Identification and Characterization of Variant Surface Glycoprotein (VSG) Genes with diagnostic potential for *Trypanosoma brucei rhodesiense*.

By

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DECLARATION

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

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DEDICATION

This work is dedicated to my loving son, Sean Mireji for always being there and giving me the strength and courage to carry out the work.

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This study was approved by the national ethical review committee.

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
BP	Base pair
BCIP	5-Bromo-4-chloro- 3- indolyl phosphate
BCT	Buffy Coat Technique
BLAST	Basic Local Alignment Search Tool
CATT	Card Agglutination Test for Trypanosomiasis
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DDT	Dichlorodiphenyltrichloroethane
DFMO	Difluoromethylornithine
DNA	Deoxyribonucleic Acid
DRC	Democratic Republic of Congo
EATRO	East African Trypanosomiasis Research Organization
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assays
EMBL	European Molecular Biology Laboratory
ES	Expression Site
ESAG	Expression site-associated gene
FAO	Food and Agriculture Organization
FP	Filter Paper
gDNA	genomic Deoxyribonucleic Acid
GPI	Glycosyl-phosphatidyl inositol

HAT	Human African Trypanosomiasis
IFAT	Indirect Fluorescent Antibody Test
IPTG	Isopropyl-β-D-Thiogalactopyranoside
KDa	Kilo Dalton
KETRI	Kenya Trypanosomiasis Research Institute
KCL	Potassium Chloride
KB	Kilo Base pair
LAT	Latex Agglutination Test
LB	Luria-Bertani
mAECT	mini-Anion-Exchange Centrifugation Technique
mHCT	microHematocrit Centrifugation Technique
mM	Millimolar
mRNA	messenger Ribonucleic Acid
NACL	Sodium Chloride
NBT	Nitro Blue tetrazolium
NCBI	National Center for Bioinformatics Information
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH"	power of hydrogen"
QBC	Quantitative Buffy Coat
ORF	Open Reading Frame
RNA	Ribonucleic Acid
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SELDI TOF MS	Surface-Enhanced Laser Desorption-Ionisation Time-of-Flight Mass Spectrometry
SIT	Sterile insect release Technique
SMART	Simple Modular Architecture Research Tool
SOC	Super Optimal broth with glucose
SRA	Serum Associated Gene
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TBS	Tris-saline buffer
TBS-T	Tris-saline buffer-Tween
TgsGP	*T. b. gambiense*-specific flagellar pocket glycoprotein
TIM	Triosephosphate isomerase
TLF	Trypanolytic Factor
TRC	Trpanosomiasis Research Centre
UNDP	United Nations Development Programme
VAT	Variable Antigen Type
VSG	Variant Surface Glycoprotein
WB	Whole Blood
WBC	White blood cell
WHO	World Health Organization
X-GAL	5-bromo-4-chloro-3-indolyl β -D-galactopyranoside

ABSTRACT

Human African Trypanosomiasis (HAT) is present in 36 sub-Saharan African countries. The disease is easier to treat in early stage than late stage when treatment with melarsoprol is responsible for significant fatality. Therefore there is need to develop a diagnostic test for early detection before progression of the disease into the late stage. Currently available tests have poor sensitivity. Thus highly sensitive methods are needed for the detection of the parasite in human blood. The surface coat of bloodstream trypanosomes including the flagellum is composed predominantly of Variant Surface Glycoprotein (VSG). African trypanosomes have evolved a complex mechanism for sequentially expressing only one VSG from a repertoire of several hundred to thousand VSGs Some VSGs occur more frequently than others. The objective of this study was to evaluate the diagnostic potential of VSGs frequently encountered in early stage of *T. b. rhodesiense* infections using blood samples from Kenya and Uganda. VSGs 3 and 4 that were previously found to be the most frequently encountered VSGs in early stage of *T. b. rhodesiense* infections were chosen as putative diagnostic VSGs candidates in this study. Sequence alignment of VSGs 3 and 4, showed high similarity between the two VSGs hence common primers were designed and used to amplify both VSG 3 and 4. 25% of 24 blood samples from Kenya and 23% of 43 blood samples from Uganda were positive for both VSG 3 and 4 in Polymerase Chain Reaction (PCR) using hotstar *Taq* polymerase. While a detection of 95.8% and 86% among blood samples from Kenya and Uganda respectively was observed in PCR using phusion *Taq* polymerase. The VSG 4 open reading frame was cloned into pRSET-A expression vector and expressed in *Escherichia coli* BL21 cells. Recombinant crude VSG 4 protein was detected by anti-VSG antibodies using positively documented human serum samples. Purified recombinant protein had a molecular weight of 55KDa which was the expected size of VSG 4 protein. BLASTn of VSGs 3 and 4 against the *T. brucei* databases revealed their top orthologs to be a putative atypical VSG, on chromosome 6 of *T. brucei* TREU927 (geneDB: Tb927.6.5450 and GenBank accession number XM840543- National Center for Bioinformatics Information NCBI) and *T. evansi* strain KETRI-JN394 clone 1A VSG messenger RNA (GenBank accession number AF317931- NCBI). However no significant matches were found on alignment of the predicted amino acid sequences of VSGs 3 and 4 with the amino acid sequences of the other *Trypanozoon* VSGs already in the public domain databases. This shows that the protein expressed is specific only to *T. b. rhodesiense*. From the results of this study it can be concluded that the primers used in this work which were designed from the sequences VSGs 3 and 4 may be used to detect *T. b. rhodesiense* infections in humans in molecular assay and the VSG 4 protein can be a good diagnostic antigen in serological assays since it is cheaper than molecular assay.

CHAPTER ONE
INTRODUCTION

1.1 Background

Trypanosomiasis is initiated by the inoculation of flagellate protozoa, known as trypanosomes by tsetse flies, of the genus *Glossina* (Hide, 1999; Gibson, 2001; Radwanska *et al*., 2002a; Gibson, 2003). *Trypanosoma brucei gambiense*, present in western and central Africa, causes chronic human African trypanosomiasis (HAT) or sleeping sickness and *T. brucei rhodesiense* which occurs in eastern and southern Africa causes acute HAT (Hide, 1999; Gibson, 2001; Radwanska *et al*., 2002a; Gibson, 2003; Picozzi *et al*., 2005). The two sleeping sicknesses foci in Uganda are discrete and are steadily converging but have not yet overlapped and are now only 150 km apart (Picozzi *et al*., 2005). *Trypanosoma brucei brucei* causes African animal trypanosomiasis (AAT) or nagana in cattle and livestock in sub-Saharan Africa (Mehlert *et al*., 1998, Mehlert *et al*., 2002). The phylogenetic relationship of these three organisms has been difficult to resolve, particularly for *T. b. brucei* and *T. b. rhodesiense*, which are morphologically indistinguishable and infect the same wild and domestic animal hosts (Gibson, 2003). *T. b. rhodesiense* and *T. b. gambiense,* both of which infect humans are also not readily distinguished by morphology alone (Gibson, 2001; Gibson, 2003).

Sleeping sickness is found in 36 countries of sub-Saharan Africa, some of which are the poorest developing countries where health systems are weak or non-existent (Papadopoulos *et al*., 2004; Picozzi *et al*., 2005, Simarro *et al*., 2008). Human infections reduce labour resources and are implicated in the

underdevelopment of the African continent (Simarro *et al*., 2008). After an epidemic from 1900 to 1940, HAT was largely eradicated in the 1960s (Picozzi *et al*., 2005). However, in the past two decades the disease has re-emerged and returned to levels comparable with those in the early 20th century (Picozzi *et al*., 2005).

The disease is clinically characterized by an incubation period of one to four weeks; an initial phase marked by a localised skin reaction at the site of the tsetse fly bite (known as a chancre), fever, enlarged lymph glands and spleen, headaches, anaemia, pain in joints and pruritus (Hide, 1999; Keiser *et al*., 2000); and a final neurological phase when the parasite crosses the blood–brain barrier and invades the central nervous system (Hide, 1999; Keiser *et al*., 2000). This leads to endocrine disorders, confusion, sensory disturbances and poor coordination (Hide, 1999; Keiser *et al*., 2000), hence the name sleeping sickness.

Early diagnosis and treatment of trypanosomiasis is essential, as treatment of late stage disease is associated with high mortality (Keiser *et al*., 2000). Curative chemotherapy approach to HAT management includes administration of Pentamidine, Suramin, Melarsoprol and Eflornithine. Pentamidine is effective against the initial phase of *T. b. gambiense* infection, while Suramin, administered intravenously is for treatment of the early phase of *T. b. rhodesiense* infection (Keiser *et al*., 2000). The only effective drug for late stage disease of both *T. b. gambiense* and *T. b. rhodesiense* infections,

melarsoprol, crosses the blood–brain barrier and is associated with a severe post-treatment reactive encephalopathy in 10% of cases of which half die (Gichuki *et al*., 1994; Keiser *et al*., 2000; Brun *et al*., 2001). Eflornithine is currently used against late stage *T. b. gambiense* infection (Raper *et al*., 2002). Timely and efficient administration of these treatments therefore critically depends on correct diagnosis.

HAT infection can be diagnosed by demonstrating the presence of the parasite by direct and indirect parasitological methods, immunodiagnosis being used to detect specific antibodies against trypanosome, or by molecular methods that search for parasite Deoxyribonucleic Acid (DNA). Multicopy genes, expression-site-associated genes (*ESAG*) 6 and 7, located upstream of the variant surface glycoprotein gene have been used to detect *T. b. gambiense* in blood samples of patients from Equatorial Guinea and Angola suspected of having sleeping sickness by using Polymerase Chain Reaction (PCR), but these have not been adopted since their results were not consistent (Morgan *et al*., 1996; Kabiri *et al*., 1999). Buffy coat/dark ground technique (BCT) diagnostic method is unable to detect low parasitaemia (Mattioli and Faye, 1996; Mattioli *et al*., 2001). Microscopy is labour intensive and can lack sensitivity under field conditions due to the routinely low parasitaemia (Picozzi *et al*., 2002).

Human serum resistance-associated (*SRA*) gene has been found to be specific to *T. b. rhodesiense* using simple PCR (Gibson *et al*., 2002; Gibson, 2005) but absent in *T. b. gambiense* whose mechanism of human serum resistance is not

known (Gibson *et al*., 2002; Gibson, 2005).The SRA gene has been found to confer resistance to the innate trypanolytic factor (TLF) found in normal human serum; thus allowing *T. b. rhodesiense* to survive exposure to normal human serum (Gibson *et al*., 2002; Gibson, 2005). SRA has been further found to be responsible for adaptation of *T. b. rhodesiense* to humans by the high conservation of both this gene and the R-ES (a VSG Expression Site specific to *T. b. rhodesiense*) in many different field isolates of this subspecies (Vanhamme and Pays, 2004; Pays, 2006).

The main immunological response to HAT is humoral, characterized by production of large amounts of immunoglobulins M and G (IgM and IgG) by B-lymphocytes (Donelson, 1988). Theoretically, these antibodies should effectively eliminate the trypanosomes, but some trypanosomes manage to evade the immune system by modifying their surface membrane by expressing a new gene from the VSG archive, a process known as antigenic variation. The trypanosome can express thousands of variants, with each new surface change (McCulloch and Barry, 1999; Donelson, 1988). This phenomenon has significantly hampered the development of an effective vaccine against HAT (McCulloch and Barry, 1999; Donelson, 1988)

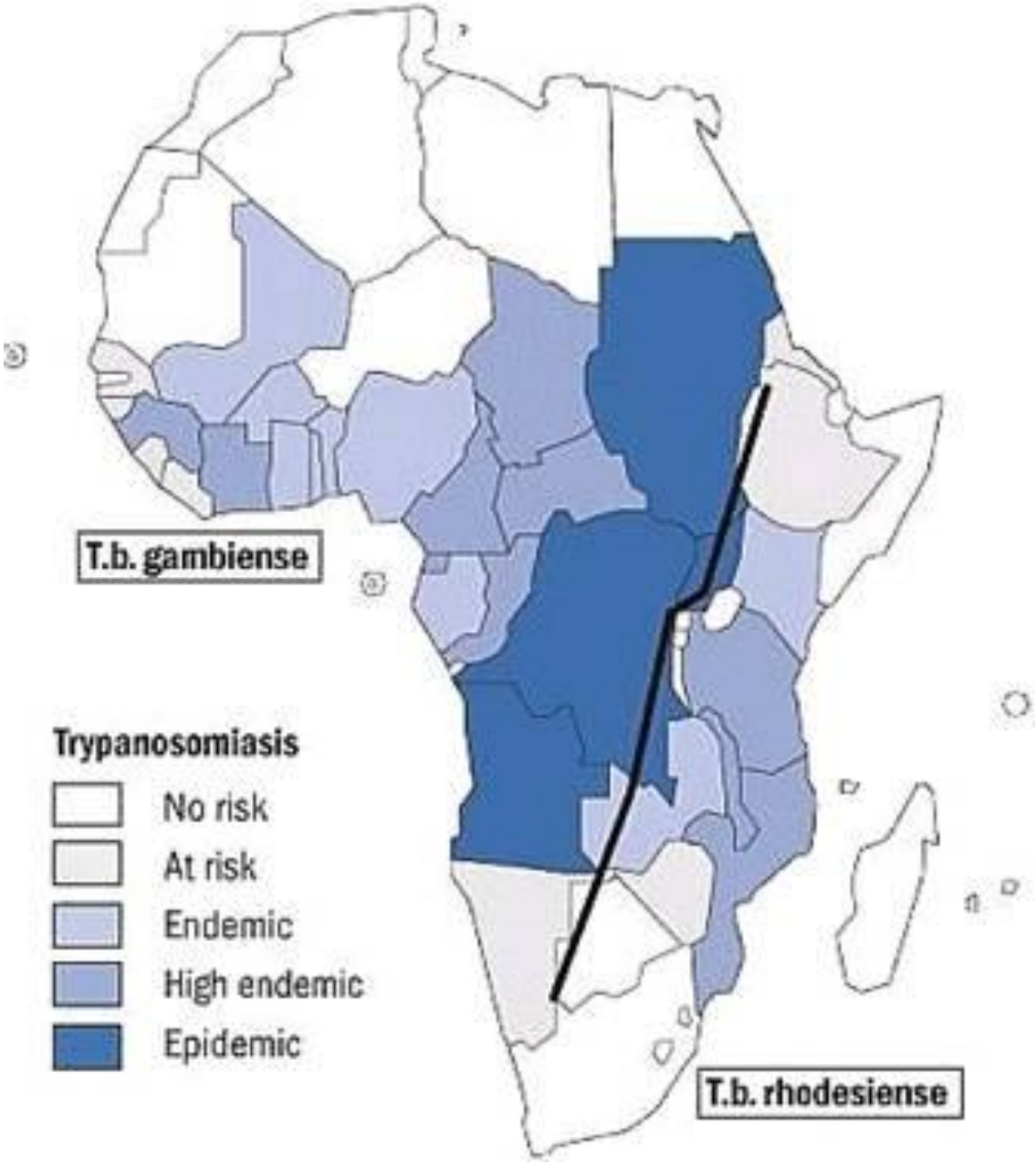


Figure1: Geographic distribution of *T. b. gambiense* and *T. b. rhodesiense* Human African trypanosomiasis in Africa.
Photo Source: WHO/CDS/CSR/ISR/2000

1.2 Justification

Sleeping sickness or Human African Trypanosomiasis (HAT) is a major obstacle to sub-Saharan African rural development; it has a devastating impact on human health and productivity. The real impact of the disease is difficult to determine since most infections occur in remote rural foci. The disease is easier to treat in the early stage than in the late stage when the treatment with melarsoprol is responsible for significant fatality in patients. Hence, there is a need to develop a diagnostic test for early detection, to enable treatment before progression to late stage disease. The East African sleeping sickness which is caused by *T. b. rhodesiense* has severe clinical symptoms and the onset of the disease is rapid, resulting in death within a few weeks or months, hence the need for a reliable sensitive and specific diagnostic test. Sleeping sickness occurs in poorly accessible rural areas amongst the poor where health facilities are scarce. Control of the disease would therefore require that its diagnosis be relatively affordable and scalable to reach large numbers within a short time. The availability of accurate, practical, sensitive and affordable screening and confirmatory tests is vital for HAT control. The current parasite demonstration, antigen and antibody detection techniques are not sensitive enough and are often not field applicable, but have the considerable advantage of being adaptable to large scale use. This study was aimed at identifying the prevalent predominant trypanosome VSGs expressed early in naturally infected persons in Kenya and Uganda. Such VSGs could then be used as raw antigens in the development of a reliable, sensitive and specific antibody detection diagnostic kit for HAT. Therefore the disease can be diagnosed and treated in the early

stage before progression into the lethal late stage, thereby improving disease control.

1.3 Research Question

The research question for this study was: Can VSGs expressed in early stage *T. b. rhodesiense* infections form a basis for a diagnostic test?

1.4 Hypothesis

The study hypothesised that frequently encountered VSGs among field isolates of *T. b. rhodesiense* can reliably be applied in diagnosis of HAT infections.

1.5 Objectives

1.5.1 General Objective

The general objective of this study was to characterize predominant *T. b. rhodesiense* variant surface glycoproteins (VSGs) expressed in from blood samples of early-stage patients in East Africa.

1.5.2 Specific Objectives

1. To investigate the prevalence of VSGs 3 and 4 in blood samples from early-stage *T. b. rhodesiense* patients from from East Africa.
2. Express and purify the VSGs 3 and 4 proteins

CHAPTER TWO

LITERATURE REVIEW

2.1 Human African Trypanosomiasis profile

The first phase of HAT is the haemolymphatic stage, during which the parasites proliferate in the bloodstream and lymph nodes after an infective tsetse fly bite, resulting into a swelling due to local inflammation (MacLean *et al*., 2004; Agranoff *et al*., 2005; Chappuis *et al*., 2005). The swelling transforms into a chancre within four weeks (Kennedy, 2004; Stich *et al*., 2002; Simarro *et al*., 2008) except in *T. b. gambiense* infections, which have no chancre formation (Burchmore *et al*., 2002). This is followed by clinical signs like malaise, headache, fever, peripheral oedema, anaemia and Winterbottom's sign (Kennedy, 2004; Stich *et al*., 2002; Simarro *et al*., 2008; Thuita *et al*., 2008). Additionally, myocarditis, pulmonary oedema, pericardial effusion, ascites, splenomegaly and hepatomegaly may also be present (Simarro *et al*., 2008; Thuita *et al*., 2008). However, most of these symptoms are also associated with other diseases common in endemic areas and are often misdiagnosed (Burchmore *et al*., 2002).

In the second phase, which is the neurological phase, the parasites invade the brain and spinal cord (Central Nervous System), usually within the first month of infection by *T. b. rhodesiense* (Gichuki *et al*., 1994; Brun *et al*., 2001; Burchmore *et al*., 2002; Thuita *et al*., 2008; Simarro *et al*., 2008). However, this period last between several months and years with *T. b. gambiense* infection (Gichuki *et al*., 1994; Brun *et al*., 2001; Burchmore *et al*., 2002;

Simarro *et al*., 2008). The CNS-invasion is accompanied by infiltration of lymphocytes, associated vasculitis and perivascular cuffing (MacLean *et al*., 2004; Simarro *et al*., 2008; Thuita *et al*., 2008). Headache becomes severe and sleep disorders, notably diurnal somnolence and insomnia are common (MacLean *et al*., 2004; Simarro *et al*., 2008; Thuita *et al*., 2008). Brain function deteriorates, culminating in coma and then death (MacLean *et al*., 2004; Thuita *et al*., 2008). Other disorders such as weight loss and endocrine abnormalities including impotence are also common (Burchmore *et al.*, 2002).

2.2 Control of Human African Trypanosomiasis

Insecticide application to eliminate the tsetse vector has been the cornerstone of HAT control. Advent of modern insecticides ushered in a massive eradication campaign of the Savannah tsetse species (Vale, 1968; Allsopp, 1984). Discovery of toxicological properties of Dichlorodiphenyltrichloro-ethane (DDT) to tsetse which was cheap, persistent and highly effective against tsetse boosted the campaign, making tsetse control increasingly dependent upon insecticides (Vale, 1968). However, insecticides appeared to provide only a temporary solution to an enduring problem (Harley, 1978), even with several other insecticides being subsequently discovered, evaluated and adopted in tsetse control programs to various extents (Allsopp, 1984). Artificial baits technology in tsetse control has been applied in southern Africa but seemed of limited use where the intention was to remove tsetse from vast invasion source and elsewhere in eastern Africa e.g. Kagera region, Tanzania (Vale, 1993). Despite intensive research into other alternatives, a practical alternative to insecticide control is still missing; with an exception of a four-year sterile

insect release (SIT) campaign on the island of Zanzibar that achieved a historic breakthrough success in complete tsetse eradication from the island (FAO, 1998). However, the success was attributable to integration with other control methods and factors peculiar to the island (FAO, 1998).

Chemotherapy against trypanosomiasis has not been sustainable mainly due to widespread and increasing resistance of trypanosomes to existing drugs (Gray, 1971; Jordan, 1986; Connor, 1994), high cost and sporadic availability of drugs in endemic areas (Jordan, 1986). Chemical compounds such as pentamidine, melarsoprol, nifurtimox and eflornithine have been used in case of human infections (Matovu *et al*., 2001). Eflornithine (Difluoromethylornithine or DFMO) has been the only new therapeutic drug used to treat the disease for over fifty years. However it is inactive against East African sleeping sickness due to innate tolerance of *T. b. rhodesiense* to the drug (Matovu *et al*., 2001). Pentamidine has been used in treatment of early stage HAT due to *T. b. gambiense*, while the treatment regimen of late stage HAT where central nervous system is affected involves the use of melarsoprol (Aksoy 2003).

Alongside efforts to reduce the spread of disease, there is also an urgent need to improve current surveillance and diagnostic procedures. Mortality can be drastically reduced when cases are diagnosed early enough to prevent progression to late-stage trypanosomiasis. Additional training and resources are needed in endemic areas for proper diagnosis and sero-surveillance.

2.3 Antigenic variation in trypanosomes

Antigenic variation is a consequence of changes in the composition of the glycoprotein, which forms a dense covering 12-15 nm thick over the entire surface of the organism (Vickerman, 1969). Through the antigenic variation process, bloodstream trypanosome populations keep "one step ahead" of the antibodies raised against their VSGs by each trypanosome expressing only one VSG on its surface at a time (Cross, 1975; Bridgen *et al*., 1976). Each antigenic type has an immunologically distinct glycoprotein, which can be readily purified (Cross, 1975).

The crucial step is the switching from expression of one VSG to another (Donelson and Rice-Ficht, 1985) since simultaneous expression of several VSG antigens can lead to proliferation of the parasite without immune control, killing the host before transmission (Cross *et al.*, 1998; Turner, 1985) while the reverse would apply with too slow switching pace, leading to immune depletion of the blood population and reduction in chance of transmission (Cross *et al.*, 1998; Turner, 1985). Antigenic variation therefore extends the chronicity of infections and enhances the chance of transmission of the parasites to new hosts. The successive and simultaneous expression of antigenically different VSGs allows the trypanosome population to evade neutralizing antibody responses, which are specific for major pre-existing variants in infected animals. The different VSGs are expressed in a hierarchical fashion, where some VSGs appear preferentially early in infection and others only later (Baltz *et al*., 1991; Donelson, 1995; Robinson *et al*., 1999).

Twenty or more potential expression sites for a VSG gene are invariably situated near a telomere, whereas the transcriptionally silent VSG genes are scattered throughout the chromosomes (Donelson, 1995; Vanhamme and Pays, 1995; Morgan *et al*., 1996; McCulloch and Barry, 1999). The active *VSG* gene in any bloodstream form of *Trypanosoma brucei* is located in a telomeric expression site (ES) (Donelson, 1995; Vanhamme and Pays, 1995; Morgan *et al*., 1996; McCulloch and Barry, 1999). The mechanisms that activate one and only one of these telomere-linked expression sites at a time in a given trypanosome are only partially understood (Donelson, 1995; Vanhamme and Pays, 1995; Morgan *et al*., 1996; McCulloch and Barry, 1999). In some cases, activation is associated either with duplicative transposition of a silent donor VSG gene to a telomeric-linked expression site or with a telomere exchange event (Donelson, 1995; Vanhamme and Pays, 1995; Morgan *et al*., 1996; McCulloch and Barry, 1999). In other cases, a silent VSG gene already at a telomere-linked site is activated *in situ* without apparent DNA rearrangement (Donelson, 1995; Vanhamme and Pays, 1995; Morgan *et al*., 1996; McCulloch and Barry, 1999). Transcription of at least some telomere-linked VSG expression sites is initiated 45-60 kb upstream of the VSG gene and proceeds through as many as nine or 10 members of different gene families called expression site-associated genes (ESAGs) (Donelson, 1995; Vanhamme and Pays, 1995; Morgan *et al*., 1996; McCulloch and Barry, 1999).

During HAT infections, there is a protracted and fluctuating course of parasitemia lapses during which a large repertoire of VSGs are expressed by the trypanosome (Nantulya *et al*., 1986; Robinson *et al*, 1999), with each relapse representing the parasite elimination by antibodies, and recrudescence appearance of a new VSG (Doyle, 1977; Auffret *et al*., 1981). Since infected hosts produce protective antibodies against the various trypanosome VSGs expressed by the parasites (Doyle, 1977; Ngaira *et al*., 1979; Masake *et al*., 1983), predominantly expressed VSGs, if common among isolates in a large geographical area, can have potential application in development of more specific and sensitive diagnostic test for trypanosomiasis.

2.4 Variant Surface Glycoprotein (VSG)

The strongly immunogenic VSG defines the variable antigen type (VAT) of an individual trypanosome and represents about 10% of its total protein content (Vickerman, 1969; Turner and Barry, 1989). Only one VSG gene is sequentially expressed at a time from a repertoire of several hundred to thousand VSG genes, in a semi-conserved manner. In the course of an infection, different VSG populations succeed each other in a hierarchical order, starting with a series of predominant VSGs (Nantulya *et al*., 1986; Baltz *et al*., 1991; Robinson *et al*., 1999).

The VSG is a glycosyl-phosphatidyl inositol (GPI) – anchored glycoprotein of around 55-65KDa with two domains (Schopf and Manfield, 1998; Wang *et al*., 2003; Chattopadhyay *et al*., 2004; Dubois *et al*., 2005; Pays, 2006; Marcello and Barry, 2007). VSGs consist of a hypervariable N-terminal domain of 350-

400 residues and a more conserved C-terminal domain of 40-80 residues that is GPI-anchored to the plasma membrane (Carrington *et al*., 1991; Hutchinson *et al*., 2003; Marcello and Barry, 2007). The C-terminal domain comprises disulphide bonds and a number of hydrophobic residues (Pays, 2006).

Normally only one species of VSG molecule is present within the trypanosome surface coat, resulting in the homogeneous display of identical surface epitopes in the exposed N-terminal regions of the molecules (Blum *et al*., 1993; Dubois *et al*., 2005). The three-dimensional structures of the N-terminal two-thirds of two VSGs have been determined by X-ray crystallography and found to be very similar rod-like shapes despite having quite different amino acid sequences (Blum *et al*., 1993; Donelson, 1988). These rod-like structures allow the homogeneous population of a single VSG to form a densely packed array on the surface, and suggest that all VSGs share a similar backbone structure from which emerge distinct epitopes derived from different groups of amino acid side chains (Blum *et al*., 1993; Donelson, 1988).

Approximately 95% of the VSGs do not properly encode protein (Barry *et al*., 2005; Marcello and Barry, 2007). Among the VSGs identified from *T. brucei* strain TREU927/4, (http://www.genedb.org/) only 5% are fully functional genes, 9% are atypical genes, 62% are full-length pseudogenes and 19% are gene fragments (Barry *et al*., 2005; Marcello and Barry, 2007). The N-terminal domain is grouped into two types, type “A” having 4-6 cysteine residues and type “B” having 10-12 cysteine residues (Carrington *et al*., 1991; Wang *et al*.,

2003). The C-terminal end is grouped into six types where types 2, 4 and 5 are single domains of 4 cysteine residues while types 1, 3 and 6 have two sub-domains each containing 4 cysteine residues (Carrington *et al*., 1991; Wang *et al*., 2003).

By virtue of their strong immunogenicity, VSGs can provide powerful diagnostic reagents provided that the tests include the correct variants (Raper *et al*., 2002). Among the 21 VSGs (designated 1-21 in order of appearance) determined from VSGs expression profiles from vervet monkeys (*Chorocebus aethiops*) infected with *T. b. rhodesiense* clones, VSGs 3 and 4 were frequently encountered in early stage infections (Masiga *et al*., unpublished). These predominant VSGs can have a potential use in the improvement of trypanosomiasis diagnosis.

2.5 Diagnosis of Human African Trypanosomiasis

Early stage of HAT is defined by the restriction of trypanosome parasites to the blood and lymph systems while at a later stage, few parasites invade the cerebrospinal fluid (CSF), accompanied with variable increase in white blood cells (WBC) in the CSF (WHO, 2003; Chappuis *et al*., 2005; Steverding, 2006). These stages are not clinically distinct (Burri and Brun, 2002), hence staging of HAT depends on examination of CSF successfully obtained by lumbar puncture, a painful, risky, and complicated procedure (Inojosa *et al*., 2006; Steverding, 2006). Treatment of HAT at this later stage in the CNS involves administration of the highly toxic melarsoprol drug, which should

therefore be preceded by appropriate positive diagnosis of the parasites in the CSF (Courtioux *et al*., 2005).

Diagnosis of the trypanosomes in tissue fluids of patients precedes initiation of chemotherapy against HAT (Fairlamb, 2003). Commonly applied diagnostic tools include microscopic identification of the parasites in thick blood smears or tissue fluid with relatively poor sensitivity, unable to detect low and fluctuating parasitemia (Fairlamb, 2003; Steverding, 2006). Detection of the trypanosome-specific antigens can provide direct evidence of a current infection status (Chappuis *et al*., 2005). Types of serological assays currently applied for diagnosis include, Enzyme Linked Immunosorbent Assays (ELISA) (Nantulya and Lindqvist, 1989), Card Agglutination Test for Trypanosomiasis (CATT) (Magnus *et al*., 1978b), latex agglutination test (LAT) (Chappuis *et al*., 2005) and Indirect Fluorescent Antibody Test (IFAT) (Chappuis *et al*., 2005). These are all capable of detecting trypanosome antibodies or antigens soon after infection (Clausen *et al*., 1998).

2.5.1 Microscopic Tests

The microhematocrit centrifugation technique (mHCT) or the Woo test (Woo, 1970), developed more than 30 years ago, is still being widely used (Chappuis *et al*., 2005). In this test capillary tubes containing anticoagulant filled three-quarters full with finger prick blood and dry end sealed with plasticine, are centrifuged in a hematocrit centrifuge for 6 to 8 minutes at high-speed, and trypanosomes are concentrated at the level of the white blood cells, between the plasma and the erythrocytes (Woo, 1970; Woo, 1971). The capillary tubes,

mounted in a special holder, are then directly examined at low magnification (x100 or x200) for motile parasites (Woo, 1970; Woo, 1971; Chappuis *et al*., 2005.). The sensitivity of mHCT increases with the number of tubes examined, with an estimated detection threshold of 500 trypanosomes/ml (Woo, 1970; Woo, 1971; Chappuis *et al*., 2005). This technique is moderately time-consuming, and the concomitant presence of microfilaria in the blood can render the visualization of the much smaller trypanosomes very difficult (Chappuis *et al*., 2005).

The quantitative buffy coat (QBC) initially developed for the rapid assessment of differential cell counts, has been extended to the diagnosis of HAT (Levine *et al*., 1989; Bailey and Smith, 1992; Truc *et al*., 1998). It has the advantages of concentrating the parasites by centrifugation and, staining the nucleus and kinetoplast of trypanosomes with acridine orange, allowing a better discrimination from white blood cells (Levine *et al*., 1989; Bailey and Smith, 1992; Chappuis *et al*., 2005). After high-speed centrifugation of the blood in special capillary tubes containing EDTA, acridine orange, and a small floating cylinder, motile trypanosomes are identified by their fluorescent kinetoplasts and nuclei in the expanded buffy coat (Levine *et al*., 1989; Bailey and Smith, 1992; Chappuis *et al*., 2005). The QBC can detect more patients with low parasitemia than the mHCT when fewer than eight capillary tubes are used and is as sensitive as the mini-anion-exchange centrifugation technique (mAECT), however the relative sophistication and fragility of the material prevents its daily usage (Truc *et al*., 1998).

Mini-anion-exchange centrifugation technique (mAECT), introduced by Lumsden *et al*. (Lumsden *et al*., 1979), based on a technique developed by Lanham and Godfrey (Lanham and Godfrey, 1970), was initially shown to be more sensitive than the thick blood film and the mHCT (Lumsden *et al*., 1981). An updated version has been described by Zillmann *et al*. (Zillmann *et al*., 1996). The technique consists of separating the trypanosomes, which are less negatively charged than blood cells, from venous blood by anion-exchange chromatography and concentrating them at the bottom of a sealed glass tube by low-speed centrifugation and examining the glass tube tip in a special holder under the microscope for the presence of trypanosomes (Zillmann *et al*., 1996; Truc *et al*., 1998; Chappuis *et al*., 2005). Whereas the large blood volume (300 µl) enables the detection of less than 100 trypanosomes/ml, resulting in high sensitivity, the manipulations are quite tedious and time-consuming (Chappuis *et al*., 2005).

2.5.2 Serological Assays

Immunofluorescence assays have been used with success for HAT diagnosis in Equatorial Guinea, Gabon and Democratic Republic of Congo(DRC), where they were shown to be highly sensitive and specific (Noireau *et al*., 1988). The availability of standardized antigen at low cost has greatly improved the reliability of the test, which can be used with serum or filter paper (FP) eluates (Magnus *et al*., 1978a). The IFAT test is generally applied in diagnosis of *T. b. rhodesiense* and rarely T. *b. gambiense* infection (Noireau *et al*., 1988). Trypanosomes are fixed onto microscope slides and later spotted with serum

under investigation; antibodies present in the serum bind to the trypanosomes, which are then detected using a fluorescent probe (Chappuis *et al*., 2005). The test is more sensitive and specific than CATT (Noireau *et al*., 1988), but the sensitivity reduces when used with impregnated filter paper (Noireau *et al*., 1988).

ELISA methods can be performed with serum, filter paper (FP) eluates, and CSF with strict standardization and quantification (Lejon *et al*., 1998). Semi-quantitative ELISA, using variable surface glycoprotein of *T. b. gambiense* as antigen, developed for the detection of antibodies of different immunoglobulin isotypes in serum and cerebrospinal fluid of sleeping sickness patients in Equatorial Guinea and Sudan, indicated increased levels of IgG and IgM, while IgA remained relatively normal (Lejon *et al*., 1998) and is a possible tool for determining the clinical stage of sleeping sickness. ELISA was shown to detect specific antibodies in the saliva from a group of 23 patients from DRC with confirmed HAT, but antibody levels were about 250-fold lower than in the serum and could not be detected by the CATT or the LATEX/*T. b. gambiense* in the vast majority of these patients (Lejon *et al*., 2003).

The CATT is a common test for *T. b. gambiense* and not *T. b. rhodesiense*, (Magnus *et al.*, 1978b), at 74–95 and 87–98% specificity and sensitivity respectively (Chappuis *et al*., 2005). This technique is particularly sensitive when applied on undiluted whole blood (CATT-WB) (Inojosa *et al*., 2006). Its specificity enhanced when performed on plasma (CATT-P) or serum diluted to 1:4, is not considered sufficient (Chappuis *et al*., 2004). Additionally, positive

predictive value (PPV) of the CATT-WB is limited due to its inability to detect low HAT prevalence, common in the majority of the patient populations infected with *T. b. gambiense* (Inojosa *et al*., 2006).

Latex card agglutination test has also been used for antibody detection in HAT using *T. b. gambiense* semipurified surface glycoprotein of Variable Antigen Type (VAT) LiTat 1.6 (Büscher *et al*., 1991). While LiTat 1.3 has been considered a suitable antigen for detecting *T. b. gambiense* in CATT, it has been shown to be absent in some *T. b. gambiense* stocks from Cameroon (Dukes *et al*., 1992). The LATEX/*T. b. gambiense* was developed as an alternative field test to the CATT and is based on the combination of three purified variable surface antigens, LiTat 1.3, 1.5, and 1.6, coupled with suspended latex particles (Büscher *et al*., 1999; Jamonneau *et al*., 2000). The test procedure is similar to the CATT, however compared to the CATT, the LATEX/*T. b. gambiense* showed a higher specificity (96 to 99%) but a lower or similar sensitivity (71 to 100%) in recent field studies conducted in several western and central African countries (Truc *et al*., 2002; Penchenier *et al*., 2003).

Papadopoulos *et al*. used proteomic signature analysis to diagnose HAT (Papadopoulos *et al*., 2004). When serum samples from 85 African trypanosomiasis patients and 146 control patients who had other parasitic and non-parasitic infections was applied to a weak cation exchange chip, and analysed with surface-enhanced laser desorption-ionisation time-of-flight mass spectrometry (SELDI TOF MS), a sensitivity and specificity of 100% and

98·6% respectively was observed (Papadopoulos *et al*., 2004). Though this approach is much more accurate than any other currently applied diagnostic test, the process requires expensive equipment and expertise, which makes it impracticable for routine use in rural Africa where the disease is prevalent.

2.5.3 Molecular Assay

Molecular diagnostic approaches to HAT have also been studied. For example, CSF-PCR assays to detect trypanosome DNA have been described for diagnosing HAT but some suffer poor reproducibility. Nevertheless, one PCR method, the detection of *T. b. gambiense* by amplification of VSG AnTat 11.17 was reported to have a sensitivity of 96% (Bromidge *et al*., 1993). An alternative diagnostic target, the receptor-like flagellar pocket glycoprotein TgsGP; has been shown to provide a specific marker for *T. b. gambiense* (Radwanska *et al*., 2002b). Clearly, PCR has real potential here but the challenge is to adapt its applicability to field conditions. With the advent of contemporary more specific and sensitive molecular diagnostic techniques, particularly PCR assay, it is potentially possible to combine this technique with other techniques to enhance the accuracy of HAT diagnosis (Gonzales *et al*., 2003) based on the VSGs that are expressed in early stage *T. b. rhodesiense* infections.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Biological Test Samples

Test blood samples from HAT infected patients were sourced in cryopreserved form from collaborating partners in a World Health Organization (WHO) supported retrospective study in Kenya and Uganda. The total number of samples collected during that study was 24 and 43 in Kenya and Uganda respectively (Tables 1 and 2).

Table 1: Test blood samples collected from Kenya

Sample No	Sample ID	Origin	Isolation Year
1	EATRO 113	Nyanza	1960
2	EATRO 151	Nyanza	1962
3	EATRO 256	Tanzania	1960
4	EATRO 465	Samia	1958
5	EATRO 739	Central Nyanza	1964
6	EATRO 1760	Tanzania	1959
7	EATRO 2101	Uganda	1969
8	EATRO 2155	Central Nyanza	1958
9	KETRI 2379	South Nyanza	1970
10	KETRI 2544	South Nyanza	1981
11	KETRI 2565	South Sudan	1982
12	KETRI 2592	Uganda	1962
13	KETRI 2594	Central Nyanza	1958
14	KETRI 2625	Bunyula, Kenya	1961
15	KETRI 3453	Tanzania	1959
16	KETRI 3637	Teso, Kenya	1999
17	KETRI 3644	Teso, Kenya	1999
18	KETRI 3741	Teso, Kenya	1999
19	KETRI 3797	Teso, Kenya	1999
20	KETRI 3798	Teso, Kenya	1999
21	KETRI 3801	Teso, Kenya	1999
22	KETRI 3803	Teso, Kenya	1999
23	KETRI 3804	Teso, Kenya	1999
24	KETRI 3928	Teso, Kenya	1999

Table 2: Test blood samples collected from Uganda

Sample No	Sample ID	Origin	Isolation Year
1	UG1	Omugo	1999
2	UG2	Omugo	1999
3	UG3	Omugo	1999
4	UG4	Omugo	1996
5	UG5	Omugo	1999
6	UG6	Tororo	1999
7	UG7	Tororo	2000
8	UG8	Tororo	2001
9	UG9	Tororo	2001
10	UG10	Tororo	2003
11	UG11	Busoga	2000
12	UG12	Tororo	2002
13	UG13	Busoga	1999
14	UG14	Tororo	1997
15	UG15	Tororo	1997
16	UG16	Tororo	1990
17	UG17	Tororo	1990
18	UG18	Tororo	1991
19	UG19	Tororo	1990
20	UG20	Tororo	1990
21	UG21	Tororo	1990
22	UG22	Busoga	1995
23	UG23	Tororo	1999
24	UG24	Tororo	2000
25	UG25	Tororo	1991
26	UG26	Tororo	1991
27	UG27	Tororo	1991
28	UG28	Tororo	1996
29	UG29	Tororo	1996
30	UG30	Tororo	1998
31	UG31	Busoga	1998
32	UG32	Busoga	1998
33	UG33	Busoga	1999
34	UG34	Busoga	1999
35	UG35	Busoga	1999
36	UG36	Tororo	2000
37	UG37	Tororo	2000
38	UG38	Tororo	2000
39	UG39	Tororo	2000
40	UG40	Soroti	2001
41	UG41	Soroti	2001
42	UG42	Soroti	2001
43	UG43	Soroti	2001

3.2 Isolation of **T. b. rhodesiense** *Genomic DNA from Biological Test Samples*

Genomic DNA from samples collected in Uganda was extracted using Puregene DNA purification kit (Gentra Systems Inc, Minneapolis, USA) according to the manufacturer's specifications. Briefly, 600µl of whole blood was added to a 1.5 ml micro centrifuge tube containing 900µl RBC Lysis Solution, inverted to mix and incubated for one minute at room temperature. The mixture was centrifuged at 16,000 x g for 20 seconds; supernatant removed leaving behind a visible white cell pellet and about 10-20µl of residual liquid to resuspend the white cells with vortexing. Cell lysis solution (600µl) was added to the resuspended cells, pipeted up and down to lyse the cells. Three (3) µl RNase A Solution was added to the cell lysate, mixed by inverting the tube 25 times and incubated at 37°C for 60 min.

The lysate solution was cooled to room temperature before adding 200µl protein precipitation solution and vortexed vigorously at high speed for 20 seconds and centrifuged at 16,000 x g for one minute. The supernatant containing the DNA was transferred into a clean 1.5 ml micro centrifuge tube containing 600µl 100% isopropanol, inverted gently 5 times and centrifuged at 16,000 x g for a minute. The remaining white DNA pellet was washed with 600µl 70% ethanol and centrifuged at 16,000 x g for a minute. DNA Hydration solution (100 µl) was added, vortexed for five seconds at medium speed to mix and incubated in a water bath at 65°C for five minutes to accelerate

rehydration. The mixture was again vortexed for five seconds at medium speed to mix and centrifuged briefly to collect the purified gDNA at the bottom of the tube.

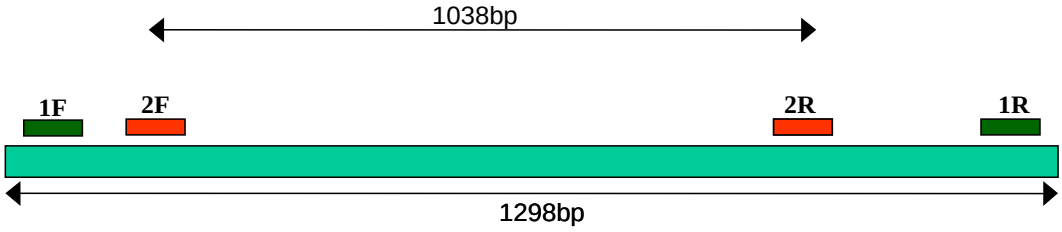
For the samples collected from Kenya a standard procedure for isolation of genomic DNA was employed (Sambrook *et al*, 1989). Briefly, 200 µl of each of the whole blood test sample was placed into eppendorf tubes, centrifuged at 6000 rpm for 10 minutes at 4 ^{o}C and the resultant supernatant discarded. The pellet was washed once with PBS buffer pH8.0 (137 mM NaCl, 2.7mM KCl and 4.3mM $NaHPO_4 7H_2O$), centrifuged at 6000 rpm for 10 minutes at 4 ^{o}C and the supernatant discarded as before. The pellet was resuspended in 200 µl of trypanosome cell lysis buffer (10mM Tris Ph8.0, 100mM NaCl, 100mM EDTA, 0.01% SDS and 100ng/ml Proteinase K) and incubated at 37 ^{o}C overnight. An equal volume of 0.1M Tris pH8.0 buffered phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) was added, the solution gently inverted several times for five minutes and centrifuged at 4000rpm for three minutes in a micro centrifuge (Epperndorf Centrifuge 5415C).

Upper aqueous layer was placed into a clean eppendorf tube. The interphase was re-extracted once and the resultant aqueous phase pooled into that in the clean tube. Approximately 0.1 volumes of 3M Sodium acetate (pH 5.2) and two volumes of ice-cold absolute ethanol were added to the DNA solution and mixed well for DNA precipitation process. The tubes were incubated overnight at –70^{o}C. The solution was centrifuged twice at 14000 rpm at 4^{o}C each for 20

minutes, with the pellet washed in one millilitre of ice-cold 70% ethanol after the first spin. The pellet was air-dried at room temperature and then subsequently dissolved in 100 µl sterile water.

3.3 Isolation of VSG Genes from Genomic DNA

Among the 21 VSGs expressed from vervet monkeys (*Chorocebus aethiops*) that were infected with *T. b. rhodesiense* clones (Thuita *et al*., 2008), two VSGs designated VSG 3 and VSG 4, were the most frequently encountered in early stage infections (Masiga *et al*., unpublished). Hence they were chosen as putative diagnostic VSGs candidates. Sequence analysis of VSG3 and VSG4 showed them to be very similar; hence, I designed primers that could simultaneously amplify the two VSG genes, this would reduce the cost of the test.



KEY:
1F: Set 1 External forward primer
1R: Set 1 External reverse primer
2F: Set 2 Internal forward primer
2R: Set 2 Internal forward primer

Figure 2: Diagrammatic presentation of the positions of primers used in PCR. Set 1 primers flank the terminal ends of VSGs 3 and 4, while Set 2 primers are internal to the Set 1 primers.

3.3.1 Amplification of VSG gene using Hotstar Taq polymerase

There is a high sequence similarity between VSG 3 and VSG 4 making it possible to use common primers to amplify both the genes. A first PCR was conducted on the genomic DNA using the following set 1 primers flanking the terminal ends of VSG 3 and VSG 4:

Forward (5'-CCTCCAAGCCCATGGCAAAAATTCGCTC-3') –1F

Reverse (5'-GTCCCTCTTTAGCCTTGCACTCCC-3') – 1R

Each PCR reaction consisted of 0.12µg of each primer, 1.2µl gDNA, 0.2mM dNTP, 3mM $MgCl_2$, and both 2.5units of HotStar *Taq* polymerase (Qiagen GmbH, Germany) and 3µl of buffer Q solution (Qiagen GmbH, Germany) in a total volume adjusted to 15µl with H_2O. The reaction mixture was placed in a thermocycler (PTC-100^{TM}, MJ Research, Inc. Watertown MA.) and incubated at 95°C for 15 minutes, then 40 cycles of 94°C for 60 seconds (denaturation), 48°C for 60 seconds (annealing), 72°C for 90 seconds (extension) and a final extension at 72°C for 10 minutes. As a negative control, a PCR mixture without gDNA template was included with each set of reactions.

Since there was no amplification in the first PCR, a second PCR was conducted using 1µl of the product of the first PCR as a template DNA, with set 2 primers, which were internal to the set 1 primers in a nested approach to PCR:

Forward (5'-GGAAGTCGACTTCAATCGG-3') – 2F

Reverse (5'-GTGACAACTCCGCCCTTGACGTCTTTACC-3') –2R

The thermo-cycling parameters (in a PTC-100^{TM}, MJ Research, Inc. Watertown MA.) were 95^{o} C for 15 minutes followed by 40 cycles of 94^{o} C for

60 seconds, 54^{o} C for 60 seconds, 72^{o} C for 90 seconds and a final extension at 72^{o} C for 10 minutes. PCR products with approximately 1 Kb size in the second PCR were indicative of presence of VSG 3 or VSG 4.

For the second PCR, the PCR products (10µl) were loaded onto ethidium bromide stained 1% agarose gels in TAE buffer (Add tris base 242g, 57.1ml glacial acetic acid and 100ml 0.5M EDTA pH8 and make to 1 liter with double distilled water to prepare 50X). A 1 Kb DNA ladder (Fermentas, Vilnius, Lithuania) was run on every gel to confirm expected size of the amplification product. Gel images were acquired with a Nikon camera (Nikon UK Limited, Surrey, UK) under ultraviolet (UV) illumination. Presence of approximately 1 Kb band represented presence of VSG 3 or VSG 4 in the second PCR. The number of positive samples was unrealistic since VSGs 3 and 4 are predominantly expressed in early stage infections, thereby necessitating the use of phusion *Taq* polymerase (New England Biolabs, Boston, USA) which is a better *Taq* polymerase than HotStar *Taq* polymerase (Qiagen GmbH, Germany). The VSG gene also being a single copy is difficult to amplify using standard PCR hence the use of high fidelity phusion *Taq*.

3.3.2 Amplification of VSG gene using phusion Taq polymerase

Amplification was conducted on the genomic DNA using the following set 1 primers flanking the terminal ends of VSG 3 and VSG 4:

Forward (5’-CCTCCAAGCCCATGGCAAAAATTCGCTC-3’) –1F

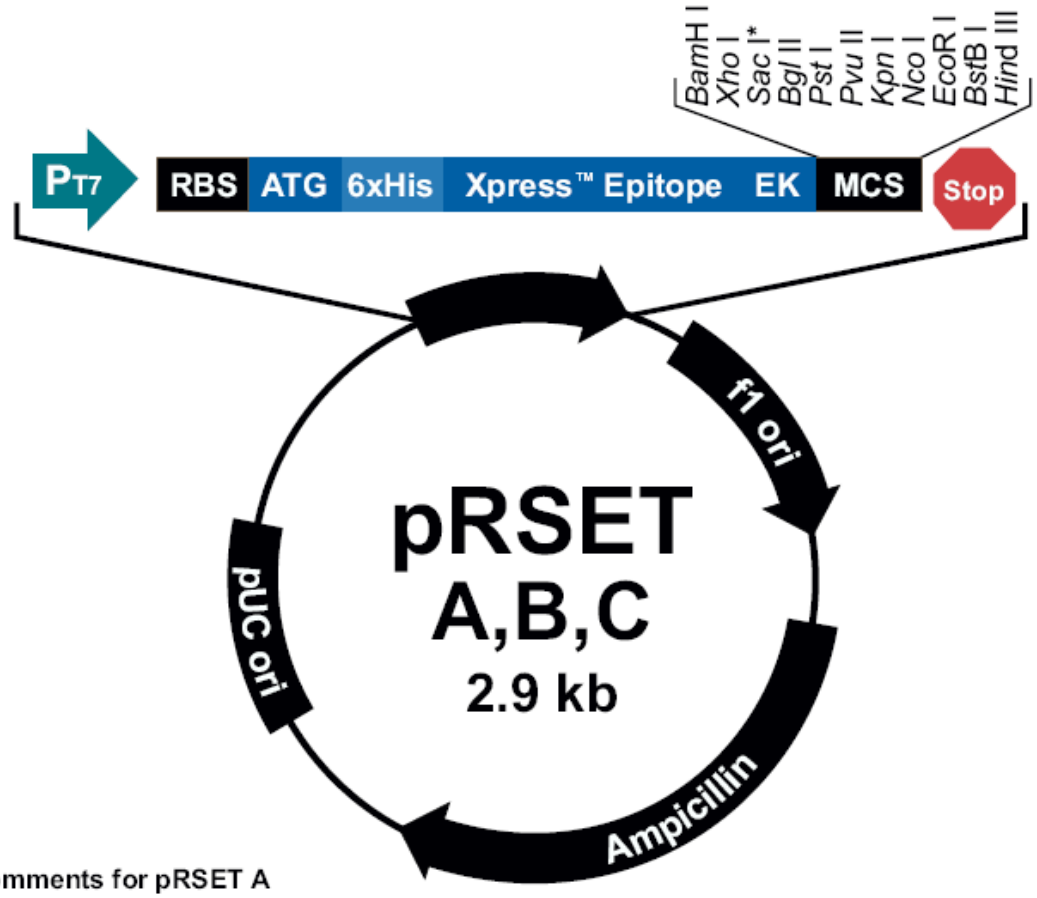
Reverse (5’-GTCCCTCTTTAGCCTTGCACTCCC-3’) – 1R

Each PCR reaction consisted of 0.12µg of each primer, 2µl gDNA, 0.2mM dNTP, 4µl HF phusion Taq buffer and 0.2µl of high fidelity phusion *Taq* polymerase (New England Biolabs, Boston, USA) in a total volume adjusted to 20µl with H_2O. The reaction mixture was placed in a thermocycler (PTC-100^{TM}, MJ Research, Inc. Watertown MA.) and incubated at 98°C for 30 seconds, then 35 cycles of 98°C for 10 seconds (denaturation), 60°C for 30 seconds (annealing), 72°C for 90 seconds (extension) and a final extension at 72°C for 10 minutes. As a negative control, a PCR mixture without genomic DNA template was included with each set of reactions.

PCR products (10µl) were loaded onto ethidium bromide stained, 1 % agarose gels in TAE buffer. A 1 Kb DNA ladder (Fermentas, Vilnius, Lithuania) was run on every gel to confirm expected size of the amplification product. Gel images were acquired with a Nikon camera (Nikon UK Limited, Surrey, UK) under ultraviolet (UV) illumination. Presence of approximately 1.3 Kb band represented presence of VSG 3 or VSG 4.

3.4 Sub-cloning of VSG 4 into pRSET-A Vector

For this experiment the reagents were limiting and also I had access to only VSG 4 clone, which was a kind donation from Dr Masiga. Open Reading Frame (ORF) specific primers with *Bam*HI and *Xho*I sites were designed to allow restriction ligation of the VSG insert into pRSET-A expression vector in the correct reading frame.



Comments for pRSET A
2897 nucleotides

*Version C does not contain *Sac* I

T7 promoter: bases 20-39
6xHis tag: bases 112-129
T7 gene 10 leader: bases 133-162
Xpress™ epitope: bases 169-192
Multiple cloning site: bases 202-248
T7 reverse priming site: bases 295-314
T7 transcription terminator: bases 256-385
f1 origin: bases 456-911
bla promoter: bases 943-1047
Ampicillin (*bla*) resistance gene (ORF): bases 1042-1902
pUC origin: bases 916-2852 (C)

A primers:

Forward (5'-GCGGATCCATGCGGCCCACCACTTTAGCAGCG-3')

Reverse (5'-GCCTCGAGCTATTAAAAAAGCAAAAATGCAAGCC-3')

Each PCR reaction consisted of 0.12 µg of each primer, 0.2µl pure plasmid, 0.2 mM dNTP, and 5µl *Taq* buffer and 0.5units of Genscript *Taq* polymerase

(Genscript Corp, New Jersey, USA) in a total volume adjusted to 25µl with H_2O. The reaction mixture was placed in a thermocycler (PTC-100^{TM}, MJ Research, Inc. Watertown MA.) and incubated at 94^{o} C for 3 minutes (Initial denaturation) followed by 35 cycles of 94^{o} C for 60 seconds (denaturation), 54^{o} C for 60 seconds (annealing), 72^{o} C for 90 seconds (extension) and a final extension at 72^{o} C for 10 minutes. As a negative control, a PCR mixture without template was included.

PCR products were loaded onto ethidium bromide stained 1 % agarose gels in TAE. A 1 Kb DNA ladder molecular weight marker (Fermentas, Vilnius, Lithuania) was run on every gel to confirm expected size of the amplification product. Gel images were acquired with a Nikon camera (Nikon UK Limited, Surrey, UK) under ultraviolet (UV) illumination. The VSG was recovered from the agarose gel using QIAGEN Gel extraction protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified product was re-suspended in 30µl triple distilled deionised water.

Twenty microlitres of the purified VSG insert was digested with 0.2units each of *BamH*I and *Xho*I (New England Biolabs, Boston, USA) restriction enzymes in five micro liters of buffer 3 (New England Biolabs, Boston, USA) and 0.2 units of Bovine Serum Albumin (New England Biolabs, Boston, USA), in a total volume adjusted to 50µl with H_2O. The digestion was conducted at 37°C for two hours followed by inactivation of the enzymes at 65°C for 20 minutes. pRSET-A vector (Invitrogen, California, USA) was also digested with the

same enzymes in the same process. The resultant digestion products were run on a 1% agarose gel as described in section 3.3.1. The linear vector and the VSG gene fragment were each excised from the gel and purified using QIAGEN Gel extraction protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions and the VSG 4 ligated into the pRSET-A vector (Invitrogen, California, USA) using T4 DNA ligase enzyme (Invitrogen, California, USA) at 4 °C overnight in a 10µl reaction according to the manufacturers instructions.

The ligation reaction mixture was added to 50 microlitres of competent DH5α *Escherichia coli* bacterial cells and the cells transformed by heat shocking for one minute at 42ºC and placed on ice for five minutes. Approximately 950µl room temperature SOC medium (Add tryptone 20g, yeast extract 5g; NaCl 0.5g 10mls, 250mM KCl 20ml 1M glucose and 5ml 2M $MgCl_2$ and make to 1 liter with double distilled water) was added to the cells and incubated in a water bath at 37^{o}C for two hours. The cells were centrifuged at 4000rpm for five minutes and the supernatant poured, leaving about 100µl to resuspend the pelleted cells. The culture was plated onto Luria-Bertani (LB)-agar (Add tryptone 10g, yeast extract 5g, NaCl 10g, agar 15g and make to 1 liter with double distilled water then adjust to pH7.0 with NaOH, filter sterilize using 0.2µm filter and autoclave)containing ampicillin at a final concentration of 100µg/ml and incubated overnight at 37^{o}C. White colonies on the plates were screened by colony PCR using the pRSET-A primers and a colony slightly scrapped with a sterile pipette tip as the template, under similar amplification

conditions previously used above. Identified positive colonies were grown overnight in 5ml liquid LB medium (Add tryptone 10g, yeast extract 5g, NaCl 10g and make to 1 liter with double distilled water then adjust to pH7.0 with NaOH and autoclave)containing ampicillin at a final concentration of 100μg/ml by incubation at 37 ^{o}C in a shaker. The bacterial cells were harvested by centrifugation at 6000X*g* for 15 minutes at 4°C. The plasmid containing the insert was then purified from the cells using QIAGEN protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

3.5 Expression of recombinant VSG 4 protein in Escherichia coli

The VSG 4 was expressed in *E. coli* cells as a protein with a Histidine tag at the amino terminus, to enable use of a nickel column in the purification of the recombinant protein from the cell extract. Competent *E. coli* BL-21 cells were transformed with the pRSETA-VSG construct and grown on LB-agar containing ampicillin at a final concentration of 100μg/ml overnight at 37°C. White colonies on the plates were randomly screened by colony PCR as in section 3.4. This was carried out using T7 promoter and insert specific primers, and the template as a colony slightly scrapped with a sterile pipette tip, under similar amplification conditions previously used above. Positive colonies were inoculated into 5ml liquid 37°C LB medium containing 100μg/ml ampicillin and grown overnight at. For expression, 100μl of the transfected cells were added to 200ml of LB medium and the cells were grown in a shaker at 30 °C to an OD_{600nm} of 0.6. To induce recombinant protein expression, 2μl of 0.1M

IPTG was added to a final concentration of 1mM, and the incubation extended for 6 hours at 30°C.

After the six hours incubation time, cells were harvested by centrifugation at 4°C at 6000Xg for 15 minutes and lysed in protein lysis buffer pH8.0 (Add 100mM NaH_2PO_4, 10mM Tris-Cl, 8M Urea and 300mM NaCl)at a ratio of five milliliters of the lysis buffer per gram weight of the pelleted cells. Whole-cell lysates were analyzed with a denaturing 8–12% gradient sodium dodecyl sulfate polycacrylamide gel electrophoresis (SDS-PAGE) system using electrophoresis running buffer (Add 25mM Tris-Cl, 192mM glycine and 0.1%v/v SDS) with protein bands visualized by silver staining. Molecular weights of the protein bands were determined by comparison with a protein standard (Fermentas, Vilnius, Lithuania). Images were acquired with a Nikon camera (Nikon UK Limited, Surrey, UK) under white light.

3.6 Detection of recombinant VSG 4 protein using anti-VSG antibodies

The cell lysates were separated on a denaturing 8 – 12% gradient Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel, together with a protein standard. The proteins were then transferred onto a nitrocellulose membrane for two hours at 169 mAmps using transfer buffer pH8.3 (Add 48mM Tris-Cl, 29mM glycine, 20% methanol and 0.037%v/v SDS). Blocking was done for one hour with 5% skimmed milk in Tris-saline buffer (TBS-T)pH7.4 (Add 25mM Tris-Cl, 137mM NaCl, 3mM KCl and 0.05%v/v Tween-20). The membrane was washed with TBS-T briefly to

remove traces of skimmed milk. The membrane was incubated for one hour with primary antibody (positive serum for human African typanosomiasis) diluted 1:500 in TBS-T. The membrane was then washed three times for 10 minutes each in TBS-T, before incubation for one hour with secondary antibody (goat anti-human IgM Alkaline phosphatase conjugated) diluted 1:1000 in TBS-T. The membrane was washed again as previously and colour developed using nitroblue tetrazolium/ bromochloroindolyl phosphate (NBT/BCIP) substrate solution (Add 330 µl of a solution of 1 tablet of NBT dissolved in 1ml distilled deionized water, 33 µl of a solution of 1 tablet of BCIP in 100% N,N-dimethylformamide and 10ml substrate buffer pH9.5 made up of 100mM Tris-Cl, 100mM NaCl and 5mM $MgCl_2$) until desired colour was obtained. Colour development was stopped by extensive washing with water. Images were acquired with a Nikon camera (Nikon UK Limited, Surrey, UK) under white light as above.

3.7 Purification of recombinant VSG 4 protein

The positive detection of the serum by the crude VSG 4 protein gave a smear thereby necessitating purification. The expressed His-tagged recombinant protein was purified using QIAGEN nickel spin columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions using wash buffer pH6.3 (Add 100mM NaH_2PO_4, 10mM Tris-Cl and 8M Urea) and elution buffer pH4.5 (Add 100mM NaH_2PO_4, 10mM Tris-Cl and 8M Urea). The purified recombinant and crude (non-induced and induced) proteins were separated on a denaturing 8 – 12% gradient SDS-PAGE gel. Proteins on the gels were

visualized by silver staining and apparent molecular weights of the protein bands determined by comparison with a protein standard (Fermentas, Vilnius, Lithuania). Images were acquired with a Nikon camera (Nikon UK Limited, Surrey, UK) under white light as above.

3.8 Sequence analysis

The nucleotide sequences of VSG 3 and VSG 4 were analysed using National Center for Bioinformatics Information (NCBI) (http://blast.ncbi.nlm.nih/) database tools. The VSG 3 and VSG 4 DNA sequences were also conceptually translated in six reading frames using European molecular biology laboratory database (http://www.ebi.ac.uk/emboss/transeq/) and annotated through a series of scanning for functional domains and motifs present through InterProScan (Mulder *et al*., 2003), Simple Modular Architecture Research Tool (Schultz *et al*., 1998; Letunic *et al*., 2006) and ProtFun (Jensen *et al*., 2002; Jensen *et al*., 2003) analyses. The sequences were further characterized by identification of orthologs in the geneDB (http://www.genedb.org/) and NCBI (http://blast.ncbi.nlm.nih/) protein databases using BLASTp (Altschul *et al*., 1997) analysis.

CHAPTER FOUR

RESULTS

4.1 Amplification of VSG gene in gDNA from biological samples

4.1.1 Amplification of VSG gene using Hotstar Taq polymerase

The presence of *T. b. rhodesiense* VSG 3 or VSG 4 in biological samples from patients infected with HAT from Kenya in the second round of PCR using VSG 3 and 4 common internal forward and reverse primers are presented in Figure 4.

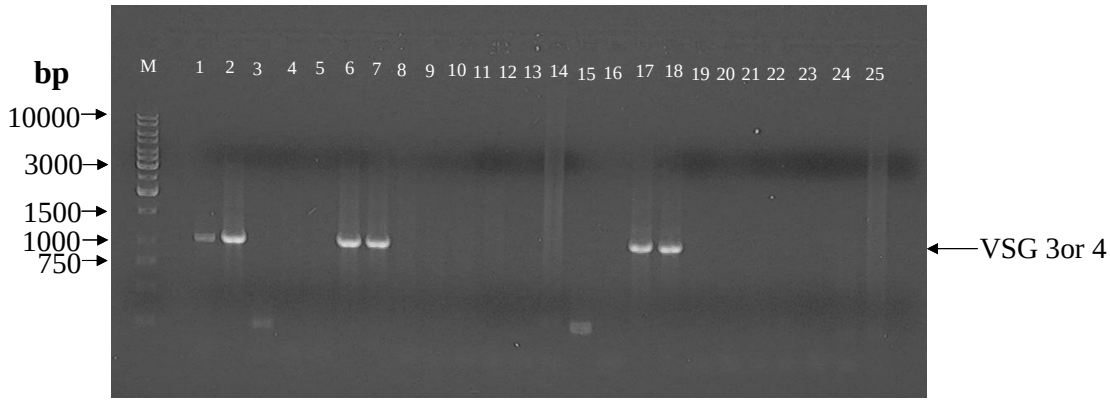


Figure 4: Ethidium bromide stained 1% agarose gel showing identification of *T. b. rhodesiense* VSG 3 or VSG 4 in biological samples from Kenya using hotstar *Taq* polymerase. Samples 1, 2, 6, 7, 17 and 18 are positive giving the expected fragment size of 1038bp. The lane marked M is a 1kb DNA ladder. Sample 25 is the negative control.

The presence of VSG 3 or VSG 4 of *T. b. rhodesiense* in biological samples from patients infected with HAT from Uganda in the second round of PCR

using VSG 3 and 4 common internal forward and reverse primers are presented in Figure 5.

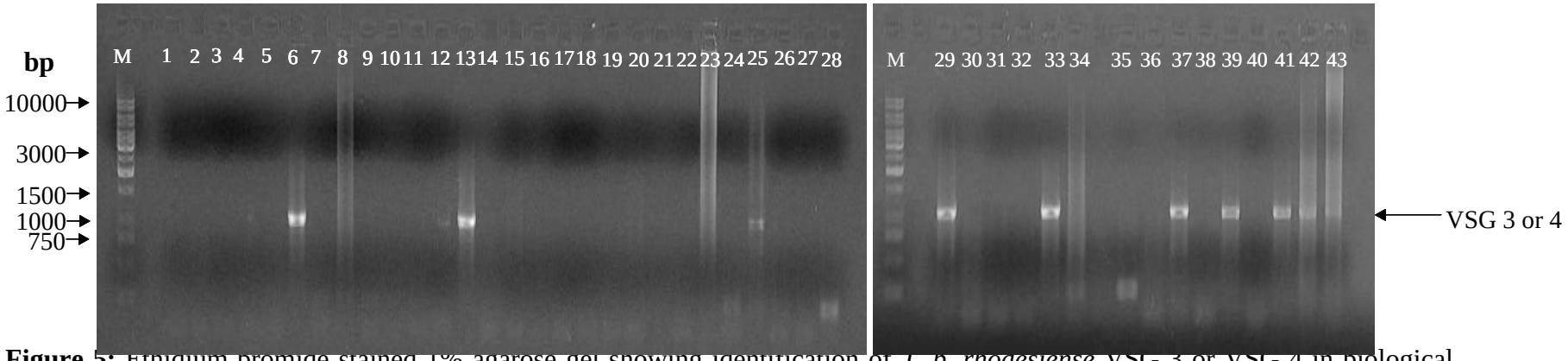


Figure 5: Ethidium bromide stained 1% agarose gel showing identification of *T. b. rhodesiense* VSG 3 or VSG 4 in biological samples from Uganda using hotstar *Taq* polymerase. Samples 6, 13, 25, 29, 33, 37, 39, 41, 42 and 43 are positive giving the expected fragment size of 1038bp. The lane marked M is a 1kb DNA ladder.

Six (25%, n = 24) and ten (23.3%, n = 43) of the samples from Kenya and Uganda respectively gave an amplified fragment of the expected size (1kb) indicating the presence of either VSG 3 or VSG 4 genes (Figure 6).

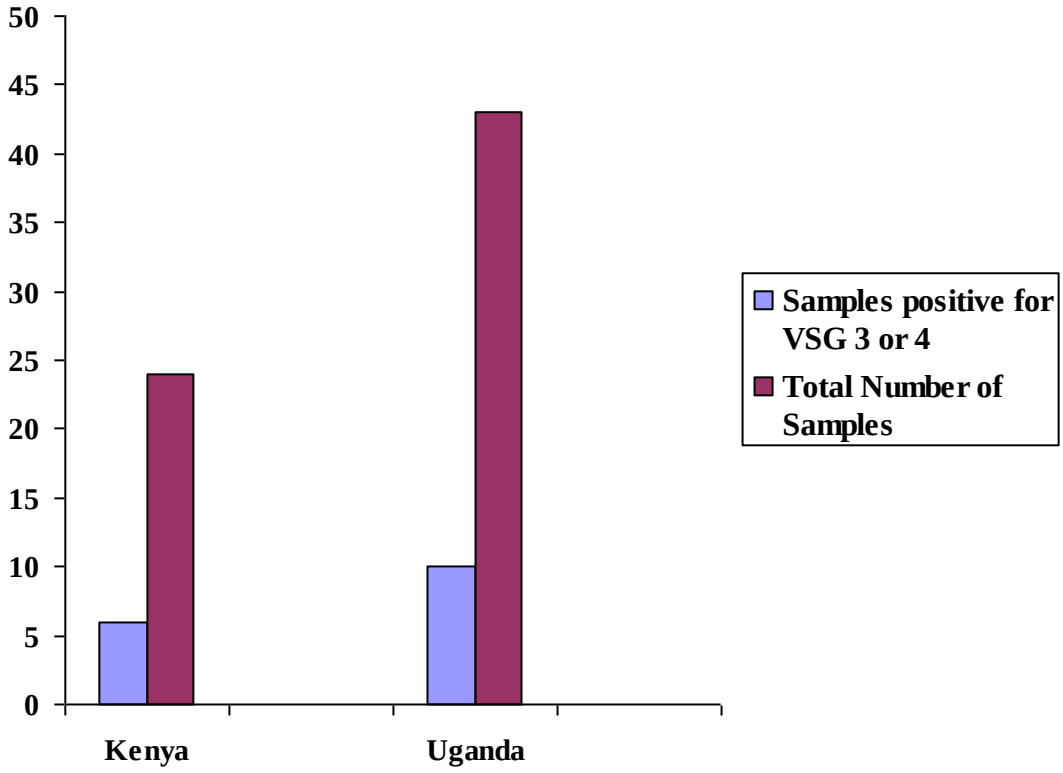


Figure 6: Chart showing the number of positive samples for the presence of VSG 3 or 4 among the Kenyan and Ugandan samples using hotstar *Taq* polymerase.

4.1.2 Amplification of VSG gene using phusion Taq polymerase

The presence of *T. b. rhodesiense* VSG 3 or VSG 4 in biological samples from patients infected with HAT from Kenya detected by PCR using phusion *Taq* polymerase and using VSG 3 and 4 common internal forward and reverse primers are presented in Figure 7.

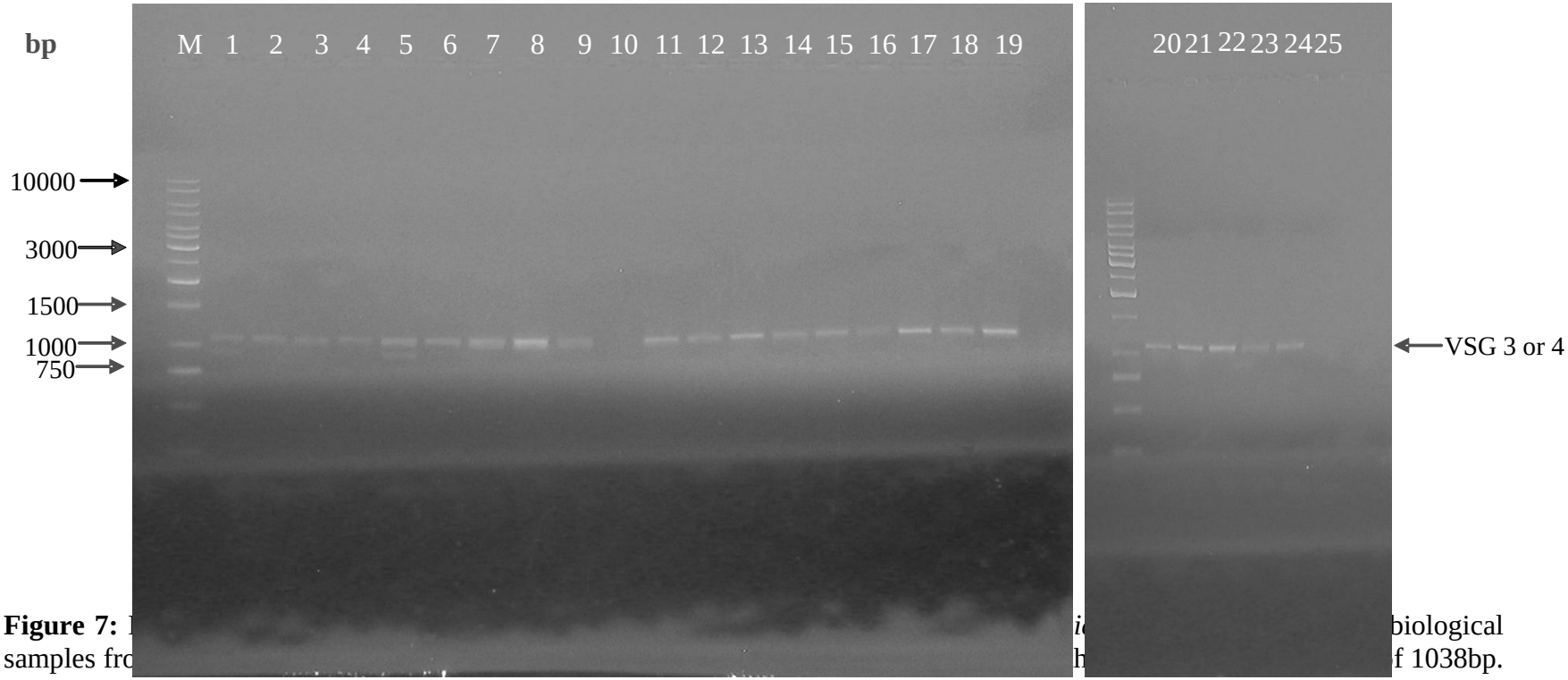


Figure 7: I *i* biological samples fro h f 1038bp. The lane marked M is a 1kb DNA ladder. Sample 25 is the negative control.

The presence of VSG 3 or VSG 4 of *T. b. rhodesiense* in biological samples from patients infected with HAT from Uganda detected by PCR using phusion *Taq* polymerase and using VSG 3 and 4 common internal forward and reverse primers are presented in figures 8 and 9.

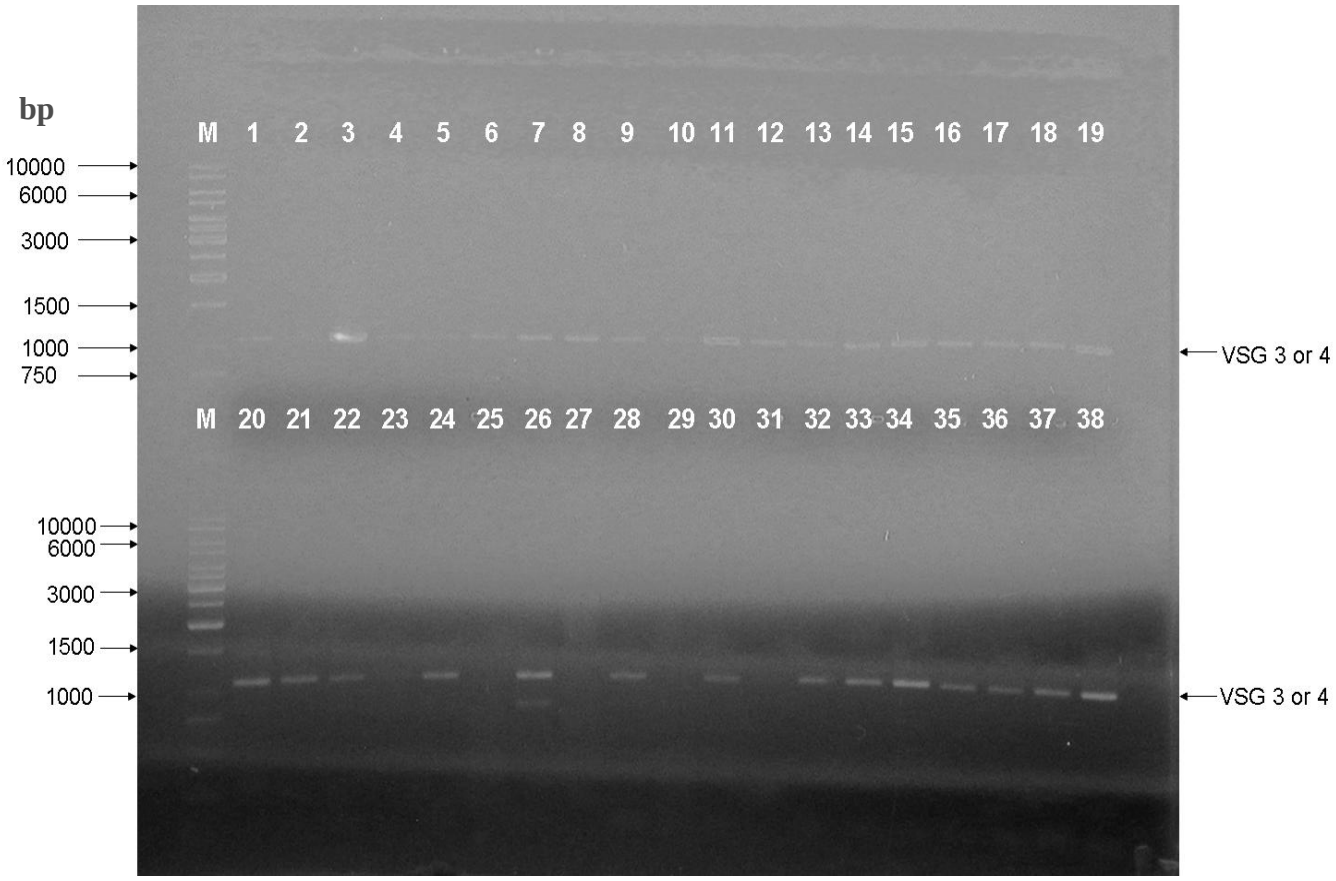


Figure 8: Ethidium bromide stained 1% agarose gel showing identification of *T. b. rhodesiense* VSG 3 or VSG 4 in biological samples from Uganda using phusion*Taq*polymerase.Samples1,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19, 20,21,22,24,26,28,30,31,32,33,34,35,36,37,38 are positive giving the expected fragment size of 1038bp. The lane marked M is a 1kb DNA ladder.

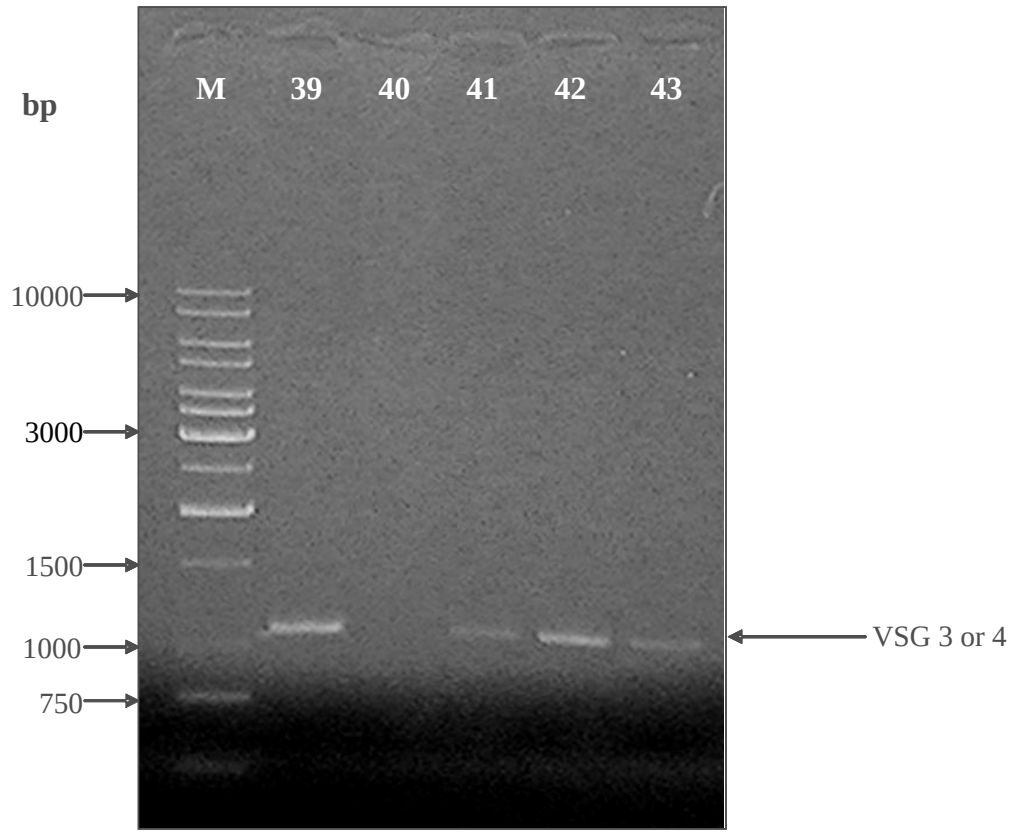


Figure 9: Ethidium bromide stained 1% agarose gel showing identification of *T. b. rhodesiense* VSG 3 or VSG 4 in biological samples from Uganda using phusion *Taq* polymerase. Samples 39,41,42,43 are positive giving the expected fragment size of 1038bp. The lane marked M is a 1kb DNA ladder.

Twenty three (95.8%, n = 24) and thirty seven (86%, n = 43) of the samples from Kenya and Uganda respectively gave an amplified fragment of the expected size (1kb) indicating the presence of either VSG 3 or VSG 4 genes (Figure 10). This was a significant improvement from that observed with hotstar *Taq* polymerase, proving that phusion *Taq* polymerase is more effective than hotstar *Taq* polymerase. This probably suggests that VSGs 3 and 4 are consistently present in biological field samples therefore making the two best candidates if a sensitive kit is used.

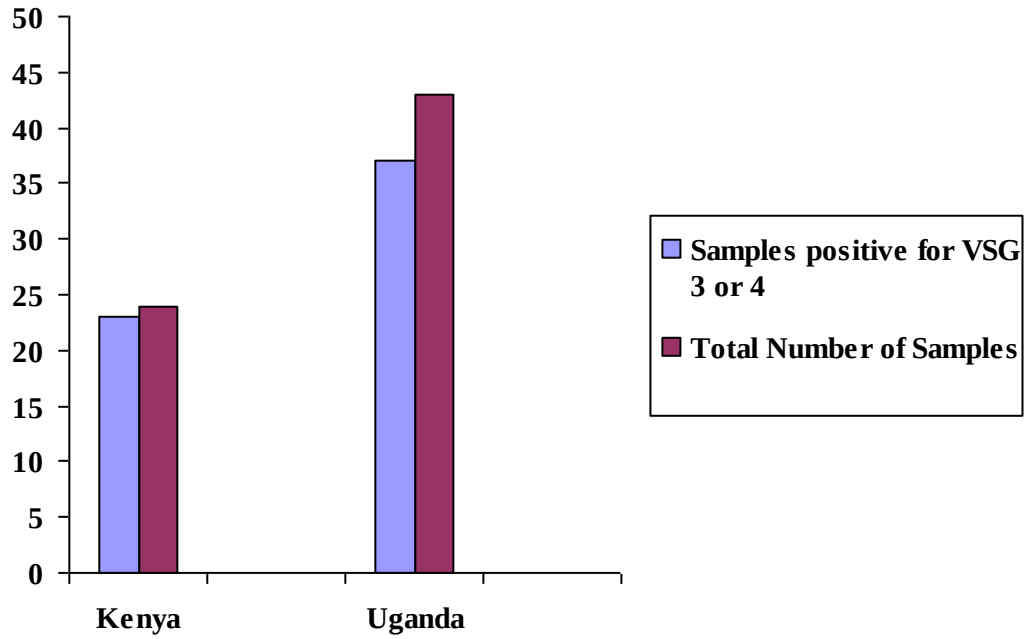


Figure 10: Chart showing the number of positive samples for the presence of VSG 3 or 4 among the Kenyan and Ugandan samples using high fidelity phusion *Taq* polymerase.

4.2 Expression analysis of recombinant VSG 4 protein

Expression of the VSG 4 protein as a histidine tagged protein was accomplished by cloning the coding region into the pRSET-A expression vector followed by subsequent expression in *E. coli* BL21 (De3). The 55-kDa 6xHis-tag protein was over expressed upon induction with IPTG.

4.3 Detection of recombinant VSG 4 protein using anti-VSG antibodies

Result of Western blot analysis of the expressed recombinant VSG 4 protein using serum positive for human African trypanosomiasis is presented in Figure 11. There was a smear within the expected range since crude protein was used, thereby necessitating purification of the crude protein so as to obtain a pure VSG 4 protein.

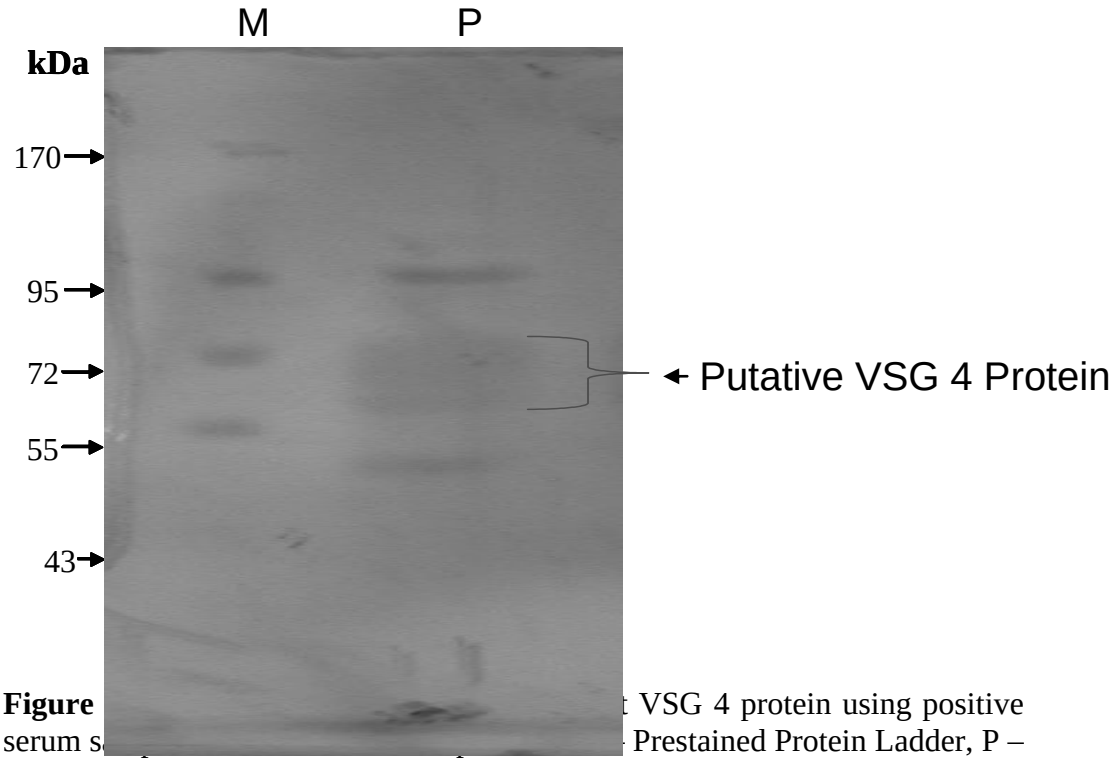


Figure t VSG 4 protein using positive serum s Prestained Protein Ladder, P – Crude VSG 4 Protein.

4.4 Purification of recombinant VSG 4 Protein

Result of purification of expressed recombinant VSG 4 protein in *E. coli* is presented in Figure 12. The recombinant VSG 4 protein was present in low concentration in the non - induced *E. coli* cells due to leakage in *E. coli* BL-21 cells, but was significantly increased upon induction with IPTG. The 55-kDa 6x histidine-tagged protein was a major component of the eluate and was recovered in relatively large quantities.

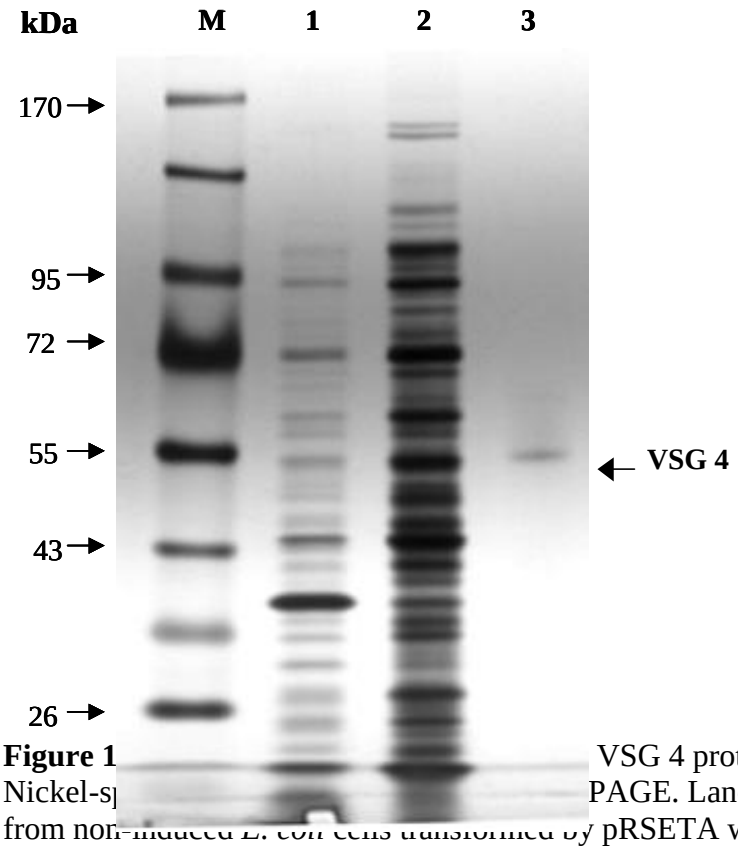


Figure 1 VSG 4 protein, from Nickel-s PAGE. Lane 1 contains proteins from non-induced *E. coli* cells transformed by pRSETA with VSG 4 gene. Lane 2 contains proteins from IPTG (1 mM)-induced *E. coli* cells transformed by pRSET-A with VSG 4 gene. Lane 3 contains purified VSG 4 protein.

4.5 Sequence analysis

4.5.1 Nucleotide Sequence analysis

VSGs 3 and 4 nucleotide sequences (Masiga *et al*., unpublished) on pairwise alignment using BLAST2Seq (Tatusova and Madden, 1999), shows VSG3 and VSG4 to be very similar with only a few mismatches (Figure 13).

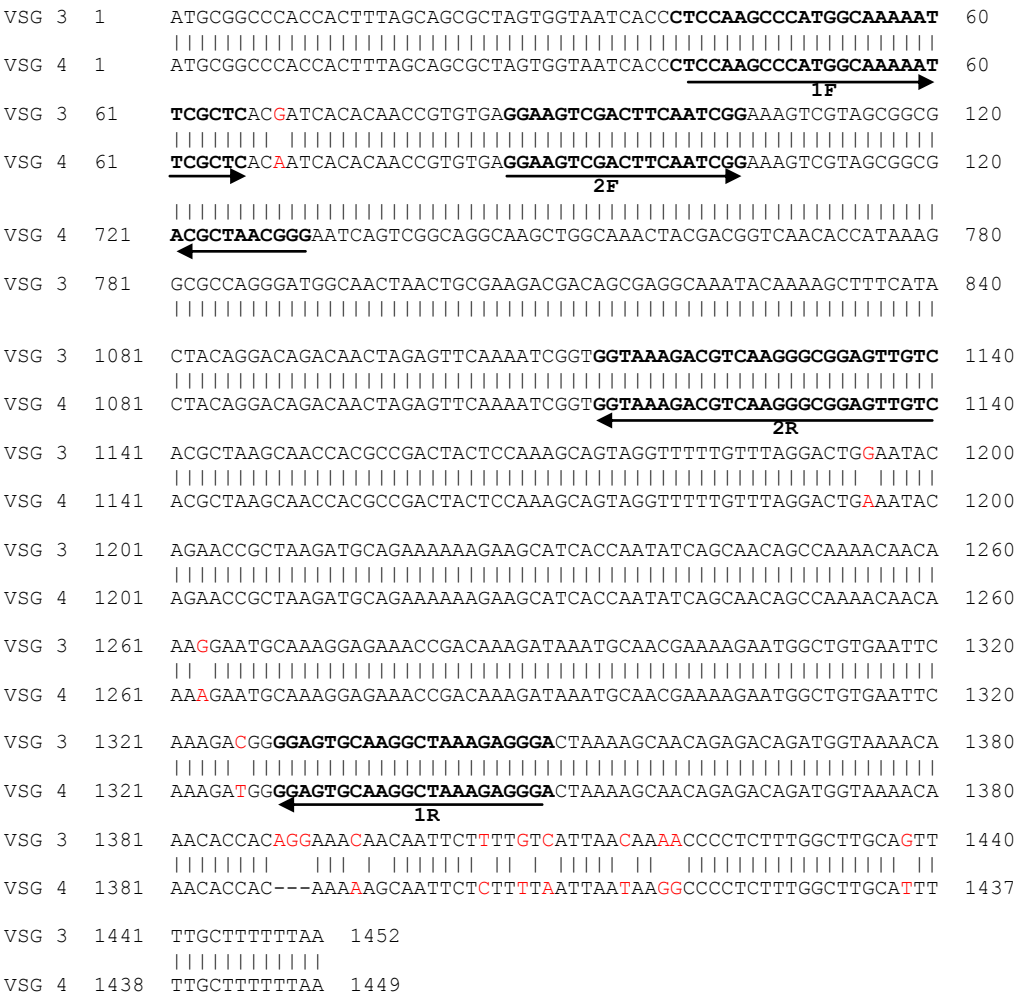


Figure 13: NCBI BLAST2 Sequence alignment of VSG3 and 4, showing the VSG gene sequences comparison. Identical nucleotides are indicated by a line in between them (|). Nonidentical nucleotides are shown in red.and are in positions 69,1263,1326,1389-1391,1395,1405,1408,1410,1416,1419-1420,1438 Primer positions are indicated in bold arrows.

BLASTn of VSG 3 against the *T. brucei* database at geneDB (http://www.genedb.org/) revealed it's top ortholog to be a putative atypical VSG, on chromosome 6 of *T. brucei* TREU927 (geneDB: Tb927.6.5450). The query VSG 3 had identity, score and E-value of 59%, 1524 and 2e-65, respectively. On the other hand for the same top ortholog; VSG 4 had identity,

score and E-value of 58%, 1450 and 4e-61, respectively. The NCBI database identified *T. evansi* strain KETRI-JN394 clone 1A VSG messenger RNA (GenBank accession number AF317931) and a putative atypical VSG, on chromosome 6 of *T. brucei* TREU927 (GenBank accession number XM840543) as putative orthologs to the VSG 3 query. The identity, score and E-value was 90%, 224 and 7e-55, respectively (GenBank accession number AF317931) and 70%, 196 and 3e-46, respectively (GenBank accession number XM840543).The two were also top orthologs of VSG 4 with identity, score and E-value of 85%, 221 and 8e-54, respectively (GenBank accession number AF317931) and 71%, 210 and 1e-50, respectively (GenBank accession number XM840543).

4.5.2 Amino acid Sequence analysis

The predicted amino acids sequences of the VSGs had a signal peptide that was identified using InterProscan (http://www.ebi.ac.uk/Tools/InterProScan/) and confirmed by SMART (http://smart.embl-heidelberg.de/) at position 1 - 21, in addition to low complexity domains at between 68-84, 198-208 and 369-380 residues. ProtFun prediction server (http://www.cbs.dtu.dk/services/ProtFun/) putatively identified VSG 3 as a cell envelope protein, but with likely cleavage site between positions 21 and 22 sites corresponding to the signal peptide boundary, 28 putative phosphorylation sites at positions 95, 101, 147, 166, 187, 215, 250, 272, 281, 301, 329, 411, 4, 5, 137, 150, 191 216, 219, 254, 258, 266, 352, 419, 427, 456, 197, 357; two putative O-glycosylated sites at positions 156 and 228, and 1 putative N-glycosylated site at position 461. No TM

helices, propeptide cleavage sites or high confidence targeting sites were predicted. BLASTp analysis of VSG 3 sequence against the *T. brucei* database in the geneDB and NCBI databases independently identified a putative atypical VSG, on chromosome 6 of *T. brucei* TREU927 (geneDB: Tb927.6.5450) as a putative ortholog to the VSG 3 query. The identity, score and E-value were 42%, 944 and 7e-100 respectively.

VSG 4 was similar to VSG 3 in all domain and motif architecture, and functional orthologs in the geneDB and NCBI, except there were differences in length of signal peptide (1-24), and lengths and positions of low complexity regions (71-87, 201-211, 372-383), and an additional putative phosphorylation site at residue 462 in VSG 4.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Detection of T. b. rhodesiense by amplification of VSG3 and VSG4.

Comparison of the two VSG 3 and 4 nucleotide sequences showed a high degree of similarity; they are almost identical, with only a few substitutions at the c-terminal end. The SRA which is a VSG significantly shorter than average (Berberof *et al*., 2001), also has a very high level of conservation with 97.9–99.7% similarity (Gibson *et al*., 2002). Hence it was possible to use one set of primers to amplify both VSG 3 and 4. This makes the VSG-PCR cheaper since only one set of primer can be designed to amplify both VSG 3 and 4, therefore not more than one PCR is carried out hence less reagents are used.

The detection level of approximately 25% using hotstar *Taq* was very low and unrealistic since both VSGs 3 and 4 are predominantly consistently expressed during early stage of HAT infection. The detection level of approximately 90% using phusion *Taq*, compares well with the specific detection of *T. brucei* ssp. by PCR using primers against conserved expression-site-associated genes (*ESAG*) 6 and 7 sequences, which detected approximately 80% of the parasitologically positive cases (Morgan *et al*., 1996; Kabiri *et al*., 1999). This detection level is surprising as the *ESAG6* and *ESAG7* DNA target sequences each occur in the trypanosome genome in about 20 copies (Morgan *et al*., 1996; Kabiri *et al*. 1999) as compared to the VSG gene, which occurs as a single copy in the genome.

Each VSG gene represented as a single copy within the genome is difficult to amplify using standard PCR as is evidenced by the low percentage of positives when using the Qiagen hotstar *Taq* polymerase. This was observed in about 75% of the 24 and 43 biological samples from Kenya and Uganda respectively, which could not be detected by the VSG-PCR even after nested PCR with Qiagen hotstar *Taq* polymerase using an aliquot of the product of the first round of PCR as template. This necessitated the use of high fidelity phusion *Taq* polymerase. This DNA polymerase possesses a 3′-5′ proof-reading endonuclease activity, its mutation rate is 50-fold lower than that of Taq DNA polymerase and 6-fold lower than that of *Pyrococcus furiosus* (Pfu) DNA polymerase (as claimed by the manufacturer). While Xiaoping *et al*. showed that the mutation rate was only about 0.01% when the phusion high-fidelity DNA polymerase was used as compared to 0.1% mutation rate of *Taq* polymerase on PCR amplification of cDNA of human polymeric immunoglobulin receptor gene (Xiaoping *et al*., 2007). Although the phusion *Taq* is more expensive, the fact that only one set of PCR is carried out means that the total cost of the diagnosis would be lower than using the hotstar nested PCR. In addition the higher sensitivity would eliminate the requirement for additional diagnostic tests usually carried out for HAT making this test more cost effective.

MacLeod *et al*. (1997) showed that, it is technically feasible to amplify a single copy sequence using nested primers by successfully amplifying the single copy triosephosphate isomerase gene (TIM) sequence of *T. brucei* using dilutions of

extracted DNA equivalent to a single genome (0.12 pg) per reaction. In addition, it has been demonstrated that it is possible to amplify a PCR product from DNA or trypanosome extracts diluted to less than a single genome equivalent (MacLeod *et al*., 1997; Kabiri *et al*., 1999; Sehgal *et al*., 2001). The detection level observed with nested amplification of TIM gene of approximately 60% is lower than that observed in this study using phusion *Taq* polymerase of approximately 90%.

The results of this study, using the high fidelity phusion *Taq* polymerase (New England Biolabs, Boston, USA), show an increase in the number of detectable positive samples significantly from 25% to 95.8% for samples from Kenya and from 23.3% to 86% for samples from Uganda. This could be attributed to polymerase ability to anchor onto the template more efficiently or strongly. Frey *et al*. (2007) observed that Phusion DNA polymerase perform much better than the mixture of the conventional enzymes, Pfu/*Taq* polymerase, with over 80% of the total 71 targets being amplified at the first attempt. These results indicate that it is possible to amplify single copy VSG sequence to detectable levels using Phusion *Taq* polymerase as evidenced by percentage increase of positive samples.

Though phusion *Taq* polymerase is more expensive than the hotstar *Taq*, it is better as a confirmatory test especially when the parasitaemia level is low, since *T. b. rhodesiense* infection is characterized with several parasitaemic waves. Also the phusion *Taq* can be used on follow-up of treatment of a

patient, due to the fact that it can amplify small amounts of DNA leading to detection of infection even when the parasite is not visible by other techniques such as microscopy. Phusion taq can also be used to assess for relapse in patients since *T. b. rhodesiense* are known to recrudescence even after two years. The results of this study demonstrate over 90% detection of infection, which is better than the detection usually achieved by other field applicable techniques including Ab-ELISA, Ag-ELISA that achieve up to 89% and 74% respectively (Davison *et al*., 1999; Monzon *et al*., 1995). These findings suggest that the detection of VSG3 and 4 would make a more sensitive and affordable diagnostic kit in the diagnosis and treatment follow-up of aparasitaemic patients.

Other groups have developed PCR tests based on *T. b. gambiense* VSG sequences (AnTat 11.17 and LiTat 1.3), but observed that some *T. b. gambiense* strains from northwestern Uganda and Cameroon remained negative in these PCRs due to the absence of the corresponding VSG genes within their genome (Enyaru *et al*., 1998; Kanmogne *et al*., 1996). Such a scenario may account for the approximately 10% that were not detected in the present study.

The specific detection of *T. b. gambiense* by PCR focused predominantly on identification of the variant specific glycoprotein (VSG) genes, LiTat 1.3 and AnTat 11.17 (Bromidge *et al*., 1993). Due to the sequence variation that exists within the VSG repertoire, there were occasional foci detected where the established molecular targets were not present within the parasite genomes

(Radwanska *et al*., 2002b). The receptor-like flagellar pocket glycoprotein TgsGP was identified as an alternative diagnostic target (Berberof *et al*., 2001; Radwanska et al., 2002b). This region was shown to be a specific marker for all *T. b. gambiense* foci and not T. b. rhodesiense (Radwanska *et al*., 2002b). A specific 308-base pair (bp) PCR product was obtained with 13 of 15 *T. b. gambiense* populations while the other 58 non-*T. b. gambiense* populations tested negative (Berberof *et al*., 2001; Radwanska *et al*., 2002b). The VSG-PCR in this study can be used specifically for the *T. b. rhodesiense* sleeping sickness just as the TgsGP-PCR has been used for the *T. b. gambiense* sleeping sickness.

Human *SRA* gene found exclusively in *T. b. rhodesiense*, has been used as a specific diagnostic tool for *T. b. rhodesiense* by comparing *T. brucei* ssp. isolates obtained from humans, animals and tsetse in HAT foci in Kenya and Uganda (Gibson *et al*., 2002, Radwanska *et al*., 2002a, Njiru *et al*. 2004; Enyaru *et al*., 2006). The *SRA* gene was shown to be present in all 44 *T. b. rhodesiense* isolates from human patients (36 isolates had been collected from Lambwe valley, Kenya and eight from Busoga, Uganda HAT foci), but absent in *T. b. gambiense* or *T. evansi* isolates (Njiru *et al*., 2004). The specificity of the SRA-based PCR tested on 97 different trypanosome populations originating from various taxonomic groups, host species, and geographic regions, showed only one of 25 *T. b. rhodesiense* samples negative and none of 72 other samples positive (Radwanska *et al*., 2002a).

Though the *SRA* gene has been shown to have a very high level of conservation (97.9–99.7% identity) in a comparison of sequences from *T. b. rhodesiense* sampled throughout its geographic range, two variants have been found (Gibson *et al*., 2002; Gibson *et al*., 2005). The northern variant is found in the majority but not all isolates of *T. b. rhodesiense* from Uganda, Kenya and Tanzania, while the southern variant is found in *T. b. rhodesiense* isolates from Ethiopia, Malawi and Zambia (Gibson *et al*., 2005).This means that more than one PCR test would have to be carried out for every isolate from the different geographical regions, as compared to only one PCR test in this study. SRA is also much less abundant than the VSG which predominantly covers the entire surface of bloodstream form trypanosomes (Pays *et al*., 2001). Therefore, *VSG* seems to be a better tool for identification of *T. b. rhodesiense* due to its abundance.

However, so far not much work has been done with variant surface glycoprotein gene of *T. b. rhodesiense* in the search for new diagnostic methods for HAT. Since the entire surface of bloodstream form trypanosomes is predominantly covered by VSGs, these VSGs represent the best theoretical candidates for diagnosis of HAT. Since VSG 3 and 4 are predominantly expressed in early stage infection (Masiga *et al*., unpublished), their use is vital for early diagnosis. This means prognosis is better as late stage HAT has very few and often toxic curative drugs.

5.1.2 Detection of crude recombinant protein using anti-VSG antibodies

Specific detection of antibody in serum positive for human African trypanosomiasis using recombinant VSG 4 as an antigen gave a smear approximately within the expected range. This supports the hypothesis that recombinant VSG protein of predominantly expressed VSG genes can be successfully used to detect early stage HAT caused by *T. b. rhodesiense*, as has been used in CATT and derivatives for *T. b. gambiense* (Büscher *et al*., 1999; Dukes *et al*., 1992) and similar tests for *Trypanosoma evansi* (Atarhouch *et al*., 2003; Gutierrez *et al*., 2000; Lejon *et al*., 2005).

RoTat 1.2, a predominant VSG expressed early during infection of susceptible animals, with the majority of *T. evansi* strains was shown to be a good candidate for diagnostic reagents (Verloo *et al*., 2000; Verloo *et al.*, 2001). The RoTat 1.2 VSG has been used as an antigen in antibody detection tests for diagnosis of *T. evansi* infections in camels (Atarhouch *et al*., 2003). This was illustrated by the diagnostic tests based on the RoTat 1.2 such as the CATT/*T. evansi* (Gutierrez *et al*., 2000), LATEX/*T. evansi* and ELISA/*T. evansi* (Verloo *et al*., 2000; Verloo *et al.*, 2001). In this same way, VSG 4 also a predominant VSG gene expressed in early stage during infections with *T. b. rhodesiense*, can also be used as an antigen in antibody detection tests for diagnosis of HAT. The smear observed with the antigen used in this study may be due to the use of crude and not purified antigen. Therefore some true positive samples may fail to be detected in the present study which employed use of crude antigen, due to its lower sensitivity. Therefore use of purified antigen needs to be

employed to enhance the signal. Magnus *et al*. (1978b) demonstrated that purified VSG from VAT *T. b. brucei* AnTat 1.8 can provide a powerful serodiagnostic reagent. This principle was later confirmed by a comparative study of different *T. b. gambiense* VATs that included LiTat 1.3 and LiTat 1.6 (Magnus *et al*., 1978b). An antibody detection ELISA for diagnosis of *T. b. gambiense* infection in man, using LiTat 1.3 and LiTat 1.6 antigens consisting of semi-purified variable surface glycoprotein also revealed high sensitivity and specificity (Büscher *et al*., 1991).

In this study to improve on the test sensitivity, in addition to using purified VSG 4 antigen, it may be good to include VSG 3 which is also predominantly expressed in early stage *T. b. rhodesiense* infection, so as to also detect those VSGs that may share similar epitopes to VSG 3. Ngaira *et al*. (2004) reported RoTat 1.2 to be absent in four *T. evansi* isolates including reference *T. evansi* KETRI 2479 in Western blot using anti-RoTat 1.2 serum. The same scenario was also reported in T. *b. gambiense* with the absence of the diagnostic LiTat 1.3 from some *T. b. gambiense* stocks in Cameroon, although this did not appear widespread (Dukes *et al*., 1992). This phenomenon led to the development of Latex/*T. b. gambiense* test that included additional antigens, LiTat 1.5 and LiTat 1.6 to the LiTat 1.3, for improved sensitivity in the diagnosis of *T. b. gambiense* (Büscher *et al*., 1999).

5.1.3 Sequence analysis

5.1.3.1 Nucleotide sequence analysis

A remarkable degree of sequence similarity was found among VSGs 3, 4 and other VSGs in geneDB and NCBI databases, with VSG 3 being very similar to VSG 4. In some cases the homology was greater between VSGs from different species than between VSGs of the same species. This was evidenced by 90% identity to *T. evansi* strain KETRI-JN394 (GenBank accession number AF317931), as compared to 70% identity to *T. brucei* TREU927 putative atypical VSG (GenBank accession number XM840543) on alignment with VSG3. The same pattern was observed in VSG 4 with 85% identity to *T. evansi* strain KETRI-JN394 (GenBank accession number AF317931) as compared to 71% identity to *T. brucei* TREU927 putative atypical VSG (GenBank accession number XM840543). Urakawa *et al*. (2001) observed that DNA sequences homologous to the diagnostic RoTat 1.2 VSG gene were present in the genomes of other trypanosomes and yet a 488 bp section of the fragment was only specific for *T. evansi* by PCR amplification. The same may be true with VSG 3 or VSG 4.

5.1.3.2 Amino acid sequence analysis

The structural features of the cloned gene were examined to verify that the gene was a trypanosome VSG. These structural characteristics include a signal peptide, an N-terminal domain, a cysteine- rich C-terminal domain and a hydrophobic tail (Rice-Ficht *et al*., 1981; Carrington *et al*., 1991). Analysis of the amino acid sequences of VSGs 3 and 4 show they have eight cysteine

residues in their N-terminal domain thus fitting the Type B categorization (Carrington *et al*., 1991). Furthermore VSGs 3 and 4 fit Type 2 C-terminus category by having four cysteine residues (Bubler *et al*., 1998, Carrington *et al*., 1991). The hydrophobic extension for this group is 17 residues long and usually starts with asparagine (Carrington *et al*., 1991; Rice-Ficht *et al*., 1998); this pattern matches VSGs 3 and 4. VSGs 3 and 4 also have a serine C-terminal residue which fits them in Type 2 class (Carrington *et al*., 1991, Rice-Ficht *et al*., 1998). This type of C-terminus category has a potential asparagine glycosylation site at five residues from the C-terminus. The two VSGs, 3 and 4 have an intact open reading frame with a typical VSG domain structure made up of N-terminal and C-terminal signal sequences. This proves that the two VSGs, 3 and 4, used in this study are trypanosome VSGs.

On alignment of the predicted amino acid sequences of the VSG 3 and 4 with the amino acid sequences of the other *Trypanozoon* VSGs already in the public domain databases, no significant matches were found. Even the amino acid sequence of the *T. evansi* strain whose DNA sequence was similar to VSG 3 and VSG 4, was not a match to the VSGs amino acids. This may indeed mean that *T. evansi* do not express VSG 3 or VSG 4. Urakawa *et al*. (2001) detected RoTat1.2 VSG gene homologous sequences in genomes of other cloned isolates of trypanosomes within the *Trypanozoon* but obtained no direct evidence that these trypanosomes do express the RoTat1.2 VSG, making it specific to *T. evansi*. It is possible that the lack of expression in other Trypanosoma species would make the use of the expressed protein specific to

T. b. rhodesiense and hence can be useful as diagnostic target in *T. b. rhodesiense* sleeping sickness.

5.2 Conclusions

From the results of this study it can be concluded that the primers used in this work; designed from the sequences VSGs 3 and 4 that are predominantly expressed in early stage HAT infection, may be used to detect *T. b. rhodesiense* infections in humans.

Also the predicted amino acid sequence of VSG 4 showed that protein expressed is specific to *T. b. rhodesiense*, therefore VSG 4 protein would be a good diagnostic candidate in serological assay which is normally cheaper than molecular assay.

5.3 Recommendations

- VSG 3 that is also predominantly expressed in early stage infection needs to be also expressed, purified and used in combination with VSG 4, so as to also detect those VSGs that may share similar epitopes to VSG3.
- Evaluation of other expression systems such as baculovirus to improve the yields.
- The specificity of these VSGs needs to be tested on other regional protozoan diseases.
- Evaluation of the utility of the recombinant protein in diagnosis using ELISA or agglutination tests.

- Evaluation of the recombinant protein as a vaccine candidate

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