POTENTIAL APPLICATION OF VARIABLE SURFACE GLYCOPROTEINS IN DIAGNOSIS OF *TRYPANOSOMA BRUCEI RHODESIENSE*

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Potential application of variable surface glycoproteins in diagnosis of *Trypanosoma brucei rhodesiense*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

I dedicate this work to my parents, Mr. Joshua Mule and Mrs. Rosemary Mule for their unwavering support, belief, guidance, devotion and prayers during my studies.

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ABBREVIATIONS

AAT	African Animal Trypanosomiasis
CATT	Card Agglutination Test for Trypanosomiasis
CIATT	Card Indirect Agglutination Test
DNA	Deoxyribonucleic Acid
ESAG	Expression Site-Associated Gene
FAO	Food and Administration Organization of the United Nations
FIND	Foundation for Innovative New Diagnostics
GPI	Glycosylphosphatidylinositol
HAT	Human African Trypanosomiasis
IPTG	Isopropyl-Beta-D-Thiogalactopyranoside
JKUAT	Jomo Kenyatta University of Agriculture and Technology
LB	Luria Bertani medium
PCR	Polymerase Chain Reaction
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TE	Tris-EDTA buffer
TLF1	Trypanolytic Factor 1
TLF2	Trypanolytic Factor 2
TNM-FH	*Tricoplusia ni* medium-formulation hink
VAT	Variable Antigen Type
VSG	Variable Surface Glycoprotein
WHO	World Health Organization
X-gal	5-Bromo-4-Chloro-Indolyl-B-D- Galactopyranoside

ABSTRACT

Sleeping sickness, also known as human African trypanosomiasis (HAT), is a neglected tropical disease caused by two subspecies of *Trypanosoma brucei* namely *T. b. gambiense* and *T. b. rhodesiense*. *Trypanosoma b. gambiense* causes chronic form of HAT (Gambian HAT) in West and Central Africa while *T. b. rhodesiense* is responsible for the acute form (Rhodesian HAT) in East and Southern Africa. In both cases, the disease evolves through clinically distinct stages namely early/stage 1 and late/stage 2. The early stage is haemolymphatic, in which parasites develop in the blood, lymph and peripheral organs. The parasites then spread to the central nervous system (CNS) (late/encephalitic stage) where they cause serious neurological disorders, and results into death if untreated. This necessitates timely diagnosis, stage determination and treatment, with drugs used dependent on the stage of the disease. However, diagnostic tools applied are limiting, suffering from low sensitivity and specificity, high costs and inapplicability in field environments in rural areas where the disease is endemic. This calls for development of improved and/or novel diagnostic tools, specifically for Rhodesian HAT, which kills in weeks or months if not treated. One potential diagnostic approach is serodiagnosis using trypanosome surface proteins, the variable surface glycoproteins (VSGs), which are highly immunogenic and expressed in an ordered fashion early during infection. In this study, two VSGs previously shown to be predominantly expressed early during *T. b. rhodesiense* infection in monkeys were expressed in bacterial and insect expression systems, and the purified recombinant diagnostic antigens used for immunological detection by applying Ouchterlony double immunodiffusion test. The recombinant proteins generated from both expression systems detected anti-VSG antibodies in a panel of sera from infected monkeys, with a detection rate of 89%, 97% and 83% by VSG3 expressed in bacterial cells, VSG3 and VSG4 expressed in insect cells respectively. In addition, sera recovered up to 15 days post infection, and 19 days when the parasite has crossed into the CNS were seropositive, suggesting potential application of VSGs in diagnosis of sleeping sickness. This is a proof of concept that VSG recombinant antigens predominantly expressed during early stage of trypanosome infection have diagnostic potential, and could form a basis for the development of a simple, cheap, robust and sensitive screening tool applicable in the field setting for early disease detection.

CHAPTER ONE

INTRODUCTION

1.1 Background

African trypanosomes (Genus: *Trypanosoma*) are single cell protozoan parasites that cause sleeping sickness or human African trypanosomiasis (HAT) in man and Nagana or animal African trypanosomiasis (AAT) in cattle; both diseases are generally called African trypanosomiasis (AT). The parasite is transmitted by tsetse fly (Genus: *Glossina*), an insect vector found in 37 countries in sub-Saharan Africa where the disease is endemic, with an estimated 70 million people (WHO, 2012; Simarro *et al.,* 2013) and 60 million cattle (Cecchi *et al.,* 2014) at different levels of infection risk. This region is over 10 million square kilometres (Cecchi and Mattioli, 2009) and represents the most arable part of the continent. HAT affects approximately 10, 000 people annually, with 3,796 new cases reported by the World Health Organization (WHO) in 2014 (WHO, 2015), a new low in 75 years. This reported HAT incidence could however be an underestimate due to inaccessibility of some of the most affected areas, poor or no health infrastructure, logistic constrains and lack of accurate diagnostic tools (Cattand *et al.,* 2001). AAT, on the other hand, affects the health and productivity of livestock in approximately 348 distinct disease loci (Cecchi *et al.,* 2014), and results in 3 million cattle deaths annually across the tsetse infested belt in sub-Saharan Africa (Vreysen, 2006).

HAT is caused by two subspecies of *Trypanosoma brucei*; *T. b. gambiense* and *T. b. rhodesiense*. *T. b. rhodesiense* is responsible for the acute form (sometimes chronic) (MacLean *et al.,* 2004) of the disease in Eastern and Southern Africa (Rhodesian HAT), whereas *T. b. gambiense* causes the chronic form of the disease in Western and Central Africa (Gambian HAT) (MacLean *et al.,* 2004; Brun *et al.,* 2010). There is however, a potential convergence of *T. b. gambiense* and *T. b. rhodesiense* foci due to the continued

expansion of *T. b. rhodesiense* foci in Uganda towards the northwest (Picozzi *et al.,* 2005) as farmers move with infected livestock. HAT progresses in two distinct clinical manifestations, an early stage known as haemolymphatic stage, in which parasites develop in the tissues, blood and lymph, and the late/ meningoencephalitic stage which occurs when trypanosomes cross the blood brain barrier and spread to the central nervous system (Odiit *et al.,* 1997; Kennedy, 2004). Nagana, on the other hand, is caused mainly by *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei brucei*. Other species such as *T. evansi*, *T. godfreyi*, *T. simiae* and *T. equiperdum* also cause AAT. Mixed infections involving two or three species have also been reported (Taylor and Authie, 2004). Nagana develops in three stages; anaemia, tissue lesions and immunosuppression. In the absence of treatment, HAT and AAT are fatal irrespective of the causative species (Pentreath, 1995).

Control strategies against AT are mainly chemotherapy to treat individuals and animals, and vector based strategies to block parasite transmission by the fly. Vector control strategies include use of insecticides (the sequential aerosol technique), bait technologies (traps and targets), push and pull technique (Hassanali *et al.,* 2008) and the sterile insect technique (SIT) (Vreysen *et al.,* 2000). Drugs in routine clinical use include pentamidine and suramin for treating early stage disease and eflornithine and melarsoprol for late stage HAT disease (Bacchi, 2009). Treatment is dependent on accurate diagnosis and staging of the disease followed by administration of appropriate drugs. Escalating costs and limitations in maintenance of tsetse control strategies, however, has hindered vector based control strategies, and resulted in the reliance of trypanocidal drugs to treat (HAT and AAT) and/or prevent the disease (AAT). The drugs are however old and faced with problems of toxicity and ineffectiveness due to resistance (Barrett *et al.,* 2007).

Presently, methods used for sleeping sickness diagnosis include examination of clinical presentations, parasitological demonstration of the parasites in body fluids, use of molecular probes for trypanosome subspecies specific nucleic acids (Masiga *et al.,* 1992) and serological assays targeting trypanosome specific antibodies (Magnus *et al*., 1978;

Lejon *et al*., 1998a; Lejon *et al.* 1998b) or antigens (Nantulya, 1997). Clinical signs such as fever, headache, joint pains, and swollen lymph nodes are non specific for HAT, necessitating the need for laboratory diagnosis. Microscopic demonstration of the parasite in body fluids is the method recommended by the WHO as the golden standard to diagnose African trypanosomiasis (Chappuis *et al.,* 2005). Although this method provides direct evidence for trypanosome presence, it is labour intensive and suffers from low sensitivity leading to approximately 30% of HAT-positive persons being missed (Chappuis *et al.,* 2005). Molecular diagnostic tools have high sensitivity and specificity, but require well equipped health facilities, constant power supply and qualified personnel, resources which are lacking in most remote rural foci where the disease is endemic (Trouiller *et al.,* 2002; Brun *et al.,* 2010). Serological tests, such as the Card Agglutination Test for Trypanosomes (CATT) that involves detection of *T. b. gambiense* LiTat 1.3 variable antigenic types (VAT) (Magnus *et al.,* 1978), require minimal equipment and are easily applicable in field conditions with limited health facilities. A similar kit is however absent for *T. b. rhodesiense* diagnosis (Radwanska, 2010). Moreover, clinical signs are insufficient for specific disease detection, as the signs are nonspecific and mimic other diseases such as malaria, enteric fever and tuberculous meningitis (Chappuis *et al.,* 2005) that co-exist in localities. The high toxicity of drugs for late stage disease, the acute nature of Rhodesian HAT and the limitations with current diagnostic tools necessitate the need for improvement and/or development of novel diagnostic tools which are simple, specific and field applicable. This study validated trypanosome surface coat, the variable surface glycoprotein (VSG), as potential sero-diagnostic candidates for Rhodesian HAT.

African trypanosomes are exclusively extracellular parasites covered by a dense surface coat monolayer, the VSG (Marcello & Barry, 2007), which shields invariant surface antigens from immune detection (Cross, 1990a, 1990b; Ferrante & Allison, 1983). The VSG is highly immunogenic, and forms a potential sero-diagnostic candidate for *T. b. rhodesiense* infection. Trypanosomes undergo antigenic variation, a mechanism which involves change of the VSG to an antigenically different one (Borst & Ulbert, 2001; Borst,

2002). The parasite's genome has 1000 - 2000 alternative and highly diverged surface antigen genes and pseudogenes, of which only a single one is expressed in any individual trypanosome cell (Lythgoe *et al.,* 2007). Past studies (Barry and Turner, 1991; Frank, 1999; Turner, 1999) have shown that despite the high diversity of antigenic variants, parasitaemia develop as a series of outbreaks, each outbreak dominated by relatively few antigenic types. The expression of dominant antigenic variants is not entirely random but rather sequential and hierarchical (Frank, 1999). The VSG ability to elicit an antibody mediated immune response, and their sequential expression from a possible >1000 genes, forms a potential diagnostic target for Rhodesian HAT.

In this study, two variant antigen types (VAT) previously shown to be predominantly expressed in early phase of *T. b. rhodesiense* in experimentally infected vervet monkeys (*Cercopithecus aethiops*) (Thuita *et al.*, 2008; Masiga *et al.,* unpublished data) were expressed in bacterial and insect expression systems and purified by immobilized metal affinity chromatography. Subsequently, the diagnostic potential of the recombinant antigens were assayed by Ouchterlony double diffusion test against 12 monkey serum infected with different *T. b. rhodesiense* strains collected at different days post infection (dpi) and two uninfected control sera samples. Both recombinant antigens generated from bacterial and insect expression systems were serologically detected by anti VSG specific antibodies in the assayed monkey sera indicating their potential use for sero-diagnosis of Rhodesian HAT, the acute form that kills in weeks or months if untreated.

1.2 Statement of the problem

Current tools used in HAT diagnosis suffer from low sensitivity and specificity, high costs, and are not applicable in the field environment. Furthermore, the *T. b. rhodesiense* causes the acute form of disease, drugs used to treat late stage disease are toxic and currently there exists no easy-to-use test for this disease. These reasons necessitate early and accurate diagnosis of the disease to evade complicated and risky treatment procedures. This need is especially critical as it is anticipated that the number of sleeping sickness

cases will fall in the next few years, making less sensitive methods unsuitable for tracking patients during the elimination phase of sleeping sickness. Therefore, there is need for identification of new diagnostic molecules for diagnosis and staging of the disease.

1.3 Justification

For improved Rhodesian HAT control, there is an urgent need for the enhancement of current and/or development of new diagnostic tools because of the following reasons. First, the clinical, parasitological and molecular methods of diagnosis relied on for *T. b. rhodesiense* have limitations. Clinical signs are non specific for HAT, parasitological approaches are low in sensitivity and specificity, while molecular approaches in which detection of parasite specific nucleic material is targeted, specifically serum resistance-associated (SRA) gene are not only difficult to apply in screening, but also expensive. Further, the required resources are not readily available in remote foci where the disease is prevalent. Secondly, being the acute form with severe clinical symptoms that results into death within weeks or months, a reliable, sensitive and ready-to-use point-of-care diagnostic tool(s) is needed for early diagnosis. Otherwise, detection at the late-stage where the only alternative treatment option, melarsoprol, is inevitable; treatment failures have been reported and results in 10% of patients developing post-treatment reactive encephalopathy (PTRE) due to the drug, half of whom die. Thirdly, the potential convergence of *T. b. gambiense* and *T. b. rhodesiense* foci in Uganda due to expansion necessitates the development of simple and sensitive detection kits specifically for *T. b. rhodesiense*. This will be essential in easy diagnosis of mixed infections and epidemiological surveys. Finally, large scale screening of animal reservoirs of *T. b. rhodesiense* is not possible using the current methods. This greatly limits surveillance and the ability to carry epidemiological studies that are required especially after the recently reported cases of Rhodesian HAT in Maasai Mara game Reserve in Kenya (Clerinx *et al.*, 2012; Wolf *et al.*, 2012), a previously unknown focus. Therefore, a VAT-antibody detection approach could allow development of a robust, simple, ready-to-use and large scale screening diagnostic kit.

1.4 Research Hypothesis

Variable surface glycoproteins expressed predominantly during early phase of *T. b. rhodesiense* infection do not elicit strong immunological responses and cannot be used as targets for diagnosis.

1.5 Objectives

1.5.1 General Objective

To evaluate the diagnostic potential of variable surface glycoproteins identified predominantly early during trypanosomiasis onset as candidates for *T. b. rhodesiense* diagnostic tool.

1.5.2 Specific Objectives

i. To generate recombinant variable surface glycoproteins predominantly expressed during early stages of *T. b. rhodesiense* infection in bacterial and insect expression systems
ii. To evaluate the diagnostic potential of the generated recombinant VSG antigens

CHAPTER TWO

LITERATURE REVIEW

2.1 African Trypanosomiasis

African trypanosomiasis is a disease that affects both humans and animals and is caused by uniflagellated single-cell extracellular parasites called African trypanosomes (genus, *Trypanosoma*). The parasite is transmitted between successive mammalian hosts by the bite of an infected tsetse fly (genus, *Glossina*), an insect vector found in 37 countries in sub Saharan Africa where the disease is endemic. In man, the disease is called sleeping sickness (or human African trypanosomiasis, HAT) and is caused by two subspecies of *T. brucei*, namely *T. b. rhodesiense* and *T. b. gambiense*. On the other hand, the disease is called Nagana or African animal trypanosomiasis (AAT) in domestic animals and is mainly caused by *T. congolense*, *T. vivax* and *T. brucei* subspp *brucei*, although other species including *T. evansi, T. equiperdum, T. simiae* and *T. godfreyi* cause AAT. *Trypanosoma vivax* and *T. evansi* also cause disease outside Africa, where they are transmitted mechanically by biting insects such as tabanids and stomoxes (Osório *et al.,* 2008; Desquesnes *et al.,* 2013).

2.1.1 African Animal trypanosomiasis (AAT)

African animal trypanosomiasis (AAT) is a parasitic disease caused by African trypanosomes. This disease affects domestic and wild animals, and is transmitted by tsetse flies (genus *Glossina*). An estimated 60 million cattle are at risk of infection in sub-Saharan Africa where the biological vector exists (Cecchi & Mattioli, 2009). This disease is endemic throughout the tsetse fly infested regions of Africa (the tsetse belt) covering over 10 million square kilometres in 37 sub-Saharan African countries (Figure 2-1).

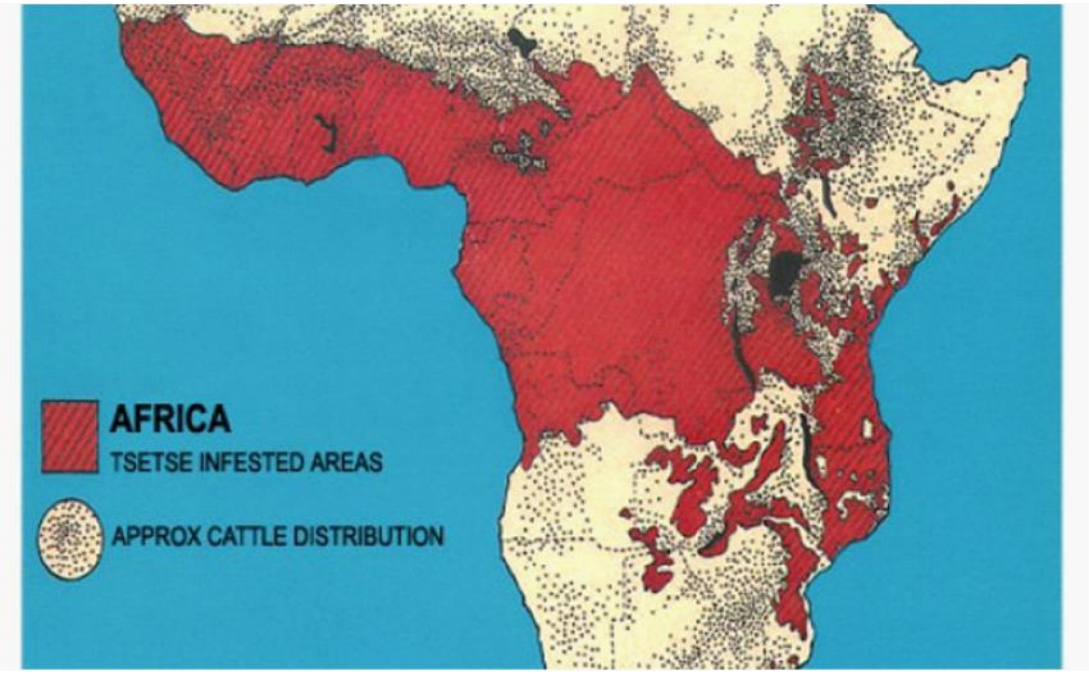


Figure 2-1. Tsetse fly and cattle distribution in Africa. Areas in red represent the tsetse infested regions where livestock keeping is constrained, the while the approximate cattle distribution is illustrated by black dotted areas. Image adopted from International Atomic Energy Agency (IAEA) (http://allafrica.com/view/group/main/main/id/00012920.html [accessed 18 Aug 2015]).

Nagana severely affects the development of agriculture in sub-Saharan Africa, contributing to heavy direct losses due to animal mortality, morbidity, reduced meat and milk productivity, and reduced calving rates and manure for fertilizing land for crop production (Vreysen 2006; Cecchi *et al.,* 2014). Nagana also prevents the integration of crop farming and livestock keeping which is crucial to the development of sustainable agricultural systems. Notably, this disease limits the exploitation of animal draught power and transport, and constrains the optimal utilization of fertile lands, reducing food security (Cecchi *et al.,* 2014). An estimated 3 million cattle deaths are reported annually as reported by Vreysen (2006). The losses incurred as a result of reduced meat and milk production, animal death, loss of draught power and costs of programs that attempt to control nagana are estimated to amount to between 0.6 and 1.2 billion US dollars each year (Budd, 1999). The overall impact on agriculture Gross Domestic Product loss due to AAT is

approximated to be 4.75 billion US dollars annually (Budd, 1999; FAO, 2007).

The major pathogenic tsetse-transmitted trypanosome species are *T. congolense*, *T. vivax*, and *T. brucei* subsp. *brucei* which affect cattle, goats and sheep, and *T. simiae* which affect pigs. *Trypanosoma congolense* (subgenus *Nannomonas*) is the single most important cause of AAT in cattle (Maré, 2004), and affects sheep, goats, horses, and pigs. *T. congolense* is grouped into three types namely savannah, Kenya coast/kilifi and forest/riverine types, which are morphologically indistinguishable. *Trypanosoma vivax* (Subgenus *Duttonella*) also affects cattle, sheep and goats but is less pathogenic for cattle than *T. congolense* (Maré, 2004). *T. brucei brucei (subgenus Trypanozoon)* causes disease in cattle, sheep and goats. It is morphologically, biochemically and antigenically indistinguishable to *T. b. rhodesiense* and *T. b. gambiense***,** but differs in host specificity; *T. b. rhodesiense* and *T. b. gambiense* affects human due to their ability to neutralize human trypanolytic serum factors TLF1 and TLF2, a characteristic lacking in the other trypanosome species (Raper *et al.,* 1996; Pays *et al.,* 2001). *Trypanosoma evansi* is mechanically transmitted by biting flies such as *Tabanidae* species and *Stomoxys*, and in Africa it mostly affects camels (Desquesnes *et al.,* 2013), causing a disease called ‘‘Surra’’, with cattle being reported as the next highly susceptible host to *T. evansi* (Mahmoud & Gray, 1980). *Trypanosoma equiperdum* affects horses and other equids, causing a disease termed as “Dourine” (Brun *et al.,* 1998; Claes *et al.,* 2005), which is transmitted during breeding. Other species such as *T. simiae,* which infects pigs, and *T. godfreyi,* a recently identified species which is pathogenic to pigs (Adams *et al.,* 2010) also cause AAT. *Trypanosoma uniforme* and *T. suis* are other less common tsetse transmitted species.

Nagana presents itself as an acute, sub acute or chronic disease (Maré, 2004) characterized by intermittent fever, anaemia, occasional diarrhoea, oedema of limbs and genitalia, and often terminates in death in the absence of treatment. The acute form of the disease causes abortion, central nervous system disorders and death, while in the chronic condition which

is the most common form of the disease and often aparasistaemic, affects the working capacity and productivity of the animals, causes severe anaemia, weight loss and infertility (Taylor & Authie, 2004).

Trypanocidal drugs available for AAT include isometamidium chloride (trade names: Samorin, Trypamidium, MandB 4180A) which is used as a prophylactic drug, while diamazene aceturate (trade name: Berenil) is used for treatment. These drugs are over a century old, and they are ineffective due to increasing resistance (Chitanga *et al.,* 2011), necessitating the need to identify novel compounds for treatment of the disease.

Clinical signs are non specific for AAT diagnosis and cannot be relied upon solely, requiring the need for laboratory confirmation. Demonstration of trypanosomes in body fluids is the standard diagnostic technique used, a technique which suffers from low sensitivity and specificity leading to half of the infected animals being missed (Nantulya, 1990). Sample inoculation in rats or mice has been used to diagnose AAT, a method that enables detection of low levels of parasites, but this method is time consuming, costly and non amenable to such species which do not infect laboratory rodents such as *T. vivax*, *T. simiae* and T. *congolense* (Nantulya, 1990). Detection of anti-trypanosome antibodies in blood samples using indirect fluorescence antibody test (IFAT) and enzyme linked immonosorbent assay (ELISA) are alternative diagnostic tests used, but suffer from low specificity because of cross reactivity with non-target parasitic diseases, require sophisticated laboratory equipment thereby limiting their application in the field (Nantulya, 1990). Detection of trypanosome antigens in circulation rather than antibodies elicited after infection enables the differentiation of current infection from successfully treated or self cure cases. Antigen detection tests using polyclonal antibodies against *T. vivax* and *T. congolense* antigens (Rae & Luckins, 1984) and monoclonal antibodies (Nantulya *et al.*, 1987) have been applied in the diagnosis of AAT using IFAT and sandwich ELISA tests. Polyclonal antibodies have however been shown to be limited by cross reactivity with non target trypanosome species and other blood parasitic animal

diseases leading to low specificities, while species specific monoclonal antibodies recorded high sensitivity (92%) and specificity (100%) with no cross reactions between trypanosome species (Nantulya *et al.* 1987; Nantulya 1990). Antigen detection tests are more sensitive than the recommended parasitological detection tests and can be applied in large scale screening, but suffer from their inability to detect infections at onset of disease before the parasites are destroyed to release the antigens for detection (Nantulya & Lindqvist, 1989).

2.1.2 Human African Trypanosomiasis (HAT)

Human African trypanosomiasis (HAT) or sleeping sickness is a disease caused by two subspecies of *Trypanosoma brucei, T. b. gambiense* and *T. b. rhodesiense. Trypanosoma b. gambiense* is responsible for the chronic form of the disease in Central and Western Africa (Gambian HAT) while *T. b. rhodesiense* causes an acute form of the disease in Eastern and Central Africa (Rhodesian HAT) (figure 2-2). There is, however, a risk of overlap of the two forms of disease in Uganda due to expansion of *T. b. rhodesiense* foci (Picozzi *et al.,* 2005), with major impacts on diagnosis and treatment of the disease (Brun *et al.,* 2010).

In the tsetse fly belt, about 70 million people are at risk of infection, and an estimated 3,796 new cases were reported in 2014 (WHO, 2015) compared to 7,214 new cases reported in 2012 (WHO, 2013). This represents a 90% reduction in cases reported as compared to the peak which occurred in 1998 when 37,991 new cases were reported (Simarro *et al.,* 2012). This could however be an underestimate as most cases occur in remote rural areas mostly in Democratic Republic of Congo, the Republic of Congo, Angola, Central African Republic and Southern Sudan (WHO, 2001; Brun *et al.,* 2010) where health infrastructures are poor, leading to most cases being missed due to lack of appropriate diagnostic tools, or unreported due to inaccessibility of some areas due to geopolitical unrests e.g. in Southern Sudan.

The clinical manifestation of HAT involves two stages. The first stage of the disease (the haemolymphatic stage) characterized by presence of the parasite in blood and lymph fluids is accompanied by fever, headache, adenopathy, joint pain and pruritus. Rapid parasite growth is countered by host immune responses, but parasite antigenic variation, a mechanism that involves the parasites continual switching from the expression of one variant surface glycoprotein (VSG) on the surface to the expression of a different immunologically distinct VSG (Donelson, 2003), enabling the parasites' persistence in the hosts blood stream.

Subsequently, the parasites cross the blood-brain barrier into the cerebrospinal fluid causing the second stage (encephalitic stage) of the disease (de Atouguia and Kennedy, 2000). At this stage, sleeping sickness causes an alteration of the circadian sleep/wake cycle, and is accompanied by endocrinological, cardiovascular and renal disorders, which, in the absence of treatment, progress to body wasting, somnolence (hypersomnia), coma and ultimately death of the patient (Kennedy, 2006, 2013).

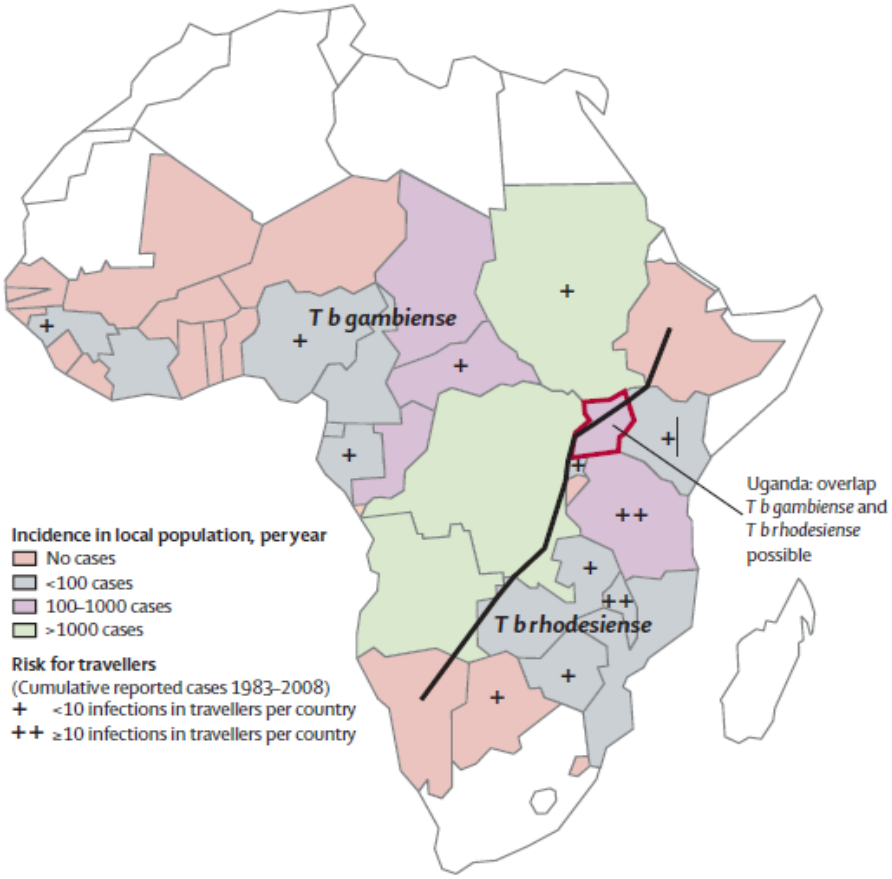


Figure 2-2. Human African trypanosomiasis (HAT) distribution, incidence and risk for travelers in sub Saharan Africa. The black line divides the Gambian and Rhodesian disease foci within the geographic distribution of tsetse fly. Image adopted from Brun *et al.* (2010).

2.2 Trypanosome life cycle

African trypanosomes are digenetic, alternating between vertebrate mammalian and invertebrate tsetse fly hosts (Figure 2-3). During a blood meal, an infected fly injects metacyclic trypomastigotes into blood and subsequently the lymphatic system of mammalian host. In the bloodstream, the metacyclic trypomastigotes transform into bloodstream trypomastigotes (morphologically slender forms) and are carried to other sites throughout the body where they continue to multiply. The blood stream

trypomastigotes express the bloodstream-stage-specific VSG coat to evade the mammalian immune response via antigenic variation (Matthews, 2005). At high densities, differentiation to morphologically stumpy forms occurs; these are division-arrested forms pre-adapted for transmission to tsetse flies (Matthews, 2005; Denninger *et al*., 2007). Upon uptake during feeding, the stumpy forms transform into procyclic forms, which are the proliferative forms in the fly's midgut, and express a surface coat called procyclin that is distinct from the bloodstream VSG coat. After establishment in the fly midgut, division is arrested and the trypanosomes migrate to the tsetse salivary gland, where they attach as epimastigote forms. These are proliferative and attached through elaboration of their flagellum. Eventually, the epimastigotes generate non-proliferative metacyclic forms, which re-acquire a VSG coat in preparation for transmission into a new mammalian host (Matthews, 2005). Theparasites cycle in the fly takes about 2 to 3 weeks (Chappuis *et al.,* 2005).

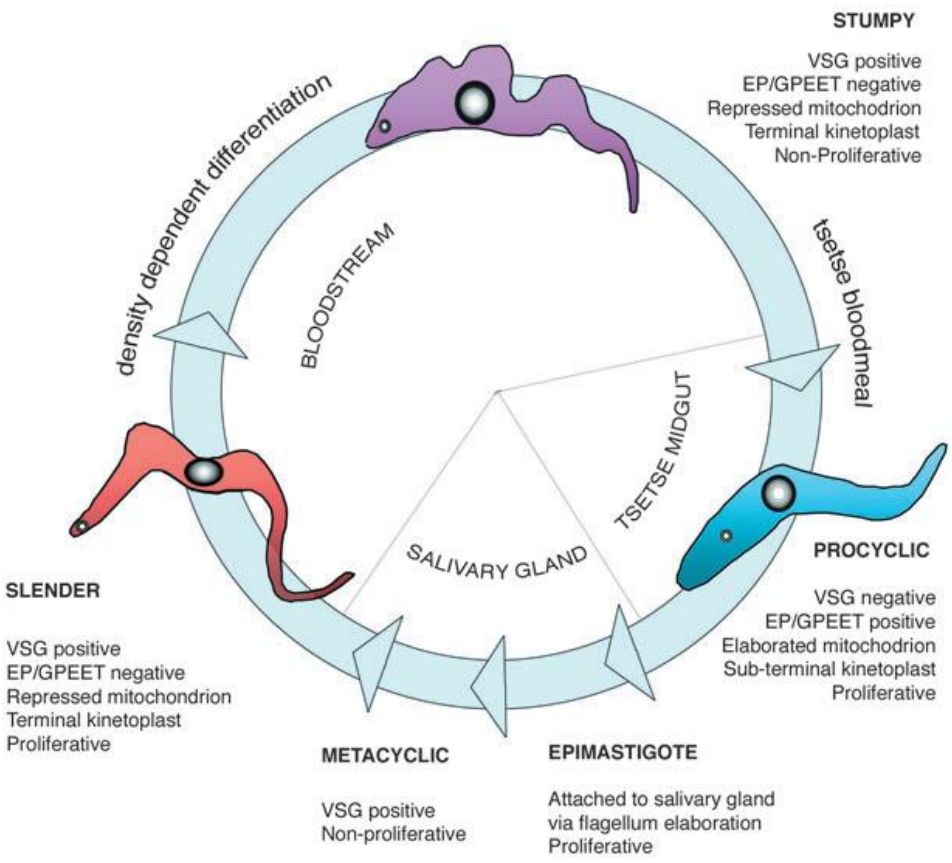


Figure 2-3. Life cycle of *Trypanosoma brucei* in mammalian host and the tsetse fly vector. The different developmental stages of trypanosomes in the mammalian blood stream (blood stream slender and stumpy forms), tsetse flies midgut (procyclic) and salivary glands (epimastigote and metacyclic stages, respectively) is illustrated, showing the surface coat at each stage. Image adopted from Matthews (2005).

2.3 Control of African trypanosomiasis

Various methods have been used to control trypanosomiasis, with such methods as vector based control strategies, screening of populations and curative treatment of infected people being used for HAT management. Management of AAT involves prophylactic and curative treatment of animals with trypanocidal drugs, the promotion of trypanotolerant cattle and suppression or eradication of the vector (Vreysen *et al.,* 2012).

2.3.1 Chemotherapy

Chemotherapy for HAT has lagged behind, with the current drugs in use being more than 50 years in existence (Chappuis *et al.,* 2005; Bacchi, 2009). Treatment of sleeping sickness is complicated and depends on the *T. brucei* subspecies involved and on the stage of the disease (Radwanska, 2010).

2.3.1.1 Drugs for Early Stage Treatment

Pentamidine is a water soluble aromatic diamine (diamidine) developed for treatment of early stage *T. b. gambiense*, leishmaniasis and pneumonia (Nok, 2003). A dosage consists of 7-10 intramuscular injections (Bacchi, 2009). This drug however causes severe side effects such as hypoglycaemia, hyperglycaemia and hypotension (Kennedy, 2006b).

Suramin is a sulphonated naphthylamine agent for treatment of early stage *T. b. rhodesiense* disease. The inability of suramin to penetrate the blood brain barrier renders this drug ineffective in the treatment of second stage disease. Suramin treatment regimen includes five intravenous injections every three to seven days for four weeks (Etet & Mahomoodally, 2012).

2.3.1.2 Drugs for Late Stage Treatment

Melarsoprol, an intravenously administered drug, is an arsenic drug used in first line therapy for late stage Gambian and Rhodesian HAT. Earlier, the standard melarsoprol regimen included two to four courses of intravenous injections, each course consisting of three injections administered after a week's break between the courses. Recently, a ten day continuous regimen was introduced for treatment of Gambian HAT, and has been adopted in many endemic countries (Kennedy, 2006b). This drug is painful to administer, destroys veins after several applications and is faced with toxicity caused by reactive arsenical induced encephalopathy in 10% of patients it is administered to, followed by pulmonary oedema and death in more than half of the cases in 48 hrs (Chappuis, 2007; Bacchi, 2009). Treatment failure rates have also been reported in DRC, CAF, Sudan and Uganda (Brun *et al.*, 2001). Melarsoprol is also administered for treatment of late stage *T. b. rhodesiense* disease. Eflornithine (α-difluoromethylornithine or DFMO) is a recently

developed alternative drug for treatment of late stage *T. b. gambiense* disease. Eflornithine drug regimen is long, and doses are given by prolonged intravenous infusion (Etet & Mahomoodally, 2012).

2.3.1.3 New Drugs in Use or Under Clinical Trials

New drugs are currently under preclinical and/or clinical trials, while a new combination therapy has been introduced for treatment of second stage Gambian HAT. A Diamine derivative (CPD-0802) entered advanced preclinical trials in mouse models for second stage HAT treatment, Nitroheterocycle derivative (fexinidazole) entered phase II clinical trials, and a Benzoxaborole derivative (SCYX 7158) entered phase I clinical trials (Barrett & Croft, 2012). These compounds are still under considerations for further development (Thuita *et al*., 2015).

The introduction of Nifurtimox-Eflornithine Combination Therapy (NECT) in 2009 for treatment of second stage *T. b. gambiense* HAT has been shown to be less toxic and more effective compared to eflornithine only treatment regimen in a preclinical study conducted in Congo (Priotto *et al.*, 2007).

2.3.2 Vector Control

Trypanosome transmission blocking by vector based control strategies is the most preferred method of controlling the disease due to increased tolerance to trypanocidal drugs in use. The adult fly is mostly targeted as it is the only accessible stage of the fly due to the absence of eggs, a larval free stage in nature and the fact that the pupa develops in the soil (Vreysen *et al.,* 2012). Removal of the vectors' preferred vegetation and the killing of host game to control tsetse flies proved to be a very efficient vector control strategy, but this strategy has become unacceptable due to its negative effect on the ecosystems.

Pour flow strategy of residual insecticides such as dichlorodiphenyltrichloroethane (DDT), dieldrin and endosulfan in areas where tsetse flies rest during dry seasons was

effective in controlling the disease, but has been limited due to logistical difficulties (supervision and planning) and labour intensiveness. The use of insecticides which often persist for long periods of time on the environment after spraying leads to the potential development of resistance to the insecticide, killing of non target insects, the potential outbreak of other pests due to elimination of their natural predators and the destruction of beneficial insects such as bees, ladybugs and beetles involved in pollination. The cumulative effect of insecticides also increases general pollution of the environment because of accumulation of the chemicals in the insecticides (Vreysen *et al.,* 2012).

The application of persistent insecticides has been gradually replaced by the aerial spraying of non residual ultra-low volume insecticides with planes, a technique known as the sequential aerosol technique (SAT) that is more environmentally friendly. The insecticide drop-size is small enough to suspend in the air but heavy enough to prevent upward drift (Vreysen *et al.,* 2012). Electrically or air driven rotary atomizers are used at a speed of 16,000 rpm to produce an aerosol of droplets of 30–40 μm. This control tactic aims at killing all adult flies in each spraying cycle through direct contact with the insecticide mist followed by all emerging flies in the subsequent cycles before they can start reproducing.

A technique that targets tsetse flies using stationary attractive devices such as cloth traps/targets that either kill the flies through tarsal contact with insecticides applied to the surface of the target or by heat or starvation after being guided to a non-return cage (Vreysen *et al.,* 2012) has been applied as a control strategy. The trap method is relatively cheap, unsophisticated and can be improved by incorporating insecticides such as pyrethroids. A live bait technique which involves insecticide treatment of livestock exploits the blood sucking behaviour of both sexes of tsetse which are killed by picking up a lethal deposit of insecticide on the ventral tarsal spines and on pre-tarsi during feeding. This method requires no sophisticated equipment, and insecticide application is rapid and easy (Vreysen *et al.,* 2012).

Sterile insect technique (SIT) involves the mass rearing of a target insect, sterilization by exposure to gamma or X rays or chemicals and the subsequent and sustained release of the sexually sterile insects into the target population (Dyck *et al.,* 2005) to outcompete the indigenous insect for the female insects resulting in no production of offsprings (Vreysen *et al*., 2013). This technique was applied in Zanzibar (Ugunja island) in the eradication of *Glossina austeni* using gamma sterilized male flies from 1994 to 1997 (Vreysen *et al.*, 2000). The SIT is environment friendly, has no adverse effects on non-target organisms, is species-specific and can easily be integrated with biological control methods such as parasitoids, predators and pathogens. There is no evidence of development of resistance to the effects of the sterile males provided that adequate quality assurance is practiced in the production process. This technique is, however, only effective when the target population density is low, requires detailed knowledge of the biology and ecology of the target insect, and the insect should be amenable to mass-rearing (Vreysen *et al*., 2013).

Combinations of repellent and attractant chemicals have also been used in push-pull tactics. Tsetse flies show a feeding preference on different vertebrate animals and appear to use push–pull signals actively to avoid some hosts and to locate those preferred. This strategy involves the use of synthetic repellents to push the flies away, and to attract them (pull) into baited traps (impregnated with insecticides). Natural and synthetic repellents such as 2-methoxyphenol, a constituent of bovid odors offer less protection to cattle compared to a repellent obtained from waterbuck, *Kobus defassa,* which is refractory to tsetse flies (Hassanali *et al.,* 2008).

One novel control strategy under development involves reducing the ability of the tsetse fly to transmit trypanosomes by expressing anti-trypanosomal molecules in Sodalis (*Sodalis glossinidius*), the commensal symbiont in the tsetse midgut. This is an environmentally acceptable, efficacious and affordable technique for the control of tsetse flies (Medlock *et al.,* 2013).

In sum, vector control strategies targeting the adult stage of the fly have recorded mixed success in controlling African trypanosomiasis, complementing the use of trypanosomal

drugs which have been limited by increasing tolerance and unacceptable toxicity. Vegetation clearance and insecticides have an adverse effect on the environment, and the application of SIT, live bait technique, push-pull technique and paratransgenesis offer a more environmentally friendly options. However, the application of vector control strategies are limited by logistical hurdles. Therefore, there is need for improvement of vector control methods, development of new safe drugs and novel diagnostic tests for African trypanosomiasis.

2.4. Diagnosis of Human African Trypanosomiasis

Despite its huge impact, human African trypanosomiasis is a neglected tropical disease, with little progresses in development of novel drugs and diagnostic tools (Papadopoulos *et al.,* 2004). Diagnosis is limited by low sensitivities and specificities of the current diagnosis procedures (Radwanska, 2010). The high toxicity of melarsoprol for stage 2 treatment necessitates high accuracy in both diagnosis and staging of the disease (Chappuis *et al.,* 2005). The approaches used in the diagnosis of HAT are discussed below.

2.4.1. Clinical Diagnosis

Clinical diagnosis entails the examination of individuals for clinical signs such as enlarged cervical lymph nodes (lymphadenopathy), excessive collection of fluids in tissues (oedema), enlarged spleen and liver (splenomegaly), fever, pruritus, development of a chancre at the site of tsetse bite and joint pains (Chappuis *et al.,* 2005; Radwanska, 2010). Early stage symptoms are experienced 1-3 weeks after the bite of an infected tsetse fly, but these clinical signs are in general insufficient for specific diagnosis of sleeping sickness, leaving most patients undiagnosed at the early stage of disease or misdiagnosed for other endemic and more frequent tropical diseases such as malaria (Kennedy, 2013). Owing to these varied clinical manifestations, diagnosis of trypanosomiasis cannot be solely based on clinical signs alone to both confirm or to stage the disease. Limitations in clinical diagnosis necessitate laboratory confirmation (Nantulya, 1990) and staging of

HAT disease using serological tests targeting anti trypanosome specific antibodies raised by patients' immune response to screen populations at risk, microscopy to demonstrate the presence of trypanosomes in body fluids and/or molecular amplification to detect parasite specific nucleic acids.

2.4.2 Parasitological Diagnosis

Parasitological diagnosis involves the microscopic detection of trypanosomes in blood, in chancre aspirates or cervical lymph node (CLN) aspirates, and cerebrospinal fluid for stage determination. Trypanosomes in the chancre are detectable days earlier than in blood by microscopy as fresh, fixed or Giemsa- stained preparations, but because the chancre disappears before most patients are tested, it is rarely used (Chappuis *et al.,* 2005). The gold standard for diagnosis of HAT recommended by the WHO is the demonstration of the presence of parasites in body fluids, a method that suffers from low sensitivity and is cumbersome. About 20 – 30% of patients are estimated to be missed by the various parasitological tests in use (Robays *et al.,* 2004; Chappuis *et al*., 2005). The standard parasitological techniques applied in the diagnosis of HAT are discussed below.

2.4.2.1 Wet Blood Films

This technique is used to examine fresh blood between a cover slip and a slide with a microscope. The parasites are seen either directly moving between the blood cells or indirectly as they cause the blood cells to move. This technique is simple and inexpensive, offering immediate diagnosis upon parasite detection. However, this technique has limited sensitivity with a detection limit as high as 10,000 trypanosomes per ml (corresponding to 1 parasite in 200 microscope fields) (Chappuis *et al.,* 2005). This technique requires fresh blood samples as the trypanosomes lose mobility after time, and cannot be used to stage the disease nor identify the trypanosome species (Chappuis *et al.,* 2005). The blood films can either be thick or thin blood films.

2.4.2.2 Microhematocrit Centrifugation Technique (mHCT)

This technique, also referred to as capillary tube centrifugation (CTC) technique or the Woo test, involves the use of capillary tubes containing anticoagulant which are filled with finger prick blood, one end sealed with plasticine and the trypanosomes concentrated by high speed centrifugation in a hematocrit centrifuge for 6-8 minutes. The parasites are concentrated at the level of leukocytes, between the plasma and the erythrocytes (Woo, 1969; Chappuis *et al.,* 2005), and the capillary tubes can be directly examined at low magnification of ×100 or ×200 for mobile parasites. This test has a detection threshold of 500 trypanosomes per ml. In addition to being time consuming, the presence of microfilaria (the pre-larval stage of *Filarioidea* in the blood of humans) limits visualization of trypanosomes.

2.4.2.3 Quantitative Buffy Coat (QBC) technique

Originally developed for the assessment of differential cell count, this technique has been used to concentrate and stain the nucleus and kinetoplast of trypanosomes with acridine orange dye, clearly discriminating the parasites from leukocytes. Blood is centrifuged at high speed in capillary tubes with ethylenediaminetetraacetic acid (EDTA) and acridine orange containing dye, enabling the discrimination of the parasites from leukocytes. Motile trypanosomes can be identified by their fluorescent kinetoplasts and nuclei in the expanded buffy coat using ultra violet light in a dark room. This technique has a high sensitivity, with 95% sensitivity for parasite concentrations of 450 parasites per ml, and can detect more patients with low parasitaemia than the mHCT (Chappuis *et al*., 2005). However, the fragility and sophistication of the materials used in this technique limits its application in the field, and thus is not used most frequently in field diagnosis.

2.4.2.4 Mini-Anion-Exchange Centrifugation Technique (mAECT)

This technique is based on use of anion exchange chromatography to separate trypanosomes from blood cells since the parasites are less negatively charged than blood

cells. The blood is first passed through anion exchange gel column, separating the parasites from the blood, with parasites collected in a sealed glass tube. The parasites are subsequently concentrated at the bottom of the sealed glass tube by low speed centrifugation. The tip of the glass tube is then examined in a special holder under the microscope for presence of parasites. The large blood volume used (300 µl) enables the detection of 100 trypanosomes per ml resulting in high sensitivity, but this technique involves tedious manipulations and is time consuming.

2.4.3 Serological Diagnosis

Tests for screening the population at risk in *T. b. gambiense* prone foci are mostly based on antibody detection against the trypanosomes' VSGs, although tests that detect trypanosome antigen against trypanosome specific antibodies have also been developed. Serological tests require minimum equipment and are easily applicable in the field environment with limited health facilities. Serological test used in the diagnosis of HAT are discussed below.

2.4.3.1 Immunofluorescence Antibody Test (IFAT)

The immunofluorescence antibody test (IFAT) is a serological test for both serum and dried whole blood on filter papers which utilizes IgG antibodies conjugated to fluorescein isothiocyanate. Before the introduction of CATT, the IFAT was the widely applied serological test in the mass screening of *T. b. gambiense* infection in the 1970s (WHO, 2001).

The test procedure includes antigen preparation by inoculating *T. b. gambiense* in guinea pig or albino rats, blood collected after the parasitaemia reaches 2-20 parasites per field; and mixed with heparine-glucose mixture and used to prepare thin blood films consisting of 5-50 parasites/field. This is followed by placing of patient serum on a glass plate with the prepared antigen, the mixture is washed with phosphate buffered saline (PBS) and fluorescein labeled antihuman at a predetermined dilution added. After washing as before,

the slides are covered with glycerine-PBS mixture and the slides viewed under UV microscopes (Wery *et al.,* 1970). IFAT is easily performed by non specialized laboratory technicians with a read out time of 10 to 30 seconds. However, this test is costly and only amenable to some laboratory settings.

2.4.3.2 CATT/*T. b. gambiense* and its variants

The card agglutination test for trypanosomiasis (CATT) is a serological test for diagnosis of Gambian HAT developed in 1978 by Magnus and colleges (Magnus *et al.*, 1978). CATT is the most widely used test applied for both passive and active screening of populations in *T. b. gambiense* endemic foci due to its simplicity and cost effectiveness. This test is based on a single lyophilized laboratory adapted strain of *T. b. gambiense* expressing predominant variable antigenic type (VAT) designated LiTat 1.3 on the parasites surface. Applied on whole blood, plasma or serum, the CATT/ *T. b. gambiense* is used in active screening of populations at risk, and seropositive cases are further confirmed by parasitological examination.

Mass production of trypanosomes expressing the variable antigenic type LiTat 1.3 antigen is achieved by culturing the parasites in laboratory rodents and the blood stream stage of the parasite extracted from the blood; a process which puts laboratory staff at risk of infection (Van Nieuwenhove *et al.*, 2012). The harvested parasites expressing VAT LiTat 1.3 are fixed, stained with Coomassie blue before being lyophilized (Chappuis *et al*., 2005). The kit consists of the antigen reagent, a control sera and a rotator; and operates by mixing blood and PBS-resuspended antigen reagent in equal volumes (one drop each), the mixture is rotated at 60 rpm for 5 minutes using the rotator and the results analyzed by macroscopic viewing, and the reported sensitivity and specificity of this test is 87-98% and 95% respectively (Chappuis et al., 2005). CATT on diluted blood is more cost effective than on whole blood (CATT/wb), but its slight complication makes it rather difficult to perform under field conditions (Elrayah *et al.,* 2007).

Micro-CATT test which permits utilization of minute amounts of dried blood samples on

filter papers or diluted blood was recommended by WHO for mass screening due to its affordability (Miezan *et al.,* 1991). This test uses micro-volumes, unlike the classical CATT/*T. b. gambiense* test (a fifth of the standard quantities of antigen and sample). This test is however less sensitive than the classical CATT/*T. b. gambiense* especially when the FP elute is older than a day or stored at 4°C (Chappuis *et al.,* 2002; Truc *et al.,* 2002). Coupled to the decrease in sensitivity with time, micro-CATT also suffers from difficulties in interpreting agglutination test results due to the micro volumes used in this test (Chappuis *et al.,* 2002; Truc *et al.,* 2002).

CATT on blood impregnated on filter papers (CATT-FP) was developed at the Department of Parasitology of the Prince Leopold Institute of Tropical Medicine, Antwerp (ITMA), is an improvement of the micro-CATT, and involves using a higher volume of the FP eluate (30 μL). CATT-FP has also been improved to enable the screening of populations in areas outside active screening radius (Chappuis *et al.,* 2002). The maiden test evaluation of this test was carried out in south-Sudan in Kajo-Keji County, and compared well with CATT on plasma, showing a sensitivity of 94% on parasitologically confirmed cases. More so, the CATT-FP test illustrated that samples could be stored at ambient temperatures (25-34 °C) and under refrigeration (2-10 °C) for upto fourteen days to eight weeks without loss of sample integrity (Chappuis *et al.,* 2002), unlike micro-CATT test whose sensitivity decreased with time. This can be attributed to the strict dry storage of the filter papers impregnated with blood, ensuring integrity of the blood samples.

Another alternative to the classical CATT/*T. b. gambiense* test, the CATT-EDTA test (Pansaerts *et al.,* 1998) is an improvement of the classical CATT/*T. b. gambiense* test; the EDTA (10mM) incorporated in the reaction buffer helps reduce complement mediated prozone effect, increasing the sensitivity of CATT. Active complement present in freshly collected blood inhibits the agglutination reaction leading to the formation of a prozone common in classical CATT test (Jamonneau *et al*., 2000). Dilution of blood also helps increase the specificity of the CATT-EDTA test (Jamonneau *et al.*, 2000). The CATT-

EDTA test was evaluated in a preliminary study conducted in Cote d'Ivoire (Jamonneau *et al.,* 2000), and showed a higher specificity (94.6%) compared to that of CATT/*T. b. gambiense* (92.5%).

A limitation of CATT/ *T. b. gambiense* screening tests is the observed absence of VAT LiTat 1.3 in some *T. b. gambiense* foci e.g. Cameroon (Dukes *et al.,* 1992), leading to false negative results. In a study carried out in Fontem, Cameroon, it was observed that the LiTat 1.3 gene was not expressed by *T. b. gambiense* strains, while a LiTat 3 like gene, expressed by some strains, elicited antibodies which could not be detected by the CATT test (Dukes *et al.,* 1992). This lowers the sensitivity of the CATT/*T. b. gambiense* in such areas where LiTat 1.3 is not expressed.

2.4.3.3 LATEX/*T. b. gambiense*

The LATEX/*T. b. gambiense* utilizes the combination of three predominant VATs, namely LiTat 1.3, LiTat 1.5 and LiTat 1.6 (VAT G16/6) of bloodstream *T. b. gambiense* parasites. The incorporation of three VATs predominantly expressed during *T. b. gambiense* infection broadens the geographic user-range of this test. Partially purified antigens are adsorbed on latex particles/beads, and equal amounts of diluted blood samples are mixed with the latex reagent and spread onto a 1.5 cm reaction zone with a dark background (Jamonneau *et al.*, 2000). Agitating the card at 70 rpm for five minutes enables the antigens to agglutinate with the VAT specific antibodies present in the blood, which are visible as white macroscopic agglutinations (Büscher *et al.* 1991; Lejon *et al.* 1998).

The specificity of LATEX/*T. b. gambiense* test was reported to be 98.1% in a study conducted in Cote d'Ivoire. Despite being highly specific, this test requires qualified personnel, is time consuming and the requirement of microplates complicates its application in the field setting (Jamonneau *et al.,* 2000). In addition, mixing more than one antigen in the same reaction reagent reduces the reactivity of the individual antigens in the final mixture, reducing the sensitivity of the test.

2.4.3.4 Card Indirect Agglutination Test for Trypanosomiasis (CIATT).

The CIATT is an antigen detection test which enables the differentiation between active and cured HAT by the detection of antigens released by non-circulating trypanosomes sequestered in the liver, spleen, lymph nodes, or CNS (Chappuis *et al*., 2005). Developed in Kenya, the card indirect agglutination test for trypanosomiasis (CIATT) or TrypTect CIATT® is a latex agglutination test for both *T. b. gambiense* and *T. b. rhodesiense* infection first described in 1997 by (Nantulya, 1997). This test detects trypanosome antigens circulating in the bloodstream using a monoclonal antibody specific to an invariant internal antigen present in both human infective *T. brucei* subspecies (Nantulya, 1997) - the antibody is coupled to latex particles, forming the reaction reagent. The assay involves mixing on the test card equal volumes (50 µl) of test reagent and serum/ plasma, and after two minutes of incubation the test card is tilted and rotated, macroscopically showing presence of agglutination when the test sample is positive.

Analysis of parasitologically confirmed 244 *T. b. gambiense* samples from Uganda and Côte d'Ivoire, and 132 *T. b. rhodesiense* samples from Uganda by the CIATT illustrated that the test was highly sensitive, recording 95.8 % and 97.7 % sensitivities for *T. b. gambiense* and *T. b rhodesiense* infections respectively (Nantulya, 1997). The antibody coupled to latex particles did not agglutinate with 193 negative sera samples assayed, illustrating the high specificity of this test (100% specificity). This study showed that the CIATT was more sensitive than some parasitological tests done in parallel (lymph node aspirates, mAECT, mHCT and CSF single and double centrifugation techniques). However, this test could not detect minute antigens in circulation, necessitating the need to test suspect cases after one to two weeks (Nantulya, 1997).

Antigen detection tests enable detection of active infection, unlike antibody detection tests which can detect persistent antibodies still in circulation months after the infection has been cleared due to treatment or self-cure. This means that the CIATT can be used in post treatment follow-up, and the ability to assess post treatment follow up with finger prick

blood could eliminate the need for lumber puncture, a painful and intrusive method undergone by patients during post treatment follow ups.

2.4.3.5 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is an immunological test that indirectly demonstrates the presence of an infecting parasite in body fluids. In contrast to CATT and LATEX tests, ELISA makes use of markers to enable the detection/visualization of antigen–antibody complexes. ELISA has been used in the serodiagnosis of sleeping sickness to detect anti-trypanosome specific antibodies in serum and CSF (Lejon *et al.* 1998) or saliva (Lejon *et al.,* 2003). ELISA has also found application in the staging of the disease, as anti-trypanosome specific antibodies or cerebrospinal IgM immunoglobulin whose levels are elevated in the CSF during infection (Greenwood and Whittle, 1973; Whittle *et al.,* 1977; Lejon *et al.,* 2002).

Trypanosome antigens are prepared by infecting laboratory rodents, and the trypanosomes are collected by differential centrifugation, resuspended in saline, lysed via sonication and centrifuged to remove insoluble cell debris (WHO, 1976). The test antigens (crude or pure) are adsorbed on microplates at different dilution factors, and diluted sera/CSF added. An enzyme conjugate (antigen specific antibody conjugated to an enzyme such as alkaline phosphatase or horse radish peroxidase) subsequently added followed by the enzymes substrate. The optical density is read at a specific wavelength depending on the substrate used, with the extinction value obtained being a measure of the quantity of enzyme-labeled immunoglobulin bound to the antigen – antibody complex (Knapen, 1977).

ELISA/*T. b. gambiense* is highly sensitive (Knapen, 1977) and specific (98.5%) (Elrayah *et al.,* 2007), cost effective and has acceptable reproducibility. However, this test suffers from variable sensitivities, and can only be applied in reference centers with highly skilled technicians, who are more often lacking in poor remote areas. In addition, cross reactions with antibodies from patients infected with Leishmaniasis is a concern, leading to false positives (Knapen *et al*., 1977). A sensitive micro-ELISA for *T. b. rhodesiense* is also

described by (Voller *et al.,* 1975), but this test too suffers from cross reaction by antibodies specific to, and antigens of *Leishmania donovani* and *T. cruzi*.

ELISA based on antigens for sleeping sickness diagnosis is sensitive and not limited by cross reactions with sera collected from patients infected with common tropical diseases. In addition, detection of trypanosome specific antigens circulating in the body fluids provides direct evidence of active infection (Komba *et al.,* 1992). Using a monoclonal antibody elicited against invariant surface protein at the procyclic stage of the parasites, (Nantulya, 1989) describes an indirect ELISA tool for diagnosis of Rhodesian HAT. In this study, the antigen ELISA was 100% specific, and 90% sensitive, and could also detect six samples which were parasitologically negative. However, this test could not detect some parasitologically confirmed cases, a limitation attributable to low amounts of antibody produced or during early stages of the disease (Nantulya, 1989).

2.4.3.6 Immune trypanolysis test (TL)

The immune trypanolysis test (TL), is an antibody-compliment mediated serological test that detects antibodies raised against specific VATs, revealing/demonstrating prior contact with the parasite. The variable glycoprotein surface coat of the trypanosomes, being highly immunogenic, elicits an immune response by the host's immune system which involves the production of antibodies specific to the VATs. These antibodies are able to opsonize, agglutinate and lyse circulating trypanosomes (Van Meirvenne *et al.,* 1995). This test can only be applied in reference laboratories, limiting its field applicability.

Immune trypanolysis test involves the intravenous inoculation of 10^7 mouse adapted bloodstream trypanosome clones expressing predominant VATs and a control VAT in complement rich cavia serum. Human plasma sample (25 µL) is mixed with 25 µL of complement rich serum, and 50 µL of 10^7 trypanosomes/mL suspension prepared from adapted bloodstream form trypanosomes in mice added. The micro-plates are mixed and incubated at room temperature for one and a half hours, and the reaction mixture examined under a phase contrast microscope (Jamonneau *et al.,* 2010).

In 1990, Isharaza and Van Meirvenne described a trypanolysis test for the detection of *T. b. rhodesiense* antibodies in 85 sera samples collected from Uganda (Isharaza & Van Meirvenne, 1990). The study used a panel of twelve *T. b. rhodesiense* specific VATs (ETat 1/1, 1/2, 1/3, 1/10, 1/14, 1/18, 1/19, UTat 3/1, 3/7, 1/1 and 4/1) and one control VAT (AnTat 25/1). All the assayed VATs were able to detect all the seropositive sera samples albeit with varying proportions. In combination, three of the VATs i.e. ETat 1/1, ETat 1/4 and UTat 1/1 showed positive reactions with all sera samples infected with *T. b. rhodesiense*. However, some parasitologically positive sera were missed by the assayed VATs; this can be attributed to low levels of anti VAT specific antibodies in circulation (Isharaza & Van Meirvenne, 1990).

Van Meirvenne and colleagues described a study where a panel of twelve predominantly expressed *T. b. gambiense* VATs were used (LiTat 1.1 to LiTat 1.10, and AnTat 11.17 and AnTat 11.20), and a control from *T. b. rhodesiense;* ETatR1 (Van Meirvenne *et al.,* 1995). This study was highly specific (100%) in different studies conducted in *T. b. gambiense* endemic countries such as Equatorial Guinea, Côte d'Ivoire, Gabon, Congo, Uganda (north west part of the country), Zaire, Sudan and Nigeria. However, the sensitivities vary depending on the sera dilution factor (Van Meirvenne *et al.,* 1995). In addition, some of the VATs included in this study were absent in some countries, narrowing the geographical user range of the test.

In 2014, Camara *et al.* evaluated the use of blood spotted filter papers as a substitute for serum or plasma in immune trypanolysis test. Blood spotted filter papers can be stored and transported under field conditions, while serum or plasma require a cold chain. Notably, their results showed that use of filter papers resulted in decline in sensitivity, but specificity was not affected (Camara *et al.,* 2014).

2.4.4 Molecular Tools

Molecular tools, specifically polymerase chain reaction (PCR), have been applied in the detection of trypanosome specific nucleic acids (Masiga *et al.*, 1992; Kabiri *et al.*, 1999;

Radwanska *et al.,* 2002a; Radwanska *et al.,* 2002b; Desquesnes and Dávila, 2002; Gibson, 2002, 2009; Adams and Hamilton, 2008). Generic primers that allow detection of multiple trypanosome species by targeting a common internal transcribed spacer (ITS) (Desquesnes *et al.*, 2001; Njiru *et al.*, 2005) exhibits interspecies size variation. While ITS amplification is more applicable in screening, it cannot distinguish the members of *T. brucei*, a limitation suffered by amplification of kinetoplast minicircle (Mathieu-Daudé *et al.*, 1994) and mini-exon repeat DNA (Ramos *et al.*, 1996), hence the need for subspecies-specific amplification.

PCR targeting single copy sequences for the diagnosis of Rhodesian HAT based on the detection of serum resistance associated (SRA) gene (Gibson *et al.*, 2002; Radwanska*et al.*, 2002a) and the diagnosis of Gambian HAT by targeting the *Trypanosoma gambiense* specific glycoprotein gene (TgsGP) (Radwanska *et al.,* 2002b) have been used on blood samples from CATT positive patients, and have been shown to have higher sensitivities and specificities than parasitological techniques. However, amplification targeting repetitive sequences is more sensitive than the amplification of single copy sequences (Masiga *et al.*, 1992; Chappuis *et al*., 2005). Masiga *et al.* (1992) applied the PCR technique to detect various trypanosome species by targeting repetitive DNA for amplification. Using oligonucleotide primers designed to anneal specifically to the satellite DNA monomer of each species/subgroup, this technique is able to accurately identify *T. simiae*, three subgroups of *T. congolense*, *T. brucei* and *T. vivax*, and using a set of more than one primer, this technique can detect mixed infections without use of radioisotopes. This method uses crude preparations of template DNA, making this technique rapid and ideal for large scale epidemiological studies. However, this technique requires qualified personnel and a constant supply of electricity, resources which are limited in most endemic regions (Masiga *et al.,* 1992). Amplification of DNA by PCR is able to accurately identify different trypanosome species and subspecies, enabling the detection of mixed infections.

Recently, the Loop Mediated Amplification (LAMP) technique has been used in the diagnosis of HAT (Njiru *et al.,* 2008; Matovu *et al.,* 2010). LAMP technique, developed in 2000 by Notomi and colleagues is a DNA amplification technique which utilizes *Bst* DNA polymerase with strand displacement activity and a set of four – six primers specific to the template DNA being targeted. An inner primer containing sequences of the sense and antisense strands initiates the amplification, and in an hour the target DNA is amplified to 10^9 copies with high sensitivity. A LAMP-based test by Kuboki *et al.* (2003) to detect *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. evansi* and *T. congolense* extracted DNA showed that LAMP was 100 times more sensitive than PCR, and *in vivo* studies on *T. b. gambiense* infected mice showed applicability of this test for disease diagnosis both in reference laboratories and in field settings. Thekisoe *et al.*(2007) designed primers targeting the 5.8S-rRNA-ITS2 sequence specific for *T. b. gambiense,* 18S *rRNA* targeting *T. congolense* and *T. cruzi,* and a VSG specific for *T. evansi* (VSG Rotat 1.2). These primers can detect 0.01 trypanosomes (1 femtogram trypanosome DNA), and the technique is rapid and simple, showing applicability in field settings (Thekisoe *et al*., 2007). In 2010, LAMP targeting random inserted mobile element (RIME-LAMP) for *T. b. gambiense* detection was evaluated, and showed higher sensitivity and specificity compared to TgSGP-PCR (Matovu *et al*., 2010). The need for four to six primers that recognize six to eight regions of the target DNA makes it an expensive diagnostic test. In addition, the need for trained personnel and power supply, make the approach inapplicable in rural settings with limited or no health facilities.

Real time nucleic acid sequence-based amplification (NASBA/self sustained sequence replication) targeting 18S rRNA of *Trypanosoma brucei* was developed for HAT diagnosis in 2008 by Mugasa *et al.* (2008) NASBA is a RNA (or sometimes DNA) detection technique that involves isothermal amplification of transcripts. It has been applied in the detection of 18S rRNA of *T. brucei* (Mugasa *et al.,* 2008). The test is sensitive, with a detection limit of 10 parasites per mL. The ease and speed of detection of PCR and NASBA products have been recently improved by the coupling of oligochromatography,

a technique whose sensitivity and specificity for *T. b. gambiense* and *T. b. rhodesiense* differed, with the later recording low sensitivity and specificity on blood samples from Uganda patients (Matovu *et al*., 2010).

The current techniques used in the diagnosis of HAT need improvement, and development of a robust, cheap, simple, ready-to-use and large scale screening diagnostic kit is urgently needed for *T. b. rhodesiense* detection as there is still no equivalent to the CATT for population screening. An alternative approach to HAT diagnosis should be centred on the detection of the parasite during its early stages of infection before the disease reaches its late stages where treatment is both difficult and risky. This study evaluated the use of two VSGs predominantly expressed during early stage of disease for early diagnosis of Rhodesian HAT.

2.5 Disease Stage Determination

After pathogen detection in blood, it is necessary to determine the state of disease progression by demonstration of parasites in the CSF or white blood cell counts in the CSF, as recommended by the WHO (WHO, 1998). Stage determination and post treatment follow up to determine cure or treatment failure are carried out by examination of the CSF after patients undergo lumber puncture, a very painful and intrusive method that prevents most people in endemic areas from being tested. According to WHO recommendations, second-stage HAT is defined by the presence in the CSF of raised white blood cells (>5 cells/µl) and/or trypanosomes and increased protein content (>370 mg/liter, as measured by the dye-binding protein assay) (WHO, 1998). The CSF white blood cell count is the most widely used technique for stage determination (Chappuis *et al.,* 2005), but the threshold for treatment is controversial, differing from country to country (Table 2-1). New born children (Sarff *et al*., 1976; Chappuis *et al*., 2005) and patients with bacterial, viral or fungal meningitis however have high levels of wbc in the CSF, and this further complicates the use of wbc counts in staging sleeping sickness disease.

Table 2-1. Different WBC/µL cut off for HAT staging in different sub Saharan countries. Different countries apply different WBC/µL thresholds for HAT stage determination, complicating the most commonly used method for stage determination and subsequent treatment.

Etiological agent	Country	WBC/µL	Reference
T. b. gambiense			
	Angola	≥20	Stanghellini and Josenando, 2001
	Cote d'Ivoire	≥20	Lejon *et al.*, 2003
	Uganda	≥20	Lejon *et al*., 2003
	Sudan	>5	Blum *et al.*, 2006
	Equatorial Guinea	≥10	Chappuis *et al.* 2005; Blum *et al.*, 2006
	Central African Republic	>5	Blum *et al.*, 2006
	Democratic Republic of Congo	>5	Balasegaram *et al.* 2006; Blum *et al.*, 2006
	Republic of Congo	>5	Blum *et al.*, 2006
T. b. rhodesiense			
	Malawi	>5	WHO, 1998; Chappuis *et al.*, 2005
	Uganda	>5	WHO, 1998; Chappuis *et al.*, 2005

Detection of trypanosomes is carried out immediately after the lumber puncture because the parasites lyse within 10 minutes in CSF. Demonstration of parasites in the CSF is simple and cheap, but suffers from insufficient sensitivity, and is a very painful and intrusive method that prevents most people in endemic areas from being tested or going back for post treatment follow up.

Staging is also determined by assaying protein concentration in the CSF. In healthy individuals, the CSF consists mainly of albumin (70%) and IgG (30%). In HAT patients the protein concentrations are elevated from 15-60 mg/L to 100-2,000 mg/L. However, protein concentrations can also be raised in first-stage illness due to diffusion in the CSF of IgG which is present in high concentrations in the serum. This technique, though simple, is limited by the difficulty in determining accurately the total protein concentration in the CSF and is no longer used in the field and requires specific reagents which are more often lacking in rural health centres (Chappuis *et al*., 2005). Demonstration of parasites in the CSF and counting of white blood cells as methods of stage determination are limited by low accuracy. In recent studies, biomarkers such as neopterin, CXCL-10, CXCL-13, ICAM-1, VCAM-1 have been studied and shown potential for disease stage determination, offering increased accuracy (Tiberti *et al.,* 2013).

Figure 2-4. Lumber puncture to collect CSF for subsequent staging of sleeping sickness. Patients with parasite in body fluids undergo lumber puncture, a process which involves withdrawing cerebrospinal fluid (CSF) for analysis of trypanosome presence to determine the stage of the disease. Image derived from FIND, 2012.

The CSF is subsequently analysed for presence of trypanosomes, while blood cell count

and protein quantities, allowing staging of the disease. Lumber puncture is a risky, painful and intrusive method, with most patients showing that prevents most people in endemic areas from being tested and/or going for follow-up to monitor treatment.

2.6 Antigenic Variation in African Trypanosomes

African trypanosomes are extracellular parasites that reside and replicate in the tissue fluids and bloodstream of their mammalian hosts, where they are constantly exposed to hosts immune system. Despite the continuous exposure to immune attack, trypanosomes evade the immune response raised by the hosts' immune system, leading to prolonged pathological manifestations. This persistence in the mammalian host is due to a phenomenon termed as antigenic variation, which involves switching the expression of one variant surface glycoprotein (VSG) on the trypanosomes surface to another immunogenically different VSG.

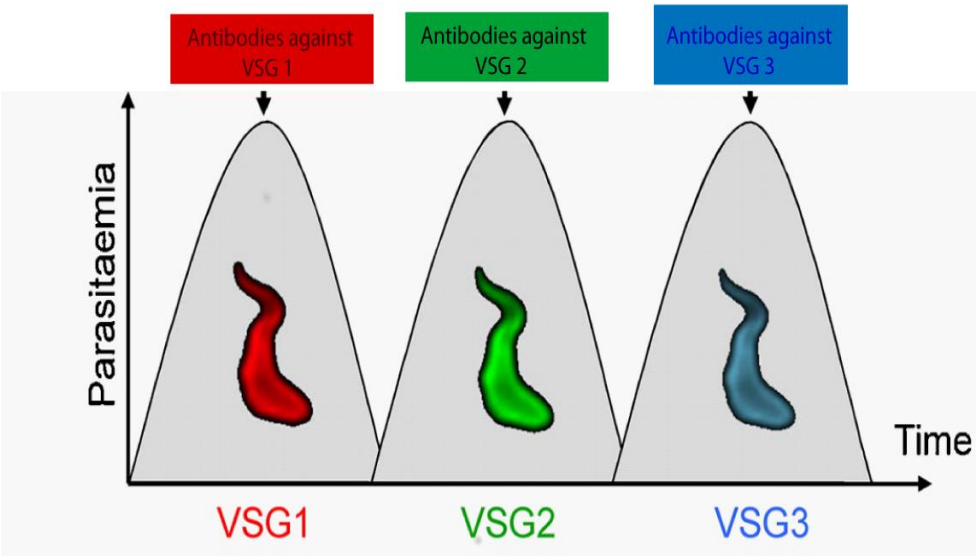


Figure 2-5. African trypanosome antigenic variation. Trypanosomes change the expression of the surface coat, the variable surface glycoprotein (VSG) to an antigenically distinct surface coat, enabling immune evasion. Image modified after Horn (2014).

The VSG is a dense cell surface coat that shields invariant surface antigens from immune recognition. Antibodies against the expressed VSG kill the trypanosomes, but part of the population survives due to stochastic switching at a rate of up to 10^{-2} to 10^{-7} switches/doubling time of 5-10 hrs (Turner & Barry, 1989). Three recombination pathways which operate in hierarchy contribute to VSG switching, a hierarchy in which telomeric VSGs are activated earliest in infections, followed by intact sub-telomeric array VSGs, then lastly pseudogenes, which are recombined to form functional VSG mosaics (Morrison *et al.*, 2009). Gene conversion is a recombination pathway copying and transferring a silent, functional VSG to the expression site (ES), replacing the resident VSG. A second recombination pathway, the reciprocal telomeric VSG exchange involves chromosome ends being exchanged by cross-over, moving a silent telomeric VSG into the active ES and retaining the previously active VSG on the other chromosome, most likely by double strand breaks (DSB) repair. The third recombination pathway involves the generation of novel, functional VSGs by combining portions of the open reading frames (ORFs) from at least two pseudogenes to generate VSG mosaics (Morrison *et al.*, 2009).

VSG genes of *T. brucei* are transcribed in telomeric loci termed VSG expression sites (ESs). The ESs active in bloodstream forms are polycistronic and contain several genes in addition to the VSG, named ES-associated genes (ESAGs). So far 12 ESAGs have been identified, some of which are present only in some ESs (Pays *et al.*, 2001). The expressed VSG is always located in an ES - found at the telomeres of the large and intermediate chromosomes, while inactive VSG genes are scattered among different chromosomes (Donelson, 2003). Each is a polycistronic unit, containing a number of ESAGs all expressed along with the active VSG (Pays et al., 2001). While there are at least 20 known expression sites, only a single one is ever active at one time. The RAD51 enzyme which participates in DNA break repair and genetic exchange in many organisms is thought to play a role in antigenic variation, but studies have shown that in its absence other trypanosome factors provide for the VSGs switching (McCulloch & Barry, 1999).

2.7. The Variable Surface Glycoprotein (VSG)

The surface coat of bloodstream form trypanosome cell is composed of a 12 nm thick glycoprotein known as the variable surface glycoprotein (VSG) (Vickerman, 1969). Approximately 10 million molecules of the monolayer enwrap the trypanosome cell, forming about 10-20% of the total bloodstream protein of the trypanosome cell (Bangs *et al*., 1997). The VSG physically protects the underlying plasma membrane from components of the hosts nonspecific immune effectors such as cytotoxic cells (Robinson *et al*., 1999). The VSG molecule is approximately 60 kDa (Horn, 2014), and is anchored to the plasma membrane by glycophosphatidylinositol (GPI). X-ray crystallography on two thirds of the N terminal domains shows that the N terminals have a conserved rod like shaped structures despite the different amino acid sequences, partly due to a conserved disulphide bonds arrangement, indicating that VSGs share a similar back bone structure from which emerge distinct epitopes from the different groups of amino acid side groups (Donelson, 2003). The C terminal regions of the VSGs are conserved (Majumder *et al.*, 1981).

2.8 Sequential Expression of VSG

The majority of VSG genes are kept silent, and those expressed are sequentially switched on (Ploeg *et al.,* 1982). Even though there are >1000 VSG genes to select from, the genes are expressed sequentially and in a hierarchy, a hierarchy in which telomeric VSGs are activated earliest in infections, followed by intact sub-telomeric array VSGs, then lastly pseudogenes, which are recombined to form functional VSG mosaics (Morrison *et al.,* 2009). At the beginning of the infective phase, VSGs are produced by parasites in the insect salivary glands. A subset of the repertoire (about 12) of the VSGs which are produced here remains during the first waves of parasitaemia in the mammal i.e. they appear preferentially early in infection (Esser *et al.,* 1982; Morrison *et al.,* 2009) before more complex genes predominate later on in the presence of a host-specific immune response. The whole repertoire is then open to expression and there is preferred but not fixed order of expression. VAT expression is reversible and hierarchical (i.e. non- random) (Turner, 1999), and the possibility that there is an initial subset of VSGs that are expressed sequentially during the early waves of parasitaemia gives hope for new methods of diagnosis. Masiga and colleagues (unpublished work) carried out a study and identified variable surface glycoproteins genes which where sequentially expressed predominantly in all four vervet monkey models tested (see also Thuita *et al.,* 2008). Two of the determined genes formed the basis of this study, and were expressed in bacterial and insect expression vectors. The recombinant proteins were serologically assayed against infected monkey sera, and showed diagnostic potential by detecting anti CSG specific antibodies in the samples assayed.

2.9. New serodiagnostic algorithms

Rapid diagnostic tests (RDTs) are fast, cheap, and easy to use on whole blood or plasma and do not require electricity or a cold chain, making them applicable in screening populations at risk under field conditions with limited resources. Furthermore, RDTs provide an alternative to the card agglutination test for trypanosomiasis in *T. b. gambiense* endemic foci. To improve specificity, synthetic and modified antigenic peptides (Van

Nieuwenhove *et al.,* 2012) that will eliminate cross reactivity due to use of whole cell lysates as in Gambian HAT kits can be used. Though this will mean increased kit costs, generation of and simultaneous use of multiple antigens in rapid diagnostic tests (RDTs) platforms are worth considering. For example, Büscher *et al*., (2013) developed HAT Sero-Strip and HAT Sero-K-SeT tests specific for *T. b. gambiense* LiTat 1.3 and LiTat 1.5; these were a dipstick and a lateral flow devices that cost about $2.50 each. Others include SD BIOLINE HAT test by FIND and a prototype that uses recombinant ISG65 and native VSG miTat 1.4 (sVSG117) developed by BBI Solutions (BBI) (Sullivan *et al.,* 2014). These attempts indicate potential for development of new and/or improved field deployable sero-kits for Gambian HAT. The knowledge will be of immense importance in diagnosis of the acute Rhodesian HAT.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Generation of Recombinant VATs in Bacterial and Insect Expression Systems

3.1.1 Recovery of Cloned Variable Antigen Types (VATs)

Previously, Masiga and his colleagues (unpublished data) had recovered 21 VAT mRNA from *T. b. rhodesiense* parasites during early onset of infections in vervet monkey models (*Cercopithecus aethiops*) (see also Thuita *et al*., 2008). These VATs mRNAs were reverse transcribed to cDNA, sequenced, cloned into pGEM-T Easy cloning vector (Promega, Madison, USA) and subsequently sub-cloned into pRSET A expression vector (Invitrogen, Carlsbad, USA). The recombinant plasmids were ultimately cryopreserved by transforming competent DH5α and BL21 pLysS *Escherichia coli* (*E. coli*) cells respectively. Two cryopreserved VAT gene isolates designated VAT3 and VAT4 were the focus of this study. Of the identified VATs, these two VATs were shown to be the most predominantly expressed in all vervet monkeys.

The recovery of the two cryopreserved VAT clones was carried out using Luria Bertani (LB) (both agar and broth) selective media (Appendix 1). The cryopreserved stabilates were aseptically streaked on freshly prepared LB agar plates with 100 µg/mL ampicillin, and cultured overnight at 37 ºC in a bench top stationary incubator (Binder GmbhH, Tuttlingen, Germany).

To confirm the presence of insert, colony PCR was carried out on randomly selected colonies using Green *Taq* DNA polymerase (Genscript, Piscataway, USA). The reaction mix consisted of 1 µL of 10 pmoles T7 universal primer and VAT3 and VAT4 reverse primers (Appendix 2), 1 µl of 10× GenScript PCR Buffer, 0.4 µL of 1.25 mM $MgCl_2$, 0.1 µL of 1 unit *Taq* DNA polymerase (GenScript Corporation, Piscataway, USA) then topped to 10 µl with PCR grade water. Template DNA for PCR was a single bacterial colony for

each gene. Amplification was done using a programmed thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research, Gaithersburg) at conditions which included an initial denaturation/ activation step at 94 °C for 3 min, 35 cycles consisting of denaturation at 94 °C for 30 sec, annealing at 54 °C for 1 min, and extension of 1 min 30 sec at 72 °C and a final extension step at 72 °C for 10 min. Following amplification, the PCR products (5 µl) were gel electrophoresed on a 1% agarose gel for 1 hr at 7 V/cm and visualized using the Kodak gel logic 200 imaging system.

3.1.2 Propagation and Purification of Recombinant Plasmids.

From the colony PCR, positive colonies for VAT3 and VAT4 each were cultured overnight in 5 mL LB broth supplemented with 100 µg/mL ampicillin at 37 °C. The bacterial cell cultures were cultured with vigorous shaking (150 rotations per minute) using the Environ – Shaker, 3597-1 (Lab-line Instruments Inc, Conroe, USA). Subsequently, recombinant plasmids were purified as described in the Quick-clean II Miniprep Plasmid Purification Kit from GenScript (Piscataway, USA) with minor modifications. Briefly, the cells were pelleted, resuspended in 250 µL resuspension buffer supplemented with RNAse A solution, lysed and neutralized using 250 µL and 350 µL Lysis buffer and Neitralizing buffers respectively. The plasmids were bound on the silica membrane, washed two times with Wash buffer and eluted in 35 µL Elution buffer. Three (3) µL of the eluted plasmids were electrophoresed as before (section 3.2).

Sequencing of the recombinant plasmids was outsourced from Macrogen Inc, Seoul, South Korea and analysed using BioEdit Sequence Alignment Editor *(*Hall, 1999*).* From the consensus sequences*,* bioinformatics analyses of the gene sequences were performed using BLASTn, BLASTp and ExPASy Translate Tools (Gasteiger *et al.,* 2003).

3.1.3 Cloning and Expression of VATs in Bacterial Cells

3.1.3.1. Amplification of VATs

The bacterial expression systems of choice were pRSET (Invitrogen, Carlsbad, USA) and

pBAD/His expression system. pBAD expression system was chosen after limited expression success using the pRSET-his expression system. VAT4 full gene and VAT4 gene lacking the signal sequence (designated F0 and F1, respectively) were cloned into pRSET B. VSG3 and VSG4 full genes were cloned into pBAD/His A expression.

The genes were amplified in a 50 µL PCR reaction volume consisting of 34.5 µl PCR grade water, 10 µl of 5X Phusion HF Buffer, 1 µL of 10 mM dNTPs (final concentration of 200 µM each), 1.5 µL of 10 pmoles forward and reverse primers (Appendix 2) (0.5µM final concentration), 1 µL of template DNA (sequenced recombinant plasmids) and 0.5 µL of 1 unit Phusion High Fidelity DNA polymerase (Thermo Scientific) to a final concentration of 0.02 U/µl. Step-up PCR was used to amplify the VAT genes using the PTC 100 Programmable Thermal Controller (MJ Research, Gaithersburg). The amplification conditions included an initial denaturation/activation step at 98°C for 30 sec, followed by 40 cycles of denaturation at 98 °C for 45 sec, annealing at 46 °C for 1 min (first 20 cycles) and 50 °C (for the final 20 cycles), and an extension step at 72 °C for 1 min 30 sec, followed by a final extension step at 72 °C for 10 min. The PCR products were electrophoresed as previously described and subsequently gel purified.

3.1.3.2 Gel Extraction of Amplified VAT Genes

Amplified VAT genes were excised and extracted as described in the QuickClean II Gel Extraction Kit (GenScript, New Jersey, USA). Briefly, the excised gel slices were weighed (not exceeding 40 mg) and 3 volume of binding buffer II to 1 volume of gel slice added. The gel slices were dissolved by incubation at 55 °C for 6 min, and mixed with one volume of research grade isopropanol to 1 volume of the gel slices. The extraction mixtures (750 µL) for each gene were transferred to separate spin columns, centrifuged at 6000 ×*g* for 1 min and flow through discarded. The DNA was bound to the matrix by adding of Binding buffer II (500 µL) to the spin columns, centrifuged at 12000 ×g for 1 min, and the flow through discarded. All subsequent centrifugations were carried out at this speed. The columns were washed by 500 µL wash buffer and centrifuged as before and the flow

though discarded. The spin columns were centrifuged for an additional 1 min to remove residual wash buffer, and the spin column transferred to sterile 1.5 microcentrifuge tubes. The genes were eluted using 25 µL of elution buffer, and 3 µL electrophoresed as previously described (section 3.2.1).

3.1.3.3 Cloning of amplified VAT genes into pGEM-T Easy Cloning Vector

pGEM-T Easy cloning system (Promega, Madison, USA) utilizes A/T- tail cloning strategy. Since Phusion High Fidelity DNA polymerase does not add an adenine (A) residue, polyadenylation of the gel purified PCR products was carried out using Green *Taq* DNA polymerase enzyme (GenScript). A reaction mix consisting of 7 µL of each gel purified product, 1 µL of 10× GenScript buffer, 1 µL of 2mM dATP and 1 µL (5 Units) of Taq polymerase from GenScript was prepared, mixed gently and incubated at 70 °C for 30 min. A ligation reaction mix of 10 µL was prepared consisting of 3 µL of the A-tailed PCR products, 5 µL of 2× Rapid ligation buffer, 1 µL pGEMT- Easy linear plasmids and 1 µL T4 DNA ligase. The ligation reactions were incubated overnight at 4 °C.

Five (5) µL of the ligation products for each gene were transferred into prechilled sterile 1.5 mL microfuge tubes and 50 µL of freshly thawed chemo-competent DH5α *E. coli* cells added, mixed gently by flicking, and incubated on ice for 20 min. The cells were heat shocked on a water bath at 42 °C for 45 sec, placed on ice for 2 min and Supper optimal broth with Catabolite repression (SOC) media (950 µL) (Appendix 1) added to the cells. The cells were cultured with vigorous shaking at 150 rpm in a shaker incubator at 37 °C (Environ – Shaker, 3597-1, Lab-line Instruments Inc, Conroe, USA) for 1 hr, and aseptically plated on LB/X-gal/IPTG plates supplemented with 100 µg/mL ampicillin (see appendix 1). The plates were incubated at 37 °C overnight in a stationary bench top incubator. The pGEM-T Easy vectors contain T7 and SP6 DNA polymerase promoters flanking a multiple cloning region within α-peptide coding region of the enzyme galactosidase. Insertional inactivation of the α- peptide allows identification of recombinants by blue - white screening on indicator plates. Recombinant colonies were

inferred by blue - white screening. Positive (white) colonies were grown overnight in 5 mL selective LB broth (supplemented with 100 µg/ mL ampicillin) with vigorous shaking (150 rpm) at 37 °C. The recombinant pGEMT-Easy vectors were purified using the Quick Clean II Plasmid Purification Kit from GenScript as previously described (see section 3.1.2) and 3µL of the purified plasmids analyzed in a 1% agarose. The recombinant plasmids were sequenced before the inserts were sub cloned into the respective expression vectors and analyzed for correct orientation using ExPASy Translate tool.

3.1.3.4 Sub-cloning into bacterial expression vectors

The purified recombinant cloning vectors (pGEMT Easy) and expression vectors (pBAD/ His A and pRSET B) were restricted using FastDigest restriction enzymes from Thermo Fischer Scientific. Restriction digestions using *Xho*I and *Kpn*I for the pBAD/ His A vector and *Bam*HI and *Xho*I for pRSET B expression vector were performed. The restriction reactions consisted of 12 µL nuclease free water, 3 µL 10 × FastDigest Green buffer, 13 µL plasmid DNA (1-2 µg) and 2 µL of FastDigest restriction enzymes in a final volume of 30 µL. The restriction components were thoroughly mixed by gentle flicking, spun down and incubated for 1 hr at 37 °C in a PTC-100 Programmable Thermal Controller. The enzymes were inactivated at 80 °C for 5 min and the digested products electrophoresed on a 1.5% agarose gel at 7V/cm for 1.5 hr. The expression vectors (pBAD/His A and pRSET B) and the VSGs inserts were gel purified as described in section 3.1.3.2.

The ligation reactions were set up in a total reaction volume of 10 µL, consisting of 5 µL of 2 × Rapid Ligation Buffer (Promega), 1 µL of double digested plasmids, 3 µL of double restricted VSG genes and 1 µL of T4 DNA ligase. The ligation reactions were incubated at 4 °C overnight. Recombinant plasmids were transformed into chemo-competent DH5α *E. coli* cells, propagated, the plasmids purified, sequenced and analysed as previously described. From the consensus sequences, the recombinant plasmid sequences were analyzed using BLASTn, BLASTp and ExPASy Translate Tool.

3.1.3.5 Expression and Analysis of VATs in Bacterial Cells

Bacterial strains used for expression were TOP10 and BL21pLysS for expressing genes cloned in pBAD and pRSET expression vectors respectively. TOP10 *E. coli* strain is able to transport the inducer L- arabinose but not able to metabolize it, while BL21 strain is able to transport the inducer isopropyl β-D-thiogalactosidase (IPTGT) inside the cells to induce expression of genes cloned downstream of a strong T7 promoter. TOP10 chemo-competent cells transformed with recombinant pBAD/His A and BL21pLysS chemo competent cells transformed with pRSET-B were grown on LB agar supplemented with 100 µg/ mL ampicillin and 34 µg/ mL chloramphenical (for BL21pLysS) and grown overnight at 37 °C on a bench top incubator. One positive colony for each was inoculated in 2 ml Supper Optimal Broth (SOB) (Appendix 1) with 100 µg/ mL ampicillin. The starter cultures were grown overnight with shaking at 150 rpm to an OD_{600} 1-2.

pBAD mock (pilot) expression study was carried out by sub-culturing 0.1 mL of the overnight culture into five 10 mL SOB fractions and grown at 37 °C with vigorous shaking to an OD_{600}= 0.5. One mL aliquot of cells was removed from each starter culture, centrifuged at maximum speed in a micro centrifuge tube for 30 sec, and the supernatant aspirated. The cell pellets were frozen at -20 °C, making the zero time point sample. The 5 fractions were induced with 10 fold serially diluted L-arabinose concentrations as shown in table 3-1 below. The cells were grown at 37 °C for 5 hrs with vigorous shaking at 225 rpm in a shaker incubator, centrifuged at maximum speed and the supernatant aspirated.

Table 3-1. Serially diluted L-Arabinose for protein expression pilot studies

Tube	Volume (mL)	Stock solution	Final concentration
1	0.1	0.002%	0.00002%
2	0.1	0.02%	0.0002%
3	0.1	0.2%	0.002%
4	0.1	2%	0.02%
5	0.1	20%	0.2%

Expression of recombinant VATs using pRSET B expression systems involved culturing 1 mL of the overnight culture, and the subsequent inoculation into 50 mL SOB. The bacterial cells were grown at 37 °C with vigorous shaking (225 rpm) to an $OD_{600\ nm}$ of 0.4-0.6. Time 0 samples were collected and pelleted as previously described. The cells were divided into two, with one fraction induced with 1 mM IPTG final concentration and the other fraction used as the uninduced expression control. The cells were grown at 37 °C with vigorous shaking at 225 rpm. One mL from the induced and uninduced samples were collected after every hour and pelleted as before and stored at -20 °C.

Protein expression analysis was done by one-dimensional sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) which involves dissociation of proteins into their individual polypeptide subunits and separation in accordance to the polypeptides size through migration through the polyacrylamide gels. Briefly, the protein samples previously collected were dissolved completely in 1× Laemmli sample buffer (see appendix 3). The samples were incubated at 95 °C for 7 min, spun down and 10 µL separated at 10 mA, 100 V on a 12% (wt/vol) polyacrylamide gel (Sambrook *et al*., 1987) for 2 hrs using the FTV 100Y Fischer Scientific electrophoresis system. The gels were fixed and stained with Coomassie Brilliant Blue staining solution (Appendix 3) and destained for visualization using white a light source.

3.1.3.6 Determination of the Solubility of the Recombinant VATs

Expressed recombinant proteins can either be soluble in the bacterial cells' cytoplasm or they may accumulate as insoluble aggregates called inclusion bodies due to aberrant folding. To determine the solubility of the recombinant VSG proteins expressed, the expressed pelleted cells were lysed completely by resuspension in 100 µL of 20 mM phosphate buffer (pH 7.2) (Appendix 3) and frozen in liquid nitrogen. The lysates were thawed at 42°C in a water bath. The freeze-thaw cycle was repeated three additional times, and the insoluble fractions pelleted in a microfuge for 10 min at maximum speed at 4°C. The supernatants were transferred into fresh 1.5 mL microfuge tubes. To 100 µL of

supernatant sample, an equal volume of 2× Laemmli sample buffer was added, and the pellet resuspended in 100 µL of 1× Laemmli sample buffer. The protein fractions were prepared and analysed as previously described (section 3.1.3.5).

3.1.3.7 Protein Scale Up and Purification

Two hundred and fifty (250) mL of SOB was inoculated with 500 µL of recombinant *E. coli* cells cultured overnight and grown to an $OD_{600\ nm}$ 0.4-0.6 at 225 rpm. The cells were induced with IPTG or L-Arabinose at concentrations and durations (in hours) determined by the pilot studies, grown at 37 °C with vigorous shaking (225 rpm) and the cells pelleted for 10 min at 3000 ×*g*. Purification was carried out as described in the ProBond ™ purification system (Invitrogen, Carlsbad, USA). Preparation of buffers used in the purification of recombinant proteins is shown in Appendix 4. The pelleted cells were resuspended in lysis buffer (Native binding buffer supplemented with 8mg Lysozyme enzyme) and incubated for 30 min on ice. The cells were lysed by sonication at high intensity using the Soniprep 150 MSE Ultrasonic disintegrator (MSE Ltd, London, UK) for six 10 second bursts with 10 second cooling intervals between each burst. The lysates were centrifuged at 3000 ×*g* for 15 min at 4°C and the supernatant transferred onto prepared ProBond ™ resin. The proteins were bound for 1 hr at room temperature using gentle agitation on a rotating wheel to keep the resin suspended in the lysate solution. The columns were washed four times with native wash buffer and clamped in a vertical position and the cap on the lower end snapped off. The proteins were eluted with 12 mL native elution buffer and collected in 1 mL fractions. Twenty (20) µL of the collected fractions were analyzed by one-dimensional SDS-PAGE as describe in section 3.1.3.5.

3.1.3.8 Dialysis and Quantification of Purified Recombinant VATs

Purified recombinant VSG proteins in 50 mM NaH_2PO_4, 0.5 M NaCl and 250 mM imidazole elution buffer were dialyzed against 10 mM PBS, pH 7.2 and 50 % sterile glycerol at 4 °C overnight in a ratio of 1 mL eluted VSG protein to 200 mL PBS-glycerol dialysis buffer. The dialyzed recombinant proteins were stored at -80 °C.

Dialyzed recombinant VSG proteins were quantified according to the Pierce™ BCA (Bicinconinic acid) protein assay kit (Pierce, Rockford, USA). Briefly, 2mg/mL bovine albumin serum standard was diluted using a diluents buffer composed of 0.1 M PBS (pH 7.2) and 100% glycerol in a ratio of 1:1 v/v to a final concentration of 250, 125, 50, 25, 5 and 0 µg/mL. The BCA working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA. One hundred (100) µl of the standards and VSG protein samples were transferred into sterile protein free test tubes, and 2 mL of the BCA working reagent added to each tube, mixed and incubated for 30 min at 60 °C using a water bath for even heat transfer. The tubes were cooled to room temperature and OD_{562nm} measured using the Shimadzu UV-Visible Spectrophotometer BioSpec-mini (Kyoto, Japan). The concentrations of the recombinant VSG genes were determined using the standard curve.

3.1.4 Cloning and Expression of VATs in Insect Cells

3.1.4.1 Amplification of VATs

pIZ/V5-His (Invitrogen, Carlsbad, USA) was the insect expression vector of choice. It utilizes Orgyia pseudotsugata Immediate Early promoter (OpIE2) from the multicapsid nuclear polyhedrosis virus (OpMNPV). The early promoters utilize the hosts' transcription machinery and do not require viral factors for activation. The forward primer included a Kozak translation initiation sequence (**G/A**NNATG**G)** while the reverse primer had an enterokinase cleavage recognition sequence (GACGATGACGATAAG) (Appendix 2). The amplification was done as described in section 3.2.1 with the exception of the annealing temperature used (48 °C and 52 °C). The amplification products were electrophoresed and gel purified as earlier described (see sections 3.1.3.1 and 3.1.3.2).

3.1.4.2 Cloning VAT genes into pGEM-T Easy cloning vector

The amplified genes were excised, A-tailed and ligated into pGEMT Easy cloning vector as described in section 3.1.3.3. Chemo-competent DH5α cells were transformed by 5 µLs of the ligation products as earlier described. Positive colonies confirmed by blue – white screening were propagated as described and the recombinant plasmids purified and

sequenced. The sequences were analyzed using BioEdit Sequence Alignment Editor. From the consensus sequences, the VAT sequences were analyzed using BLASTn, BLASTp and ExPASy Translate Tool.

3.1.4.3 Sub-cloning of VAT genes into the pIZ/V5-his expression vector

The recombinant pGEMT-T Easy plasmids and pIZ/ V5-His expression vectors were restricted as described in section 3.3.3 with FastDigest *Bam*HI and FastDigest *Xho*I restriction endonucleases. The restricted expression vector and VAT genes were gel extracted and ligated as earlier described and the recombinant plasmids propagated, purified and sequenced as earlier described.

3.1.4.4 Culturing *Spodoptera frugiperda* (Sf21) Insect Cells

Cryopreserved Sf21 cells (Invitrogen, Carlsbad, USA) in freezing medium (Appendix 5) were cultured in a 25 cm^2 culture flask with 4 mL complete TNM FH medium (Appendix 5) supplemented with penicillin-streptomycin at a final concentration of 100 U/mL and 100 µg/mL respectively. The cells were evenly distributed across the flasks surface and viewed under an inverted microscope. The cells were allowed to attach for 45 min inside a sterile 27 °C, non- humidified, non- CO_2 incubator (Eppendorf- New Brunswick Galaxy 48 S incubators, Edison, USA). After attachment, the medium with floating non-adherent cells was replaced with 5 mL of fresh complete TNM-HH medium supplemented with penicillin-streptomycin. The cells were cultured at 27 °C until they reached mid log phase at about 90% confluence (approximately 3.8×10^6 cells). The cells were scaled up to approximately 1.1×10^7 cells in a 75 cm^2 culture flask and grown to confluence (approximately 1.1×10^7 cells).

3.1.4.5 Transient Expression in Insect Cells

The cells were sloughed off with fresh medium as described before, and 1.2×10^6 cells transferred into each well of a 6 well 60 mm tissue culture dishes. The cells were incubated in a 27 °C incubator for 45 min to allow the cells to fully attach to the bottom of the dishes to form a monolayer of cells. The cells were subsequently viewed under an inverted

microscope to verify adherence. Transfection mixtures were prepared in sterile 1.5 mL microcentrifuge tubes, and consisted of 1 mL Grace insect media, Unsupplemented, 2 µg/µL plasmid constructs (pIZ/V5-His, VSG 3-pIZ/V5-His, VSG 4-pIZ/V5-His and GFP -pIZ/V5-His) and 20 µL Cellfectin II reagent (Invitrogen, Carlsbad, USA). The transfection mixtures were gently mixed and incubated at room temperature for 15 min. The medium from the cells was carefully removed without disrupting the monolayer, the cells washed completely with Grace insect media, Unsupplemented, and each transfection mixture added to a separate well. Three replicates for each transfection were included.
The culture dishes were incubated at room temperature for 5 hr on a side-to-side, rocking platform at 2 side to side motions per min. Complete TNM-FH medium (2 mL) was added to each well and the dishes placed in a humidified 27 °C incubator. The cells and medium were collected separately at 48, 72 and 96 hrs post-transfection and assayed for expression of the target recombinant VSG proteins and fluorescence.

3.1.4.6 Testing for Expression of recombinant VATs

Twenty (20) µL of cells transfected with pIZ/V5-His empty vector and pIZ/V5-His GFP constructs were fixed onto glass slides using an equal volume of 4% formaldehyde. The cells were viewed under Light-emitting Diode (LED) and fluorescence using the automated iMIC microscope (Till Photonics, Gräfelfing, Germany) Phantom camera.

Cells and medium from wells containing Sf 21 cell lines transfected with pIZ/V5-His empty vector, pIZ/V5-His – VAT3 and pIZ/V5-His - VAT4 constructs were harvested and assayed for expression using one dimension 12% SDS-PAGE. The cells were resuspended in 100 µL of 1X Laemmli sample buffer, while 100 µL of the medium was mixed with 100 µL of 2x Laemmli sample buffer. The samples were boiled for 10 min and 10 µL loaded onto a 12% SDS polyacrylamide gel. Electrophoresis was carried out at 10 mA and 100 V as previously described. The gels were fixed and stained with Coomassie Brilliant blue and destained to visualize the proteins.

3.1.4.7 Scale-up, Purification and Quantification of Recombinant VATs

Sf21 cells were cultured in three 75 cm^2 culture flasks using Sf-900™ SFM to confluence. The cells were transfected as before with 2µg pIZ/V5-His empty vector, pIZ/V5-His – VAT3 and pIZ/V5-His - VAT4 constructs, and incubated at 27 °C for 96 hr. The cells and the media were harvested separately. The cells were resuspended in 8 mL Native binding buffer (Appendix 4), the cells lysed by two freeze thaw cycles and passed four times through an 18-gauge needle and centrifuged at 3000 ×g for 15 min. Proteins in the medium were dialyzed against Native binding buffer as well. The lysate and dialyzed secreted proteins were bound for 30 min at room temperature on a rotating wheel. The resin was settled by low speed centrifugation (800 ×g) and the supernatant discarded. The column was washed and the proteins eluted as described before (section 3.3.5.4). Subsequently, the eluted proteins were analyzed by one dimensional SDS PAGE, and subsequently dialyzed against 0.1M PBS – 100% glycerol overnight as previously described and stored at -80°C. Dialyzed recombinant VSG 3 and VSG 4 antigens were quantified using the BCA protein assay as described in section 3.1.3.8.

3.2 Evaluation of the Diagnostic Potential of the Generated Recombinant VATs

3.2.1 Infection of Monkey disease models and sera collection

Previously, Thuita *et al*. (2008) had infected nine monkeys with four different *T. b. rhodesiense* strains (with varying pathogenicity and collected from different disease foci in Kenya and Uganda). Briefly, nine vervet monkey models (*Chlorocebus aethiops*) were infected via a single bite of tsetse infected with *T. b. rhodesiense.* The monkeys were randomly allocated to four experimental groups, and each group infected with a different strain of *T. b. rhodesiense* as follows;

Table 3.2: A table showing the different *T. b. rhodesiense* strains used for infecting monkeys belonging to four experimental groups, and their years of isolation.

T. b. rhodesiense strain	Geographic region of strain isolation	Year of strain isolation	Monkeys per experimental groups	Monkey tags
3741	Busoga, Kenya	2003	3	476,515,536
3804	Bukhayo, Kenya	1989	2	523, 579
3801	Busia, Kenya	1989	2	556,574
3804	Tororo, Uganda	1972	2	554,555

Blood was harvested from monkeys from all the experimental groups after the 3rd, 7th, 11th, 15th days post infection (dpi) representing early stage of disease and 19th dpi representing onset of late stage disease. The collected blood was stored at -80°C. Twelve sera samples available at *icipe's* cryo-bank were used in this study, with each *T. b. rhodesiense* strain represented. Sera collected from two uninfected monkeys were used as controls in the immunoassays.

3.2.2 Serum and Immunodiffusion Gel Set-up

Archived monkey blood samples infected with *T. b. rhodesiense* were thawed on ice and centrifuged at 1000 ×g for 15 min using the 5417R Eppendorf bench top centrifuge. The serum was carefully aspirated from the pelleted erythrocyte cells and transferred to sterile 1.5 mL micro tubes. Sera from two uninfected monkeys were included as controls in this study, and were prepared as described above.

One gram of agarose, molecular grade, was dissolved in 50 mL 0.1 M PBS (pH 7.2) supplemented with 0.03% sodium azide (w/v), and dissolved completely by boiling, then cooled to 55 °C. Three (3) g of polyethylene glycol (PEG) was dissolved in 50 mL 0.1 M PBS (pH 7.2) with supplemented with 0.03% (w/v) sodium azide and dissolved completely by warming it to 55 °C. The agarose and PEG solutions were mixed in equal

volumes to make a final concentration of 1% and 3% respectively, and the gel cast on clean dry glass plates. Holes were punctured on the gel using a gel punch and a water vacuum pump used to carefully aspirate the gel from the punctured holes.

3.2.3 Evaluation of Diagnostic Potential via Ouchterlony Immuno-assays

The expressed purified recombinant antigens (15 µL) were loaded in the central well and the same volume of neat monkey serum loaded in the peripheral/ outer wells, separated by a well of 15 µL 0.1 M PBS, pH 7.2. Negative controls of monkey sera collected from none-infected monkeys were also assayed. Care was taken to avoid over filling or under filling the wells. The agarose plates were placed in moist air tight plastic containers, and incubated for 24-48 hrs at 37°C. The formation of white precipitin lines was monitored by viewing the plates in a dark field light viewing box. For permanent results, the plates were stained using Coomassie brilliant blue after rinsing the plates overnight with 0.1 M PBS, pH 7.2 and drying/ pressing the agarose overnight. The plates were viewed and photographed.

CHAPTER FOUR

4.0 RESULTS

4.1 Generation of Recombinant VATs in Bacterial and Insect Expression Systems

4.1.1 Expression of Recombinant VATs in Bacterial Cells

The amplification of VSG genes from recovered cryopreserved bacterial colonies by colony PCR showed DNA bands of approximately 1.5 kb product for both VAT3 and VAT4 in a 1% ethidium bromide stained agarose gel (Figure 4-1A), confirming presence of these genes in the cryopreserved bacterial stabilates. Sequence analysis confirmed the sizes of the VSG genes to be 1452 and 1449 basepairs respectively. BLASTn and BLASTp resulted in hits closely similar to trypanosome VSGs. Amplification of VAT3 and VATs4 genes with primers having restriction sites to enable cloning of the genes into bacterial expression vectors (both pRSET and pBAD) gave approximately 1.5 kb for full genes (Figure 4-1B), while amplification of VSG 4 without the signal peptide sequence yielded a 1382 bp product. After cloning, restriction of recombinant pGEMT-Easy and pRSET B plasmids showed two bands at approximately 3 kb and 1.5 kb for the linearized pGEMT Easy/pRSET B plasmids and VSG genes respectively (Figure 4-1C). Sequence analysis by ExPASy translate tool confirmed successful cloning of VAT3 and VAT4 genes into pBAD/His A at *Xho*I and *Kpn*I and pRSET/His B at *Bam*HI and *Xho*I sites. Moreover, the genes were shown to be cloned in frame with the N terminal six histidine tags (Figure 4-1D), necessary for subsequent purification after expression of the recombinant proteins. The gene sequence results of VATs will not be presented here, because Masiga and colleagues, still have interests in these sequences.

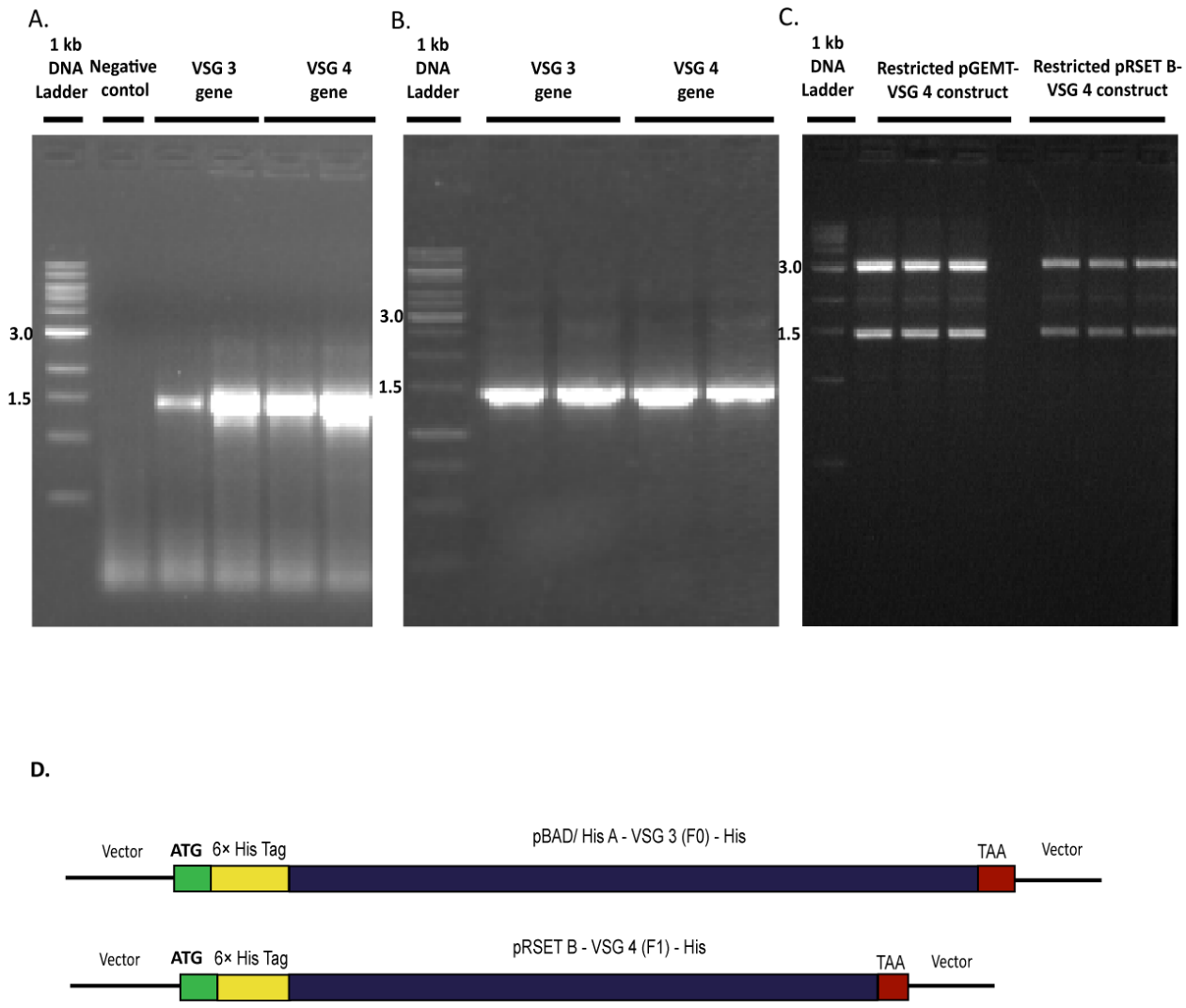


Figure 4-1: Results of VAT3 and VAT4 amplification, cloning and bioinformatics analysis. (A) Amplification of cryopreserved bacterial stabilates by colony PCR and (B) Subsequent amplification with primers for cloning into pBAD or pRSET bacterial expression vectors produced 1.5 kb products. (C). Restriction digestion of recombinant pGEMT Easy and pRSET/ His B vectors after cloning. (D) A schematic representation of recombinant bacterial expression vectors after sequence analysis, showing successful cloning of the genes in frame with the N- terminal 6 his tag.

Recombinant VATs were successfully expressed after transformation of competent *E. coli* cells and induction with 1M IPTG or 0.02% L-Arabinose for pRSET B and pBAD/His A

expression vectors respectively and grown for 4 hrs post induction, as shown in figure 4-2 below. Purification of the recombinant 6× his-tagged VSG 3 and VSG 4 proteins expressed using pRSET and pBAD expression vectors using immobilized metal affinity chromatography was also successful as shown in panels (B) and (C) respectively.

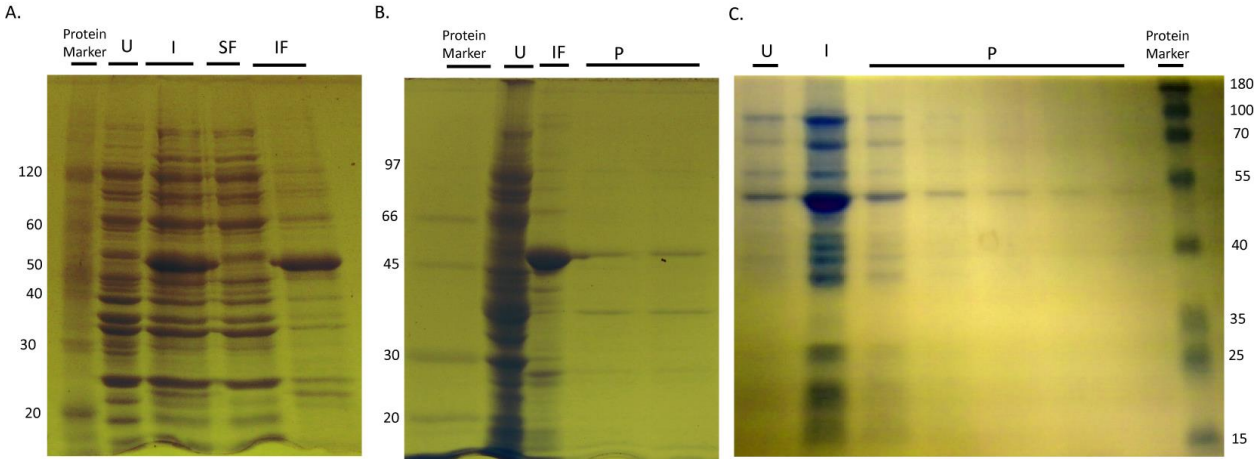


Figure 4-2: SDS-PAGE analysis of expression and purification of recombinant VAT3 and VAT4 in bacterial cells. **(A)** Recombinant VAT4 (full gene) expressed in pRSET B bacterial expression system gave a product of approximately 55 kDa. **(B)** VAT4 without the signal peptide, (F1), expressed using pRSET B expression system gave a product of about 45 kDa. **(C)** Full length VAT3 recombinant was expressed using pBAD/His A expression system, similarly giving a product at about 55 kDa. U, Uninduced total cell lysate control; I- Induced total cell lysate; SF, Soluble fraction; IF, Induced insoluble fraction; P- Purified recombinant VSG protein.

4.1.2 Expression of Recombinant VATs in Insect Cells

Amplification of VAT3 and VAT4 genes with primers for cloning into *Bam*HI and *Xho*I sites of the pIZ/V5-His insect expression vectors were successful, as confirmed by agarose gel electrophoresis of amplified products, restriction of the recombinant plasmids with *Bam*HI and *Xho*I restriction endonucleases, and subsequent sequence analysis (Figure 4-3). Analysis of the sequenced recombinant plasmids by ExPasy Translate Tool confirmed that the two genes were in their correct ORFs and had been cloned in frame with the C terminal histidine tag.

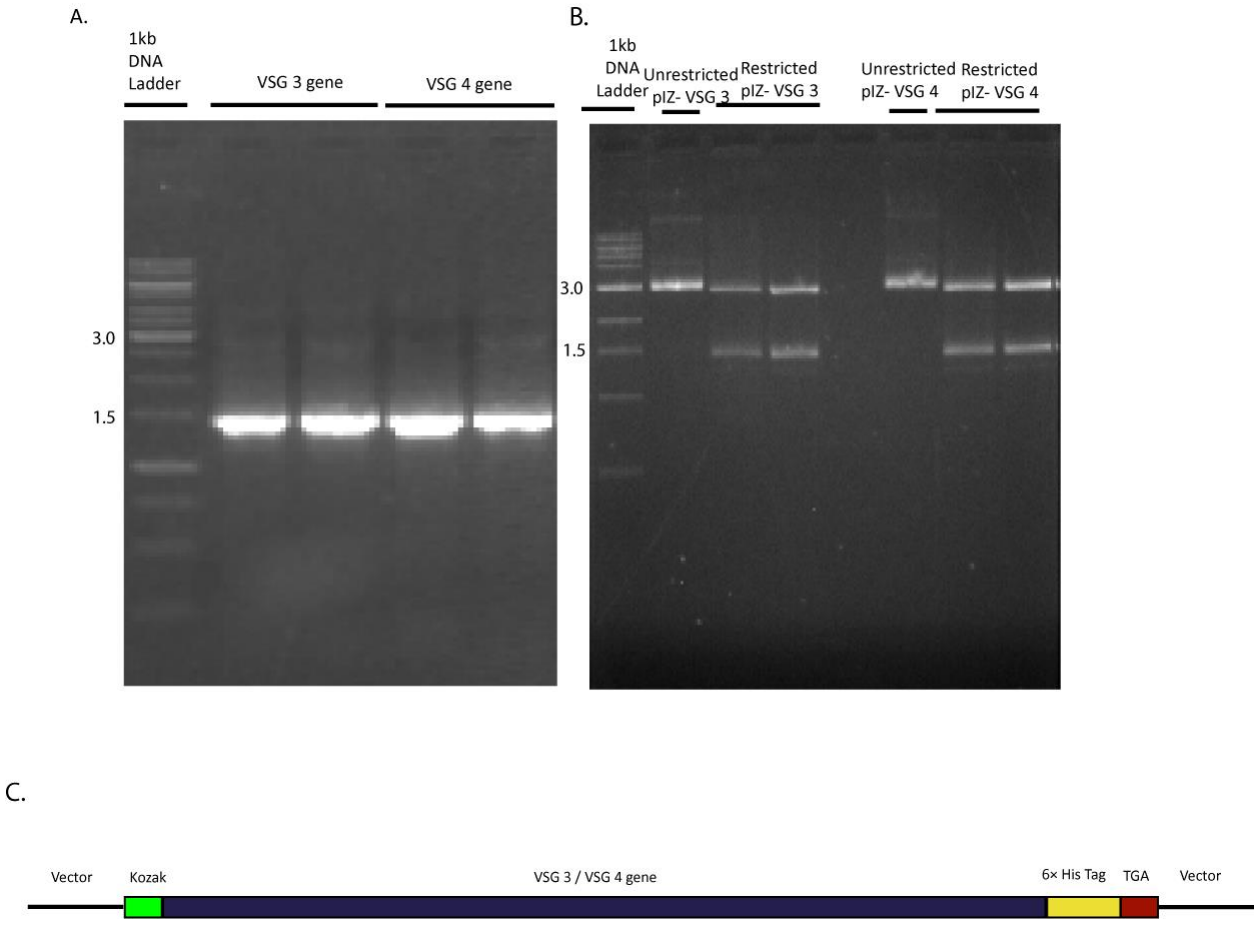


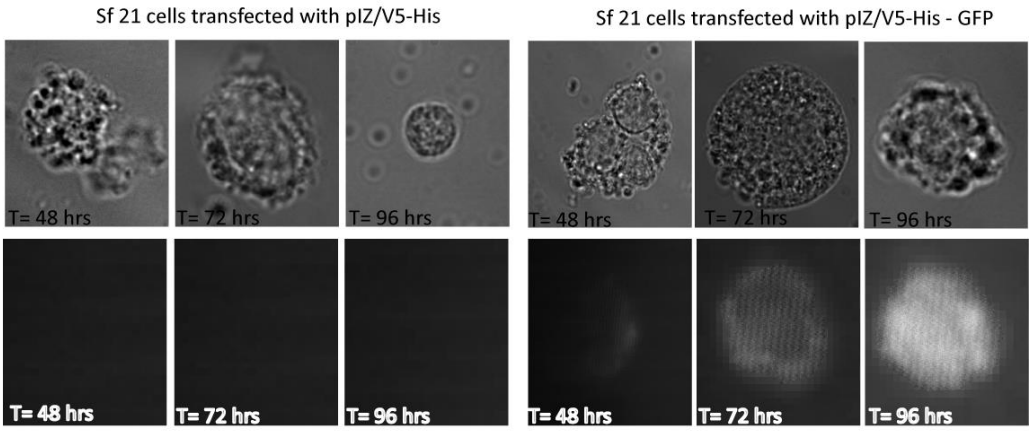
Figure 4-3: Agarose gel results of VAT3 and VAT4 gene amplification and bioinformatics results of recombinant pIZ/V5-his constructs. (A). Amplification of VAT genes produced approximately 1.5 kb DNA products. (B). Restriction digestion of recombinant pIZ/V5-his VAT3 and pIZ/V5-his VAT4 constructs produced two bands at 3 kb and 1.5 kb, representing vector DNA and VSG DNA inserts respectively. (C) A schematic representation of recombinant pIZ/V5-His – VSG 3 or pIZ/V5-His – VSG 4 constructs after sequence analysis.

A green fluorescent protein gene (GFP) cloned into pIZ/V5-His expression vector included as a positive control for expression was analyzed for both transfection efficiency and expression in Sf 21 cell lines. The images of Sf 21 cells transfected with pIZ/V5-His – GFP recombinant construct were compared to images of Sf 21 cells transfected with an empty pIZ/ V5-His expression vector (Figure 4-4A). The cells transfected with an empty

expression vector did not emit fluorescence under UV light at 48, 72 and 96 hrs post transfection. Cells transfected with a recombinant pIZ/V5-His carrying the GFP gene fluoresced under UV light, with the fluorescence increasing with time.

Sf 21 cells transfected with recombinant pIZ/V5-His – VSG 3 and pIZ/V5-His – VSG 4 constructs and pIZ/V5-His only control by lipid mediated transfection, harvested after 48, 72 and 96 hrs post transfection and analyzed for expression by SDS-PAGE, and showed that VSG proteins were expressed and extracellularly secreted into the growth media (Figure 4-4B). The recombinant proteins were approximately 55 kDa. Media from cells transfected with pIZ/V5-His only control did not show the prominent band at 55 kDa.

A.



B.

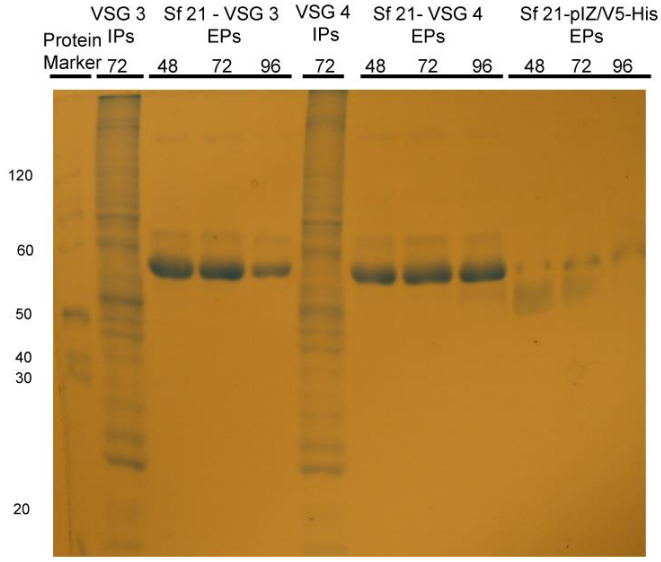


Figure 4-4. Microscopy and SDS PAGE results of recombinant protein expression in insect cells (Sf21 cell line. (A). Sf 21 cells transfected with pIZ/V5-His – GFP recombinant construct showed fluorescence under UV light, illustrating successful transfection and expression of the green fluorescent protein using the pIZ/V5-his insect expression vector. (B) Expression of VAT3 and VAT4 recombinant antigens showed a 55 kDa protein in the secreted fraction. Protein ladder is shown next to the gel image. IPs-intracellular proteins, EPs-Extracellular proteins.

4.2 Evaluation of the Diagnostic Potential of the Expressed Recombinant VATs

4.2.1 Serodiagnostic Potential of Recombinant VAT3 and VAT4

Following expression and purification, recombinant VAT3 and VAT4 were dialyzed and serologically assayed against archived monkey serum infected with *T. b. rhodesiense*. Twelve monkey sera samples infected with four different *T. b. rhodesiense* strains collected 3, 7, 11, 15 and 19 days post infection were assayed against the recombinant VATs expressed in bacterial and insect expression systems. Formation of precipitin lines of immuno-recognition between the expressed recombinant variable surface antigens and anti VSG specific antibodies in the sera samples was observed as shown in figure 4-5. Sera collected from two uninfected healthy monkeys did not form immuno-precipitin lines, illustrating the specificity of the recombinant antigens.

From the immunoassays, the detection rates (in percentage) were calculated for the recombinant VATs against the assayed infected monkey sera by dividing the number of times the recombinant VATs formed precipitin lines of immunorecognition by the total number of infected sera samples assayed then multiplied the ratio by 100. Recombinant VAT3 and VAT4 expressed in bacterial cells had detection rates of 88% and 0% respectively, while VAT3 and VAT4 expressed in insect cells had detection rates of 97% and 83% respectively.

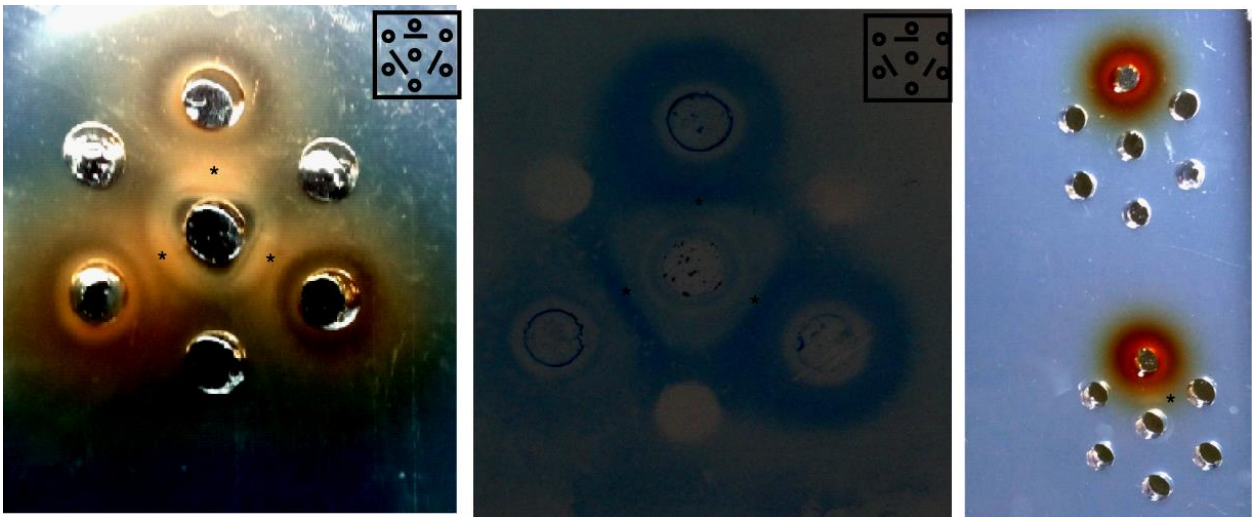


Figure 4-5. Ouchterlony double diffusion results showing immune recognition of recombinant VATs and sera collected from *T. b. rhodesiense* infected monkeys. Precipitin lines formed between **(A)** recombinant VAT3 expressed in bacterial cells and **(B)** recombinant VAT4 expressed in insect cells against sera collected from infected monkeys **(C)** Precipitin lines did not form when sera collected from uninfected monkey was reacted against VAT3 expressed in bacterial cells (upper pattern), while the same antigen formed precipitin lines against sera collected from an infected monkey (lower pattern).

4.2.2 Potential Application of VATs for Early Disease Detection

Twelve monkey sera samples infected with four different *T. b. rhodesiense* strains collected 3, 7, 11, 15 and 19 days post infection were assayed against the recombinant VATs expressed in bacterial and insect expression systems. The serological assays were repeated three times. The formation of a white precipitin lines, indicating immuno-recognition between the expressed antigens and anti-VSG specific antibodies, were observed after a 24 hr incubation, and are represented by (+) in Table 4-1 below. Absence of immuno-recognition is represented by (-). Sera collected from uninfected monkeys did not form precipitin lines with all the expressed recombinant antigens.

The sera samples assayed were collected 3, 7, 11 and 15 dpi (representing early stage disease) and 19 dpi (representing onset of late stage disease) from monkey models infected by the bite of a single infected tsetse fly to mimic the natural infection route in man. Four

strains of *T. b. rhodesiense* collected from different geographic regions in Uganda and Kenya were used to infect the monkey models (Thuita *et al*., 2008). Precipitin lines were formed between VSG specific antibodies and the expressed recombinant VATs from sera samples collected as early as 3 dpi through to the 15th dpi representing the early stage/haemolymphatic stage of the disease, and 19 dpi when the parasite had crossed into the CNS (Table 4-1).

Table 4-1: Diagnostic performance of recombinant VATs against monkey sera samples infected with four different *T. brucei rhodesiense* KETRI strains collected at different days post infection (dpi). Uninfected sera controls are also shown. Each immunoassay was repeated three times. + represents immunorecognition between recombinant VATs and anti-trypanosome specific antibodies in the serum, while – represents absence of immunorecognition for each experiment.

Monkey experimental group	*T. b. rhodesiense* strain	Geographic region of strain isolation	Days post infection (dpi)	Bacterial expression system		Insect expression system	
				VSG 3	VSG 4	VSG 3	VSG 4
552	KETRI 3741	Busoga, Kenya	3	+++	---	+++	++-
554	KETRI 3928	Tororo, Uganda	3	+++	---	+++	+++
554	KETRI 3928		7	+-+	---	+++	+++
555	KETRI 3928		3	+++	---	+++	-++
555	KETRI 3928		7	+-+	---	+++	+-+
555	KETRI 3928		11	+++	---	+++	+++
555	KETRI 3928		15	+++	---	+++	+++
555	KETRI 3928		19	-++	---	-++	-++
556	KETRI 3804	Bukhayo West, Kenya	3	+++	---	+++	+++
574	KETRI 3804		3	-++	---	+++	+++
574	KETRI 3804		7	+++	---	+++	+-+
657	KETRI 3801	Busia, Kenya.	7	+++	---	+++	+++
716	Uninfected		N/A	---	---	---	---
720	Uninfected		N/A	---	---	---	---

CHAPTER FIVE

5.0 DISCUSSION

5.1 Generation of Diagnostic Recombinant VATs in Bacterial and Insect Expression systems

Recombinant VAT3 and VAT4 were successfully generated in bacterial and insect cells and subsequently purified as histidine tagged fusion proteins (the yields are shown in Appendix 6, Table A-2). The VATs expressed from full length VSG genes were of the same molecular weight irrespective of the expression systems, suggesting that post translational modification possibly did not occur in insect cells. Expression in bacterial system was fast (between 3–4 hours post induction), reproducible and used inexpensive culture medium. On the contrary, expression in insect cells was time consuming (3-4 weeks of cell culture followed by 48-96 hrs post transfection) and used expensive culture medium. Overall comparison of the two expression systems however indicates that expression in insect expression system results in more recombinant protein yields and an easier purification procedure. Removal of the fusion tag from the purified recombinant proteins is possible using enterokinase enzyme, however immuno-recognition of the 6 histidine tagged VATs by antibodies in the sera samples assayed suggests that the histidine tags (both N and C terminally placed) do not alter epitope conformations during folding of the proteins in the two expression systems.

A VAT diagnostic tool based on recombinant antigens offers advantages over the use of native antigens. For example, the CATT/*T. b. gambiense* mass screening tool is based on natively produced LiTat 1.3 VSG antigen. The diagnostic antigen is mass produced by infecting laboratory rodents using human infective *T. b. gambiense* parasites expressing LiTat 1.3 VSG. The use of recombinant antigens eliminates the need to culture human infective trypanosomes. Moreover, generation of recombinant antigens is cheaper and easy to standardize, ultimately leading to affordable diagnostic tools (Rogé *et al.*, 2014).

Several diagnostic antigens have been expressed in heterologous expression systems and successfully applied in diagnosis of human diseases, further increasing confidence in the recombinant antigens assayed in this study. For instance, a fungal disease termed paracoccidioidomycosis (PMC) caused by *Paracoccidioides brasiliensis*, has been shown to be diagnosed using an exocellular glycoprotein gp43 expressed in *E. coli* cells (Diniz *et al.*, 2002). Insect expression systems have also been used in the generation of diagnostic antigens. In 2001, Urakawa and colleagues expressed a variable surface glycoprotein specific to *T. evansi* in Sf 21 cells, and showed their potential in diagnosis of *Surra* disease in livestock.

5.2 VATs Have Diagnostic Potential for Antibody Detection

This study set out to evaluate the diagnostic potential of two VSGs predominantly expressed during early stages of *T. b. rhodesiense* infection as potential candidates for diagnosis. Immunorecognition of the purified recombinant antigens by anti-VSG specific antibodies in monkey sera infected with *T. b. rhodesiense* illustrated the diagnostic potential of the recombinant antigens assayed (Figure 4-5; Table 4-1). The interaction is specific between VAT epitopes and anti VSG specific antibodies, and results in the formation of precipitin lines. Recombinant VAT4 expressed in bacterial cells did not react with the sera samples assayed, probably due to low yields (see Table A-2), or aberrant folding of the recombinant VAT. Non reactivity of expressed recombinant VATs with sera from uninfected infected monkey controls indicates the specificity of these antigens.

Monkey models have been shown to mimic HAT clinically, immunologically and pathologically (Schmidt & Sayer, 1982), validating the use of sera from vervet monkeys in this study as a model for HAT disease. The ability of the expressed recombinant antigens to be immunologically recognized by antibodies from *T. b. rhodesiense* infected monkeys gives confidence that they can be potent diagnostic markers for screening specific anti VAT3 and anti VAT4 antibodies in human serum samples. Furthermore, the two VSGs assayed were shown to be predominant in the four different strains of *T. b. rhodesiense*

isolated from different locations, increasing the geographic user range of the VSG diagnostic candidates.

The confirmation of diagnostic potential of the individual recombinant antigens offers a valuable alternative to parasitological demonstration of parasites in body fluids, the recommended diagnostic gold standard by WHO, which suffers from low sensitivity and specificity, leading to approximately 30% of individuals being missed (Chappuis *et al*., 2005). Even though antibody detection tools do not offer direct evidence for the presence of trypanosomes, a VAT based diagnostic tool able to detect antibodies elicited upon contact with the parasite could form more potent diagnostic tools for disease detection compared to parasitological tools which are in addition not amenable to mass screening. The ability of expressed recombinant VATs to show immuno-reactivity with sera collected three days post infection (dpi) up to 15 dpi demonstrate potential application in early diagnosis of sleeping sickness caused by *T. b. rhodesiense*. The mean peak parasitaemia in the monkey models used was 3-4 days, with an overall median of parasite penetration into the CSF at 16 dpi (Thuita *et al.*, 2008). Early treatment of sleeping sickness caused by *T. b. rhodesiense* is essential since treatment of late stage disease is risky and complicated due to the toxicity of Melarsoprol, the only drug option available (Kennedy, 2013). The assayed recombinant VATs show promise as ideal diagnostic candidates for early disease detection. Moreover, detection of specific VSG 3 and VSG 4 antibodies at 19 days, which represents the late stage of the disease when the parasite has crossed into the CNS, suggests potential application of assayed VATs in diagnosis of sleeping sickness during both stages of the disease. However, antibody detection tests are limited by the fact that as antibodies remain in circulation days after their production by host's immune system, detection of circulating antibodies instead of antigens could lead to false positives after treatment or self cure. One way of resolving this limitation would be combing the VSGs with other diagnostic candidates such as ISG 65 and ISG 75 (Ziegelbauersq & Overaths, 1992). These invariant surface glycoproteins have been reported to be highly immunogenic, eliciting production of specific antibodies, and are potent diagnostic

molecules for sleeping sickness (Sullivan *et al*., 2013; Sternberg *et al*., 2014). Parasitological tests that also demonstrate parasites during active infection can be applied following screening to confirm and/or discriminate active infection from cured cases.

In the absence of a serological tool for screening populations at risk and animal reservoirs of *T. b. rhodesiense*, this study describes the potential application of recombinant VSG antigens applicable in distinguishing mixed infections, especially in Uganda in combination with the CATT/*T. b. gambiense* mass population screening tool. Currently, no equivalent of the CATT/ *T. b. gambiense* serological screening diagnostic tool is available for screening populations at risk of *T. b. rhodesiense* infection (Chappuis *et al*., 2005). The potential convergence of Gambian and Rhodesian HAT in Uganda (Picozzi *et al.*, 2005) complicates diagnosis, treatment and epidemiological studies of the disease, and the introduction of a sensitive, robust and field applicable serodiagnostic tool for screening *T. b. rhodesiense* infections would facilitate early and accurate diagnosis and thus safe and correct treatment in foci where the two form of the disease occur. The CATT/*T. b. gambiense* and LATEX/*T. b. gambiense* are serological tests based on lyophilized trypanosomes expressing LiTat 1.3 and purified LiTat 1.3, 1.5 and 1.6 variable surface antigens, respectively. These are predominantly expressed VSGs which agglutinate with *T. b. gambiense* specific antibodies. A VAT – antibody test for *T. b. rhodesiense* based on predominant variable surface antigens could form the basis for development of a similar serodiagnostic tool to screen both humans and animal reservoirs of sleeping sickness, and improve surveillance and epidemiological studies impacting control strategies. Serological assays are cheap, amenable to field conditions and do not require material resources.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMENDATIONS

6.1 CONCLUSIONS

This study delivers proof of concept that assayed VAT recombinant antigens predominantly expressed during early onset of *T. b. rhodesiense* infection have diagnostic potential. The diagnostic potential of each separate recombinant VAT was confirmed by ouchterlony double immunodiffusion test on sera collected from monkeys infected with different *T. b. rhodesiense* strains and two negative sera samples. VSG3 and VSG4 recombinant antigens could form the basis for development of a simple, cheap, robust and sensitive diagnostic tool applicable in the field setting. Moreover, detection of the expressed variable surface antigens by antisera collected at 3-15 dpi from monkey models infected with different *T. b. rhodesiense* strains suggests that these antigens have potential to be important candidates for consideration in development of a novel diagnostic tool for early disease detection. However, further evaluation and testing is required with a large panel of HAT positive and negative sera collected from endemic and non endemic areas, and sera collected from patients suffering from other infections which co-exist in sub Saharan Africa such as malaria and Leishmaniasis to assay for cross reactivity.

6.2 RECOMMENDATIONS

This study suggests that recombinant VAT expressed predominantly by *T. b. rhodesiense* have diagnostic potential for early case disease detection as shown by ouchterlony test, a simple serological test. Moving forward, a larger panel of VSGs expressed predominantly during early stages of infection should be assayed, both as single antigens and/or in combination, to form a more robust panel of antigens which could be included in combination to develop a serodiagnostic tool. This will improve the geographic user range for this test, as different strains of *T. b. rhodesiense* exist (Barry & McCulloch, 2001).

Secondly, screening the recombinant antigens with a large panel of human sera from

endemic and non-endemic areas needs to be carried out, with a more sensitive serological test such as ELISA before development and deployment of rapid kits based on these antigens. This will enable the determination of the sensitivity, specificity with the current diagnostic tools currently available for the diagnosis of *T. b. rhodesiense* sleeping sickness.

Thirdly, the diagnostic performance of the assayed variable surface antigens could be improved by testing them in combination with other highly immunogenic trypanosome antigens. Two predominantly expressed invariant surface glycoproteins, ISG65 and ISG75, expressed specifically in bloodstream form trypanosomes (Ziegelbauersq and Overaths, 1992), form good combination antigens to improve the sensitivities and specificities of the two assayed variable surface antigens. ISG65 and ISG75 have been used in combination for diagnosis of Gambian HAT (Sternberg *et al.*, 2014), and could be used in combination with the assayed recombinant antigens in this study to form a basis for the development of a cheap, simple, robust and field applicable diagnostic tool for diagnosis of *T. b. rhodesiense* HAT.

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APPENDICES

Appendix 1: Bacterial Culture and Transformation media

a) Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution 0.1M contains 1.2g IPTG dissolved in 50ml distilled water and stored at 4°C

b) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) 2ml was prepared by dissolving 100g of 5-bromo 4-chloro 3-indolyl β-D-galactoside in 2ml of N, N'-dimethyl-formamide. It is stored at -20°C in an aluminium foil covered container.

c) Luria-Bertani (LB) medium: 10g Bacto-tryptone, 5g Bacto-yeast extract and 5g NaCl was dissolved in a litre of distilled water and its pH adjusted to pH 7 using NaOH. This was then autoclaved before use.

d) LB plates with ampicillin, IPTG and X-Gal: 15g agar was added to a litre of LB medium then autoclaved. This was allowed to cool to 50°C before ampicillin was added to a final concentration of 100µg/ml.

e) 2M Mg^{2+} stock: Prepared by mixing 20.3g $MgCl_2 \bullet 6H_2O$ and 24.65g $MgSO_4 \bullet 7H_2O$ and distilled water to a final volume of 100ml then filter sterilized.

f) Supper Optimal Broth: 100 mL was prepared by mixing 2g of bacto-tryptome, 0.5g bacto-yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml 2M Mg^{2+} stock (filter sterilized) and distilled water to a final volume of 100ml.

g) Super Optimal broth with Catabolite repression (SOC) medium: 100ml was prepared by mixing 2g of bacto-tryptome, 0.5g bacto-yeast extract, 1ml 1M NaCl, 0.25ml 1M KCl, 1ml 2M Mg^{2+} stock (filter sterilized), 1ml 2M glucose (filter sterilized) and distilled water to a final volume of 100ml.

Appendix 2: Primer sequences

Table A-1: Sequences of primers used to amplify VAT3 and VAT4 genes for subsequent cloning into pRSET, pBAD and pIZ/V5-His expression system in frame with the 6-his tag

Gene and Vector	Primer Sequence (5' to 3')
T7 Promoter	TAATACGACTCACTATAGGG
VSG 3-pBAD/His A	**For**: GTG*CTCGAG*ATGCGGCCCACCACTTTAG **Rev**: GGCGGTACCATTAAAAAAGCAAAACTGC
VAT4-pBAD/His A	**For**: GTG*CTCGAG*ATGCGGCCCACCACTTTAG
	Rev: GGCGGTACCATTAAAAAAGCAAAACTGC
VAT4-pRSET B (F0)	**For**: CAGGATCCGATGCGGCCCACCAC
VAT4-pRSET B (F1)	**For**:GCGGATCCAATCACACAACCGTGTGAGGAAG
	Rev: ACTCGAGCCATTAAAAAAGCAAAACTGC
VAT3-pIZ/V5-His	**For**: GCGGGATCCATGCGGCCCACCACTTTAGC **Rev**:GC*CTCGAGCG*ATCCTTATCGTCATCGTCAAAAAGC AAAACTGCAAGCC
VAT4-pIZ/V5-His	**For**: GCGGGATCCATGCGGCCCACCACTTTAGC **Rev**:C*CTCGAGCG*ATCCTTATCGTCATCGTCAAAAAGCA AAACTGCAAGCC

Appendix 3: SDS-PAGE Buffers

Electrophoresis/Running Buffer: 1000mL was prepared by mixing 15.10 g Tris base, 94 g of Glycine and 5.0g SDS. The pH was 8.3 without adjusting.

a) Laemmli sample buffer: 10 mL 4×stock solution was prepared by mixing 2 mL 0.2M Tris HCl, pH 6.8, 0.8g SDS, 4 mL 100% glycerol and 8 mg bromophenol blue and 0.4 mL 14.7M β- mercaptoethanol to make 50 mM Tris HCL, 2% SDS, 10% glycerol , 0.002% bromophenol blue and 1% β- mercaptoethanol.

b) Separating buffer: To make 100 mL 1.5M Tris HCl, 18.15g of Tris base was

dissolved in 90 mL H2O, pH adjusted to 8.8 then topped to 100 mL.

c) Stacking gel buffer: To make 100 mL of 5 × 1M Tris HCl, 12.114g of Tris base was dissolved in 90 mL H20, the pH adjusted to 6.8 and topped to 100 mL.

d) Coomassie brilliant blue staining solution: To prepare 100 mL, 50 mL H_2O was mixed with 40 mL methanol and 10 mL Glacial Acetic Acid. 0.2g Coomassie brilliant blue was dissolved completely and the staining solution filtered using a whatmann filter paper.

e) Phosphate Buffered Saline (PBS): To prepare 100 mL of 1× PBS, 8g of NaCl, 0.2g of KCl, 1.44g of Na2HPO4 and 0.24g of KH2PO4 were dissolved in 80 mL H2), the pH adjusted to 7.4, topped to 100 mL and autoclaved.

Appendix 4: Protein Purification Buffers

a) Native Purification Buffer: To prepare 100 mL 5X Native Purification Buffer (250 mM NaH_2PO_4 and 2.5M NaCl), 3.5g sodium phosphate, monobasic and 14.6g sodium chloride were dissolved in 90 mL H2O. The pH was adjusted to 8.0 and the volume topped to 100 mL.

b) Imidazole: To prepare 100 mL 3M Imidazole, 20.6 g Imidazole, 8.77 mL Stock Solution A (200 mM sodium phosphate, monobasic (NaH_2PO_4), 5 M NaCl and 1.23 mL of Stock Solution B (200 mM sodium phosphate, dibasic (Na_2HPO_4) and 5 M NaCl were added to 80 mL H_2O. The ph was adjusted to 6.0 and the final volume brought to 100 mL.

c) Native Binding Buffer: To prepare 30 mL native binding buffer, 30 mL 1× Native Purification Buffer and 100 µL of 3 M Imidazole, pH 6.0 were mixed well and the pH adjusted to 8.0.

d) Native Wash Buffer: To prepare 50 mL Native wash buffer, 50 mL of 1× Native Purification Buffer and 335 µL mM of 3M Imidazole, pH 6.0 were mixed and the pH adjusted to 8.0.

e) Native Elution Buffer: To prepare 15 mL, 13.75 mL of 1× native purification buffer was mixed with 1.25 mL of 3 M Imidazole, pH 6.0. The pH was adjusted to 8.0.

Appendix 5. Insect cell culture media

a) Complete TNM-FH media: To prepare 100 mL, 90 mL of Grace insect media, supplemented was mixed with 10 mL heat inactivated fetal bovine serum (FBS). 1 mL pen-strep was added to give a final concentration of 100 U/mL penicillin and 100 µg/mL streptomycin.

b) Freezing medium: To make 100 mL of freezing medium, 70 mL of complete TNM-FH medium was mixed with 10 mL of heat inactivated FBS and 20 mL of 100% glycerol.

Appendix 6. Quantities of the purified Recombinant VATs

Table A-2: Quantification results of Purified recombinant VATs expressed in bacterial and insect cells

Recombinant protein	OD_{562nm}	Concentration (µg/mL)
VSG 3 pBAD/His A	2.529	157.335
VSG 4 pBAD/His A	2.382	84.605
VSG 4 pRSET/His B (F0)	2.171	74.383
VSG 4 pRSET/His B (F1)	1.482	68.945
VSG 3 pIZ/V5-His	2.4215	120.3975
VSG 4 pIZ/V5-His	2.392	108.471