# PRODUCTION OF INFECTIVE *Trypanosoma brucei brucei* EXPRESSING GREEN FLUORESCENT PROTEIN FOR LOCALISATION OF TRYPANOSOMES IN BRAIN OF INFECTED RODENTS

**Kayamba Mbanza Yves**

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## DECLARATION AND RECOMMENDATION

### DECLARATION

I, hereby declare that this research thesis is my original work and has not been submitted wholly or in part for any award in any other institution of learning.

Kayamba Mbanza Yves

Signature:…… Date:……..

### RECOMMENDATION

We wish to confirm that this research thesis was carried out under our supervision and has been presented with our approval for examination as per the Egerton University regulations.

Prof. Raphael Ngure, Egerton University

Signature:…… Date:……

Prof. Bruno Kubata, African Research for Health Initiative

Signature:…….. Date:…….

Prof. Michael Duszenko, University of Tuebingen

Signature:……. Date:…….

## DEDICATION

I dedicate this thesis to my parents, Arsene Kayamba Kavunga and Helene Fitidi Nsaka, since without their guidance and unfaltering support; I would not be the person that I am today.

To my mother Henriette Lubanzadio Wambi thanks for the support and encouragement throughout my academic education.

To my wife Marie de Lourde Okolo for her immeasurable love and encouragement to carry out this study.

To all my family members and friends, you have been there to help me to choose the right way to be the person that I am today.

## COPYRIGHT

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| BBB | Blood brain barrier |
| BCB | Blood cerebrospinal fluid barrier |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| eGFP | Enhanced green fluorescent protein |
| HAT | Human African trypanosomosis |
| IFIB | Interfaculty Institute of Biochemistry |
| PCR | Polymerase chain reaction |
| VSG | Variant surface glycoprotein |

**ABSTRACT**

Human African trypanosomosis (HAT) is caused by subspecies of *T. brucei* found in East, Central and West Africa, with *T. b. rhodesiense* and *T. b .gambiense* causing sleeping sickness in human through the bite of a tsetse fly of the genera *Glossina*. The treatment of CNS infection is difficult and at the same time the biology of late stage trypanosome infection is poorly understood. In addition, the parasites hideout in the brain and their mode of survival and multiplication in the brain remains elusive. The current study aimed at production of infective *T. b. brucei* expressing green fluorescent protein to track the trypanosomes in infected rodents to provide a better understanding of how the parasite establishes infection of the CNS and thus causes pathogenesis within the brain. Results from the study indicate that it was possible to extract the *eGFP* gene, cloning it to *pJET1.2* subcloning vector and thereafter into *pLEW100* an expression vector before transfecting the gene into the trypanosome genome. This resulted in the production of infective trypanosome parasite expressing green fluorescent protein that can be used to track the trypanosomes in brains of trypanosome infected rodents during various stages of infection in order to provide a better understanding of how the parasite establishes infection in the CNS and thus causes pathogenesis within the brain. The tool can thus be used to connect CNS infection and scientific evidence that can be used to devise new strategies for the management of late stage African sleeping sickness.

# CHAPTER ONE
# INTRODUCTION

## 1.1 Background Information

Human African trypanosomosis (HAT) is a vector-borne parasitic disease caused by protozoan parasite belonging to the genus *Trypanosoma* transmitted through tsetse flies bites. HAT occurs in 36 sub-Saharan countries which correlate to distributional areas of the tsetse fly. WHO estimates that over 60 million people living in some 250 foci are at risk of contracting the disease (WHO, 2000) while over 400,000 new cases of the disease are being reported mainly from villages in Angola, the Democratic Republic of Congo (DRC) and Southern Sudan with prevalence of up to 50% (Kioy *et al.,* 2004). The disease had been successfully controlled by a combination of approaches, including treatment of patients and active vector control (WHO, 1998). Since the 1970s, the disease has re-emerged as a new epidemic of immense proportions which has received little attention from the international community (August *et al.,* 2002). HAT exists in two forms with different clinical presentations and epidemiology caused by morphologically indistinguishable subspecies of *T. brucei.*

Trypanosomes develop in the blood and lymphatic system. At this stage of infection, symptoms are unspecific and similar to those of flu thus comprising of persistent headache, immunosuppression, joint pains and intermittent fever attacks. The early stage of trypanosomiasis is treated with drugs such as suramin and pentamidine, which are usually well tolerated and produce only limited side effects. However, the current emergence of resistant strains against these drugs is further complicating the treatment of the disease even at this stage (Delespaux & de Koning, 2007; Kagira & Maina, 2007). After weeks, months, or sometimes years, trypanosomes establish themselves within the brain, where they become inaccessible to drugs such as suramin and pentamidine that cannot cross the blood brain barrier (BBB). During brain infection, the primary symptoms range from diurnal sleep attacks, nocturnal insomnia and lymphadenopathy to headache, general weakness, aggressiveness, miscarriage in pregnant women and behavioral changes. The final phase is characterized by progressive mental deterioration and general body wasting. Death results usually from concurrent infections such as pneumonia or from malnutrition.

At this late stage of sleeping sickness, patients are often treated with the - 4 - trivalent arsenical melarsoprol that can cross the BBB to eliminate the trypanosomes present within the central nervous system (CNS). However, melarsoprol therapy is associated with serious adverse effects such as reactive arsenical encephalopathy which is fatal in up to 10% of all patients given the drug and chemical cellulitis that is caused by leakage of the injected propylene glycol solvent from the vein into the surrounding tissues (Pepin *et al.*, 1994; Kennedy, 2004). Alternatively, patients can also be treated with difluoromethylornithine (Eflornithine®), an inhibitor of ornithine decarboxylase and a drug that is effective against all stages of the *T. b. gambiense* infection. However, Eflornithine is not commonly available and is considered to be too expensive for routine use, requires hospitalization of the patient and works against *T. b. gambiense* but not *T. b. rhodesiense* that causes the acute form of sleeping sickness (Burri & Brun, 2003). Since treatment of trypanosomiasis depends on the infection stage, it is essential to accurately establish a diagnosis process that would enable clinicians to determine the stage of the disease and decide on the type of the drugs to be used.

The trypanosomes are transmitted by different species of tsetse flies, which have differing predilections as breeding sites. Western and Central African sleeping sickness, caused by *T. b. gambiense*, is now a re-emerging, threat to some 60 million people in West and Central Africa as well as some parts of East Africa (WHO, 1998).

Eastern African sleeping sickness caused by *T. b. rhodesiense*, is a zoonosis with an extensive animal reservoir in ungulates, including game animals. Sporadic human cases may occur in areas where people intrude into tsetse infested habitats as a result of civil unrest (Smith *et al.*, 1998). A cluster of HAT among European tourists visiting national parks in Northern Tanzania brought this zoonosis to the attention of the medical community in the West, highlighting its importance not only as a plague for people in endemic areas but also as a threat to travelers in rural Africa (Ripamonti *et al.*, 2002). *T. brucei* spread by the tsetse fly in sub-Saharan Africa causes humans sleeping sickness.

Treatment strategies aim at developing drugs that can impede penetration of trypanosomes into the brain and/or eliminate trypanosomes once they are inside the brain parenchyma, but the drugs should have lower toxicity than the ones presently in use (Masocha *et al*., 2007).

## 1.2 Statement of the Problem

Human African trypanosomosis is of medical and veterinary importance since it causes huge mortality and morbidity in human and animal populations, yet limited success in the treatment regimen and the current view that trypanosomes re-enter the blood from their hideout in the brain demand that brain stage trypanosomes be studied in order to put an end to relapse cases. After a variable time of persistent blood parasitaemia, trypanosomes enter the brain resulting in CNS infection. Imaginative speculations have proposed that the parasites cross the BBB or the blood cerebrospinal barrier (BCB) by an unknown mechanism. In addition, the parasites hideout in the brain as well as their mode of survival and multiplication in the CNS remains elusive. The current study aimed at production of infective *T. b. brucei* expressing green fluorescent protein to be used to track the trypanosomes in infected rodents in order to provide a better understanding of how the parasite establishes infection in the CNS and thus causes pathogenesis within the brain. The information will be used in drug development and thereby forestall relapse infection.

## 1.3 Objective

### 1.3.1 Main objective

To develop infective *T. b. brucei* expressing green fluorescent protein for localisation of trypanosomes in brain of infected rodents.

### 1.3.2 Specific objectives

1. To isolate the green fluorescent protein gene from *E. coli.*
2. To electroporate the green fluorescent protein gene into infective trypanosome genome.
3. To locate the green fluorescent protein expressing trypanosomes in brain of infected rodents.

### 1.4 Hypotheses

1. Green fluorescent protein gene can not be isolated from *E. coli.*
2. Green fluorescent protein gene can not be transferred to infective trypanosome.
3. It is not possible to locate green fluorescent protein expressing trypanosomes in brain of infected rodents.

### 1.5 Justification

Limited success of the current treatments for HAT has led to investigations on the biology of trypanosomes in the CNS in order to understand the mechanism of survival of the parasite in the brain and CSF with the aim of devising new strategies for the development of new anti-trypanosomal drugs for late stage African sleeping sickness. Since treatment of trypanosomosis depends on the infection stage, it is essential to accurately establish a diagnosis process that would enable clinicians to determine the stage of the disease and decide on the type of the drugs to be used. Although it appears that encephalitis as the morphological expression of the late stage is a consequence of parasitic infiltration of brain tissue, the question of how the trypanosomes get into the brain, which is important for therapy, has not been answered. Haematogenous infiltration of the majority of organs by parasites in the early phase of the disease does not occur in the brain so that delayed invasion of brain tissue by haematogenic pathways does not seem probable. These considerations clearly justify the need for systematic experiments to analyze the details of brain infection in trypanosomosis and thus come up with better understanding of trypanosome biology in CNS infection. This scientific evidence can be used to develop treatment regimes for late stage human trypanosomosis.

### 1.6 Expected Outputs

1. Develop a fluorescent trypanosome tool for use in understanding CNS trypanosome biology.
2. Thesis for a Master of Science in Biochemistry.
3. Publications in peer review journals.

# CHAPTER TWO
# LITERATURE REVIEW

## 2.1 Human African Trypanosomosis

Human African trypanosomosis constitutes a serious public health threat in Africa, particularly in East and Central Africa, where approximately 60 million people are at risk of contracting the disease (WHO, 1998). Although there are thousands of new cases every year in countries such as the DRC, Angola and Uganda, no noteworthy progress has been recorded in the development of new drugs to fight the condition (WHO, 2000). Sleeping sickness occurs in remote rural areas where health systems are weak or non-existent. The disease spreads in poor settings with displacement of populations due to war and poverty being important factors leading to increased trypanosome transmission (Smith *et al*., 1998). These parasites are divided into several species of which the human infective *T. brucei* is the most important. This species contains three members namely *T. b. gambiense, T. b. rhodesiense* and *T. b. brucei* with the former two subspecies causing human sleeping sickness, whereas the latter is not human infective.

HAT takes two forms, depending on the parasite involved. *T. b. gambiense* is found in West and Central Africa and represents more than 90 percent of reported cases of sleeping sickness. It causes a chronic infection which can be infective for months or even years without major signs or symptoms appearing but later on the patient progresses to an advanced disease stage when the central nervous system is affected (Mehlitz *et al*., 1982). *T. b. rhodesiense* is found in Eastern and Southern Africa and represents less than 10 percent of reported cases and causes an acute infection with first sign being observed after a few months or weeks and later the disease develops rapidly and invades the CNS.

## 2.2 Pathogenesis and Clinical Signs

Trypanosomosis is transmitted when an infected tsetse fly bites a host thereby leading to multiplication of the parasite in subcutaneous tissues, blood and lymph (August *et al.*, 2002). This amplification of the parasite within the host system is also characterized by parasites crossing the blood brain barrier to infect the central nervous system (Enanga *et al.*, 2002) and in some instances; mother-to-child infection may occur where the trypanosome crosses the placenta and infect the fetus, causing perinatal death (Dumas & Bisser, 1999).

Other methods of transmission include infection through blood sucking insects. However, it is difficult to assess the epidemiological impact of transmission through other blood sucking insects (WHO, 1998). The first stage of the disease, known as a haemolymphatic phase, entails bouts of fever, headaches, joint pains and itching. The second stage, known as the neurological phase, begins when the parasite crosses the BBB and invades the CNS. This is when the brain involvement signs and symptoms of the disease appear and include confusion, sensory disturbances and poor coordination (Kennedy, 2004). Disturbance of the sleep cycle, which gives the disease its name, is an important feature of the second stage of the disease (Lean *et al.,* 2004).

Investigating the mechanisms by which parasites penetrate the cerebral blood vessels and enter the brain parenchyma is therefore of fundamental importance for understanding the neuropathogenesis of African trypanosomosis and for developing new treatment strategies. In experimental animals infected with the rodent pathogenic strain namely *T. b. brucei*, parasites appear early during infection in the choroid plexus and other circumventricular organs that lack a BBB (Schultzberg *et al.,* 1988). At later stages, the parasites penetrate the BBB and enter the brain parenchyma, as revealed by double immunohistochemical labeling of parasites and brain endothelial cells in a rat model of the chronic disease (Mulenga *et al.,* 2001).

### 2.3 Treatment of HAT

The type of treatment depends on the stage of the disease. Drugs used in the first stage of the disease are less toxic, easier to administer and more effective (Kennedy, 2004). The earlier the identification and treatment of the disease condition, the better the prospect of a cure following treatment. Treatment success in the second stage depends on drugs that can cross the BBB to reach the parasite. Most of the drugs are quite toxic and require technical knowhow to administer (Dumas *et al*., 2000).

Four drugs are registered for the treatment of sleeping sickness and are provided free of charge to endemic countries through a WHO private partnership with Sanofi-Aventis and include pentamidine, melarsoprol, eflornithine and suramin.

#### 2.3.1 First stage treatments

Pentamidine is one of the first drugs discovered in 1941 that is commonly used for the treatment of the first stage of *T. b. gambiense* sleeping sickness (Doua *et al.,* 1996). Despite a few undesirable effects, it is well tolerated by patients.

The second is suramin which is effective for treatment of the first stage *T. b. rhodesiense* infection (Voogd *et al.,* 1993). Other current drugs introduced against human and veterinary African trypanosomosis therapy and the introduction of nifurtimox and DB289 (Delespaux & de Koning, 2007).

### 2.3.2 Second stage treatments

During the late stages of African trypanosomosis, parasites lodge in privileged sites within the central nervous system, causing encephalitis. The biological nature of such parasites and the mechanisms by which the parasite causes CNS pathogenesis are unknown (Pepin *et al.,* 1994). Melarsoprol an arsenic derivative, discovered in 1949 is used in both early and late stage forms of HAT infection. Melarsoprol treatment is accompanied by a post treatment reactive encephalopathy that results in 10% fatality. However an increase of resistance to the drug has been observed in several foci particularly in Central Africa (WHO, 2000). Eflornithine is another drug that is less toxic than melarsoprol and is effective against *T. b. gambiense* though the regime is strict and difficult to apply (Pepin *et al.,* 1994).

Despite the many decades of use of most of the trypanocides, very little of their mode of action is known. This may in part be because most of the drugs act on multiple targets once inside the cell and they derive their selective action on the parasite from selective accumulation by the pathogen. Loss of this capacity for drug uptake by the trypanosome would thus be a major cause for drug resistance. There is evidence that eflornithine acts additively with non-permeating drugs in late-stage infections and suppresses the encephalitis.

The nature of these drug interactions is not known but understanding them is critical to the development of new approaches to chemotherapy. In the past decade, drug discovery has proceeded along biochemical target-based approaches with little success (Kennedy *et al.,* 2002).

## 2.4 Mechanisms of Immune Evasion by Trypanosomes

The immune responses and pathogenesis that occur in a mammalian host infected with African trypanosomes are complex and poorly understood. From an immune perspective, a trypanosome is a package of thousands of invariant antigens surrounded by 10 million copies of single variant surface antigens (VSGs) (Vickerman, 1985).

The surface coat of the bloodstream form of African trypanosomes is arranged as a dense monolayer of homodimers on the parasite surface. The ability of the surface coat of trypanosomes to undergo continuous antigenic variation results in the parasite escaping attack of the host's immune response (Cross, 1990). The surface antigens change every 8-10 days after the trypanosome enters the vertebrate host. Specific antibodies produced by the host are no longer effective owing to the appearance of new VSGs. The exchange of VSG is based on the sequential expression of several hundred different VSG genes (Van der Ploeg *et al.,* 1982). The actively transcribed gene is always located in a VSG expression site, which is in a polycistronic transcription unit on the telomere of a chromosome.

**2.5 Mechanisms Used by Trypanosomes to Cross the Blood Brain Barrier**

The life cycle of African trypanosomes has been extensively studied and consists of three distinct stages within both, the mammalian host and the insect vector (Vickerman, 1965). Trypanosomes are taken up in the tsetse fly blood meal. Within the digestive tract of the fly procyclic parasites are generated. They populate the midgut of the insect and give rise to formation of the epimastigote form found primarily in the salivary gland. Eventually, the human infective metacyclic forms appear and are transferred during the next blood meal. Within human blood, the cell cycle arrested metacyclic form spontaneously converts to the fast dividing slender form which finally, via a poorly defined intermediate stage, differentiates to the cell cycle arrested stumpy form.

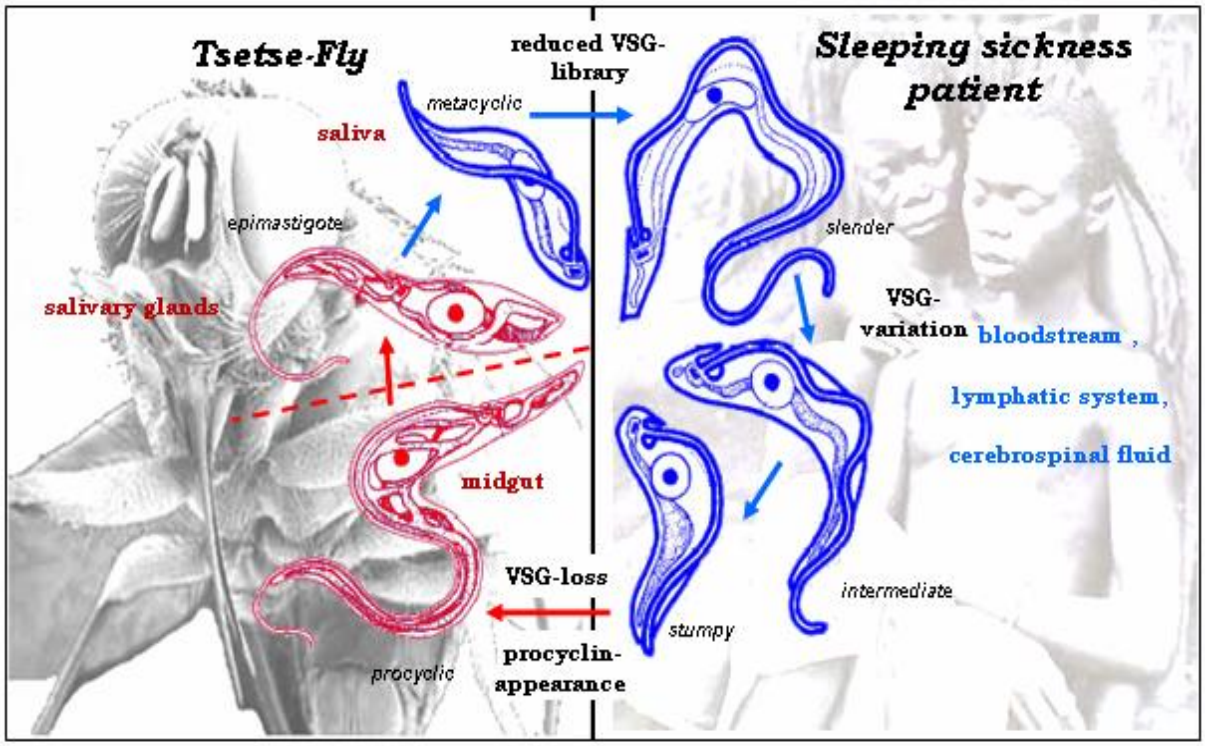


**Figure 1 :** Life cycle of *Trypanosoma brucei* (Vickerman, 1969).

### 2.6 Diagnosis of HAT

HAT is a lethal parasitic infection with neurological involvement. Examination of the CSF plays an essential role in diagnosis, selection of treatment and post-treatment follow-up (Lejon & Büscher, 2005). Alternatively, the increase in the number of lymphocytes in CSF is used as an indicator of brain infection (Lejon & Büscher, 2005). However, this diagnostic process is limited by the fact that an increased amount of lymphocytes in CSF may also be indicative of brain infection by non-trypanosomal organisms. To overcome the problem more sophisticated PCR techniques to look for trypanosome DNA in CSF have been proposed (Kennedy, 2004). Unfortunately, here too, the sophistication of the PCR does not allow the technique to be reliably applied in remote endemic areas and under field conditions. Understanding of the CNS infection is that after a variable time of persistent blood parasitaemia, trypanosomes enter the brain. Imaginative speculations have proposed that the parasites cross the BBB or the BCB by an unknown mechanism (Schmidt, 1983; Masocha *et al.*, 2004).

In addition, the parasites hideout in the brain as well as their mode of survival and multiplication remains elusive. Furthermore, there is no biochemically or genetically based conclusive evidence of existence of slender, stumpy or distinct blood forms predominately in the brain. In addition, the parasites density regulation mechanism in the brain remains speculative. In blood, antibody formation against the variant surface glycoprotein and the mechanism of antigenic variation leads to an oscillating population which is further controlled by apoptosis of the stumpy form (Duszenko *et al.*, 2006).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Extraction of Enhanced Green Fluorescent Gene

The research work was carried out at the Interfaculty Institute of Biochemistry (IFIB), Germany where an expression plasmid *pRS416eGFPMCA1* which is a plasmid (*pRS416*) with a gene for metacaspase marked *(MCA1)* with enhanced green fluorescent protein *(eGFP*) gene was extracted from a culture of *E. coli* using midi plasmid purification method (Nucleobond® Kit) as described by the manufacturer.

### 3.1.1 PCR products analysis

The full length of *eGFP* gene was amplified from a plasmid DNA by Hot Star Taq® PCR with sense primer (5'-AAGCTTATGGTGAGCAAGGGCGAG-3') and antisense primer (5'-GGATCCTTACTTGTACAGCTCGTCCATGC-3') in the same PCR mixture to increase the amount of *eGFP*. A volume of 0.5µl from 1ng template DNA 732µg/µl as a concentration (diluted 1: 1000 of extracted DNA) was taken and mixed with 24.5µl of a PCR-mix containing 12.5µl Taq polymerase master mix, 1µl antisense primer, 1µl sense primer, 5µl of 10X AccuPrime PCR buffer I and 5µl of distilled water. All amplifications were carried out in a thermal cycler. Cycling conditions were done for 15 minutes at 94°C to activate the enzyme, denaturation for 1 minute at 94°C, followed by 25 amplification cycles. Primer template annealing was done at 57°C for 1 minute while polymerization was done at 72°C for 2 minutes. A final elongation step was carried out for 10 minute at 72°C (Masiga *et al.,* 1992). Agarose gel electrophoresis was performed to visualize the PCR products.

In solution, the phosphates on the DNA are negatively charged and the molecule therefore migrated to the positive pole. A volume of 25µl of the PCR product, 4µl of Bromophenol and 5µl of 1kb DNA ladder molecular marker (Fermentas, Germany) were subjected to electrophoresis in a 1% agarose gel at 80V, 200mA for 45 minutes (Masiga *et al.,* 1992). The gels were stained with 1.6µl of ethidium bromide and visualized under ultraviolet light.

### 3.1.2 Gel extraction of *eGFP* Gene and Expression Vector *pLEW100*

Gel extraction or gel isolation was performed to isolate *eGFP* from an agarose gel following agarose gel electrophoresis for two purposes. After extraction, fragment of interest was enzymatically ligated in simple steps into *pJET1.2* subcloning vector (Uzcátegui *et al.*, 2004) using 0.3g of agarose gel to make a volume of 900µl of solubilization and binding buffer. The *eGFP* gene was extracted and then ligated with the expression vector *pLEW100* using 0.2414g of agarose gel to make a volume 724µl of solubilization and binding buffer. The same procedure was carried out to extract *pLEW100* following agarose gel electrophoresis before being enzymatically ligated with *eGFP* gene using 0.7g of agarose gel and 2100µl of solubilization and binding buffer.

The DNA fragment was incubated at 50°C for 10 minutes or until the gel slice was completely dissolved and the tube vortexed every 2–3 minutes during the incubation. After the gel slice had dissolved completely, the color of the mixture was checked to see whether it was yellow being similar to solubilization and binding buffer without dissolved agarose. The QIAquick spin column was placed in a provided 2ml collection tube. The DNA was bound and applied to the QIAquick column and centrifuged for 1 minute. The flow-through was discarded and QIAquick column was placed back in the same collection tube. A volume of 0.5ml of solubilization and binding buffer was added to QIAquick column and centrifuged for 1 minute. This step was done to remove all traces of agarose. Thereafter, a volume of 0.75ml of wash buffer was added to wash the sample into the QIAquick column and centrifuged for 1 minute. The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 minute at 13,000rpm. The QIAquick column was then placed into a clean 1.5ml micro-centrifuge tube. To the eluted DNA, a volume of 50µl of elution buffer (10mM Tris·Cl, pH 8.5) was added to the center of the QIAquick membrane, stood for 1 minute and the column centrifuged for 1 minute (QIAquick gel extraction kit from QIAgen).

### 3.2 Ligation of *eGFP* into *pJET1.2* Subcloning Vector

A volume of 10µl of 2X reaction buffer, 2µl of non-purified PCR products, 7µl of nuclease-free water and 1µl of DNA blunting enzyme were taken to make a total volume of 20µl, vortexed briefly and centrifuged for 3 seconds. The mixture was incubated at 70°C for 5 minutes in a thermal cycle and thereafter chilled briefly on ice.

To set up the ligation reaction, 1µl of *pJET1.2/*blunt subcloning vector (50ng/µl) and 1µl T4 DNA ligase was then added, vortexed briefly and centrifuged for 3 seconds. The ligation mixture was incubated at room temperature (22°C) for 5 minutes and thereafter the time extended up to 60 minutes. The ligation mixture was then directly used for bacterial transformation.

### 3.3 Transformation Using One Shot® Top10 Competent Cells Protocol

The vial(s) of the ligation reaction(s) was centrifuged briefly and placed on ice. One 50µl vial of One Shot® cells was thawed on ice for each ligation/transformation. A volume of 5µl of each ligation reaction were pipetted directly into the vial of competent cells and mixed by tapping gently on the side. The vial was placed on ice for 30 minutes and then incubated for exactly 30 seconds in the 42°C water bath. The vial was removed from the 42°C bath and placed on ice. A volume of 250µl of Soc medium was added to the ligation reaction mixture and competent *E. coli* cells prepared, the vial was shaken at 37°C for exactly 1 hour at 225rpm in a shaking incubator. A volume of 100 and 200µl was spread from each transformation vial on separate, labeled Lysogeny broth agar plates. The remaining transformation was stored at 4°C. The plate(s) were inverted and incubated at 37°C overnight. Colonies were then selected and analyzed by plasmid isolation, PCR or sequencing.

### 3.4 Plasmid DNA Isolation

After a good number of bacterial colonies had grown, they were *miniprepped* to harvest the plasmid DNA. Two tubes of 1.5ml of overnight bacteria culture were filled and centrifuged at 13,000rpm for 3 minutes to harvest the bacterial cells. Pelleted bacterial cells were resuspended in a volume of 250µl re-suspension buffer and transferred to a microcentrifuge tube. A volume of 250µl lysis buffer (200mM NaOH, 1% SDS) was added and mixed thoroughly by inverting the tube 4–6 times.

A volume of 350µl neutralization buffer was added and mixed immediately and thoroughly by inverting the tube 4–6 times. The content was thereafter centrifuged for 10 minutes at 13,000rpm in a table-top microcentrifuge.

The supernatants were applied to the QIAprep spin column by decanting or pipetting then followed by centrifugation for 30–60 seconds and the flow-through discarded.

The QIAprep spin column was washed by adding a volume of 0.5ml binding buffer and centrifuging for 30–60 seconds. The flow-through was discarded and the QIAprep spin column washed by adding a volume of 0.75ml wash buffer followed by centrifugation for 30–60 seconds. The flow-through was discarded and centrifuged for an additional 1 minute to remove residual wash buffer. The QIAprep column was then placed in a clean 1.5ml microcentrifuge tube and to elute the DNA, a volume of 50µl elution buffer (10mM Tris·Cl, pH 8.5) was added to the center of each QIAprep spin column, stood for 1 minute and then centrifuged for 1 minute.

### 3.5 Digestion of the Plasmid *pJET1.2eGFP*

Restriction enzymes BamHI and HindIII were used to excise the gene of interest (*eGFP*) from the plasmid. The insert was purified in order to isolate it from background junk. A volume of 19.7µl of 3.660µg of plasmid DNA, 0.3µl of BamHI (10units/µl), 0.6µl of HindIII (10units/µl), 3µl of buffer BamHI and 6.4µl of water were put together to make a total volume of 30µl in a PCR tube for digestion at 37°C during 4 hours. The expected plasmid DNA was sent to the University clinic of Tuebingen for sequencing using chain termination method developed by Sanger (1977).

### 3.6 Digestion and Gel Extraction of the Expression Vector *pLEW100* Using QIAquick Gel

Restriction enzymes were used to digest the expression vector at the right place in order to allow the gene of interest to be ligated at the restriction sites. The expression vector used was already open but it was digested to make sure that it was pure to be used then finally extracted from the gel.

A volume of 10µl of 1µg of *pLEW100* was digested with 1µl of BamHI (10units/µl), 1µl of HindIII (10units/µl), 2µl of buffer BamHI and 6µl of water were mixed together to make a total volume of 20µl in a PCR tube for digestion at 37°C for 2 hours.

### 3.7 Ligation of *eGFP* Gene into *pLEW100*

An overnight ligation was performed at 22°C in a thermo cycler with 1µl of *eGFP* with 65ng/µl concentration, a volume of 3.6µl of *pLEW100* (360ng/µl) using a molar ratio insert-vector of 3:1.

This was then mixed with 1µl of T4 DNA ligase (5units/µl), 2µl of 10x T4 DNA ligase buffer and 12.4µl of nuclease free water to make a total volume reaction of 20µl (Uzcátegui *et al.*, 2004).

### 3.8 Transformation of *pLEW100eGFP* into One Shot® TOP10 Competent Cells Protocol (Invitrogen™)

The plasmid is often transformed into a bacterium like *E. coli*. Ideally when the bacterium divides the plasmid should also be replicated. In the best case scenario, each bacteria cell should have several copies of the plasmid. The vial(s) of the ligation reaction(s) was centrifuged briefly and placed on ice. One 50µl vial of One Shot® cells was thawed on ice for each ligation/transformation. A volume of 5µl of each ligation reaction was pipetted directly into the vial of competent cells and mixed by gently tapping the vial.

The mixture was incubated into ice for 30 minutes and thereafter incubated for exactly 30 seconds in the 42°C water bath. The vial was then removed from the 42°C bath and placed on ice. A volume of 250µl of Soc medium was added to the ligation reaction mixture and competent *E. coli* cells prepared, the vial was shaken at 37°C for exactly 1 hour at 225rpm in a shaking incubator. A volume of 350µl was spread from the transformation vial on separate labeled LB agar plates. The plate(s) were inverted and incubated at 37°C overnight. Colonies were selected and analyzed for plasmid isolation, PCR or sequencing (Uzcátegui *et al.*, 2004).

### 3.9 Plasmid Isolation Using the Nucleobond® Kit

After a good number of bacterial colonies had grown, they were harvested using the Nucleobond® Kit to get a huge amount of plasmid for a later electroporation.

An overnight bacterial culture was set up by inoculating the appropriate volume of LB medium (plus Ampicillin) with a single colony picked from a freshly streaked plate. The culture was shaken overnight for 12–16 hours. The saturated culture was centrifuged at 13,000rpm for 15 minutes at 4°C and the supernatant was carefully discarded.

The pellet of bacterial cells was then resuspended in a volume of 4ml of resuspension buffer plus RNase (50mM Tris-HCl, 10mM EDTA, 100µg/ml RNase A).

A volume of 4ml of lysis buffer (200mM NaOH, 1% SDS) was added to the suspension and then mixed gently by inverting the tube 6–8 times. The mixture was incubated at room temperature for 5 minutes. A volume of 4ml of neutralization buffer (2.8M KAc, pH 5.1) was added to the suspension and then the suspension mixed gently by inverting the tube 6–8 times during which a heavy, flocculent precipitate was formed. The suspension was placed on ice for 5 minutes. The suspension was centrifuged at 13,800rpm for 10 minutes at 4°C. The suspension was filtered by placing the Nucleobond folded filter in a small funnel and the filter pre-wetted with a few drops of equilibration buffer (100mM Tris, 15% ethanol, 900mM KCl, 0.15% Triton X-100, adjusted to pH 6.3 with $H_3PO_4$). The lysate was loaded onto the wet filter and the flow through collected.

A Nucleobond AX 100 (Midi) column was equilibrated with a volume of 2.5ml of equilibration buffer to allow the column to empty by gravity flow. The flow through was discarded and the cleared lysate loaded onto the Nucleobond column to allow the column to empty by gravity flow. The column was washed with a volume of 10ml of wash buffer (100mM Tris, 15% ethanol, 1.15M KCl, adjusted to pH 6.3 with $H_3PO_4$) and the flow through discarded. The plasmid DNA (*pLEW100eGFP)* was eluted and collected with a volume of 5ml of elution buffer (100mM Tris, 15% ethanol, 1M KCl, adjusted to pH 8.5 with $H_3PO_4$). A volume of 3.5ml of isopropanol, pre-equilibrated to room temperature was added to precipitate the eluted plasmid DNA.

The suspension was centrifuged at 13,800rpm for 30 minutes at 4°C. Carefully the supernatant was discarded. Ice-cold 70% ethanol was added to the pellet and the suspension vortexed briefly and thereafter centrifuged at 13,000rpm for 5 minutes at room temperature.
Carefully ethanol was removed from the tube with a pipette tip to allow the pellet to dry at room temperature for 10 minutes. The pellet was redissolved with a volume of 100µl of resuspension and storage buffer (10mM Tris·Cl, pH 8.0; 1mM EDTA).
The expected plasmid DNA was sent to the University clinic of Tuebingen for sequencing using chain termination method developed by Sanger (1977).

### 3.10 Digestion of the *pLEW100eGFP* Plasmid Vector with NotI

The restriction enzymes NotI were used to linearize the plasmid thus allowing it to get into the genome of the trypanosome after being electroporated.

The total mass of 167.384µg of *pLEW100eGFP* was taken and digested with 2µl of NotI (10units/µl) (2x over digestion 20units), 10µl of 10x orange buffer, a volume of 98µl of *pLEW100eGFP* and 40µl of water to make a total volume of 150µl in a PCR tube for an overnight digestion at 37°C for 16-19 hours, 20 minutes at 80°C, cooled down at 4°C then stored at -20°C for a later electroporation (Uzcátegui *et al.*, 2004).

### 3.11 Electroporation of Antat 1.1

Electroporation is a novel gene transfection technique which entails brief, high intensity pulses to create transient pores in the cell membrane to facilitate the entry of the exogenous molecules like DNA, RNA and proteins. Once the expression vector is inside the Antat1.1, the protein that is encoded by the gene is expressed by the cellular-transcription and translation machinery.

A volume of 30ml culture HMI9 in a culture tube (medium) was used and put into the incubator at 37°C and 5% $CO_2$. Two Antat1.1 stabilates were taken and filled into a flacon of 15ml and filled with HMI9 up to 10ml, centrifuged at 3,000rpm for 5 minutes. The plasma was discarded and the buffy coat transferred to the culture tube then placed into the incubator, set for electroporation by electroporation 2x pulse (1400V, 25µF) and 22 hours regeneration of the strain (Uzcátegui *et al.*, 2004).

### 3.12 Cultivation and Selection of Trypanosome Clones by Tetracycline Resistance

The entire content of trypanosome culture tube was placed into 0.65% agar-HMI9-tetracycline-24 wells plates, a volume of 800µl was plated out and mixed with 0.05µl tetracycline/ml. Cultivation and selection of *eGFP* expressing trypanosome clones by tetracycline resistance *in vitro* and counting of cells using a light microscope was carried out after 5 days.

### 3.13 Fluorescent Microscopy

The fluorescent microscope was used to check up successful green fluorescent trypanosomes that were later used to infect rodents. Pictures were made and analyzed using an Olympus BH2 fluorescent microscope and Biosis imaging software (Uzcátegui *et al.*, 2004).

### 3.14 Infection of Rodents with Antat 1.1

One or two stabilates were thawed to infect the rodents. The rodents were anaesthetised with a mixture of $O_2/CO_2$ before infection. The rodents were then intra-peritonealy infected with $2x10^7$ trypanosomes.

# CHAPTER FOUR

# RESULTS

## 4.1 PCR Amplification of *eGFP*

The results for the gel for *eGFP* gene amplified from *pRS416eGFPMCA1* products are presented in figure 2. Each and every lane from A to D contained the same amount of PCR reaction put into each well. This gel shows the presence of the expected bands size of 5778bp from bands 1 to 4 for *pRS416MCA1* and of 732bp from bands 5 to 8 for *eGFP* gene which have been amplified. The *eGFP* gene was modified by adding to the sticky ends the forward primer a sequence for the restriction enzyme HindIII and to the reverse primer a sequence for the restriction enzyme BamHI for a later ligation.

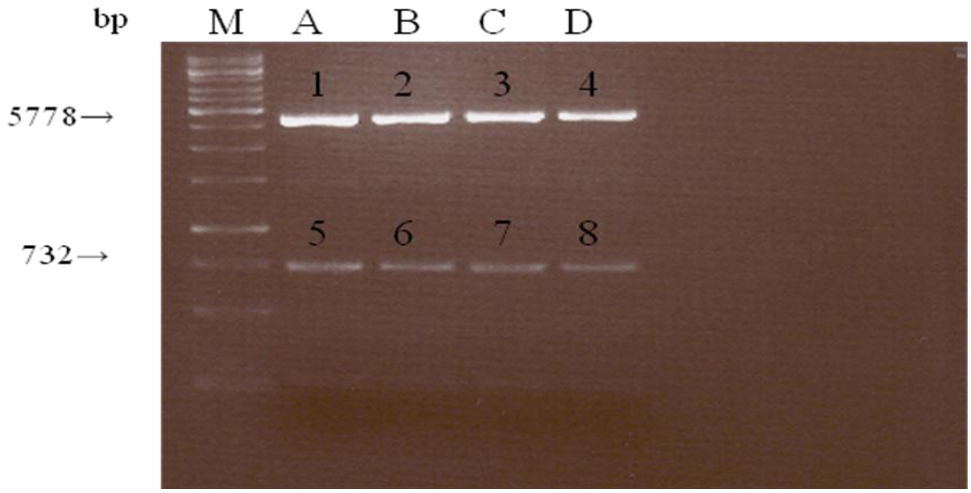


**Figure 2**: A 1% agarose gel electrophoresis confirming sizes of *eGFP* amplified by hot star Taq Master Mix PCR from *pRS416eGFPMCA1* using HindIII added to the forward primer & BamHI to the reverse primer. M: Molecular marker; 1kb DNA ladder (Fermentas). Lanes 1 to 4: *pRS416MCA1* and 5 to 8: *eGFP*.

### 4.2 Production of *pJET1.2 eGFP* and Digestion with BamHI and HindIII to Isolate *eGFP*

The results of the products of the gel for digestion of *pJET1.2eGFP* after being ligated and transformed into *E. coli* are presented in figure 3. Into each lane (A to B) was put the same amount of plasmid isolation content of *pJET1.2eGFP*. The two lanes A to B showed bands 1 and 2 with a molecular weight size of 2974bp which coincide with bands for the subcloning vector *pJET1.2* and bands 3 and 4 coinciding with bands size of molecular weight 732bp for *eGFP* after being digested by BamHI and HindIII to isolate *eGFP* for a later ligation.

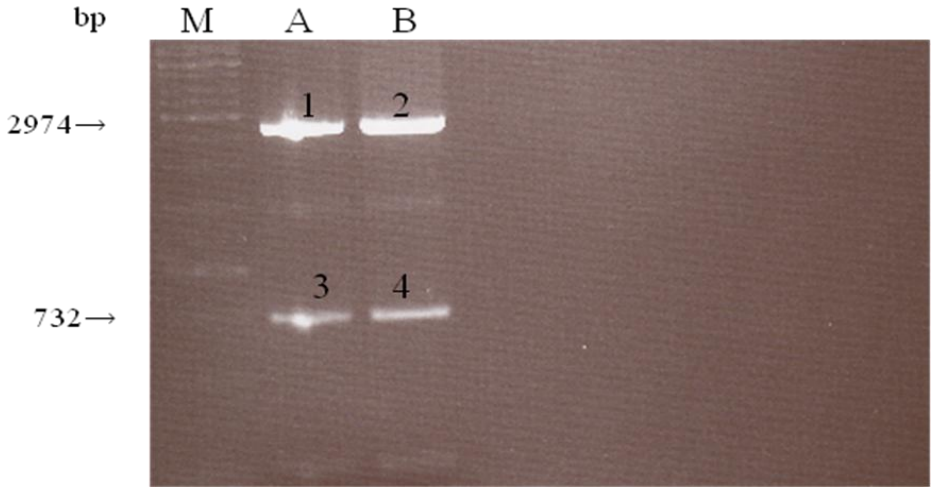


**Figure 3**: A 1% agarose gel electrophoresis showing the digestion of the recombinant *pJET1.2 eGFP* DNA with BamHI and HindIII. M: Molecular marker; 1kb DNA ladder (Fermentas). Lanes 1 to 2: *pJET1.2* and 3 to 4: *eGFP*.

### 4.3 Digestion of Expression Vector *pLEW100* with BamHI and HindIII

The results for digestion of *pLEW100* products are presented in figure 4. Into each and every well was put the same amount of the digestion reaction of *pLEW100*. The expression vector *pLEW100* was opened before being digested with BamHI and HindIII that coincides to the size 5563bp bands labeled 1 to 3.

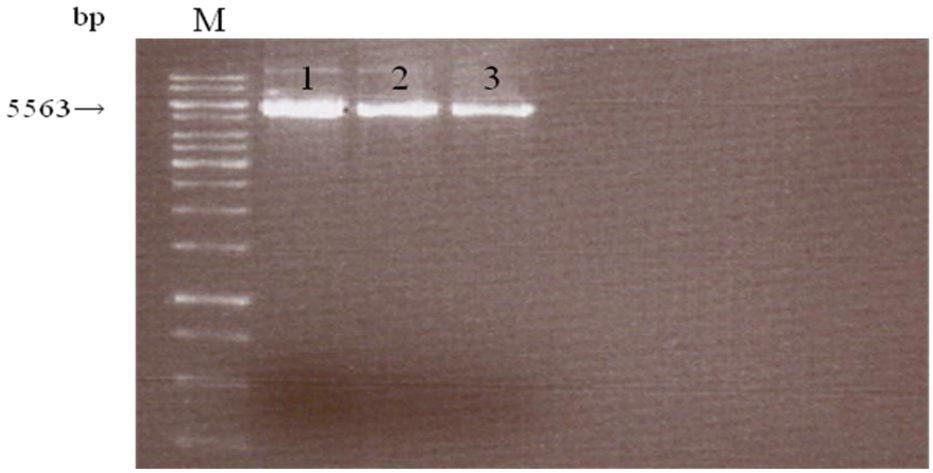


**Figure 4**: A 1% agarose gel electrophoresis showing the digestion of the expression vector *pLEW100* digested with BamHI and HindIII. M: Molecular marker of 1kb DNA ladder (Fermentas). Lanes 1 to 3: *pLEW100*.

### 4.4 Ligation of *pLEW100eGFP*

The results for the ligation reaction of *pLEW100* and *eGFP* products are presented in figure 5. Into each and every well for lanes 1 and 2 contained the same amount of the ligation reaction content of *pLEW100eGFP* sealed with T4 DNA ligase. The addition of the insert into that expression vector DNA made a total size of 6295bp used to check for the effectiveness of that ligation.

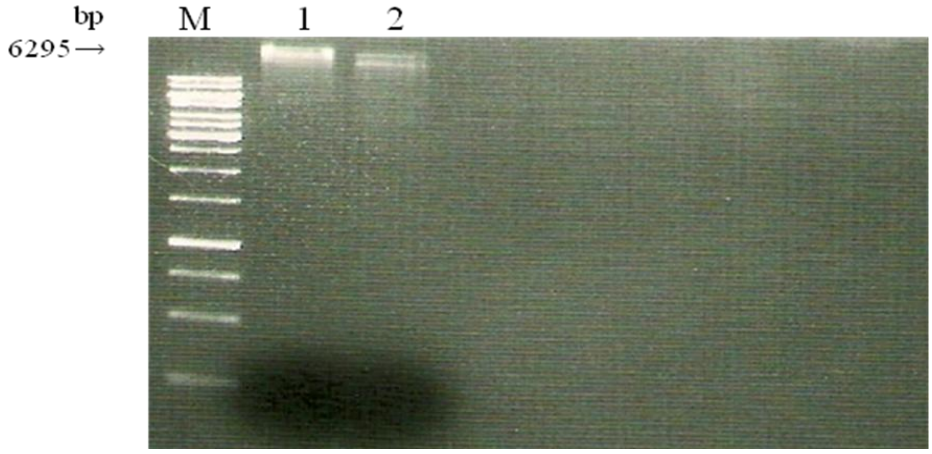


**Figure 5**: A 1% agarose gel electrophoresis showing the ligation reaction of *pLEW100* and *eGFP*. M: Molecular marker; 1kb DNA ladder (Fermentas). Lanes 1 and 2: *pLEW100eGFP*.

### 4.5 Positive Control of *eGFP* into *pLEW100* Using Hot star Taq Master Mix PCR

The results for the gel products are presented in figure 6 showing the presence of the expected bands for bands 1 and 2 with a molecular size of 732bp for the positive control of the insert *eGFP* into *pLEW100* using a direct culture of *E. coli* then amplified by Hot star Taq Master Mix PCR.

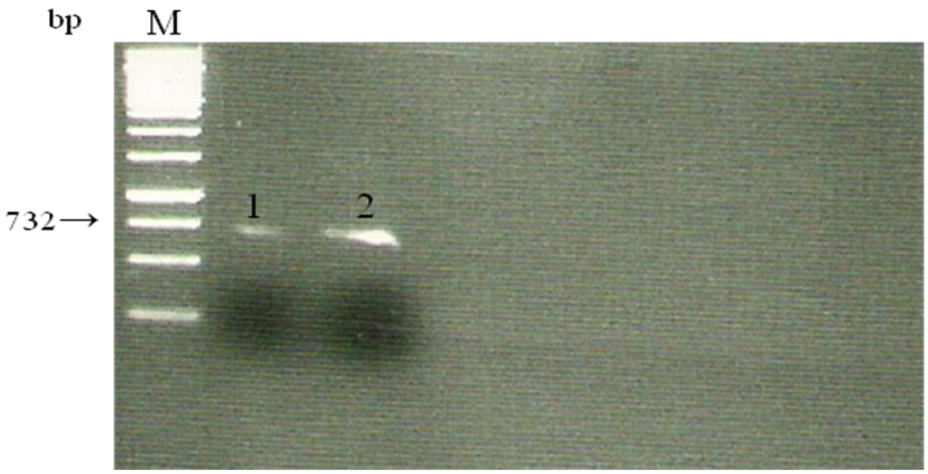


**Figure 6**: A 1% agarose gel electrophoresis showing the positive control of *eGFP* into *pLEW100.* M: Molecular marker; 1kb DNA ladder (Fermentas). Lanes 1 and 2: *eGFP*.

### 4.6 Positive Control of *eGFP* into *pLEW100* by Digestion with BamHI and HindIII

The results for the gel products are presented in figure 7 showing in lanes A and B bands 1 and 2 *pLEW100* of molecular size 5563bp and the presence of the expected bands 3 and 4 molecular size of 732bp for the positive control of the insert *eGFP* into *pLEW100* after digestion with the restriction enzymes BamHI and HindIII.

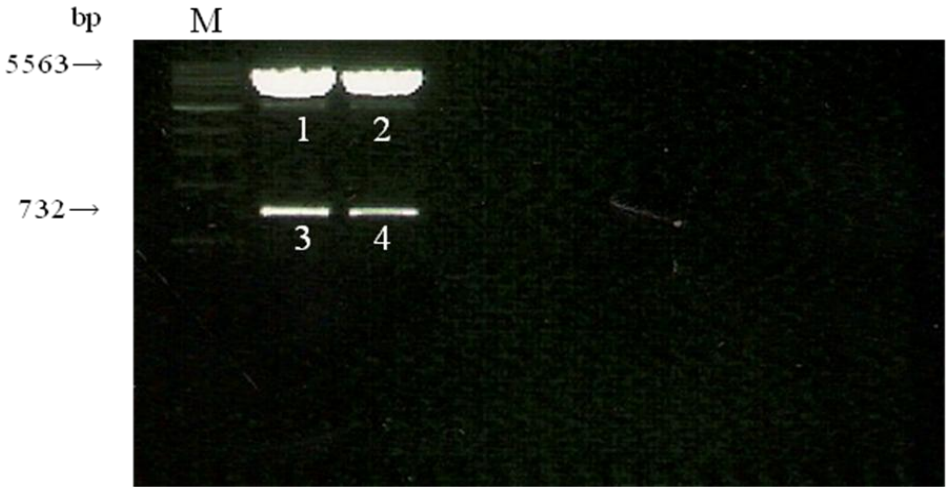


**Figure 7**: A 1% agarose gel electrophoresis showing the positive control of *eGFP* into *pLEW100* following digestion with BamHI and HindIII. M: Molecular marker; 1kb DNA ladder (Fermentas). Lanes 1 to 2: *pLEW100* and 3 to 4: *eGFP*.

### 4.7 Intraperitoneal Infection of *Mastomys natalensis* and *In vivo* Control of Fluorescence

The result from the fluorescent microscope shows the *in vivo* control of the fluorescence of the trypanosomes after being electroporated with *pLEW100eGFP* and infected *Mastomys natalensis*. The 22$^{th}$ days, blood from the tail was picked and checked to control the fluorescence of trypanosomes expressing green fluorescent protein. The brain contained trypanosomes showing green fluorescence named a,b and c.

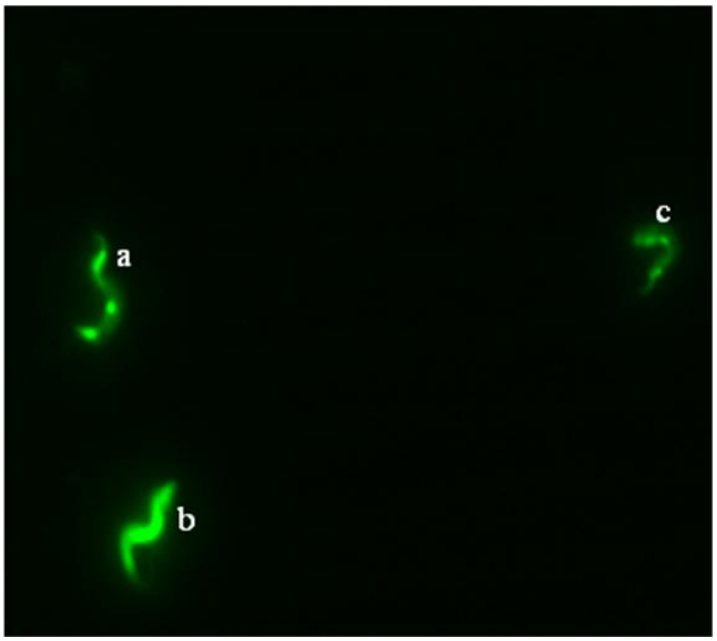


**Figure 8**: Fluorescence microscopy of bloodstream stage trypanosomes smear of *Mastomys natalensis* infected with *T. b. brucei* expressing green fluorescent protein (a, b & c).

### 4.8 Transversal Section of Unstained Rodent Brain Infected with Trypanosome

The result from any sliced part of the brain section shows an infected rodent after 21 days post infection, time that trypanosomes are expected to be seen in the brain of infected *Mastomys natalensis.* Slide shows presence of trypanosomes within the brain of infected rats.

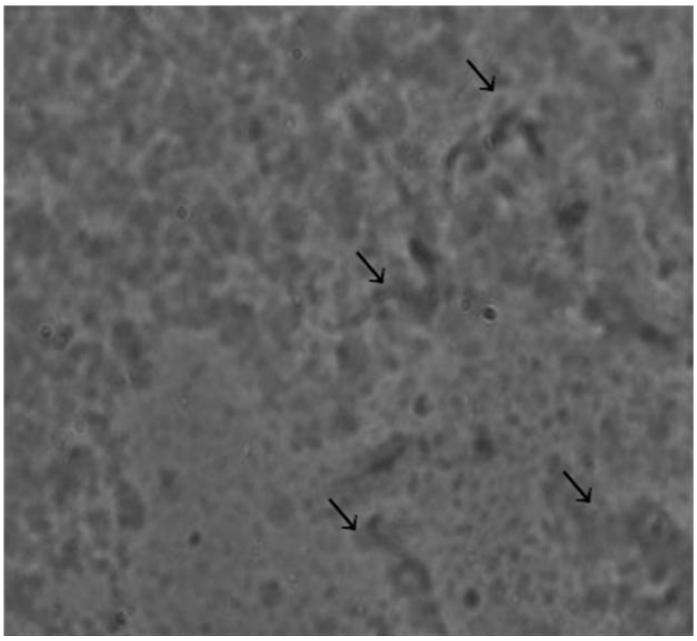

**Figure 9**: Localisation of *pLEW100eGFP* in tranfected *T. b. brucei* brain form into the parenchyma of *Mastomys natalensis* brain without stainning.↘: Infiltrated trypanosomes parenchyma.

### 4.9 Sliced Brain Slides Stained with Bisbenzimide

The result from the fluorescent microscope shows a *Mastomys natalensis* sliced brain tissue stained with bisbenzimide showing *T. b. brucei* trypanosome after 21 days post infection. In most of the stained slides, trypanosomes were easily visible. Few trypanosomes were seen in the third ventricle but never near the neurons.

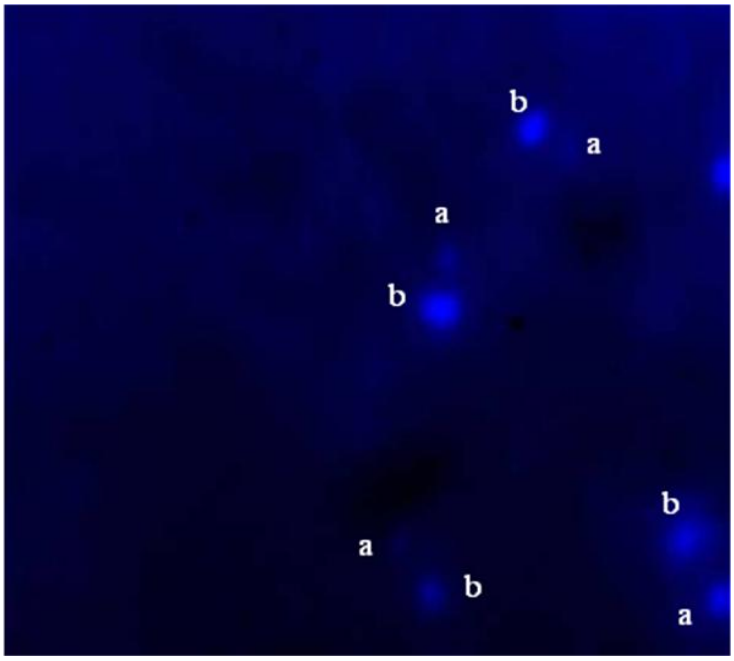


**Figure 10**: Localisation of *pLEW100eGFP* in tranfected *T. b. brucei* brain form expressing green fluorescent protein stained with bisbenzimide (a: kinetoplast and b: nucleus).

# CHAPTER FIVE
# DISCUSSION AND CONCLUSION

## 5.1 Discussion

Chronic infection with human African trypanosomosis leads to invasion of the CNS with trypanosomes leading to CNS symptoms (Enanga *et al.,* 2002) and thereafter CNS disturbances (Simarro *et al.,* 2008) and death if untreated (WHO, 2003).The trypanosome CNS biology is poorly understood. In addition, the parasites hideout in the brain as well as their mode of survival and multiplication remains elusive. This has led to a leg in the development of drugs for CNS trypanosome infection treatment. This is further complicated by the fact that few drugs available for the treatment of CNS trypanosome infection are toxic (Pepin & Milrod, 1994; Maclean *et al.,* 2006). Treatment of late stage infection is difficult and usually leads to re-invasion of the systemic system (Kennedy, 2004). There is therefore a need to develop tools that can be used to study and understand the trypanosome CNS infection biology with the aim of using the knowledge gained in developing new drugs for the treatment of late stage CNS infection.

The current understanding of the mechanism of the CNS infection is scanty. It is however known that after a variable time of persistent blood parasitaemia, trypanosomes enter the brain. Imaginative speculations have been proposed that the parasites cross the BBB or the BCB by an unknown mechanism (Schmidt, 1983; Masocha *et al.,* 2004). However experimental *T. b. brucei* infection of rodents has indicated that parasites appear early during infection in the choroid plexus and other circumventricular organs that lack a BBB (Schultzberg *et al*., 1988). At later stages, the parasites penetrate the BBB and enter the brain parenchyma, as revealed by double immunohistochemical labeling of parasites and brain endothelial cells in a rat model of the chronic disease (Mulenga *et al.*, 2001). A larger number of parasites appear in the white matter and in the septal nuclei rather than in the cerebral neocortex or the roof of the cerebral cortex that forms the part of the mammalian brain.

The current study therefore attempted to produce infective *T. b. brucei* expressing green fluorescent protein to be used to locate trypanosomes in brain of infected rodents. The said parasites would be used to investigate the biology of trypanosomes in the CNS, the mechanisms used by trypanosomes to regulate their density in the brain and in the CSF in order to establish the late stage of African trypanosomosis.

Results from the cloning expression study demonstrated that *eGFP* gene was increased following amplified by PCR technique. The *eGFP* gene was cloned into subcloning plasmid vector *pJET1.2* then into the expression plasmid vector *pLEW100* under control of T7 promoter. The procedures used in the lab to clone *eGFP* gene have been demonstrated to be robust as shown in previous studies (Uzcátegui *et al.,* 2004). Results obtained in this study showed the successful transfection of Antat1.1 by *pLEW100eGFP* resulting in *eGFP* expressing trypanosome clones by tetracycline resistance *in vitro* after 5 days of culture. These cells clearly showed the presence of *eGFP* within the trypanosomes cellular membrane an observation similar to that described for yeast cells expressing the glycerol facilitator from *E. coli* (Hedfalk *et al*., 2004).

A study using 20 velvet monkeys infected with *T. b. rhodesiense* (Schmidt, 1983) revealed 3 phases of brain infection, namely development of chronic meningitis after parasitisation of the CSF, progression of parasites from the leptomeninges to blood vessels entering the brain and ultimately the spread of parasites from these vessels into brain parenchyma and thus development of encephalitis. However, several animals in that study died before reaching encephalitic phase. Although this was hardly a systematic study, the data clearly indicated that certain foci exist in the brain where trypanosomes accumulate in fairly large numbers. Furthermore, studies using artificial BBB model system, proposed that brucipain, a parasite-specific cysteine protease and calcium signaling are required to open the tight junctions between the endothelial cells (Nikolskaia *et al.*, 2006). In addition, protease-activated receptor signaling has recently been found to play a role in *T. b. rhodesiense* traversal of human brain endothelial cells *in vitro* and models of how this signaling event may lead to BBB dysfunction have been proposed (Grab *et al*., 2009). At the same time interferon gamma has been noted to plays an important role in regulating trypanosome trafficking into the brain (Masocha *et al.,* 2007) and that accumulation of parasites in the brain is regulated by cytokines and chemokines (Kristensson *et al.*, 2010). However, these data were inconclusive and left unanswered the question whether penetration of the BBB is only one or even the only physiological way for the parasite to enter the brain and also did not indicate the precise sites where parasite multiply and enter the brain and thus establish CNS infection. The current study developed fluorescent trypanosomes that were infective to rodents.

At the same time it was possible to demonstrate the trypanosomes using fluorescent microscopy within the brains of infected rodents at 21 days post infection. Such green fluorescent expressing trypanosomes at various stages of infection can be used as a useful tool to provide scientific evidence on biology of CNS trypanosome infection such as the sites where trypanosomes multiply and any trypanosome density changes during CNS infection.

## 5.2 Conclusion

This study concludes that it is possible to produce infective *T. b. brucei* parasites expressing green fluorescent protein which can be used to track the trypanosomes in brains of trypanosome infected rodents. The parasites can thus be used to track trypanosomes during various stages of infection in order to provide a better understanding of how the parasite establishes infection in the CNS and thus causes pathogenesis within the brain. The information will be used in drug development and thereby forestall relapse infection.

## 5.3 Recommendations

A detailed systematic analysis study at various stages of infection needs to be done in order to get a clear understanding on the location and densities of parasites changes during the course of infection. In this way it will be possible to define where trypanosomes enter the brain and whether parasites grow in clusters in defined areas of the brain or if they are more or less dispersed. At the same time the results of such a study may reveal whether brain located parasites are different from blood stage trypanosomes.

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