGRAMME AGAINST AFRICAN TRYPANOSOMIASIS (PAAT)

The Programme Against African Trypanosomiasis (PAAT) was endorsed in 1997 by the FAO Conference. By combining the forces of FAO, WHO, the International Atomic Energy Agency (IAEA) and the Organization of African Unity (OAU)/Inter-African Bureau for Animal Resources (IBAR), the programme seeks to:

1. ensure a harmonious, sustainable approach towards improved human health and sustainable socio-economic and agricultural development of tsetse-infested areas;
2. promote and coordinate international alliances and efforts assisting in harmonized interventions against trypanosomiasis and tsetse flies;
3. achieve integrated trypanosomiasis control in Africa;

PAAT is primarily concerned with the development and application of science-based standards for assessing the economic, social and environmental benefits and costs of Trypanosomiasis and Tsetse (T&T) management. It studies and analyses the balance between human needs in terms of food security and livelihood sustenance and the preservation of natural resources and prevention of environmental degradation. The integration of T&T intervention into the general process of agricultural development and production provides the opportunity to maximize the benefits for the rural poor while minimizing the negative effects on the environment.

2.6.4 NOVEL AND POTENTIAL STRATEGIES FOR MANAGEMENT OF TRYPANOSOMIASIS

Traditionally, control of the vector populations has been an effective disease management strategy. Recent strategies involves those vector control strategies been fortified by research in insect biology and in insect–pathogen interactions, as well as thedevelopment of transgenic technologies. In addition to insect population reductionmethods, disease control via selective elimination of pathogens in insects can nowbe explored. Some of these strategies include:

a. Tsetse immunity

Tsetse flies, both in the field and in the laboratory, demonstrate a remarkable ability to resist infection by pathogenic trypanosomes. In fact, current evaluations, derived by microscopic examination of midguts of tsetse populations from the field, and by performing polymerase chain reaction (PCR) with trypanosome-specific primers, indicate that approximately 1–10% of flies contribute to disease transmission (Aksoy *et al.*, 2003). Tsetse's midgut is a hostile environment containingproteolytic digestive enzymes, potent antimicrobial substancesand a chitinous peritrophic matrix that trypanosomes mustcross before an infection can be established.

The mechanisms involved in parasite elimination arelikely based on tsetse immune responses (Gibson and Bailey,2003) and may include lectin agglutination, lectin-like activities,antioxidant activity and antimicrobial peptide activity (AMP) (Hao *et al.*,2001).

Tsetse genomics can unravel host–parasite interactions.

b. Tsetse Genomics

The tsetse genomics can unravel insect- pathogen interaction.Recently,through a collaborative international effort, the two largestchromosomes of *T. b. brucei*have been sequenced andothers are in the final stages of assembly (http://www.genedb.org) (El-Sayed *et al.*,2003). The

Whole GenomeSequence (WGS) analysis of the tsetse species *Glossinamorsitans morsitansis* also underway. The availability ofgenomics information can lead to the development of newcontrol strategies aimed directly at the fly or at its abilityto transmitparasites(vector competence) (Aksoy *et al.*, 2005).The availability of the tsetsegenome sequence will enhance understanding of itsolfactory biology, which will in turn improve currently applied trapping technologies; will be important for tsetse's population genetics, which isessential for the application of current tsetse control tools; and tsetse's immunology, which in turn can helpdevelop new approaches to combat trypanosome developmentand transmission biology.

c. Tsetse Symbionts Genomics

Most insects are involved in some form ofsymbiosis with microbial companions (Ishikawa, 2003). Throughthese intimate associations, insects have been able to expand their niches into otherwise ecologically unfavorableenvironments. The tsetse fly is no exception tothese symbiotic relationships. It has at least three established symbiotic organisms. Given the relatively sterilenature of their blood-specific diet, tsetse'sresident microbial community appears to be more restricted compared to insects that feed on multiple diets.Two of the tsetse symbionts, genus *Wigglesworthia*and *Sodalis glossinidius*are bothmembers of the family *Enterobacteriaceae* and are mostprevalent in tsetse's midgut tissue, while the third symbiont, genus*Wolbachia* belongs to α-Proteobacteria, and is found only incertain natural populations of tsetse (Werren*et al.*, 1995). Although the role of *Wolbachia*in tsetse isunknown, in other insects, *Wolbachia*infections can cause avariety of reproductive abnormalities, the most commonof which is called "cytoplasmic incompatibility" (CI).

In comparison with severalfree-living microbes, *Wigglesworthia's*genome has suffereda drastic reduction to approximately 700 kb (Akman *et al.*, 2002). Its putative transcriptome, based on

WGS analysis,indicates that this organism's symbiotic role may be toprovide its tsetse host with vitamins that are not present inthe vertebrate blood meal. This hypothesis is based onthe presence of several vitamin biosynthesis pathways,including those that produce biotin, thiazole, lipoic acid,FAD (riboflavin, B2), folate, pantothenate, thiamine (B1),pyridoxine (B6), protoheme and nicotinamide (Akman *et al.*, 2002). In further support of this theory, previous experimentsdemonstrated that tsetse fed on a diet of antibiotics (whichkilled their *Wigglesworthia*) became sterile (Nogge, 1976).When these flies were fed a combination of B vitamins, thisloss of fecundity was partially reversed (Nogge, 1981; Nogge1976).

Both of these symbionts mentioned and their 'perceived' roles provide potentials for control and management of tsetse flies and ultimately trypanosomiasis, by exploring their genomes.

d. Insect Transformation

Advanced insect science technologies promise the development of new vector control tools that will curb vectorcompetence. This goal has been fortified by the developmentof genetic transformation systems.By using tissue specificexpression systems, transgene products can be expressed where they will most adversely affect parasite viability;that is, gut,salivary gland or hemolymph (Kokoza *et al.*,2001). The viviparous reproductivebiology of tsetse has hindered the application of this transformation technology. However, *Sodalis*can beexploited, as it lives in tsetse's midgut inclose proximity to pathogenic trypanosomes. This allows forthe expression of foreign gene products that could affectparasite viability. This symbiont - based transformationsystem is referred to as "**paratransgenesis"** (Beard *et al.*, 1993; Beard and Aksoy, 1998).

e. Effector Molecules

Two types of effector molecule have been identified in several insects, and are worthy candidates for expression in tsetse. The first type is antimicrobial peptides produced by insects when challenged by a pathogen. These substances are an innate component of numerous insect immune systems, and may be largely responsible for pathogen-refractory phenotypes in many vector species. Four of these peptides – attacin, cecropin, defensin and diptericin – have been identified in tsetse (Hao *et al.*, 2001; Boulanger *et al.*, 2002). One distant antimicrobial peptide of particular interest, called BMAP-27, is produced by bovine neutrophils. Experiments by Haines *et al.* (2003) revealed that BMAP-27 is highly lethal to both bloodstream-form and procyclic-form trypanosomes. These results indicate that this peptide will be very useful in paratransgenesis experiments.

The second type of effector molecule of interest is transmission-blocking agents capable of disrupting parasite development and pathogenicity by binding proteins necessary for these events to occur. For practical purposes, these molecules can be expressed as target-specific single-chain antibody fragments from one gene. Some of these molecules have already been tested in insects, where they have successfully reduced or eliminated pathogen infections. For example, an antibody (TB7/8.1.48) fed via a bloodmeal to *T. b. brucei*-infected tsetse completely eliminated the development of infective metacyclic trypanosomes in the salivary glands.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 MATERIALS AND REAGENTS

3.1.1 EQUIPMENTS

Equipments, used during the research are:

EQUIPMENT	COMPANY	MODEL NUBER
CO2 incubator	Biorad, USA	Nil
Geldoc 200 imaging system	Biorad, USA	geldoc 200/2008435
Laminar biosafety hood	Biorad, USA	Nil
Microscopes	De-applegate	Nil
MultiGeneII centrifuge	Labnet	115V
Roche Molecular random cycler (version 3.5)	Quiagen	Cat No: 9001550
Thermocycler	Biorad, USA	2029241
Vortexer	Biorad, USA	BR-2000
QuantGene 1300 spectrophotometer	GE Healthcare	28-9182-13

3.1.2 REAGENTS

All reagents were of molecular grade and purchased from reputable companies.

2% Glucose solution

20% Fetal Bovine Serum (Sigma Aldrich)

Antibiotics (penicillin- streptomycin solution)

Acupower greenstar q-PCR premix (Bioneer)

cDNA synthesis premix (Bioneer)

Hotstart RT-PCR premix (Bioneer)

PCR hotstart premix (Bioneer)

RNA extraction kit (DNA land scientific greenbio research)

Chlorofoam

Complete RPMI 160 media with L-Glutamine (Sigma Aldrich)

Ethanol (75%)

Formalin

Giemsa stain (%5)

L-Cysteine (Sigma Aldrich)

Sterile phosphate buffer saline

β-mercaptoethanol (Sigma Aldrich)

3.1.3 PRIMERS

Primers used for the research, which were obtained from genebank (for housekeeping or internal control) and designed EP primers are:

GAPDH primer pair (forward and reverse)

Forward primer: 5´- CTCGGCGTTGAGTACGTGAT- 3´

Reverse primer: 5´-GCTCGCGTGGGTTATAGTCA- 3´

Synthesized EP gene primer pair (novel)

Forward primer: 5´-GAAGGACCAGAAGACAAGGGT - 3´

Reverse primer: 5´-AACAGCAGGAAACAAACACGAT- 3´

3.2 METHODS

3.2.1 DETERMINATION OF GENETIC HOMOLOGY BETWEEN THE EP PROCYCLIN GENE OF *Trypanosomabrucei* AND *Trypanosomacongolense*

The EP-procyclin gene of *Trypanospma brucei* with ascension number AF353628.1 was generated from the NCBI gene bank (http://www.ncbi.nlm.nih.gov). This was aligned with the entire genome of *Trypanosoma congolense* which was obtained from the TriTrypD genebank, using the BLAST and clustalW tools of the EXPASY genebank. Blast searches were performed using the entire open Reading Frames (ORFs) of the *T. Brucei* EP procyclin geneas a probe, default search parameters, and Expect value (E value) taken to be significant at < 0.19. The sequences were also aligned using the Lalign software v2.1.30.

3.2.2 PRIMER DESIGN

Conserved regions of *Trypanosoma congolense* sequence identified were marked out, and the site with the best homology used to design primer pairs, using the PICK PRIMER tool of NCBI. The best of the primer pairs with melting temperature at 59º wasgeneratedand then synthesized at Bioneer Company, USA.

3.2.3 COLLECTION OFINFECTED BLOOD

The *Trypanosoma congolense;* savannah strain was obtained from an infected mouse from the Nigerian Institute for Trypanosomiasis and Onchocerciasis Research (NITR), Kaduna- Nigeria. This was done by sedating the animal with chloroform in a sterile desiccator, then pinning the animal to a sterile board and cleaning its surface with ethanol-soaked cotton wool. The blood containing life parasites was collected directly from the heart into sterile heparin bottles and mixed gently to avoid clotting. Wet mount microscopy was carried out to ensure the animal was infected before the collection was done(Codija *et al*., 1993).

3.2.4 INITIATION OF CULTURE

In a $25cm^3$ culture flask, 5ml of the media was introduced followed by 1.5ml 2% glucose solution. 50µl of the Penicillin/Streptomycin solution, 1.5ml of FBS, 0.5ml of 0.001mM L-Cysteine and 0.5ml β- Mercaptoethanol (0.1%) were added to the media and gently mixed using a micro pipette. The Media and other nutrients were then incubated at 37º and 5% CO_2 for 10minutes to attain uniformity. 1.0ml of blood containing life parasites (7/field of microscopic observation) was then added to the culture media and incubated at 37º and 5% CO_2.

The culture was done in duplicates and maintained for 48hours, with periodic observations which was done at 6, 12, 24 and 48hours after initiation of culture. Thereafter the culture was terminated.

Parasites from the dissected midguts were also cultured using the same media and protocol as the blood stream forms of the parasites. This was also done in duplicates and observed over a 24hour period.

3.2.5 PARAMETERS OBSERVED DURING CULTURE

a. Viability and motility

Viability of the parasites was monitored as a measure of the motility of the cultured parasites since the flagella is very important to the survival of the parasites in whatever form and also helps in immune response evasion (Broadhead *et al.*, 2006). This was done using the inverted microscope to view parasites from the culture flasks. Motility was monitored using the light microscope by wet mount microscopic observation procedures. A cover slip was then used to cover the fluid and viewed at 10X magnification. (Leak,*et al.*, 1993).

b. Morphology/Cell Density

The cell density and morphology of the cultured parasites were determined by the thick smear and staining technique. A thick smear of the culture was made on slides, allowed to air dry and then stained with Giemsa. This was allowed to stay for 10minutes and then washed with water. The stain is added and allowed to stay for another 2minutes, washed and allowed to air dry. Immersion oil was then put on the stain and viewed under a light microscope at 10X. The parasites were then counted visually and average taken per field. (Codija *et al.*, 1993)

c. Infectivity of Cultured Parasites

0.5ml aliquots of culture were injected into laboratory animals intra-peritoneally according to the method described by Wei *et al.* (2011). Three animals were used for each time point. The animals were monitored for parasiteamia for 42days; this was done by taking blood from the tail of the animals and smearing on slides. This process was carried out against a control group of animals that were transfected with non-cultured parasites, from an infected animal.Time taken

for the cultured parasites to establish infection in wistar rats was monitored as a measure of their viability and infectivity as previously described (Hirumi,*et al.,* 1980).

d. Gene Expression

Aliquots (1ml) of cultured parasites were taken at each time point observation (0, 6, 24 and 48hours) after initiation of culture, placed in eppendorf tubes and stored at -4^{oc}. These were later used for gene expression studies by extracting total RNA;carrying cDNA synthesis of 1ng of total RNA in a final volume of 20µl; and running qPCR in a Roche Molecular Rotor-gene cycler (version 3.5). Reaction mixtures contained 1µl reaction buffer, 2.5mM $MgCl_2$, 0.05mM deoxynucleotides, 0.1µM gene-specific primesr, 1U of Taq polymerase (Fisher Biotech), a 1: 10000 dilution of SYBR Green I (Molecular Probes) and 2µl of sample. RNA extraction was done in triplicate for each time point, while sample at 0hrs was used as positive control. Expression was estimated using the relative quantification method, and calculated using the second derivative method.

This procedure was also done for procylic parasites after culturing to determine if the EP gene is present or if culturing affects the expression of this gene.

3.2.6 CONVENTIONAL PCR OF cDNA TO TEST THE EFFECTIVENESS OF EP PROTEIN AND GAPDH HOUSE KEEPING GENE PRIMERS

Synthesized EP gene primer pair and the Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) primers as internal control or house-keeping gene were diluted for 100 reactions with the following volumes of Diethyl-pyrocarbonate DEPC (RNAse- free treated water):

EP gene reverse primer = 127µl

EP gene forward primer = 132.8µl

GAPDH reverse primer = 104.2µl

GAPDH forward primer =105.4µl

These were further aliquoted and diluted serially to give 1pm concentration of the primers.

0hr and 48hr samples were used for RNA extraction, quantification and cDNA synthesis to optimize the primer pairs. Concentrations and volumes of the samples used are: 240nm; 2µl and 32.6nm; 16µl respectively. These were then inserted in a thermocycler for 25 PCR cycles, using 59º annealing temperature (as indicated for the gene of interest primers). Afterwards, the amplicons were run on gel against a 500bp ladder and viewed.

3.2.7 RNA EXTRACTION AND QUANTIFICATION OF CULTURED BLOOD STREAM FORMS OF *Trypanosoma congolense*

Extraction was done using the Bioneer RNA extraction kit as follows: In eppendorf tubes marked a, b and c for each of samples 0hr, 6hr, 24hr, 48hr and containing 400µL of solution compose of β Mercaptoethanol and RX (lysing) buffer; 200µl of samples were aliquoted. These were then vortexed for 5secondsand incubated for 10mins. Isopropanol was then added (as a fixative), vortexed and the contents transferred into hi-band (mi-Total RNA) columns.This was spinned at high speed (13,000rpm) for 5minutess. The filtrate was discarded and column transferred to another holding tube.400µl of washing buffer 1 was added, spinned at high speed for 2minutes and samples placed on ice pack (to avoid denaturation).Filtrate was discarded after which 500µl of washing buffer II was added and spinned for 1minute at maximum speed. The column was again transferred to a 1.5ml RNAse free microfuge collection tube and 30µl of elution buffer added. Incubation was carried out for 5minutes, T for 1minute at high speed and the samples immediately placed on ice.

*All samples were extracted in triplicate and all instruments were cleaned using DNA-off solution. Reactions were carried out in a biosafety II hood.

RNA quantification was done immediately using the GeneQuantpro 1300 spectrophotometer at absorbance at 260nm (A_{260}). The equipment was blanked with DEPC water. 5µl of extracted RNA was put in a separate cuvette from the one used for blanking, after thorough cleaning. It was then placed in the machine and the RNA command button pressed. This gave concentration of RNA and ratio, which was used to calculate amount RNA (as given in table 4.4) to be used for cDNA synthesis, as given below:

Volume = $1000 \div concentration$

This procedure was repeated for each individual sample.

3.2.8 REVERSE TRANSCRIPTASE PCR OR COMPLEMENTARY DNA (cDNA) SYNTHESIS

Principle: using the polymerase chain reaction, template RNA is transcribed to a more stable cDNA.

From the quantification Table obtained in Table 4.4, 0.5µg of cDNA was synthesized using Hotstarts cDNA synthesis kit (Bioneer U.S.A), as follows:

The following were added to the cDNA hot start (pelleted) premix tubes: calculated volume of RNA + 1µl forward primer + 1µl reverse primer + required amount of water to give total of 20µlfor all samples. All tubes were spinned for 2seconds and placed in the multigene II machine for synthesis.Thesewere then incubated at 42º for 1hour, 94º for 5minutes and holding temperature at 4º.

3.2.9 REAL-TIME PCR (QPCR)

Principle: Real-time PCR is carried out in a thermocycler with the capacity to illuminate beam of light and detect fluorescence. It 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).

qPCR was done using thee Bioneer reaction kit and performed with a light cycler real time PCR system (Roche Germany version 3.5).

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 20 µL with ddH_2O. The reaction mixtures were loaded into the capillaries, capped and centrifuged for 30 seconds. The qPCR cycle conditions were set at : 45 cycles, with each cycle at; 59º annealing temperature for 30seconds, 94º denaturation temperature for 5minutes and 72º extension temperature for 5minutes.

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 extract the crossing point values (Cp) (for individual samples The real-time PCR data were analysed using the $2^{-\Delta\Delta CT}$ relative quantification method of livark and schmittgen, (2001), and P value less than 0.05 ($P< 0.05$) was consider significant.

CHAPTER FOUR

4.0 RESULTS

4.1 IDENTIFICATION OF EP- PROCYCLIN GENE IN *Trypanosoma congolense*

The EP- procyclin gene sequence generated from the NCBI gene bank is given in figure 4.1.

The homology between homology between EP procyclin gene of *T. brucei* and a section of the genome of *T. congolense,* is given in figure 4.1. This shows regions where there was alignment between the two sequences, and regions where the sequences were complementary to each other (according to the purine – pyrimidine bonding structure of bases). It also gives a sequence similarity of 44.9% and a bit score of 31.7.

>gi|14485017|gb|AF353628.1| Trypanosoma brucei brucei surface protein EP1-2 procyclin precursor (EP1-2) gene, EP1-2-EP/PAG2 allele, complete cds

ACTTCAATTACACCAAAAAGTAAAATTCACAATGGCACCTCGTTCCCTTTATCTACTCGCTGTTCTTCTG

TTCAGCGCGAACCTCTTCGCTGGCGTGGGATTTGCCGCAGCCGCTGAAGGACCAGAAGACAAGGGTCTTA

CTAAGGGAGGCAAAGGCAAAGGCGAGAAGGGAACCAAGGTCAGCGCCGACGATACCAATGGCACTGACCC

CGACCCTGAACCCGAACCCGAACCCGAACCCGAACCTGAACCTGAACCTGAACCCGAACCTGAACCTGAA

CCTGAACCTGAACCTGAACCCGAACCGGAACCTGAACCTGAACCCGAGCCGGAACCTGAACCAGAACCTG

AACCAGAACCTGAACCTGGTGCTGCAACGCTGAAATCCGTTGCACTTCCGTTCGCAATCGCGGCTGCTGC

TCTCGTTGCCGCATTCTAAGCGGATGCAAGCGTGTAAAGCGCCTCGGAGGAACGAAACCCTTTGAAAAGG

TTCCTTTCATTTATATCGCCTCCATATGGTGCATCGTGTTTGTTTCCTGCTGTTTCTTGTAAAACAAGTG

TGGACATTCATTTAATATTTTTTCGTTATATTTTTTTGGTGACATCCTTTCTAATGCCTTATTAACCATC

GCCTGAGACCCACAGCCCTGTAGATTTCTGTGATGTTTCGGTTGCGTATTCCATAATTTTAAGCGTTTCA

CTTCTATTTTTTTTCATTCCTTTGAATTTGGATCTTAAAA.

Figure 4.1: EP-procyclin sequence of *trypanosome brucei*.



Figure 4.2:Section of homology between EP gene sequence of *T. brucei* and *T. congolense* genome. This was generated using the clustalW tool of EXPASY genebank and the LALIGN software (version 2.1.30.

Key:

CAEQ01000355.1 = sequence for *Trypanosoma congolense* genome.

AF353628.1 = sequence for EP gene

***** = sequences that align together

: = sequences that are complimentary to each other (i.e. A to T and G to C and vise-versa)

. = sequences where purines and pyrimidines appear but not complimentary to each other

4.2 PARAMETERS OBSERVED DURING IN VITRO CULTURE OF BLOOD STREAM FORMS OF *Trypanosoma congolense*

4.2.1 Cell Density of Parasites

Table 2 gives the cell density of the parasites at different time points after culturing. As the culturing progressed, the cell density decreased to as much as 50.8% and 31.1% in flasks 1 and 2 respectively by 6 hours. After stabilizing at these values for the next 24 hours, the cell density further decreased by more than 50%.

Table 4.1: Cell density of parasites per milliliter, at different time points.

TIME (Hours)	**NUMBER OF PARASITES/mL**	**DROP IN CELL DENSITY (%)**
OHRS	4.05	
12HRS	2.05	68.9
24HRS	2.05	34.45
48HRS	0.95	17.25

Cell density determined using the light microscope. Table gives average number of parasites per ml in exponential of 10^4, as counted in two slides.

4.2.2 Viability and Motility of Cultured Parasites

The viability and motility of the parasites in culture are presented in table 4.2. From the table, it can be seen that the parasites were viable and hence motile throughout the duration of the culture except cells at 48hrs after culture. Cells at 0hrs of culture were very motile; those at 6hrs had a reduced motility. At 12hrs after initiation of culture, the motility of the parasites had picked up and become steady, this also started to drop as at 24hrs of culture. At 48hrs of culture, the parasites were not motile. Parasites cultured from the midgut of flies were not motile after 24hrs of culture.

Table 4.3 gives the infectivity of both cultured and uncultured parasites. This was carried out as a measure of viability of the parasites. The table showed the days in which parasitemia came up in the infected animals and the days the different animals died after infection. The animals were monitored for a period of 42days.

Group A animals were infected with cultured parasites after 6hrs of culture. The table shows that 2 out of 3 of the animals showed parasitemia at 22days post-infection and both died on the 38th day, while 1 showed at day 26 and died after 42 days.

Group B animals were infected with cultured parasites after 24hrs of culture. 2 of the animals showed parasitemia at day 22; one dying at day 38, and the other at day 42. While the 3rd animal showed parasitaemia at day 18 and died at day 34.

Group C animals served as the control and were infected with non-cultured parasites from an infected animal. One animal showed parasitemia after 10days post-infection and died at day 34. The other 2 animals both showed parasitemia after 14 days; with one dying at day 42 and the other dying after day 42.

Table 4.2: Motility of parasites at different time points

Time Point (Hrs)	**Motility**
0	Very motile
6	Reduced motility
12	Steady motility
24	Slightly reduced motility
48	No motility

Very Motile = parasites were strongly active

Reduced motility = parasites were active

Slightly reduced and steady motility = parasites were passively active

Table 4.3: Level of parasitemia of cultured parasites in animals post infection.

		AVERAGE NUMBER OF PARASITES DAY POST INFECTION								
GROUP	**ANIMAL**	**10**	**14**	**18**	**22**	**26**	**30**	**34**	**38**	**42**
A	1	-	-	-	4	12	21	22	*24	-
	2	-	-	-	-	10	15	17	17	22
	3	-	-	-	7	8	19	22	*27	
	Average				**5.5**	**10**	**18.3**	**20.3**	**22.7**	**22**
B	1	-	-	-	1	5	9	17	17	*20
	2	-	-	-	3	5	8	13	*14	-
	3	-	-	1	2	7	12	*17		
	Average			**1**	**2**	**5.7**	**9.7**	**15.7**	**10.3**	**20**
C	1	3	7	17	22	34	39	*47		
	2	-	4	10	19	28	40	53	58	*63
	3	-	2	5	13	26	31	39	44	59
	Average	**3**	**4.3**	**10.7**	**18**	**29.3**	**36.7**	**46.3**	**51**	**61**

Note: Animals were infected with cell densities at the various timepoints after culture.

Group A = animals infected with parasites from culture at 6hrs after culture

Group B = animals infected with parasites from culture at 24hrs after culture

Group C= animals infected with cultured parasites (i.e. from another infected animal)

* shows number of parasites per field as at the time animal died

- = no parasites seen.

4.2.3 Morphology of Parasites using the Light Microscope

The morphology of the cultured parasites was observed using the light microscopes. Figure 4.2 gives the morphology of the parasites at different time points (starting from 0hr to 48hrs) after culturing. From the results it was observed that there was a shortening in length of the parasites.

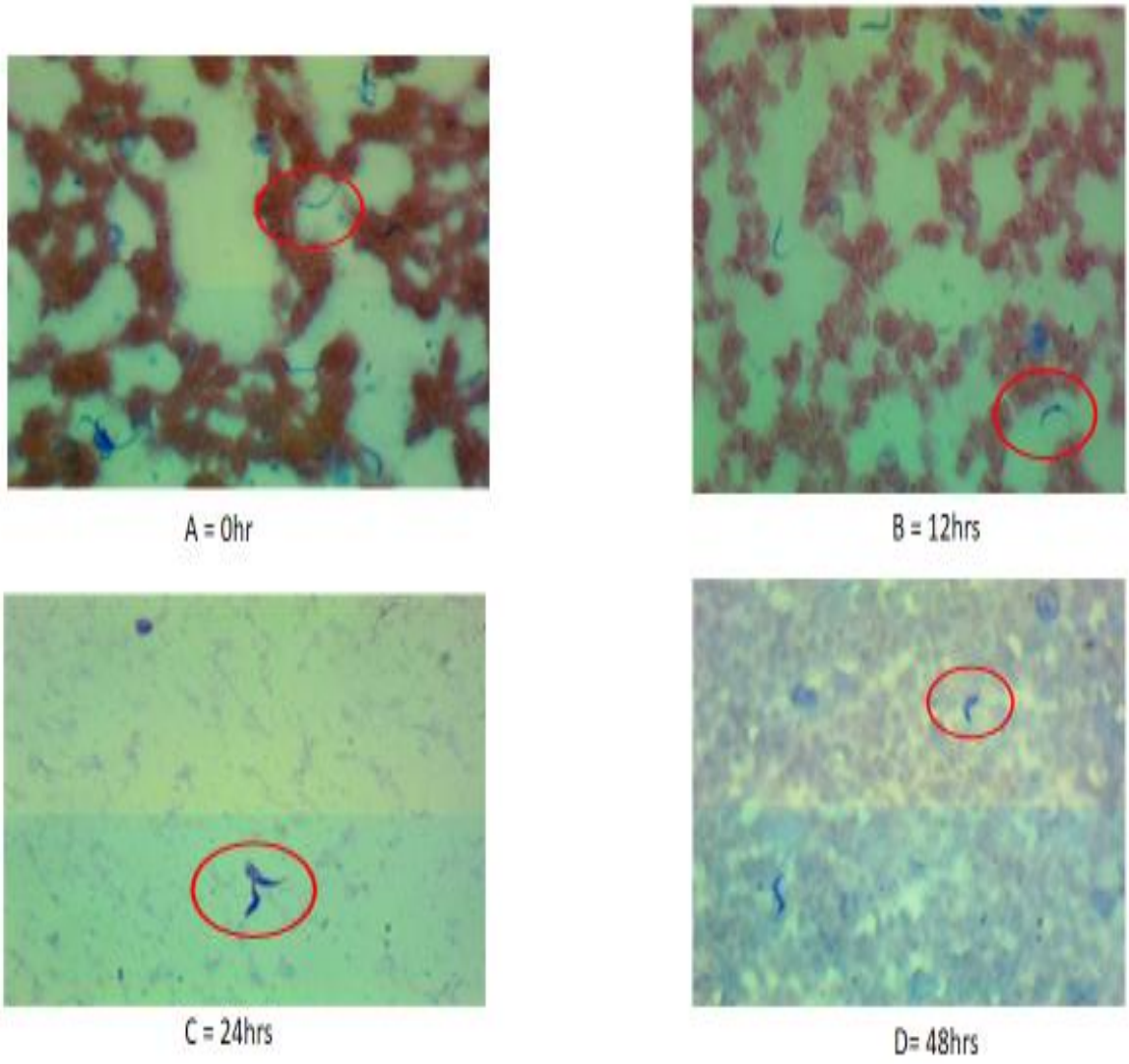


Figure 4.3: Morphology of parasites at different time-points (0hr, 12hrs, 24hrs and 48hrs) after culture.

Morphology obtained by staining with giemsa and viewed at X10. Results show a shortening in length of parasites at each time point.

A=0hrs, B =12hrs, C =24hrs, D = 48hrs.

◯ = indicates parasites.

4.3 EXPRESSION PATTERN OF EP PROTEIN IN *Trypanosoma congolense*

4.3.1 Conventional PCR to test effectiveness of House-keeping gene (GAPDH)

Plate 4.ishows gel electrophoresis band of the PCR optimization using the GAPDH (house-keeping gene) primer pairs,while Appendix iishows the graphical representation of both primers after RT-PCR optimization.

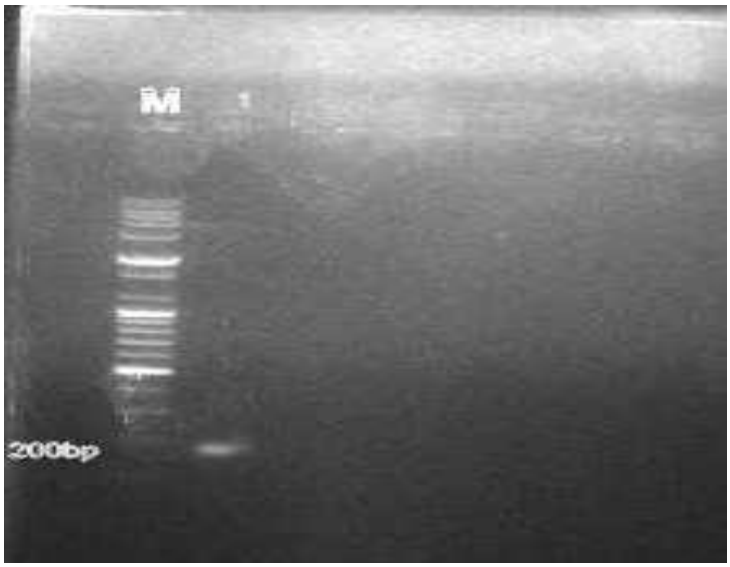


Plate 4.i: PCR amplification of GAPDH gene using its specific primers.

KEY:

Lane M= marker (500bp)

Lane 1 = GAPDH gene

Note: The bandwidth of 200bp corresponds to previously used and synthesized G3PDH primers.

4.3.2 PCR and Gel Electrophoresis

Conventional PCR of the EP- protein gene using the designed EP primer pairs, is given in Plate 4.ii. This gave band size of 309bp, which is relatively similar in size to that previously discovered in both tsetse midgut and *T. brucei*.

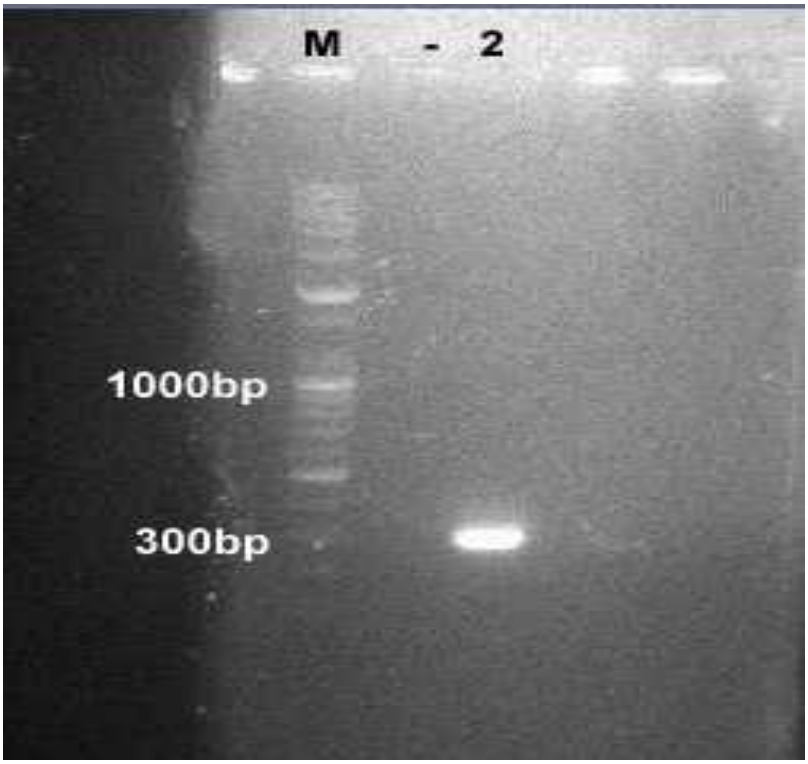


***Plate 4.ii*:** Band showing effectiveness of designed EP primers amplified from cultured*T. congolense* cDNA.

KEY:

Lane M= marker (100bp plus)

Lane2 = EP gene

\- = Negative control

Note: The Bandwidth of 309bp is similar to that synthesized for *Trypanossoma brucei* and that of the tsetse midgut.

Table 4.4: Quantification of extracted RNA

SAMPLE (Hrs)	RNA CONCENTRATION (ng/µl)	PURITY	VOLUME REQUIRED (µl)
0A	180.6	4.2	5.5
0B	169.2	3.9	5.9
0C	154.2	3.8	6.4
24A	135.8	3.9	7.3
24B	143.7	1.4	7
24C	86.6	2	11.5
48A	99.6	1.2	12.1
48B	92.9	1.1	10
48C	82.2	2.3	10.7

4.3.2 QRT-PCR and Gene Expression

RNA extracted was quantified and used to calculate the amount to be used for cDNA synthesis (as seen above in table 4.4). These were then used to carry out qPCR. The qPCR amplification curves of both EP and GAPDH primers with all samples are given in figure 4.3. The visual aid at the edge indicates the color code for each sample and primer used; where E represents the EP primers and G represents the G3PDH primers.

The result obtained from the amplification curves (Cp values), were used to calculate the fold change (expression) of EP-procyclin gene using the method described by Livark and Schimitgen (2001). The result analyzed, was used to plot a graph of expression (fold change) of EP- protein gene ($_2^{-\Delta\Delta}c_p$) against timepoints (Hrs)as shown in Figure 4.4. From the chart, it can be seen that the gene in the parasites at 0hrs of culture had little or no expression, while 24hrs had the highest expression level of about 48.8%. Parasites at 48hrs exhibited a much reduced expression level than that of 24hrs, with a value of 2.4%.

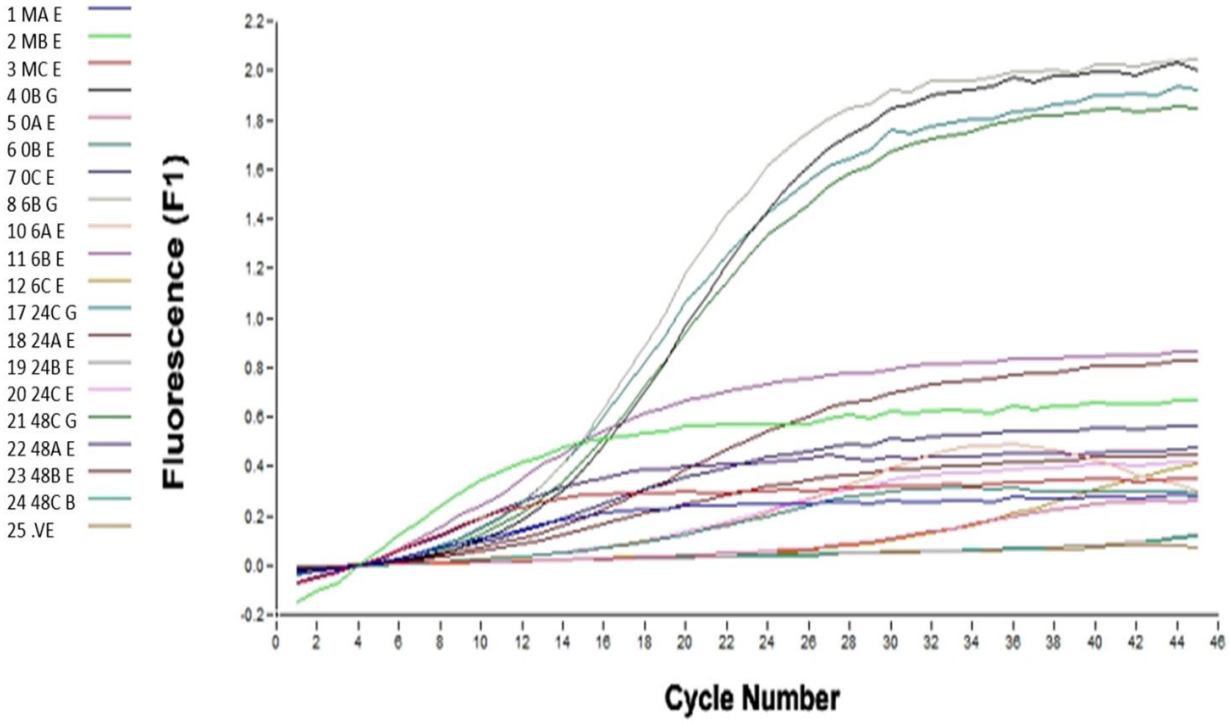


Figure 4.4: Amplification curves of qPCR used for relative quantitative expression of EP-protein gene

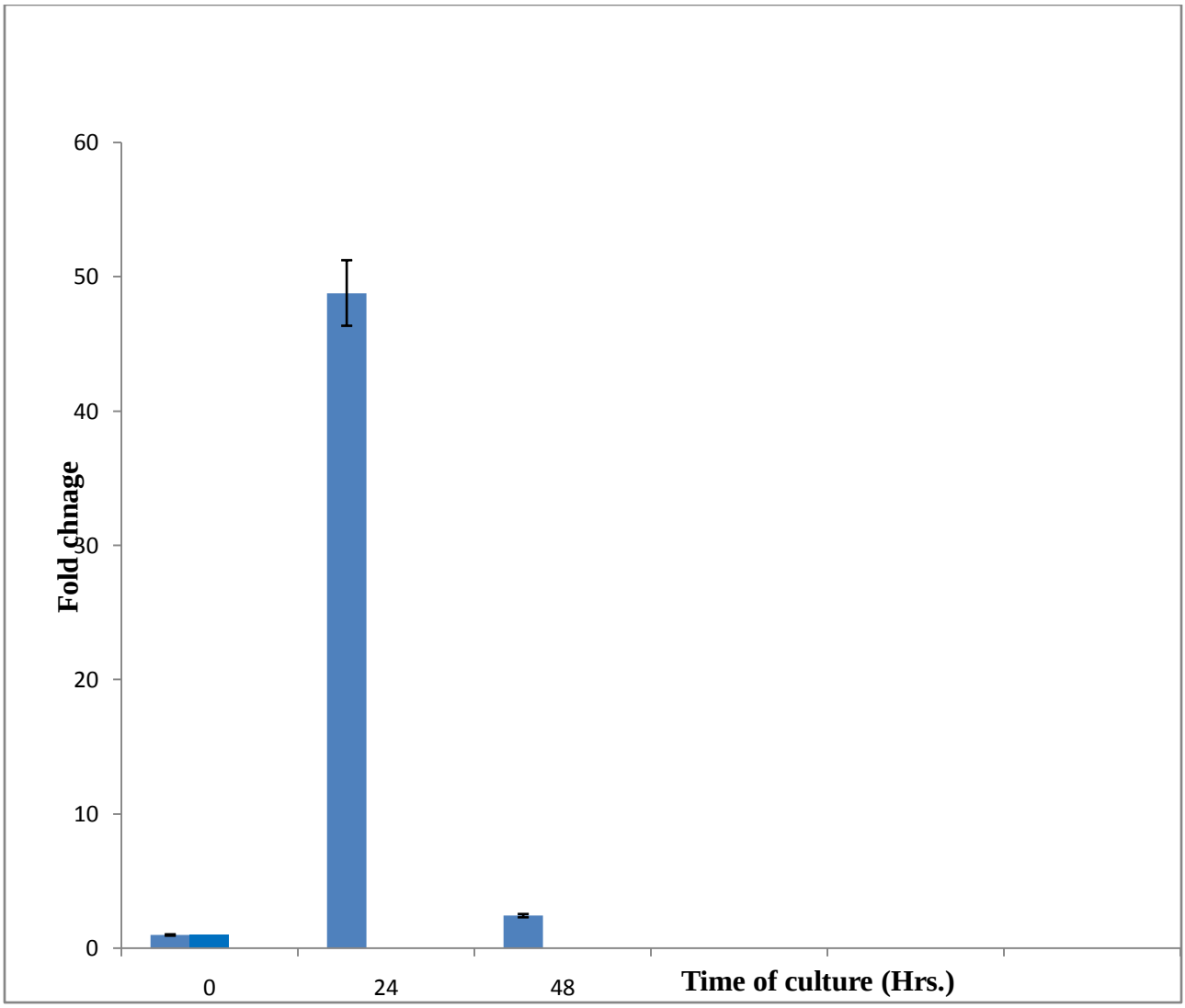


Figure 4.5: Expression levels of EP-Procyclin gene in cultured *T.congolense*.

Average C_T- values used for expression:

1. For EP gene; 0hrs =13.18, 24hrs =14.5, 48hrs = 13.23
2. For GAPDH gene; 0hrs =14.33, 24hrs = 10.04, 48hrs = 13.54

CHAPTER FIVE

5.0. DISCUSSION

African animal trypanosomiasis, caused bytrypanosomes, is an important disease of domestic livestock in sub-Saharan Africa. Resistance to immune responses and drugs used in the treatment of this disease, due to the changing VSG and procyclic EP coats, have been shown to be very critical to the survival of the trypanosomes in both mammalian and insect hosts. Targeting and inhibiting these surface coat proteins could undoubtedly break the transmission sequence (pathway) of the protozoan from the vector to the host (animals and humans). Therefore, detection of the presence and understanding the expression pattern of surface proteins, particularly the EP protein in *T. congolense* can provide a template for the development of new strategies and treatment regimen in the control and management of trypanosomiasis in animals. This provided the spur for this research. The work set out primarily to determine the expression of EP – procyclin gene in cultured Bloodstream Forms of *Trypanosoma congolense* parasites.

In doing this, the EP – procyclin sequence was first identified in *T. congolense* by carrying out a BLAST and Pairwise sequence alignment between the EP sequence of *T. brucei*and the genome of *T. congolense.* Data gotten using clustalW tool of EXPASY genebank and the LALIGN software revealed a significant similarity of 44.9% between these two sequences, with a bit score of 20.4 and an E-value of 0.15. This indicates related structural similarity between both genes and also strongly suggests similar functions between the identified gene in *T. congolense* and *T. bruceiEP-procyclin* gene, as pairwise sequence alignments are great predictors of gene structural similarities when significant homologs are found to share more than 40% identity (Pearson, *et al.,* 2013). The conserved regions from this homology were used to design primers using the "PICK PRIMERS" tool of the NCBI gene bank. These were used for gene expression.

An *in vitro* culture of the parasites was carried out and maintained for 48hours. The culture medium used supported the growth of *T. congolense* bloodstream form, as the parasites appeared to be viable and motile for the duration of the culture, even though there was an observed decrease in the motility of the cultured parasites as time of culture progressed as shown in table 4.2. Reduction in motility at a point in the culture could be attributed to the fact the parasites were adapting to their new environment, while non-viability and motility at the end of the culture was because of depletion of nutrients in the culture media. The culture also showed a decrease in cell density of the parasites as seen in table 4.1. Thus, indicating an optimum time for culture of *T. congolense* to be between 12 and 24hours.

To assess if the cultured cells retained viability, the parasite motility and presence of flagella was assessed, (as motility is also an indirect measure of the viability (Ratson, *et al.*, 2009)). Aliquots from different time points of the culture was used to infect apparently healthy mice according to the method described by Wei *et al*., (2011). The results demonstrated that the cultured *T. congolense* retained viability and infectivity. Parasites that were not cultured were able to infect their hosts as expected showing early between 10 to 14days after infection, with the animals dying much later at 42 days and beyond than the test groups, as also observed by other studies (Hirumi and Hirumi, 2009; Wei *et al.*, 2011). On the other hand, those parasites that were cultured were able to establish infection although at a slightly non-significant delay compared to the control group (between 14 to 18days) with faster mortality rates (between 34 to 38days).

Morphologically, we observed a shortening of the length of the parasites from the long slender forms to stumpy forms as seen in figure 4.2. The picture shows a shortening in length of the cultured parasites at each time point, which tallies with the findings of other researchers (Otigbuo and Woo, 1987; Zeigelbauer, *et al.*, 1990; Schwede, *et al.*, 2012). PCR is a molecular

technique, used to test the effectiveness of primer pairs by amplifying the gene of interest and giving band sizes when run on gel. Amplification of the sequence obtained using the synthesized primers (as shown in plate 4.i), revealed a band size of 309bp, relatively similar in size with the previously reported band size of 320bp to the EP sequence of *T. brucei* and that from the tsetse midgut, as discovered by Haines, *et al.* (2010).

In this study, QPCR technique was employed to monitor the expression of the EP protein gene, using reversed transcribed (cDNA) extracted RNA from the cultured bloodstream forms of *T. congolense*, at different timepoints after culture. The QPCR result analyzed from this study showed that, there was significant p (< 0.05) expression level of an EP procyclin-like gene as seen in figure 4.4. The expression peaked at 24 hours of culture with a 48.8-fold change, compared to results obtained at the beginning and at the end of the culture (0hours and 48hours). These had a 1-fold change and 2.4-fold change expression levels respectively. The results demonstrates for the first time the presence of *EP-procyclin-like* gene in cultured bloodstream form of *T. congolense* savannah strain.

The observations that cultured bloodstream forms of *T. congolense* rapidly expressed EP-*procyclin*-like gene during the early developmental phase of the culture when the long slender bloodstream form differentiated to short stumpy form was similar to reports by Vasella *et al.*, (2000) who demonstrated the expression of *EP-procyclin* coat in Agarose plate cultured *T. brucei* pleomorphic strain AnTat 1.1.

CHAPTER SIX

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6.1 SUMMARY

To carry out the study, a blast of the already discovered EP gene sequence in *T. brucei* with that of the genome of *T. congolense* was carried out, primers were designed from the conserved regions, an*in vitro* culture of the blood stream forms of the parasites was carried out and an expression study done, to determine if this EP gene is present. EP – *procyclin* – like gene was identified and amplified from *T. congolense* after *in vitro* cultivation. This gene was expressed in cultured bloodstream forms and optimally so by 24 hours post culturing.

6.2 CONCLUSION

The research was able to adapt a culture model to grow*Trypanosoma congolense*. It was able also to identify a sequence similar to the EP- procyclin sequence of *T. brucei,* contained in the genome of *T. congolense* from the alignment results.From the research, we were able to isolate a gene that is similar in size to the EP- procyclin gene of both *T. brucei* and that from the tsetse midgut.

Evidences from the research demonstrated the expression of *EP- procyclin-like*gene in cultured bloodstream forms (BSF) of *T. congolense*. The expression of *EP- procyclin* gene was monitored for the duration of the culture, revealing a peak at 24hours of culture.

6.3 RECOMMENDATION

Having carried out this research, the researcher would like to make the following recommendations for further work:

1. The correlations among the results obtained (that is the peaks of motility, cell density, shortening of the parasites and the expression of the gene), which were observed at around the same time-points of 12 to 24 hours could be further verified.
2. An SDS-PAGE and sequencing of the transcript identified could be carried out.
3. The expression of this protein could be explored *in* vivo and in other trypanosoma species, as the expression of this protein implies that it could be modified and the yield increased for vaccine development.
4. The mechanism of action of the EP protein and other surface proteins in host evasion in the trypanosomes, should be further worked on and studied, as this will go a long way in developing effective strategies that will be used in combatting this neglected tropical disease.

Appendices

Appendix i:

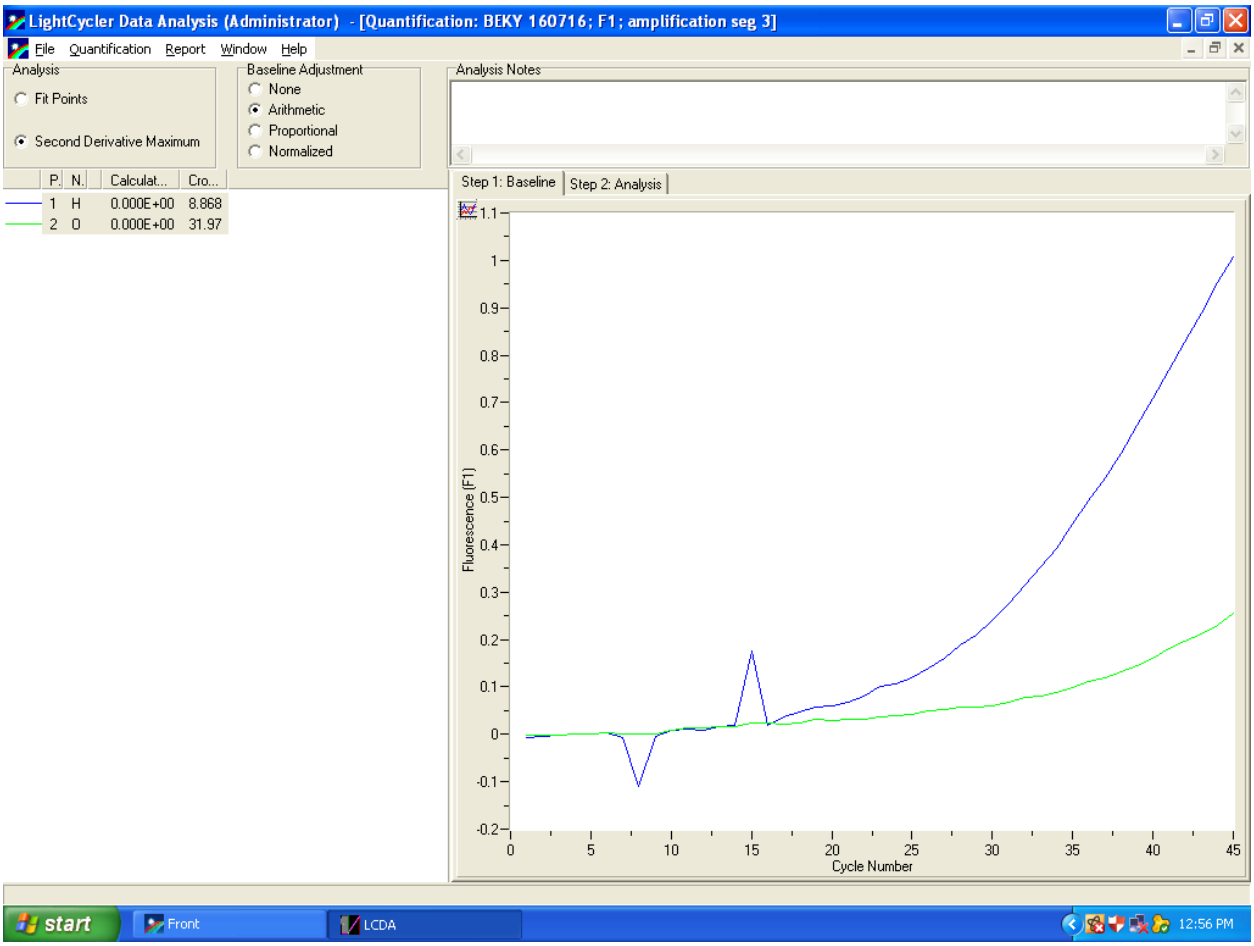
PREPARATION OF CULTURE MEDIA COMPONENTS

- 1.7612g of L-cysteine was dissolved in 10ml PCR grade water to give a 1M solution. This was serially diluted thrice to give a 0.001mM solution of L-cysteine.
- 0.2g of glucose was dissolved in 10ml PCR grade water to get 2% glucose solution.
- 49.95ml PCR grade water added to 5µl (D/W) of β-mercaptoethanol to make 1% β-mercaptoethanol.

***IN VITRO* CULTURE**

Culture media constituents were RPMI 1640 media, β- Mercaptoethanol, L-Cysteine, Glucose, fetal Bovine Serum (FBS), and Antibiotics (Penicillin/Streptomycin solution).

All preparations were carried out in a Laminar flow hood and micro-filtered using a 0.22µ filter. This was done at the DNA Labs in Kaduna.



Appendix ii: Graph of expression levels of GD3PH and EP gene primers after qPCR optimization**.**

REFERNCES

Acosta-Serrano A, Cole RN, Mehlert A, Lee MG, Ferguson MA. (1999). The procyclin repertoire of Trypanosoma brucei. Identification and structural characterization of the glu-pro-rich polypeptides. J Biol Chem 274: 29763–29771.

Acosta-Serrano, A., Vassella, E., Liniger, M., Renggli, C. K., Brun, R., Roditi, I., & Englund, P. T. (2001). The surface coat of procyclic Trypanosoma brucei: programmed expression and proteolytic cleavage of procyclin in the tsetse fly. *Proceedings of the National Academy of Sciences*, *98*(4), 1513-1518.

Akman L, Yamashita A, Watanabe H, Oshima K, Shiba T, Hattori M, Aksoy S (2002) Genome sequence of the endocellular obligate symbiont of tsetse, Wigglesworthia glossinidia. Nature Genetics **32**: 402-407.

Aksoy S, Berriman M, Hall N, Hattori M, Hide W, Lehane M (2005) A case for a Glossina genome project. Trends in Parasitology **21**: 107-111.

Aksoy S, Gibson WC, Lehane MJ (2003) Interactions between tsetse and trypanosomes with implications for the control of trypanosomiasis. Advances in Parasitology 53: 1–83.

Alberts, Bruce (2008). Molecular biology of the cell. New York: *Garland Science*. ISBN 0-8153-4105-9.

Allsopp, R. (1984). Control of tsetse flies (Diptera: Glossinidae) using insecticides: a review and. *Bulletin of Entomological Research*, *74*(1), 1-23.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389-3402.

Artursson P.; Palm K.; Luthman K. (2001). "Caco-2 monolayers in experimental and theoretical predictions of drug transport". *Advanced Drug Delivery Reviews*. **46** (1–3): 27–43. doi:10.1016/s0169-409x(00)00128-9. PMID 11259831

Barry JD, McCulloch R (2001) Antigenic variation in trypanosomes: Enhanced phenotypic variation in a eukaryotic parasite. Adv Parasitol 49:1–70.

Beard CB, O'Neill SL, Mason P et al. (1993) Genetic transformation and phylogeny of bacterial symbionts from tsetse. Insect Molecular Biology **1**: 123-131.

Bengaly Z., Sidibe I.,Ganaba R., Desquesnes M., Boly H. & Sawadogo L. (2002). Comparative pathogenicity of three genetically distinct types of Trypanosoma congolense in cattle: clinical observations and haematological changes. Vet. Parasitol.,108, 1–19.

Boulanger N, Brun R, Ehret-Sabatier L, Kunz C, Bulet P (2002) Immunopeptides in the defense reactions of Glossina morsitans to bacterial and Trypanosoma brucei brucei infections. Insect Biochemistry and Molecular Biology **32**: 369-375.

Broadhead, R., Dawe, H. R., Farr, H., Griffiths, S., Hart, S. R., Portman, N., & Gull, K. (2006). Flagellar motility is required for the viability of the bloodstream trypanosome. *Nature*, *440*(7081), 224.

Brun, R., Blum, J., Chappuis, F., & Burri, C. (2010). Human african trypanosomiasis. *The Lancet*, *375*(9709), 148-159.

Butikofer P, Vassella E, Boschung M, Renggli CK, Brun R, Pearson TW, Roditi I: Glycosylphosphatidylinositol-anchored surface molecules of Trypanosoma congolense insect forms are developmentally regulated in the tsetse fly. Mol Biochem Parasitol 2002, 119(1):7–16.

Carabarin-Lima, A., González-Vázquez, M. C., Rodríguez-Morales, O., Baylón-Pacheco, L., Rosales-Encina, J. L., Reyes-López, P. A., & Arce-Fonseca, M. (2013). Chagas disease (American trypanosomiasis) in Mexico: an update. *Acta tropica*, *127*(2), 126-135.

Chandra M, Liniger M, Tetley L, Roditi I, Barry JD (2004) Tsetse EP, a gut protein from the tsetse Glossina morsitans, is related to a major surface glycoprotein of trypanosomes transmitted by the fly and to the products of a Drosophila gene family. Insect Biochemistry and Molecular Biology 34: 1163–1173.

Chappuis, F., Loutan, L., Simarro, P., Lejon, V., & Büscher, P. (2005). Options for field diagnosis of human African trypanosomiasis. *Clinical microbiology reviews*, *18*(1), 133-146.

Checkley, A. M., Pepin, J., Gibson, W. C., Taylor, M. N., Jäger, H. R., & Mabey, D. C. (2007). Human African trypanosomiasis: diagnosis, relapse and survival after severe melarsoprol-induced encephalopathy. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *101*(5), 523-526.

Coustou, V., N. Plazolles, F. Guegan & T. Baltz (2012) Sialidases play a key role in infection and anaemia in Trypanosoma congolense animal trypanosomiasis. Cell Microbiol, 14, 431-45.

De Clercq E (October 2005). "Recent highlights in the development of new antiviral drugs". *Curr. Opin. Microbiol.* ***8*** *(5): 552–60.* doi:10.1016/j.mib.2005.08.010. PMID 16125443.

Desquesnes, M. (2004). *Livestock trypanosomoses and their vectors in Latin America*. OIE (World Organisation for Animal Health).

Duffy, T., Bisio, M., Altcheh, J., Burgos, J. M., Diez, M., Levin, M. J.,& Schijman, A. G. (2009). Accurate real-time PCR strategy for monitoring bloodstream parasitic loads in Chagas disease patients. *PLoS neglected tropical diseases*, *3*(4), e419.

Duszenko, M., Ferguson, M. A., Lamont, G. S., Rifkin, M. R., & Cross, G. A. (1985). Cysteine eliminates the feeder cell requirement for cultivation of Trypanosoma brucei bloodstream forms in vitro. *Journal of Experimental Medicine, 162*(4), 1256-1263.

Duszenko, M., Mühlstädt, K. and Broder, A. (1992). Cysteine is an essential growth factor for Trypanosoma brucei bloodstream forms. Mol. Biochem. Parasitol. 50, 269-274.

Dyer, N. A., C. Rose, N. O. Ejeh & A. Acosta-Serrano (2013). Flying tryps: survival and maturation of trypanosomes in tsetse flies. Trends Parasitol, 29, 188-96.

El-Sayed, N. M., Ghedin, E., Song, J., MacLeod, A., Bringaud, F., Larkin, C., & Tweedie, A. (2003). The sequence and analysis of Trypanosoma brucei chromosome II. *Nucleic acids research, 31*(16), 4856-4863.

Eyford, B. A., Ahmad, R., Enyaru, J. C., Carr, S. A., & Pearson, T. W. (2013). Identification of Trypanosome proteins in plasma from African sleeping sickness patients infected with T. b. rhodesiense. *PLoS One, 8*(8), e71463.

Fadiga, M., Jost, C., & Ihedioha, J. (2013). Financial costs of disease burden, morbidity and mortality from priority livestock diseases in Nigeria: Disease burden and cost-benefit analysis of targeted interventions.

Ferguson, M. A. J., Murray, P., Rutherford, H. and McConville M. J. (1993). A simple purification of procyclic acidic repetitive protein and demonstration of a sialylated glycosyl-phosphatidylinositol membrane anchor. Biochem. J. 291, 51-55.

Finelle, P. (1974). African animal trypanosomiasis. IV. Economic problems. *World animal review.*

Gargas M.L.; Burgess R.L.; Voisard D.E.; Cason G.H.; Andersen M.E. (1989). "Partition-Coefficients of low-molecular-weight volatile chemicals in various liquids and tissues". *Toxicology and Applied Pharmacology. **98**:87–99.*doi:10.1016/0041-008x(89)90137-3.

Gbem, T.T., M. Waespy, B. Hesse, F. Dietz, J. Smith, G.D. Chechet, J.A. Nok., S. Kelm (2013) Biochemical diversity in the Trypansoma congolense trans-sialidase family. PLOS Neglt Trop Disease, 7(12): e2549. Doi: 10.1371/journal.pntd.0002549. Gibson, W. C., J. R. Stevens, C. M. Mwendia, J. N. Ngotho & J. M. Ndung'u (2001) Unravelling the phylogenetic relationships of African trypanosomes of suids. Parasitology, 122, 625-31.

Gibson W, Bailey M (2003). The development of Trypanosoma brucei within the tsetse fly midgut observed using green fluorescent trypanosomes. Kinetoplastid Biology and Disease 2: 1– 13.

Gray MA, Cunningham I, Gardiner PR, Taylor AM, Luckins (1981). AG: Cultivation of infective forms of Trypanosoma congolense from trypanosomes in the proboscis of Glossina morsitans. Parasitology, 82:81–95.

Gray MA, Ross CA, Taylor AM, Luckins (1984). AG: in vitro cultivation of Trypanosoma congolense: the production of infective metacyclic trypanosomes in cultures initiated from cloned stocks. Acta Trop, 41:343–353.

Haines LR, Jackson AM, Lehane MJ, Thomas JM, Yamaguchi AY, et al. (2005). Increased expression of unusual EP repeat-containing proteins in the midgut of the tsetse fly (Glossina) after bacterial challenge. Insect Biochemistry And Molecular Biology 35: 413–423.

Haines LR, Lehane SM, Pearson TW, Lehane MJ (2010). Tsetse EP Protein Protects the Fly Midgut from Trypanosome Establishment. PLoS Pathog 6(3):e1000793. doi:10.1371/journal.ppat.1000793.

Hamilton, P. B., Adams, E. R., Malele, I. I., & Gibson, W. C. (2008). A novel, high-throughput technique for species identification reveals a new species of tsetse-transmitted trypanosome related to the Trypanosoma brucei subgenus, Trypanozoon. *Infection, Genetics and Evolution*, *8*(1), 26-33.

Hao, Z., Kasumba, I., Lehane, M. J., Gibson, W. C., Kwon, J., & Aksoy, S. (2001). Tsetse immune responses and trypanosome transmission: implications for the development of tsetse-based strategies to reduce trypanosomiasis. *Proceedings of the National Academy of Sciences*, *98*(22), 12648-12653.

Hirumi, H., & Hirumi, K. (1991). In vitro cultivation of Trypanosoma congolense bloodstream forms in the absence of feeder cell layers. *Parasitology*, *102*(02), 225-236.

Hirumi, H., Doyle, J. J., & Hirumi, K. (1977). African trypanosomes: cultivation of animal-infective Trypanosoma brucei in vitro. *Science*, *196*(4293), 992-994.

Hirumi, H., Martin, S., Hirumi, K., Inoue, N., Kanbara, H., Saito, A., & Suzuki, N. (1997). Cultivation of bloodstream forms of Trypanosoma brucei and T. evansi in a serum-free medium. *Tropical Medicine & International Health*, *2*(3), 240-244.

Holmes, P. H. (1997). New approaches to the integrated control of trypanosomosis. *Veterinary parasitology*, *71*(2-3), 121-135.

Holmes, P. H., & Scott, J. M. (1982). Chemotherapy against animal trypanosomiasis. *Tropical medicine research studies series*.

Holzmuller, P., Grébaut, P., Cuny, G., & Biron, D. G. (2010). Tsetse flies, trypanosomes, humans and animals: what is proteomics revealing about their crosstalks?. *Expert review of proteomics*, *7*(1), 113-126.

Hursey, B. S., & Allsopp, R. (1983). Sequential applications of low dosage aerosols from fixed-wing aircraft as a means of eradicating tsetse flies (Glossina spp) from rugged terrain inZimbabwe. *Department of Veterinary Services, Tsetse and Trypanosomiasis Control Branch*.

Ishikawa H (2003) Insect symbiosis: An Introduction. In: Miller TA (ed.) Insect Symbiosis, pp. 1–22. CRC Oress, Boca Raton, FL.

Jackson D. G, Smith D. K, Luo C, Elliott J. F (1993). Cloning of a novel surface antigen from the insect stages of Trypanosoma brucei by expression in COS cells. J Biol Chem;268(3):1894–900.

Jackson, A. P., H. C. Allison, J. D. Barry, M. C. Field, C. Hertz-Fowler & M. Berriman (2013) A cell- surface phylome for African trypanosomes. PLoS Negl Trop Dis, 7, e2121.

Jackson, P. R., Wohlhieter, J. A., Jackson, J. E., Sayles, P., Diggs, C. L., & Hockmeyer, W. T. (1984). Restriction endonuclease analysis of Leishmania kinetoplast DNA characterizes parasites responsible for visceral and cutaneous disease. *The American journal of tropical medicine and hygiene*, *33*(5), 808-819.

Jones, T. W. & A. M. Davila (2001) Trypanosoma vivax--out of Africa. Trends Parasitol, 17, 99-101.

Jordan AM: Trypanosomiasis control and African Rural Development. 1986, Longman Group Ltd, UK**Google Scholar**

Jordan, A. M. (1985). The vectors of African trypanosomiasis: Research towards non-insecticidal methods of control. *British medical bulletin*, *41*(2), 181-186.

Keating, J., Yukich, J. O., Sutherland, C. S., Woods, G., & Tediosi, F. (2015). Human African trypanosomiasis prevention, treatment and control costs: a systematic review. *Acta tropica*, *150*, 4-13.

Kinoshita T., Inoue N. (2000) Curr. Opin. Chem. Biol. 4:632–638.

Kokoza VA, Martin D, Mienaltowski MJ, Ahmed A, Morton CM, Raikhel AS (2001) Transcriptional regulation of the mosquito vitellogenin gene via a blood meal-triggered cascade. Gene **274**: 47-65.

König, E., Delius, H., Carrington, M., Williams, R. O. and Roditi I. (1989). Duplication and transcription of procyclin genes in Trypanosoma brucei. Nucl. Acids Res. 17, 8727-8739.

Krafsur, E. S. (2009) Tsetse flies: genetics, evolution, and role as vectors. Infect Genet Evol, 9, 124-41.

Kuzoe, F.A.S. 1991. Perspectives in research and control of African trypanosomiasis. Ann. Trop. Med. Parasit., 85:33-41.

Leak, S. G. A., Mulatu, W., Authié, E., d'Ieteren, G. D. M., Peregrine, A. S., Rowlands, G. J., & Trail, J. C. M. (1993). Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia 1. Tsetse challenge and its relationship to trypanosome prevalence in cattle. *Acta Tropica*, *53*(2), 121-134.

Lozano, R (15 December 2012). "Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010." Lancet. ***380*** *(9859):2095–128. doi:10.1016/S0140-6736(12)61728-0. PMID 23245604.*

Luckins, A. G. (1992). Methods for diagnosis of trypanosomiasis in livestock. *World animal review*, *70*(71), 15-20.

Lundkvist, G. B., Kristensson, K., & Bentivoglio, M. (2004). REVIEWS-Why Trypanosomes Cause Sleeping Sickness-African trypanosomes induce alterations in hypothalamic sleep-regulatory and circadian-timing systems to cause a fragmented sleep and wakefulness. *Physiology*, *19*, 198-206.

Maudlin, I. (2006). African trypanosomiasis. *Annals of Tropical Medicine & Parasitology*, *100*(8), 679-701.

Miralles, D. M., Marın, C., Magán, R., Fernández-Ramos, C., Entrala, E., Cordova, O., & Sánchez-Moreno, M. (2002). In vitro culture and biochemical characterization of six trypanosome isolates from Peru and Brazil. *Experimental parasitology*, *102*(1), 23-29.

Montagna, G. N., J. E. Donelson & A. C. Frasch (2006) Procyclic Trypanosoma brucei expresses separate sialidase and trans-sialidase enzymes on its surface membrane. J Biol Chem, 281, 33949-58.

Morrison, L. J., Marcello, L., & McCulloch, R. (2009). Antigenic variation in the African trypanosome: molecular mechanisms and phenotypic complexity. *Cellular microbiology*, *11*(12), 1724-1734.

Mowatt, M. R., Wisdom, G. S. and Clayton, C. E. (1989). Variation of tandem repeats in the developmentally regulated procyclic acidic repetitive proteins of Trypanosoma brucei. Mol. Cell. Biochem. 9, 1332-1335.

Nagamune, K., A. Acosta-Serrano, H. Uemura, R. Brun, C. Kunz-Renggli, Y. Maeda, M. A. Ferguson & T. Kinoshita (2004) Surface sialic acids taken from the host allow trypanosome survival in tsetse fly vectors. J Exp Med, 199, 1445-50.

Nagamune, K., Nozaki, T., Maeda, Y., Ohishi, K., Fukuma, T., Hara, T., & Kinoshita, T. (2000). Critical roles of glycosylphosphatidylinositol for Trypanosoma brucei. *Proceedings of the National Academy of Sciences*, *97*(19), 10336-10341.

Nogge G (1976). Sterility in tsetse flies (Glossina morsitans West-wood) caused by loss of symbionts. Experientia 32: 995–996.

Nogge G (1981). Significance of symbionts for the maintenance of an optimal nutritional state for successful reproduction in haematophagous arthropods. Parasitology 82: 101–104.

Nok, A. J. & E. O. Balogun (2003). A bloodstream Trypanosoma congolense sialidase could be involved in anemia during experimental trypanosomiasis. J Biochem, 133, 725-30.

Oberle, M., Balmer, O., Brun, R., & Roditi, I. (2010). Bottlenecks and the maintenance of minor genotypes during the life cycle of Trypanosoma brucei. *PLoS pathogens*, *6*(7), e1001023.

Omotainse, S. O., Kalejaiye, J. O., Dede, P. M., & Dadah, A. J. (2004). The current status of tsetse and animal trypanosomiasis in Nigeria. *Vom Journal of Veterinary Sciences*, *1*(1), 1-7.

Onyiah, J. A. (1997). African animal trypanosomosis: An overview of the current status in Nigeria. *Trop. Vet*, *15*, 111-116.

Otigbuo I.N, Woo P.T.K (1987). T.b.brucei: A culture medium reverting a monomorphic to a pleomorphic stain. Trans. Royal Society Tropical Medical Hygiene 81: 408-410. Press; 1986.

Pearson, T. W. (2001). Procyclins, proteases and proteomics: dissecting trypanosomes in the tsetse fly. *TRENDS in Microbiology*, *9*(7), 299-301.

Programme Against African Trypanosomiasis. Tsetse and Trypanosomiasis Information. PAAT 2007. Vol. 30, Part 2.

Quignot N.; Hamon J.; Bois F. (2014). Extrapolating in vitro results to predict human toxicity, in In Vitro Toxicology Systems, Bal-Price A., Jennings P., Eds, *Methods in Pharmacology and Toxicology series. New York, USA: Springer Science.* pp. 531–550.

Ralston, K. S., Kabututu, Z. P., Melehani, J. H., Oberholzer, M., & Hill, K. L. (2009). The Trypanosoma brucei flagellum: moving parasites in new directions. *Annual review of microbiology*, *63*, 335-362.

Roditti, I., Furger, A., Ruepp, S., Schürch, N., & Bütikofer, P. (1998). Unravelling the procyclin coat of Trypanosoma brucei. *Molecular and biochemical parasitology*, *91*(1), 117-130.

Roditti, I., Schwarz, H., Pearson, T. W., Beecroft, R. P., Liu, M. K., Richardson, J. P., ... & Williams, R. O. (1989). Procyclin gene expression and loss of the variant surface glycoprotein during differentiation of Trypanosoma brucei. *The Journal of Cell Biology*, *108*(2), 737-746.

Roditti I, Lehane MJ (2008) Review. Interactions between trypanosomes and tsetse flies.*Curr Opin Microbiol; 11(4):345-51.*

Ross CA: Trypanosoma congolense: differentiation of metacyclic trypanosomes in culture depends on the concentration of glutamine or proline. Acta Trop 1987, 44:293–301.

Rothman, S. S. (2002). Lessons from the living cell: the culture of science and the limits of reductionism. *New York: McGraw-Hill*. ISBN 0-07-137820-0.

Rudenko, G., Cross, M., & Borst, P. (1998). Changing the end: antigenic variation orchestrated at the telomeres of African trypanosomes. *Trends in microbiology*, *6*(3), 113-117.

Ruepp, S., Furger, A., Kurath, U., Renggli, C. K., Hemphill, A., Brun, R., & Roditi, I. (1997). Survival of Trypanosoma brucei in the tsetse fly is enhanced by the expression of specific forms of procyclin. *The Journal of cell biology*, *137*(6), 1369-1379.

Sam-Wobo, O. A. Idowu, A. B. Idowu, C. F. Mafiana And S. O, (2009). African Journal of Biotechnology V8 (17), pp. 4179-4182.

Schöni, R., Jenni, L., & Brun, R. (1982). Cyclical transmission of in vitro cultivated bloodstream forms and procyclic trypomastigotes ofTrypanosoma brucei brucei byGlossina morsitans morsitans. *Zeitschrift für Parasitenkunde*, *68*(1), 1-5.

Schwede, A., Macleod, O. J., MacGregor, P., & Carrington, M. (2015). How does the VSG coat of bloodstream form African trypanosomes interact with external proteins? *PLoS pathogens*, *11*(12), e1005259.

Sharma, R., Peacock, L., Gluenz, E., Gull, K., Gibson, W., & Carrington, M. (2008). Asymmetric cell division as a route to reduction in cell length and change in cell morphology in trypanosomes. *Protist*, *159*(1), 137-151.

Shaw, A. P. M., Torr, S. J., Waiswa, C., Cecchi, G., Wint, G. R. W., Mattioli, R. C., & Robinson, T. P. (2013). Estimating the costs of tsetse control options: an example for Uganda. *Preventive veterinary medicine*, *110*(3), 290-303.

Simarro, P. P., Diarra, A., Postigo, J. A. R., Franco, J. R., & Jannin, J. G. (2011). The human African trypanosomiasis control and surveillance programme of the World Health Organization 2000–2009: the way forward. *PLoS Negl Trop Dis*, *5*(2), e1007.

Stephen, L. E. (1986). Trypanosomiasis: a veterinary perspective. *Wheaton, London*, 1-551.

Steverding, D. (2008). The history of African trypanosomiasis. *Parasites & vectors*, *1*(1), 1.

Swallow, B. (1998). PAAT position paper: impact of trypanosomosis on African agriculture. *Food and Agriculture Organization-World Health Organization-International Atomic Energy Agency-Organisation of African Unity/Interafrican Bureau for Animal Resources (FAO-WHO-IAEA-OAU/IBAR), Rome*.

Swallow, B. M. (2000). Impacts of trypanosomiasis on African agriculture. In *PAAT Technical and Scienti® c Series, FAO*.

Takken, W., & Weiss, M. (1978). The sterile insect technique for control of tsetse flies in Africa. *IAEA BULLETIN*, *20*(3), 21.

Treumann, A., Zitzmann, N., Hülsmeier, A., Prescott, A. R., Almond, A., Sheehan, J., & Ferguson, M. A. (1997). Structural characterisation of two forms of procyclic acidic repetitive protein expressed by procyclic forms of Trypanosoma brucei1. *Journal of molecular biology*, *269*(4), 529-547.

Turner MJ. The biochemistry of the surface antigens of the African trypanosomes.Br Med Bull 1985;41:137–43.

Udenfriend S., Kodukula K. (1995) Annu. Rev. Biochem. 64:563–591.

Urwyler S, Vassella E, Van Den Abbeele J, Renggli CK, Blundell P, et al. (2005) Expression of procyclin mRNAs during cyclical transmission of Trypanosoma brucei. PLoS Pathog 1(3): e22.

Utz S, Roditi I, Renggli CK, Almeida IC, Acosta-Serrano A, Butikofer P: Trypanosoma congolense procyclins: Unmasking cryptic major surface glycoproteins in procyclic forms. Euk Cell 2006, 5(8):1430–1440.

Vassella E, Den Abbeele JV, Bu¨ tikofer P, Renggli CK, Furger A, et al. (2000). A major surface glycoprotein of Trypanosoma brucei is expressed transiently during development and can be regulated post-transcriptionally by glycerol or hypoxia. Genes Dev 14: 615–626.

Vassella E, Probst M, Schneider A, Studer E, Renggli CK, et al. (2004) Expression of a major surface protein of Trypanosoma brucei insect forms is controlled by the activity of mitochondrial enzymes. Mol Biol Cell 15:3986–3993.

Vickerman, K. (1985). Developmental cycles and biology of pathogenic trypanosomes. *British medical bulletin*, *41*(2), 105-114.

Vignais, Paulette M.; Pierre Vignais (2010). Discovering Life, Manufacturing Life: How the experimental method shaped life sciences. *Berlin: Springer*. ISBN 90-481-3766-7.

Wei G., Bull H., Zhou X., Tabel H., (2011). Intradermal infections of mice by low numbers of African Trypanosomes are controlled by innate resistance but enhance susceptibility to reinfection. J/infect Dis 203: 418-429. Doi:10.1093/infdis/jiq051.

Weiss, B. L., Attardo, G. M., Pais, R., Wang, J., & Aksoy, S. (2007). Novel strategies targeting pathogen transmission reduction in insect vectors: Tsetse-transmitted trypanosomiasis control. *Entomological Research*, *37*(4), 231-237.

Werren JH, Windsor D, Guo LR (1995) Distribution of Wolbachia among neotropical arthropods. Proceedings of the Royal society of London Series B Biological sciences **262**: 197-204.

Woo, P. T., & Hauck, L. (1987). The haematocrit centrifuge smear technique for the detection of mammalian Plasmodium. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, *81*(5), 727-728.

Ziegelbauer K, Quinten M, Schwarz H, Pearson TW, Overath P (1990) Synchronous differentiation of Trypanosoma brucei from bloodstream to procyclic forms in vitro. Eur J Biochem 192: 373–378.