RECOMBINANT EXPRESSION OF, AND CHARACTERISATION OF ANTIBODIES AGAINST VARIABLE SURFACE GLYCOPROTEINS: LITAT 1.3 AND LITAT 1.5 OF *TRYPANOSOMA BRUCEI GAMBIENSE*

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PREFACE

The experimental work described in this dissertation was carried out in Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Pietermaritzburg campus), from January 2012 to December 2013, under the supervision of Prof. Theresa H. T. Coetzer and co-supervision of Dr PR Vukea.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

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Dr P. R. Vukea (Co-supervisor)

DECLARATION-PLAGIARISM

I, Sanele Michelle Mnkandla, declare that

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2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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ABSTRACT

Human African Trypanosomiasis (HAT), also known as sleeping sickness is one of the many life threatening tropical diseases posing a serious risk to livelihoods in Africa. The disease is restricted to the rural poor across sub–Saharan Africa, where tsetse flies that transmit the disease, are endemic. Sleeping sickness is known to be caused by protozoan parasites of the genus *Trypanosoma brucei*, with the two sub-species: *T. b. gambiense* and *T. b. rhodesiense*, responsible for causing infection in humans. The disease develops in two stages, firstly, the infection is found in the blood and secondly, when the parasites cross the blood-brain barrier entering the nervous system. To date, no vaccines have been developed, however, there is a range of drugs and treatments available which depend on the type of infection (*T. b. gambiense* or *T. b. rhodesiense*) as well as disease stage.

The trypanosome parasites have a two-host life cycle i.e. in the mammalian host as well as the tsetse fly vector. Throughout the cycle, the parasite undergoes changes, one of them being the acquisition of a variable surface glycoprotein (VSG) coat prior to entry into the human host bloodstream. Once in the host, the infection progresses and through a phenomenon known as antigenic variation, the parasite expresses a different VSG coat periodically, enabling the parasites to constantly evade the host's immune response, facilitating their survival. The VSG genes coding for the proteins are activated by an intricate process involving the encoding of a gene which is kept silent, until activated in one of several expression sites. Despite the constant switching of VSG surface coats, there are VSG forms that are predominantly expressed in *T. b. gambiense* namely VSGs LiTat 1.3, LiTat 1.5 and LiTat 1.6 which are used in diagnostic tests, as antigens to detect antibodies in infected sera of HAT patients. The acquisition of these VSG antigens is, however, of high risk to staff handling the parasites, and so the first part of the study was aimed at cloning, recombinantly expressing and purifying the two VSGs known to be recognised by all gambiense HAT patients: LiTat 1.3 and LiTat 1.5, for possible use as alternative antigens in diagnostic tests.

The genes encoding both VSGs, LiTat 1.3 and LiTat 1.5, were first amplified from either genomic or complementary DNA (gDNA or cDNA), cloned into a pTZ57R/T-vector and sub-cloned into pGEX or pET expression vectors prior to recombinant expression in *E. coli* BL21 DE3 and purification by Ni-affinity chromatography. Amplification and subsequent cloning yielded the expected 1.4 kb and 1.5 kb for the *LiTat 1.3* and *LiTat 1.5* genes respectively. Recombinant expression in *E. coli* was

only successful with the constructs cloned from cDNA, i.e. the pGEX4T-1-cLiTat 1.3 and pET-28a-cLiTat 1.3 clones. Purification of the 63 kDa cLiTat 1.3_{His} protein following solubilising and refolding did not yield pure protein and there were also signs of protein degradation. For comparison, expression was also carried out in *P. pastoris* and similar to the bacterial system, expression was only successful with the LiTat 1.3-SUMO construct yielding a 62.7 kDa protein. Purification of LiTat 1.3_{SUMO} also surpassed that of cLiTat 1.3_{His} with no degradation.

The diagnostic tests based on VSGs LiTat 1.3 and LiTat 1.5 as antigens operate by binding with antibodies in infected sera, to confirm infection. These antibody detection tests have their limitations, hence an alternative would be antigen detection tests, which use antibodies to detect the respective antigens in infected sera. The second part of the study therefore involved antibody production, where chickens were immunised with the native VSGs LiTat 1.3, LiTat 1.5 as well as recombinant RhoTat 1.2 (a VSG expressed in *T. evansi*). Antibody production was analysed by ELISA and characterised by western blotting, prior to immunolabelling of *T. b. brucei* Lister 427 parasites. The chicken IgY showed a response to the immunogens, and were able to detect their respective proteins in the western blot. Interestingly, the anti-nLiTat 1.3, anti-nLiTat 1.5 and anti-rRhoTat 1.2 antibodies were able to detect their respective VSGs on the *T. b. brucei* trypanosome parasites in the immunofluorescence assay, thus demonstrating cross reactivity. As the antibodies showed specificity, they could potentially detect antigens in infected sera of HAT patients in an antigen detection based test.

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ABBREVIATIONS

2xYT	2 x yeast extract, tryptone
A_{280}	absorbance at 280 nm
A_{405}	absorbance at 405 nm
BCA	bicinchoninic acid
BMGY	buffered glycerol complex medium
bp	base pairs
BSA	bovine serum albumin
CATT	card agglutination test for trypanosomiasis
cDNA	complementary DNA
CNS	central nervous system
DIC	differential interference contrast
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
g	relative centrifugal force
gDNA	genomic DNA
HCT	haematocrit centrifugation technique
HRPO	horseradish peroxidase
IgY	immunoglobulin Y
kDa	kilo-Dalton
LAMP	loop mediated isothermal amplification
mAECT	mini anion exchange centrifugation technique

MCS	multiple cloning site
MWCO	molecular weight cut off
o.n.	overnight
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
QBC	quantitative buffy coat
RT	room temperature (approximately 25°C)
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
TBS	Tris-buffered saline
VAT	variable antigen type
VSG	variable surface glycoproteins
YDP	yeast extract, peptone, dextrose

CHAPTER 1:

LITERATURE REVIEW

1.1 Introduction

Human African Trypanosomosis (HAT) also known as sleeping sickness is caused by protozoan parasites of the species *Trypanosoma brucei* sensu lato (s.l) (Maudin *et al.*, 2004). The disease is restricted to the poor rural sub–Saharan Africa where tsetse flies of the *Glossina* spp., that transmit the disease, are endemic. Human populations living in these areas that depend on agriculture, fishing or hunting are exposed to the tsetse flies and hence the disease (Brun *et al.*, 2009; Kristensson *et al.*, 2010). *T. brucei* s.l. is divided into three subspecies. *T. brucei gambiense* and *T. brucei rhodesiense* are the two subspecies responsible for causing infection in humans, while the *T. brucei brucei* subspecies is non-human infective, but infects cattle (Maudin *et al.*, 2004; Geiger *et al.*, 2010; Jackson *et al.*, 2010). Although they are morphologically similar, the human-infective strains are distinguished from each other by their disease distribution pattern. *T. b. gambiense* is endemic in both Western and Central Africa and is responsible for the chronic form of the disease, while *T. b. rhodesiense* is endemic in East Africa and accounts for the acute form of the disease (Blum *et al.*, 2007; Geiger *et al.*, 2011).

The HAT disease evolves through clinically distinct stages. The haemolymphatic stage is the first stage where the infection is in the blood. The second stage is the meningoencephalitic stage where the parasites invade the central nervous system (CNS) through the blood brain barrier (Geiger *et al.*, 2011). In order to assign treatment to patients suspected of being infected, accurate diagnosis must be done. The gold standard for diagnosis involves serological screening of serum or finger prick blood samples, parasitological confirmation by microscopically examining blood samples for trypanosomes as well as examining the cerebrospinal fluid following a lumbar puncture, to detect the stage of the disease the patient is in, before giving treatment (Koffi *et al.*, 2006). Treatment is based on the disease stage i.e. whether the parasites are in the blood or brain of the patient and the range of drugs used is limited and most have severe side effects (Brun *et al.*, 2009). To overcome these challenges, new promising drugs have been synthesized which are currently going through clinical trials in patients with HAT (DND*i*, 2012b; DND*i*, 2012a).

Within both the human and tsetse fly hosts, the parasite undergoes changes that are necessary for its successful transmission. Infection of the human host begins with the bite of an infected tsetse fly during its blood meal (Baral, 2010). However, before transmission to the human host, the trypanosome parasite expresses a surface coat made of procyclin molecules which are shed off in the salivary gland of the tsetse fly and a variable surface glycoprotein (VSG) coat is acquired (Pays and Nolan, 1998). Once inside the human host they undergo morphological changes to facilitate inter-host transfer of the parasite to various organ systems including the lymphatic system and the central nervous system, as well as facilitate transmission to the tsetse fly (Holzmuller *et al.*, 2008).

Various VSGs have been identified and classified and three particular VSGs of variable antigen type (VAT) LiTat 1.3, LiTat 1.5 and LiTat 1.6 have been identified in *T. b gambiense* infection and are of great importance as they are extensively used in diagnostics to identify infected individuals (Büscher *et al.*, 1999; Büscher *et al.*, 2013; Yansouni *et al.*, 2013). Trypanosomes express only one VAT at a time and then switch to another through antigenic variation, a process which facilitates their survival from destruction by the immune system as it prevents antibody recognition (Baral, 2010). In addition to antigenic variation, *T. b. gambiense* and *T. b. rhodesiense* parasites secrete substances that interfere with the host immune system, and the two have also shown resistance to destruction by the trypanolytic factor (TLF) found in human serum (Cox, 2001; Gibson, 2002). All these mechanisms fuel the survival and persistence of infection in the host. (Elrayah *et al.*, 2007). Recently, two lateral flow rapid diagnostic tests HAT Sero-Strip and HAT Sero-K-SeT have been developed with the aim of improving sensitivity, and specificity of the CATT test as well as introduce the testing of one individual at a time, i.e. single patient reagents as opposed to the kits for batch tests (Büscher *et al.*, 2013).

1.2 Epidemiology

Human African Trypanosomosis has been restricted to specific areas (foci) where epidemics have resulted since 1900. The Great Epidemic of 1900 in East Africa was the first epidemic recorded which targeted the Busoga focus in Uganda and Kenya and an estimated 500 000 people were infected (Hide, 1999). The second epidemic occurred between 1920 and 1949 and because of the negative impacts the disease had, the colonial administrators at that time established control programmes targeted at vector control which involved the use of designated traps and bush clearing, screening

people at risk as well as treatment and follow up of HAT patients (Steverding, 2008; Kristensson *et al.*, 2010). This action resulted in a decrease in the incidence of the disease. However when the countries where HAT was endemic gained independence, health services were no longer the main concern as there was political reorganisation as well as movement of populations. This had a negative effect on health services which resulted in decreased surveillance and consequently re-emergence of the disease (Simarro *et al.*, 2008; Kristensson *et al.*, 2010). The re-emergence occurred in the late 1990's in DRC, Angola, Central African Republic, southern Sudan and Uganda (Brun *et al.*, 2009).

The geographical conservation of HAT was observed during one of the epidemics in the Tororo District of Uganda in 1988, where *T. brucei* parasites were isolated from humans, cattle and tsetse flies. Using molecular tools, the isolates were tested for human serum resistance and the human isolates were clearly distinguished from the non-human isolates. Furthermore, the human isolates were seen to fall under a homogenous group genetically dissimilar with human isolates from another focus, Luangwa Valley in Zambia (Maudin *et al.*, 2004). The difference in the genetics observed within the human isolates, which demonstrated how strains are conserved within a focus, was due to the expression of a serum resistance associated gene (*SRA*) (Gull, 2003; Maudin *et al.*, 2004). This gene is expressed in *T. b. rhodesiense* and this disease form is endemic in Eastern and Southern Africa (Fig. 1). *T. b. gambiense* lacks the *SRA* gene and the Gambian form of the disease is prevalent in countries in Central and West Africa (Chappuis *et al.*, 2005; Brun *et al.*, 2009). This clear-cut separation could, however, soon change because studies carried out in Uganda, where both *T. b. rhodesiense* and *T. b. gambiense* are endemic, have shown the spread of *T. b. rhodesiense* from its foci in the south-east of the country towards the *T. b. gambiense* foci in the northwest resulting in an overlap (Maudin *et al.*, 2004; Brun *et al.*, 2009).

Unlike *T. b. rhodesiense*, *T. b. brucei* lacks the *SRA* gene and is not infective to humans, but infective to cattle (Maudin *et al.*, 2004; Kristensson *et al.*, 2010). The use of the *SRA* gene as a marker proved vital in order to distinguish between the two, because parasite analysis of non-human isolates (cattle) revealed the presence of *T. b. rhodesiense* trypanosomes. *T. b. rhodesiense* has therefore been classified as a zoonosis i.e. transmitted from animals to humans (Fig. 1). The animal acts as a reservoir harbouring the parasite but does not get sick from it (Maudin *et al.*, 2004; Kristensson *et al.*, 2010). The close interaction of the people and their livestock therefore contributes to disease transmission resulting in the maintenance of the

human-infective-trypanosome strain (Maudin *et al.*, 2004; Simarro *et al.*, 2013). *T. b. gambiense* on the other hand is anthroponotic i.e. it is only transmitted from human to human (Kristensson *et al.*, 2010).

The prevalence of HAT differs from one country to another, but through efforts from WHO in collaboration with non governmental organisations to control the disease, there has been a decrease in the number of reported cases. For more than ten years, countries that were previously endemic e.g. Gambia have been disease free and WHO showed a decline in the number of newly reported cases which were 17 616 in 2004 which further reduced to less than 10 000 in 2010 and 7 000 in 2012 (Courtin *et al.*, 2008; WHO, 2012; Krishna and Stitch, 2013). Currently, the WHO Roadmap on neglected tropical diseases (NTDs) intends to eliminate HAT as a public health problem by the year 2020 (WHO, 2012).

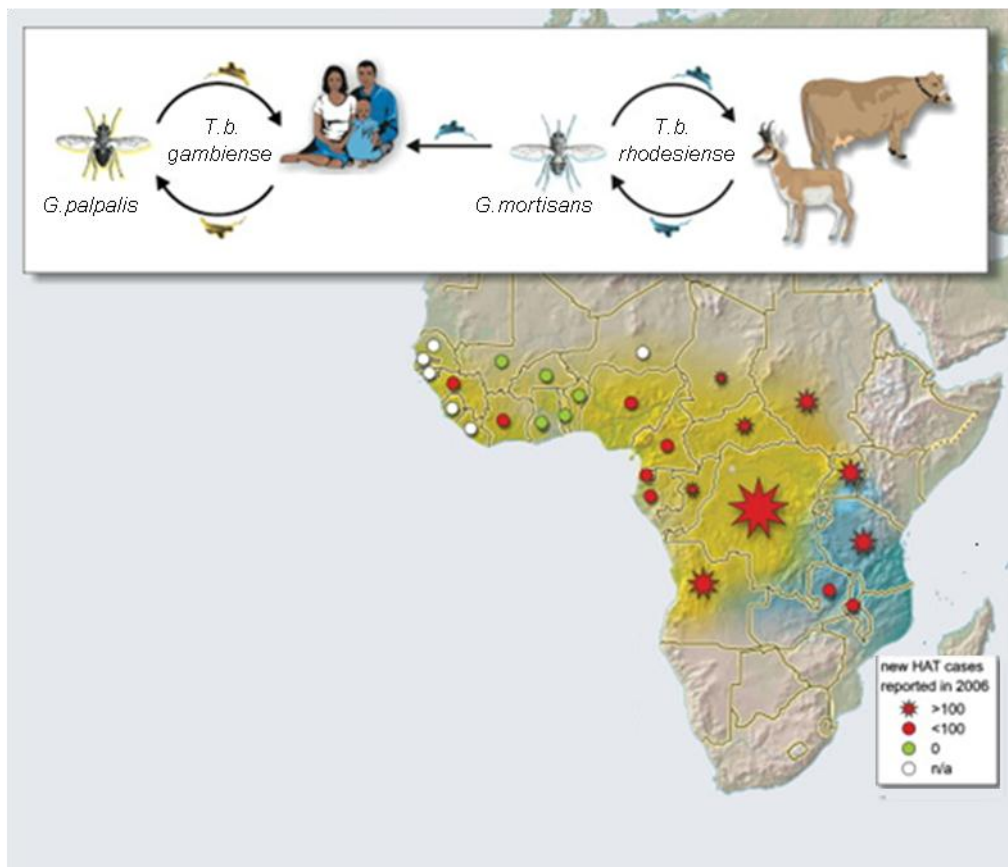


Fig. 1.1. African trypanosome transmission and epidemiology. The top diagram illustrates the transmission of the parasites. *T. b gambiense* which is anthroponotic is transmitted to the human host by the *G. palpalis* group of the tsetse fly while *T. b rhodesiense* a zoonosis whose life cycle involves cattle, which do not get infected, is transmitted to the human host by the tsetse fly of the *G. mortisans* group. The bottom diagram shows the areas which are endemic for HAT for each of the sub species: blue shading shows *T. b. rhodesiense* and the yellow shading *T. b. gambiense*. The symbols illustrate the number of cases reported by each country in 2006. Adapted from (Kristensson *et al.*, 2010).

1.3 Classification and morphology of the *T. b. brucei* parasite

Parasitic protozoa of the genus Trypanosoma responsible for trypanosomosis in mammals are divided into two groups; the Salivaria and Stercoraria (Fig. 2). In the Salivaria group, parasites develop in the midgut of the vector and move to the salivary gland and infection is via saliva as the vector injects trypanosomes when it bites during its bloodmeal (Haag *et al.*, 1998; Maudin *et al.*, 2004; Baral, 2010). In the Stercoraria group, the trypanosomes develop in the intestinal tract of the vector and infection is via contamination through faeces which are released when the host feeds (Haag *et al.*, 1998; Maudin *et al.*, 2004; Baral, 2010). Unique to all the species of the Salivaria group, is their ability to evade destruction by the immune system of their hosts due to the expression of VSGs which are involved in antigenic variation (Haag *et al.*, 1998).

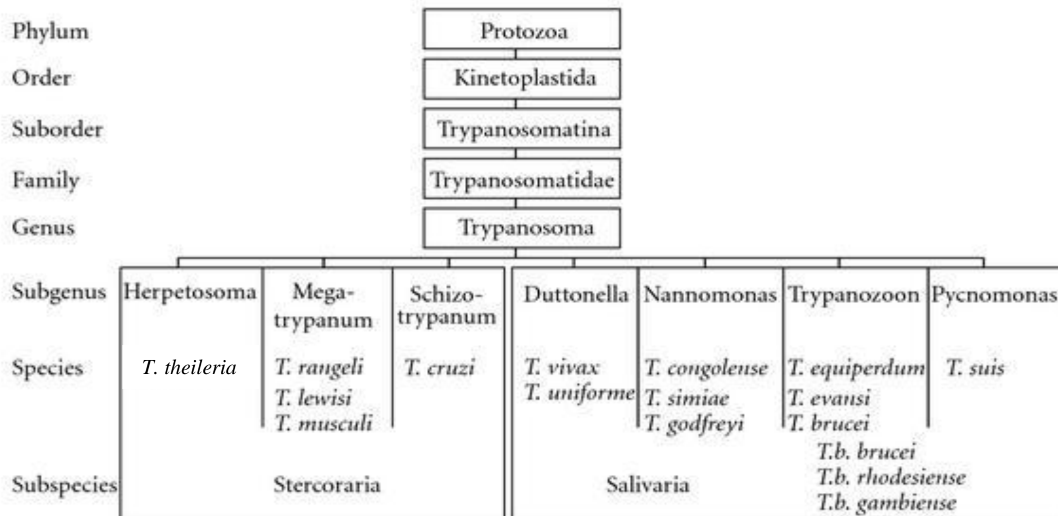


Fig. 1.2. Classification of Trypanosomes (Baral, 2010).

Trypanosome cells are well adapted to living and moving in the blood plasma or tissue fluid of the host. Apart from the general features found in other groups of organisms e. g. Golgi, and endoplasmic reticulum, trypanosomes have some special features common to the order Kinetoplastida (Lopes *et al.*, 2010).

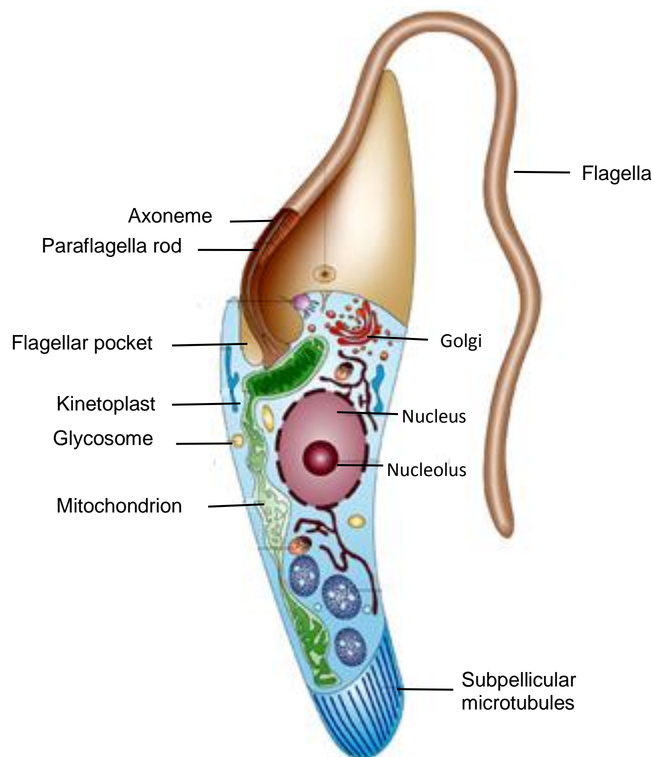


Fig. 1.3. Features of the *T. brucei* parasite. Adapted from (Vargas-Prada, 2010).

The *T. brucei* cell body is 11-42 µm in length, roughly cylindrical in shape and has tapered anterior and posterior ends (Maudin *et al.*, 2004; Ralston and Hill, 2008). There are three forms the parasite exists as: the long slender from, short stumpy form and the intermediate form which interchanges throughout the parasite's life cycle (Maudin *et al.*, 2004). The cell surface has a dense surface coat of VSGs that predominate in the bloodstream stage of mammalian hosts. These VSGs are then replaced by procyclins in the procyclic stage once the parasite enters the tsetse fly after a blood meal (Pays and Nolan, 1998). Also present in low abundance, are invariable surface glycoproteins (ISGs) and various receptors such as transferrin receptor for iron uptake which is important for the survival of the trypanosome. The shape and form of the trypanosome cell is maintained by their internal cytoskeleton (Fig. 3) which consists of sub pellicular microtubules attached to the inner surface of the membrane (Kohl and Gull, 1998; Lopes *et al.*, 2010).

T. brucei is characterised throughout its life cycle by the possession of a flagellum (Fig. 3). Each trypanosome parasite has a single flagellum that emerges from a basal body in the cytoplasm through an invagination of the plasma membrane called the flagellar pocket, a region where endocytosis/exocytosis takes place (Ogbadoyi *et al.*, 2003; Baral, 2010). The flagellum is made of a flagellar axoneme, which lies parallel to

the paraflagella rod–a lattice like structure that runs along the whole length of the flagellum (Kohl *et al.*, 1999). The flagellum is attached to the cell body at the flagellum attachment zone (FAZ) and continues towards the anterior end of the body as a 'whip-like' free flagellum (Kohl and Gull, 1998; Rocha *et al.*, 2006; Ralston and Hill, 2008; Lopes *et al.*, 2010). The trypanosome uses the flagellum in cell motility, attachment of parasite to host surface as well as morphogenesis and cell division, influencing disease pathogenesis and transmission (Ralston and Hill, 2008; Lopes *et al.*, 2010).

The nucleus has a diameter of ~3 µm with a nuclear envelope of many nuclear pore complexes (Ersfeld *et al.*, 1999). Apart from the nucleus, the parasite has another DNA structure known as the kinetoplast-the organelle responsible for the name of the Order Kinetoplastida. The kinetoplast lies at the base of the flagellum and is located in the single long mitochondrion of the parasite (Fig. 3) (Ersfeld *et al.*, 1999). The kinetoplast DNA (kDNA) is made up interlocked DNA circles: mini circles (1 kb) and maxi circles (22 kb) (El-Sayed *et al.*, 2000; Lopes *et al.*, 2010). These DNA circles play different roles in the coding and editing of mitochondrial mRNA, which is translated into various components of the mitochondrial oxidative phosphorylation system for ATP production (Salavati *et al.*, 2012). Production of ATP is also achieved through glycolysis in the glycosome (Fig. 3) enabling the parasite to take advantage of the abundance of glucose in the blood (Lopes *et al.*, 2010).

1.4 *T. b. brucei* parasite interaction with host and vector

Once metacyclic trypomastigotes are injected during a bloodmeal, trypanosomes multiply at the site of the bite prior to entry into the lymphatic system and bloodstream resulting in the build-up of metabolic waste and cell debri forming a 'chancre' (Baral, 2010). As the cycle continues, the metacyclic trypomastigotes first develop into proliferative long slender forms which replicate by asexual division, switching their VSG coats and maintaining the infection in the host as illustrated in Fig. 4 A and B (Roditi and Lehane, 2008; MacGregor *et al.*, 2011; Pollitt *et al.*, 2011). As the parasites grow, the slender form secretes a parasite–derived factor known as stumpy induction factor (SIF) which induces differentiation into short stumpy forms (Fig. 4 B). The short stumpy forms are neither replicating nor productively switching their VSG coat and so are irreversibly involved in cell cycle arrest in the bloodstream and are infective to the tsetse fly (Fig. 4B) (Kristensson *et al.*, 2010; MacGregor *et al.*, 2011; Pollitt *et al.*, 2011).

After the short stumpy forms are taken up from the human host by the tsetse fly, the life cycle continues in the insect, where the ingested trypomastigotes go through changes

in the midgut of the fly, losing their VSG surface coat and expressing their own surface proteins known as procyclic acidic repetitive proteins (PARPs) (Baral, 2010; Gadelha *et al.*, 2011). As the procyclic trypomastigote leaves the midgut, it transforms into epimastigotes with a surface coat consisting mainly of a bloodstream alanine rich protein called BARP (Gadelha *et al.*, 2011). The epimastigotes eventually transform into metacyclic trypomastigotes where they acquire a VSG coat. Acquisition of the VSG coat is important for the parasite because the procyclic and epimastigote forms are sensitive to the immune response such as complement-mediated lysis (Gadelha *et al.*, 2011).

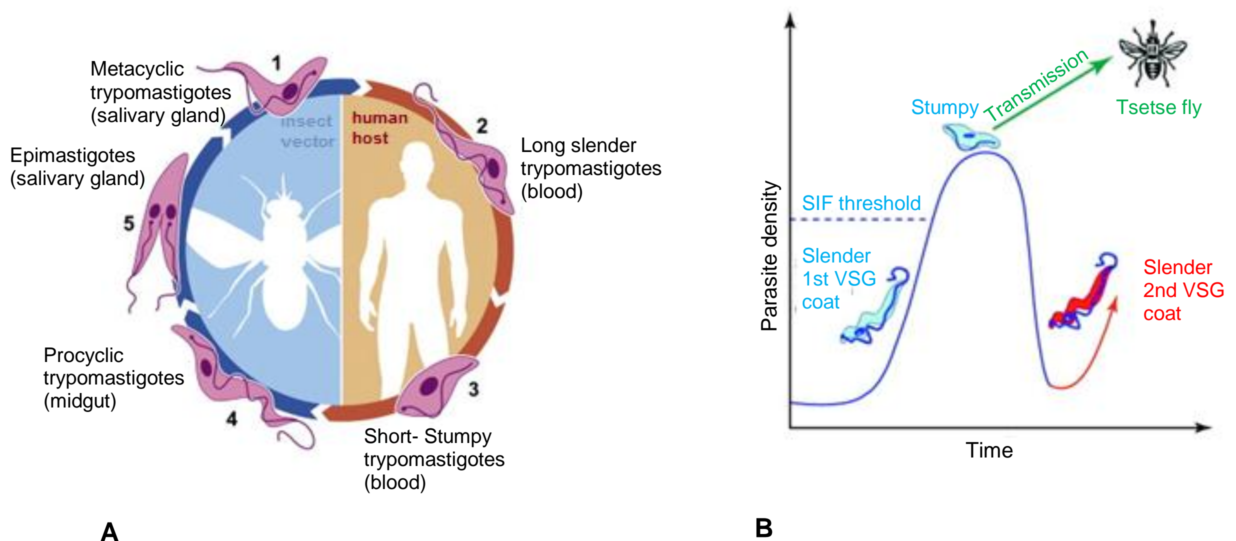


Fig. 1.4. *T. brucei* life cycle showing the different parasite forms that occur during the different stages in vector and host. A) Parasite life cycle. B) Parasitaemia wave, showing the switching of VSG coats (blue to red) in the slender form, as well as the stage (stumpy form) where parasites are transmissible from host to the tsetse fly vector to continue the life cycle in (A). Adapted from (Vargas-Prada, 2010; Pollitt *et al.*, 2011).

1.5 The major surface glycoprotein: VSG

The VSG polypeptide found on the surface of *T. brucei* comprises ~400-500 amino acid residues and forms two domains: the N-terminal domain (300-400 residues) and the C-terminal domain (40-80 residues). The VSG is attached to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor on the C terminal residue (Fig. 5 B) (El-Sayed *et al.*, 2000; Schwede and Carrington, 2010). Through X-ray crystallography, the 3D structure of two VSG N-terminal domains: MITat 1.2 (Fig. 5A) and ILTat 1.24 were determined. Despite the differences from each other in primary sequence, they showed some conservation of the tertiary structure as the proteins folded in the same way (Carrington *et al.*, 1998; Hutchinson *et al.*, 2003; Schwede and Carrington, 2010).

The N-terminal domain is a homodimer with a long axis of ~15 nm with two α-helices in each monomer giving the domain its elongated 'dumb bell' shape, and lies perpendicular to the plane of the plasma membrane (Fig. 5 B and C). The 'dumb bell' like shape allows the VSG to pack tightly on the cell surface, preventing immune recognition of invariant proteins and conserved regions that may be recognised by the immune system (Pays and Nolan, 1998; Hutchinson *et al.*, 2003; Schwede and Carrington, 2010). The N-linked oligosaccharides present on all VSGs have been suggested to also contribute to the tight packing of VSGs by increasing the volume, thus forming a protective barrier (Schwede and Carrington, 2010). These VSG homodimers represent more than 95% of the externally exposed cell surface protein and they represent 15-20% of the total cell protein with ~ 5×10^6 VSG dimers per cell (Hutchinson *et al.*, 2003).

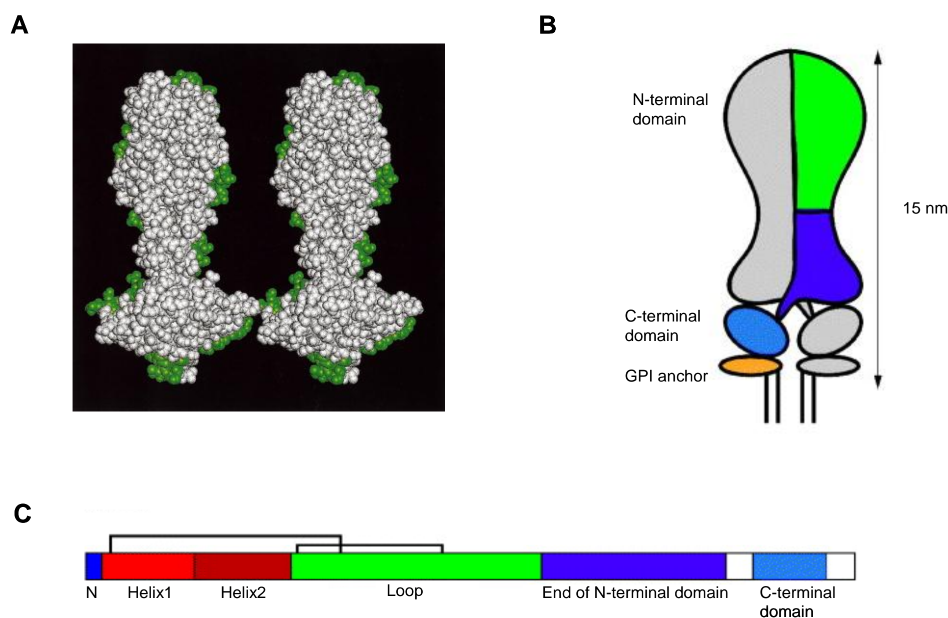


Fig. 1.5. Structure of VSG. A) Representation of two VSG dimers of MITat 1.2, one monomer is shown in white and another shown in green within each dimer. B) image shows a VSG dimer with one monomer in grey, and the other in different colors corresponding to the linear representation of a VSG MITat 1.2 monomer in (C). Adapted from (Carrington *et al.*, 1998; Schwede and Carrington, 2010).

The classification of VSGs is based on the pattern of conserved cysteine residues. Nuclear Magnetic Resonance spectroscopy has shown that each domain has independently conserved cysteines and there is little conservation in primary sequence identity in the N-terminal domain as compared to the C-terminal domain (Hutchinson *et al.*, 2003; Weirather *et al.*, 2012). The similarities between the sequences in the C-terminal domain do not compromise antigenic variation because the epitopes

recognised by antibodies are found on the N-terminal domain (Pays and Nolan, 1998; Schwede and Carrington, 2010). The N-terminal domain has three patterns of cysteines (A, B, C) and the C-terminal domain has six patterns (1-6) where types 2, 4 and 5 are single domains while types 1, 3 and 6 are di-domains (Schwede and Carrington, 2010). Any VSG can therefore be characterised as a combination of domains (A1, A2, B1 etc.) (Donelson, 2003).

Two of the predominant VSG forms of *T. b. gambiense* are VSGs of variable antigen type (VAT) LiTat 1.5 and LiTat 1.3. Their names are derived from the antigenically pure cloned *T. b. gambiense* populations of VAT LiTat 1.5 and LiTat 1.3 (Mehlert *et al.*, 2002; Van Nieuwenhove *et al.*, 2012a). On the live trypanosome, these VATs appear early during infection and induce a strong and specific immune response in most patients, hence their use in serological tests for diagnostic purposes (Van Nieuwenhove *et al.*, 2011). The 3D structures of these VSGs have not been resolved yet, however, the *VSG LiTat 1.5* gene has been sequenced and the cysteine rich N-terminal domain has been classified as type A (Van Nieuwenhove *et al.*, 2012a; Van Nieuwenhove *et al.*, 2012b).

1.5.1 VSG expression and antigenic variation

The trypanosome nuclear genome consists of three classes of chromosomes: megabase chromosomes (1-6 Mb), intermediate chromosomes (200-900 Kb) and mini chromosomes (50–150 Kb). The megabase chromosomes are diploid in the nucleus whereas the intermediate chromosomes and mini chromosomes are of uncertain ploidy (El-Sayed *et al.*, 2000; Baral, 2010). There are ~100 mini chromosomes which house up to 200 *VSG* genes in the subtelomeric region while ~1 000 *VSG* genes are found in subtelomeric tandem gene arrays in the megabase chromosomes (Barry *et al.*, 2005). Of these *VSG* genes only 7% code for known functional VSGs while 66% are pseudogenes, containing mutations such as stop codons/frame shifts. The remaining 9% are atypical genes i.e. they are complete genes but lack some elements required for correct folding and 18% are gene fragments most of which encode the C-terminal domains of the VSGs (Barry *et al.*, 2005; Weirather *et al.*, 2012).

VSG transcription is restricted to the sub telomere region in the megabase chromosomes in sites known as *VSG* expression sites (*VSG* ESs) (Barry *et al.*, 2005; Stockdale *et al.*, 2008; Baral, 2010). There are 20 *VSG* ESs and each expression site consists of a single VSG gene, a promoter and eight genes known as expression site-associated genes (*ESAG*s) (Fig. 6). The promoter directs expression in the ES and the

ESAGS, which lie between a stretch of 70 bp repeats (upstream of *VSG*) and the promoter, code for different proteins that go on the cell surface. Although transcription occurs simultaneously in all *VSG* ESs, only one VSG is expressed at a given time, while the others are silenced (Stockdale *et al.*, 2008; Baral, 2010; MacGregor *et al.*, 2011).

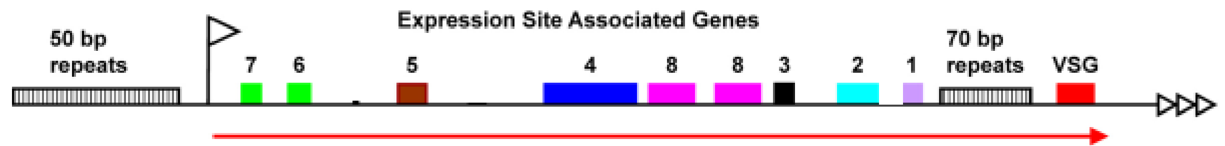


Fig. 1.6. Schematic representation of a *VSG* gene expression site. The flag indicates the promoter, coloured boxes indicate *ESAG*s. The arrow represents transcription and the expression sites located at telomeres are indicated with triangles. (Adapted from http://rudenkolab.co.uk/Research-intro.html [Accessed 5 April 2013]).

The switching of one *VSG* gene to another in the actively transcribed telomeric *VSG* ES is described as antigenic variation and is achieved through any of the four mechanisms that include gene conversion, telomere exchange, transcriptional switch and *VSG* gene arrangements. (Borst and Fairlamb, 1998). In gene conversion, an actively transcribed *VSG* gene is replaced by a copy of another *VSG* gene present on a silent telomere or a non-telomeric gene (Fig. 7 A) (Pays and Nolan, 1998). Telomere exchange or reciprocal recombination occurs by a crossing over in the 70 bp repeat region resulting in a transcribed *VSG* gene in one telomere replaced by another *VSG* gene present on a silent telomere (Fig. 7 B) (Baral, 2010). Transcriptional switch, also known as *in situ* activation is the third mechanism whereby the active expression site is switched off and a silent expression site activated (Fig. 7 C). Because *VSG* genes are transcribed as polycistronic units an *ESAG* switch occurs as well (Pays and Nolan, 1998; Baral, 2010). The fourth method entails *VSG* gene rearrangements where in contrast to in situ activation, neither the expression sites nor associated *ESAG*s are changed but only the *VSG* gene undergoes modification (Baral, 2010).

Antigenic variation has shown to be one of the strategies the parasite uses to evade the immune system.

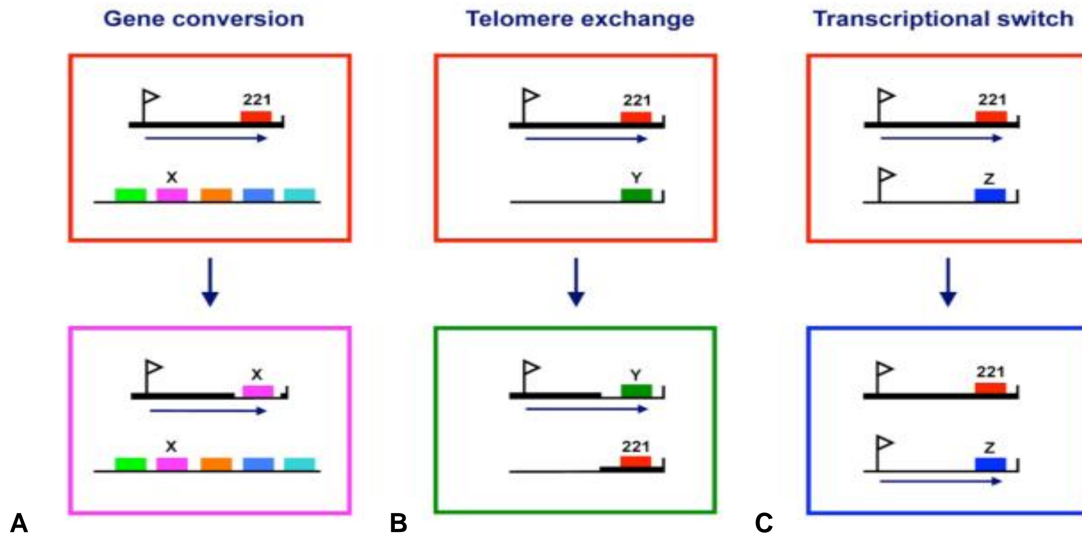


Fig. 1.7. Schematic illustration of the mechanism of VSG switching. Trypanosome parasites are indicated by the large boxes and the colour indicates the corresponding colour of the expressed *VSG* gene. The *VSG* genes are represented by the small filled boxes. One VSG is actively transcribed in the telomeric *VSG* expression site (ES). The flag represents the ES promoter and the arrow, the direction of transcription. (A) In gene conversion, an active *VSG* gene is replaced by a copy of another *VSG* gene previously silent on a telomere or non-telomeric gene. (B) The telomere exchange mechanism allows telomeric *VSG*s to be exchanged with the gene present in the active *VSG* ES and there is no loss of DNA sequences. (C) During a transcriptional switch, there is inactivation of the former ES and the simultaneous activation of a new one, from the repertoire (http://rudenkolab.co.uk/Research-intro.html [Accessed 5 April 2013]).

1.6 Parasite evasion of the immune system

Once the parasite enters the host, an appropriate immune response is generated by the host. While most parasites evade killing by the immune system through various mechanisms discussed later in this section, some face the consequences of destruction by the immune system.

The key cells involved in the immune response are Th (T helper) cells which differentiate into Th1 and Th2 cells (Cox, 2001). *T. b. gambiense* and *T. b. rhodesiense* trypanosomes secrete factors known as trypanosome derived lymphocyte triggering factor (TLTF) and trypanosome macrophage activating factor (TMAF) (Cox, 2001). The former factor induces lymphocytes i.e. Th1 cells to produce cytokines, mainly interleukin 2 (IL-2) that drives the immune response towards the production of cytotoxic T cells and IFN-γ which in turn leads to the classical activation of macrophages, while TMAF also stimulates macrophages (Reiner and Seder, 1995; Inoue *et al.*, 1999; Cox, 2001; Holzmuller *et al.*, 2008).

The classically activated macrophages secrete pro-inflammatory molecules such as nitric oxide (NO), which have direct trypanostatic and trypanocidal properties (Biemann, 1990; Holzmuller *et al.*, 2008). Following destruction of the parasites, the classically activated macrophages are down regulated together with the pro-inflammatory cytokines to prevent pathology. This results in the activation of Th2 cells which release type II cytokines such as IL-4 and IL-5 which are anti-inflammatory leading to the rise of alternatively activated macrophages which do not cause pathology (Inoue *et al.*, 1999; Cox, 2001; Baral, 2010). Release of type II cytokines also leads to the activation of B-cells for antibody production and elevated IgM production (Barrett *et al.*, 2003). Once the antibody recognises the epitope of VSG, opsonisation occurs and the immune complex is phagocytosed and destroyed in the liver by the macrophages (Baral, 2010).

The immune system also makes use of trypanolytic factor (TLF) found in the blood of healthy humans which makes them naturally resistant to infection by some African trypanosomes such as *T. b. brucei* and *T. congolense* (Milner and Hajduk, 1999; Vanhamme and Pays, 2004). The TLF exists as a heterogeneous subclass of high density lipoprotein (HDL) TLF-1, or exists as an unstable high molecular weight protein complex TLF-2 (Milner and Hajduk, 1999; Baral, 2010). Common in both TLF-1 and TLF-2 are two lipoproteins: heptoglobin-related protein (Hpr) and apolipoprotein 1 (APOL 1) and evidence has shown that APOL 1 is the active trypanolytic component of TLF (Fig. 8) (Pays *et al.*, 2006).

Apolipoprotein 1 is a secreted protein with three domains: pore forming domain, membrane addressing domain and serum resistance associated (SRA) interacting domain (Baral, 2010). Once the APOL 1, associated with high density lipoprotein (HDL) particles comes into contact with the bloodstream trypanosome, it is internalised by the parasite via HDL receptor mediated endocytosis in the flagellar pocket (Fig. 8) (Molina Portela *et al.*, 2000; Baral, 2010). The APOL 1-HDL complex is targeted for the lysosome and the acidic pH causes a conformational change, resulting in dissociation of APOL 1 from HDL and association of APOL 1 with the lysosomal membrane. At this point the pore forming domain causes formation of pores leading to Cl^- ion influx from the cytoplasm to the lysosomal lumen and subsequent movement of water into the lysosome (Fig. 8) (Pays, 2006; Baral, 2010). This action results in uncontrolled osmotic swelling of lysosomes and increased intracellular pressure which damages the plasma membrane and ultimately kills the parasite (Baral, 2010; Gadelha *et al.*, 2011).

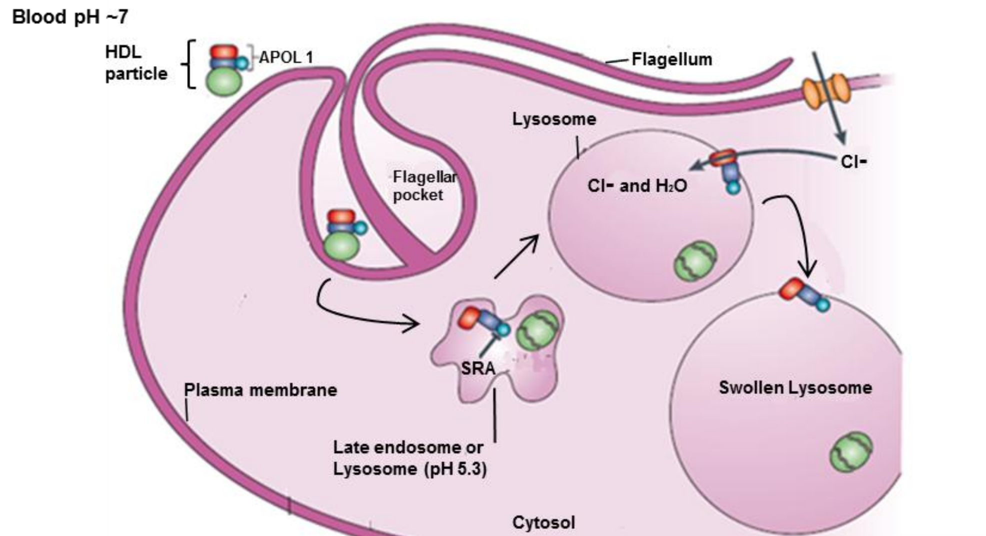


Fig. 1.8. Apolipoprotein 1 (APOL 1) action in trypanolysis. The APOL 1 comprising three domains: pore-forming domain (red), membrane addressing domain (blue) and SRA interacting domain (light blue), forms a complex with HDL particles (green) from the blood. Once the complex is endocytosed through the flagellar pocket, it moves to the late endosome/lysosome where pH is acidic. This low pH results in dissociation of APOL 1 from HDL, and APOL 1 binds to the lysosomal membrane (through the membrane addressing domain) forming pores (through the pore forming domain) which result in an inflow of Cl^- ions and water from the cytoplasm into the lysosome, swelling the lysosome and consequently rupturing the plasma membrane . Adapted from (Pays *et al.*, 2006).

It is, however, a different story for *T. b. gambiense* and *T. b. rhodesiense* as the two trypanosome species are resistant to TLF, which is the reason why they are able to infect humans (Gibson, 2002).The resistance of *T. b. rhodesiense* is a result of the expression of the *SRA* gene (Gull, 2003). This gene encodes a protein similar to VSG except for the region coding for a surface-exposed epitope that is missing because of an in-frame deletion (Gibson, 2002; Baral, 2010). The SRA protein, just like the VSG, has N-terminal α-helices, but the helix is used to neutralise the human serum trypanolytic factor by coil-coil interaction at the SRA interacting domain of APOL 1, which results in the failure of APOL 1 to continue with trypanolysis, rendering the TLF useless for its destruction allowing parasite survival in the host (Fig. 8) (Pays, 2006; Baral, 2010; Kieft *et al.*, 2012). *T. b gambiense* lacks the *SRA* gene, but it somehow exhibits resistance to TLF as isolates of this strain maintained viability as well as infectivity after incubation with human serum (Gibson *et al.*, 2010).

Antigenic variation described earlier plays a role in evasion and maintaining a persistent infection (Pays and Nolan, 1998; Bisser *et al.*, 2006). Epitopes of the same antigenic type (homotype) exposed on the surface of VSGs are detected by the host immune system which then makes antibodies against it. However as this first variable antigen type is eliminated by the immune system, a new second variable antigen type

is expressed and becomes the new homotype resulting in a new wave of parasitaemia (Stockdale *et al.*, 2008; Baral, 2010). In addition to evasion, the parasite thus makes use of the immune system to control its own growth by triggering an antibody response which will lower the number of parasites, allowing a constant tolerable number of extracellular parasites surviving in the blood.

In addition to TLF resistance and antigenic variation, the trypanosome parasite also avoids killing by the immune system through antibody clearance on their cell surface by hydrodynamic flow force. Antibodies bind to the homogenous VSG coat layer, forming an IgG-VSG complex (Engstler *et al.*, 2007; Baral, 2010). As the flagellum propels the cell in the direction away from the flagellar pocket, the IgG-VSG immune complex moves in the opposite direction towards the flagellar pocket by hydrodynamic drag. This leads to the immune complex being internalised at the flagellar pocket by endocytosis (Engstler *et al.*, 2007; Ralston and Hill, 2008; Gadelha *et al.*, 2011). This clearance was found to occur much faster in the stumpy form of trypanosomes because of the accelerated rate of endocytosis. Once endocytosed, the antibodies are targeted to the lysosome for degradation while VSGs are recycled to the cell surface (Gadelha *et al.*, 2011).

All these strategies allow for the survival and persistence of trypanosome infection in the body i.e. being 'a step ahead' of the immune system.

1.7 Clinical features of sleeping sickness

T. b. gambiense HAT is characterised as a chronic disease and infection could last for up to three years evenly divided between the two stages, while *T .b. rhodesiense* HAT is acute and death occurs within weeks or months (Blum *et al.*, 2007). One of the first symptoms observed in the first stage of HAT is the chancre formation at the site of the bite, commonly seen in *T .b. rhodesiense* rather than in *T. b. gambiense* (Maudin et al., 2004). This chancre spontaneously resolves within a few weeks, and in African patients the chancres are absent at the time of diagnosis (Baral, 2010; Geiger *et al.*, 2011).

One to three weeks after the infective bite, fever develops which is accompanied by headaches, fatigue and anaemia (Maudin *et al.*, 2004; Ehrhardt *et al.*, 2006). These correspond to the type I inflammatory reaction (Stijlemans *et al.*, 2007). Enlarged neck glands (Winterbottom's sign) are also observed in *T. b. gambiense* infection which last over weeks or months until they shrink. The Winterbottom's sign is not observed in

T. b. rhodesiense infection (Maudin *et al.*, 2004). Other clinical signs include skin rashes, oedema (swelling of eyelids and puffy face) as well as endocrine disorders. The disorders result in sterility and abortion in women, while men experience impotency (Maudin *et al.*, 2004; Ehrhardt *et al.*, 2006).

Weeks or months after the onset of these symptoms, the trypanosomes cross the blood brain barrier and invade the CNS, characterising the second stage of the infection. A number of mechanisms of how blood brain barrier traversal occurs have been proposed. It has been suggested that the presence of the parasites triggers an immune response resulting in the release of cytokines and proteases during inflammation that would degrade the intercellular matrix allowing passage through the barrier (Lonsdale-Eccles and Grab, 2002). *In vitro* studies have also shown that such localised inflammatory responses result in the release of parasite proteases such as the cysteine protease, brucipain, which was found to induce calcium-mediated transmembrane signalling in the human blood brain barrier model made of human brain microvascular endothelial cells. This signalling ultimately rendered the endothelial cells permissive to traversal of parasites (Lonsdale-Eccles and Grab, 2002; Nikolskaia *et al.*, 2006; Grab and Kennedy, 2008). Neurological changes are largely seen in *T. b. gambiense* as compared to *T. b. rhodesiense*, and the most common symptom is the change in sleeping pattern, which is where the disease gets its name sleeping sickness (Watson *et al.*, 2012). Other areas affected are the psychiatric, motor and sensory regions and these are seen in the patient as mental confusion, tremor, paralysis and general motor weakness (Brun *et al.*, 2009)

1.8 Diagnosis of HAT

1.8.1 Serological screening

Serological tests are used for the screening of populations at risk for *T. b. gambiense* infection to identify those patients with a positive response to trypanosome infection and who are carrying trypanosome specific antibodies (Koffi *et al.*, 2006). There are three types of individuals that control teams are faced with in the field: healthy people with negative serological tests; HAT cases with positive serological and parasitological tests; and people with positive serology but no parasites detected during parasitological examination (negative parasitology). The latter group of individuals are known as aparasitemic serological suspects and in such cases, those that remain untreated may

become a potential reservoir that is responsible for the persistence of transmission and re-emergence of HAT (Koffi *et al.*, 2006; Elrayah *et al.*, 2007).

There are two major serological tests that have been developed for the screening of *T. b gambiense.* The first is the card agglutination test for trypanosomosis/*T. b gambiense* (CATT/*T. b. gambiense*) which is a fast and simple agglutination assay used to detect anti-*T. b gambiense* antibodies in blood, plasma or serum of HAT patients and has been used for over 20 years (Büscher *et al.*, 1999; Kaboré *et al.*, 2011). The antigen used is a lyophilised bloodstream form of *T. b. gambiense* VAT LiTat 1.3 prepared by extracting trypanosomes from infected blood of rats, fixed and stained with Coomasie blue and finally freeze dried (Jamonneau *et al.*, 2000). The field kit contains the antigen reagent, control sera and card rotator. A drop of the reagent is mixed with one drop of blood, and shaken on the rotor for 5 min and the results are easily seen by naked eye (Chappuis *et al.*, 2005). The specificity of CATT when tested on whole blood is limited thus resulting in false positives but this has been overcome by carrying out serial dilutions on whole blood as well as plasma, increasing sensitivity and specificity (Elrayah *et al.*, 2007). Recently, two lateral flow rapid diagnostic tests HAT Sero-Strip and HAT Sero-K-SeT have been developed with the aim of improving sensitivity, and specificity of the CATT test as well as introduce the testing of one individual at a time, i.e. single patient reagents as opposed to the kits for batch tests (Büscher *et al.*, 2013; Yansouni *et al.*, 2013). Currently, these tests are under evaluation in the Democratic Republic of Congo (Tiberti *et al.*, 2013).

The second serological test is the indirect latex agglutination test which uses three purified surface antigens of VATs LiTat 1.3, LiTat 1.5 and LiTat 1.6 coupled with suspended latex particles (Büscher *et al.*, 1999). This test was developed due to the limited test sensitivity of a single VSG antigen i.e. LiTat 1.3 in CATT, especially due to reports of some strains of *T. b. gambiense* lacking the gene coding for LiTat 1.3 (Penchenier *et al.*, 2003; Deborggraeve and Büscher, 2010). The Latex/*T. b. gambiense* test procedure is similar to CATT and also uses the rotator, however, the mixed VSG reagent shows high sensitivity and specificity with antibodies in sera, recognising different VSG epitopes (Büscher *et al.*, 1999). The ELISA/*T. b. gambiense* test follows the same principle as the Latex/*T. b gambiense* test, the only difference being that the antigens are fixed in an ELISA plate. The use of sophisticated equipment used in the ELISA/*T. b. gambiense* test, however, limits its use to reference laboratories for remote testing of the samples collected in the field during surveys (Van Meirvenne *et al.*, 1995).

These serological tests unfortunately have drawbacks. Firstly, the use of native VSG antigens which have non-specific epitopes may cause cross-reactions with animal trypanosomes and thus decrease the specificity of the test (Van Nieuwenhove *et al.*, 2011). Secondly, the VSGs are obtained by culturing infective *T. b. gambiense* parasites in laboratory rodents which poses a risk of infection to the staff (Van Nieuwenhove *et al.*, 2012a). To overcome these challenges, Van Nieuwenhove and colleagues, through peptide phage display, identified mimotopes for epitopes of the predominant forms of *T. b. gambiense* VSGs: LiTat 1.5 and LiTat 1.3 that might replace the native proteins used in serological tests (Van Nieuwenhove *et al.*, 2011). These mimotopes were selected through the use of only three monoclonal antibodies which could have resulted in other diagnostic mimotopes being missed. They then improved their work by using polyclonal human antibodies from sera from HAT patients in the selection process of the peptide phage display. These antibodies were affinity purified for VSG LiTat 1.3 and LiTat 1.5, and were able to identify different mimotopes as well as the corresponding amino acid residues that take part in epitope formation (Van Nieuwenhove *et al.*, 2012a). The diagnostic potential of these mimotopes was established but before they can replace the native proteins in the current diagnostic tests, improvements such as identifying key residues necessary for binding with antibody must be considered (Van Nieuwenhove *et al.*, 2012a).

1.8.2 Parasitological confirmation

Trypanosome infection should always be confirmed by parasite detection before starting treatment (Lejon *et al.*, 2003). The simplest method is the microscopical examination of blood from a finger prick, which has a detection limit of 10 000 trypanosomes per ml and sensitivity can be improved to 5 000 trypanosomes per ml by the use of thick blood films (Wastling and Welburn, 2011). This technique, however, is time consuming (10-20 min per slide) and also requires expertise to recognise the parasite which is frequently deformed in this preparation and unlike *T. b. rhodesiense*, *T .b .gambiense* infection has a lower density of trypanosomes and can be difficult to detect (Chappuis *et al.*, 2005; Wastling and Welburn, 2011).

For easier microscopic detection as well as improving sensitivity, the parasite may be concentrated through various techniques such as haematocrit centrifugation (HCT), quantitative buffy coat (QBC) as well as mini anion exchange centrifugation technique (mAECT) (Migchelsen *et al.*, 2011). The HCT technique entails the examination of the area above the buffy coat in micro-haematocrit tubes. The QBC technique is a

modification of HCT which involves the use of tubes developed for malaria diagnosis, and the buffy coat is stained with acridine orange, resulting in fluorescence detection of the motile stained trypanosomes (Smith *et al.*, 1998). The mAECT which is particularly useful in the examination of the cerebrospinal fluid involves chromatography, concentration followed by viewing. The parasites are separated from venous blood in a gel column by anion exchange chromatography; trypanosomes are then collected in a sealed glass tube and concentrated at the bottom of the tube by low speed centrifugation and the motile trypanosomes are viewed under low magnification (http://www.finddiagnostics.org/programs/hat-ond/hat/parasite_detection/mAECT/index.html [Accessed 10 August 2012]).

A molecular approach has also been taken for parasite detection through the polymerase chain reaction (PCR) which amplifies nucleic acids for diagnostic purposes. The technique has a unique combination of sensitivity and specificity (Deborggraeve and Büscher, 2010; Abdul-Ghani *et al.*, 2012). Earlier molecular studies on *T. b. gambiense* isolates from patients living in known areas of Gambian trypanosomosis showed that *T. b. gambiense* forms a genetically homogenous group which is different from other *T. brucei* species (Godfrey *et al.*, 1987). This group is known as Group 1, and these isolates show resistance to the trypanolytic factor in human serum thus maintaining viability and infectivity (Gibson *et al.*, 2010). The genetic homogeneity of Group 1 led to the identification of the single copy glycoprotein *TgsGP* gene through PCR amplification using specific primers that targeted the sequences at the 5' end and 3' end of VSG genes in *T. brucei*. Unlike the *SRA* gene in *T. b. rhodesiense*, *TgsGP* does not confer resistance to human serum (Radwanska *et al.*, 2002).

Since PCR is carried out on blood samples, it has been found that it can also be carried out on blood samples collected on Whatman FTA cards (Cox *et al.*, 2010). This technique, however, has limitations as it cannot be applied in resource-poor settings lacking thermocyclers and reliable electricity supplies (Wastling and Welburn, 2011). Isothermal DNA amplification has helped in alleviating this challenge, as there is no use of a thermocycler during amplification (Gill and Ghaemi, 2008). Loop mediated isothermal amplification (LAMP) as well as nucleic acid sequence based amplification (NASBA) are the two isothermal DNA amplification methods that have been developed (Wastling and Welburn, 2011; Abdul-Ghani *et al.*, 2012).

1.8.3 Stage determination

Individuals that are both serologically and parasitologically positive or are aparasitemic, but showing convincing neurological symptoms undergo lumbar puncture to examine the cerebrospinal fluid (CSF) for staging (Deborggraeve and Büscher, 2010). The WHO recommendations for diagnosing late stage HAT are one or both of: raised white blood cell count (> 5 cells/µl) or presence of trypanosomes in the CSF (Kennedy, 2012b). After collecting the CSF, a cell count is performed in a cell counting chamber ensuring that the count is carried out quickly to prevent lysis. If the cell count is less than 20 cells/µl, the counting procedure must be repeated and the average calculated. Patients with 6-20 cells/µl in the CSF are said to be in the early second stage or intermediate stage of the disease. These patients together with those showing or having trypanosomes in the lymph node or blood should be treated as having second stage HAT. The CSF proteins in healthy individuals consist of mainly albumin (70%) and IgG (30%) originating from the serum. In HAT patients the CSF is elevated ranging from 100-200 mg/litre. The WHO protein concentration threshold of 370 mg/litre was said to be too low and should be increased to 750 mg/litre to reveal blood-brain barrier impairment as well as degeneration of nervous tissue (Chappuis *et al.*, 2005).

Based on the findings by Greenwood and Whittle, that in the second stage of *T. b. gambiense* sleeping sickness patients, the CSF has high concentrations of IgM, Lejon and colleagues used this intrathecal IgM synthesis as a marker for diagnosis of second stage disease and developed a latex agglutination assay for IgM quantification of sleeping sickness (Greenwood and Whittle, 1973; Lejon *et al.*, 2002). This latex/IgM agglutination test allows simple detection of IgM in CSF in the field as well as in the staging of the disease (Lejon and Büscher, 2005).

Concerning *T. b. rhodesiense* HAT, there is no screening technique equivalent to the CATT used for *T. b. gambiense*, however, a simple agglutination test for trypanosomosis as well as a trypanosomosis agglutination card test are used. Some ELISA based as well as immunofluorescence serological tests are used, however, their sensitivity is variable, and can only be carried out by highly trained technicians in reference centres. Field screening therefore relies on clinical signs and symptoms. Diagnostic confirmation and staging of *T. b. rhodesiense* HAT are based on the same methods as for *T. b. gambiense* HAT (Chappuis *et al.*, 2005).

1.9 Treatment

At present all drugs are donated to the WHO by the manufacturers, however, treatment of HAT has not been successful due to factors such as diminishing supplies of useful drugs as well as the development of drug resistance by the parasite (Jennings *et al.*, 2002; Brun *et al.*, 2009). Discussed below are some of the drugs used.

Pentamidine isethionate (2-hydroxyethanesulfonic acid salt) is a human trypanocide developed in the 1930s and this drug is used for treatment of first stage disease caused by *T. b. gambiense* and is relatively well tolerated (Akpa *et al.*, 2008; Dorlo and Kager, 2008). It is, however, inefficient in the second stage when trypanosomes have invaded the central nervous system since it does not cross the blood-brain barrier (Lejon *et al.*, 2003). It is given intramuscularly for a week or by intravenous infusion in saline over 2 h. The side effects of intramuscular injection are site pain, transient swelling, abdominal pain and gastrointestinal problems and hyperglycaemia (Brun *et al.*, 2009). Suramin (Bayer 205 or Germanin), a naphthalene urea derivative of trypan blue, is a drug used for treatment of first stage *T. b. rhodesiense* infection. Similar to Pentamidine, Suramin can not cross the blood brain barrier (Legros *et al.*, 2002; Kristensson *et al.*, 2010; Steverding, 2010). Treatment lasts up to 30 days but caution should be taken when administering treatment as the compound deteriorates quickly in air and so should be injected immediately after dilution in distilled water. Side effects are bone marrow toxicity with agranulocytosis and thrombocytopenia as well as peripheral neuropathy (Brun *et al.*, 2009).

Second stage disease caused by both forms of the *T. brucei* sub species, are treated by melarsopol, an organo-arsenical drug which can cross the blood brain barrier (Kennedy, 1999). *T. b. gambiense* treatment requires ten injections on consecutive days as recommended by the International Scientific Council for Trypanosomosis Research and Control (Brun *et al.*, 2009). *T. b. rhodesiense* treatment on the other hand, takes longer and uses more complex treatment schedules. In both instances, the patient has to be monitored carefully during treatment. Melarsoprol is highly toxic and is associated with up to 70% mortality. Treatment can provoke severe adverse side effects such as encephalopathic syndrome characterised by deterioration over hours from mild symptoms to coma and is common in *T. b. rhodesiense* than in *T. b. gambiense* infection (Caffrey *et al.*, 2001; Checkley *et al.*, 2007). Another trend seen with the use of melarsopol is relapses which have been reported for 1–10% of the patients treated (Onyango *et al.*, 2000). Other side effects are skin reactions such as maculopapular eruptions and severe ones being bullous eruptions (Brun *et al.*, 2009).

Eflonithine (α-difluoromethylornithine, DFMO), an inhibitor of ornithine decarboxylase is another drug used for stage two treatment (Balasegaram *et al.*, 2009; Steverding, 2010). Studies have shown that this treatment has reduced mortality compared to melarsopol, hence recommended for first line treatment for the second stage *T. b. gambiense* disease. Treatment lasts for two weeks because of the short half life of injection, with four short infusions per day. The numerous infusions, however, limit its use in the poor resource areas but it is more effective and the safety profile is more favourable than that of melarsoprol (Inojosa *et al.*, 2006; Balasegaram *et al.*, 2009). Side effects include fever, unusual bleeding, stomach pain, vomiting as well as convulsions (Simarro *et al.*, 2008). Attempts to improve efficacy and simplify administration were made through drug combination therapy (Simarro *et al.*, 2008). Oral nifurtimox-a drug used in the treatment of Chagas disease combined with intravenous (IV) eflornithine, showed an improvement in the curing of disease, as a Phase III trial reported 96.5% of 143 patients with the late stage gambiense were cured (Kennedy, 2012a).

The adverse side effects as well as the excruciating pain of IV administration makes treatment unpleasant and so drugs such as Fexinidazole attempt to overcome these drawbacks (Samant and Sukhthankar, 2011). Fexinidazole is a member of the azole class of compounds which showed effective *T. brucei rhodesiense* inhibitory activity as well as low cytoxicity, curing chronic sleeping sickness in the brain within two weeks in animal models of sleeping sickness. Phase I human trials of this drug which began in 2009 have been successfully completed, and has entered Phase II/III clinical study in patients with late stage HAT in the Democratic Republic of Congo (DND*i*, 2012b; Kennedy, 2012b). The study initiated by Drugs for Neglected Diseases initiative (DNDi) aims to test the efficacy and safety of Fexinidazole with tablets taken once, daily for ten days. If the trials are successful, Fexinidazole would be the first oral drug to treat stage 2 sleeping sickness caused by *T. b. gambiense*, as well as stage 1 sleeping sickness caused by *T. b. rhodesiense* (DND*i*, 2012b). This will provide a simplified way for treatment and care of patients.

Another promising drug that has entered the Phase I trial is an orally active oxaborole drug SCYX-7158 (DND*i*, 2012a; Kennedy, 2012b). The compound showed good in vitro activity against *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*. Pharmacokinetic characterisation of SCYX-7158 showed that the drug is bioavailable across species, and is able to cross the blood brain barrier to appropriate concentrations in the brain and cerebrospinal fluid of rodents, curing the murine model CNS-stage HAT (Jacobs *et al.*, 2011). Once the drug successfully goes through Phase

I trials, the treatment will be advanced by DND*i* to Phase II trials in sub-Saharan African countries (DND*i*, 2012a). This drug will be of great benefit in the rural areas, where advanced clinical settings are not available.

1.10 Objectives of present study

The first and crucial step involved in HAT diagnosis is serological screening, and the commonly used tests (e.g. CATT/*T. b. gambiense*, LATEX/*T. b. gambiense*, HAT Sero-Strip) are based on the VSGs predominantly expressed in *T. b. gambiense* (Maudin *et al.*, 2004; Van Nieuwenhove *et al.*, 2011; Büscher *et al.*, 2013). Since the VSGs of VAT LiTat 1.3 and LiTat 1.5 are said to be 'recognised by almost all *gambiense* HAT patients' (Van Nieuwenhove *et al.*, 2012a), these VSGs, rather than other VATs e.g. LiTat 1.6 are incorporated in serological tests. The acquisition of the antigens is, however, a major challenge as it involves the handling of cloned populations of the human infective *T. b. gambiense* which poses a serious health risk to the manufacturing staff (Van Nieuwenhove *et al.*, 2012a). For this reason, it is necessary to develop safer methods to obtain the antigens.

The first objective of the present study was therefore to clone, recombinantly express and purify the VSGs LiTat 1.3 and LiTat 1.5 for use in antibody detection serological tests. To this end expression was carried out in both the bacterial and yeast expression systems, followed by the purification of VSG LiTat 1.3. The results obtained are presented in chapter 2.

An alternative to antibody detection is the detection of antigens in serological screening. Such tests not only distinguish between current and past infections, but they simultaneously detect the presence of parasites (Chappuis *et al.*, 2005). The TrypTect CIATT test has been reported which detects antigens from both *T. b. gambiense* and *T. b. rhodesiense* infection, however, it uses a monoclonal antibody (Nantulya, 1997). The second objective of the present study was therefore to produce polyclonal antibodies in chickens, against purified *T. b. gambiense* native VSGs LiTat 1.3 and LiTat 1.5 as well as *T. evansi* recombinant VSG RhoTat 1.2, that could potentially be used in antigen-detection serological tests. The antibodies were characterised by ELISA, dot and western blot and finally used in immunofluorescence studies and the results obtained are presented in chapter 3. Finally, all the results obtained for this study are summarised and discussed in chapter 4.

CHAPTER 2:

CLONING, RECOMBINANT EXPRESSION AND PURIFICATION OF VSGs LiTat 1.3 AND LiTat 1.5

2.1 Introduction

The causative agents of HAT, namely *T. b. gambiense* and *T. b. rhodesiense* express 10^7 copies of VSGs on their surface, once they are in the bloodstream of mammalian hosts. These VSGs are known to switch from one VAT to another through antigenic variation making 10^{-2} to 10^{-7} switches per generation time of 5-10 h (Weirather *et al.*, 2012). This frequent switching facilitates the evasion of parasites from the host's immune system, prolonging infection (Lillico *et al.*, 2003). Before the antigenic variation phenomenon was known, initial vaccine trials were based on targeting VSGs. After realising that this approach would lead to failure in the control of the disease, effective diagnosis became the cornerstone in the control of HAT (Cornelissen *et al.*, 1985; Maudin *et al.*, 2004).

Diagnosis of HAT can either be indirect, e.g. based on serological test results, or direct, where body fluids such as blood are microscopically examined for the presence of trypanosomes (Maudin *et al.*, 2004). Serological indirect evidence of infection is based on the detection of specific antibodies or parasite antigens during the screening of individuals that may be infected (Elrayah *et al.*, 2007). The most commonly used serological tests are based on the native VSGs which express VATs LiTat 1.3, LiTat 1.5 and LiTat 1.6, recognised by almost all *T. b. gambiense* HAT patients. These are used in the CATT/*T. b. gambiense* (LiTat 1.3) and Latex/*T. b. gambiense* (LiTat 1.3, LiTat 1.5 and LiTat 1.6) (Maudin *et al.*, 2004; Van Nieuwenhove *et al.*, 2012a). The diagnostic test production involves growing clone populations of *T. b. gambiense* bloodstream forms of each VAT in rats, separating the trypanosomes from the blood, solubilising and purifying the VSGs. This process has disadvantages, one of them being the risk of infection to the manufacturing staff, as they handle infective *T. b. gambiense* trypanosomes (Büscher *et al.*, 1999; Van Nieuwenhove *et al.*, 2012a).

In an attempt to overcome the challenges of using native proteins in diagnostic tests, recombinant protein expression technology can be employed. Among the different systems for recombinant protein production, yeasts, which are single-celled eukaryotic fungal organisms are well characterised and have the ability to carry out post-translational modification processes such as glycosylation (Demain and Vaishnav,

2009). The *Pichia pastoris* strain has been used in the expression of trypanosomal proteins and recently, expression of *Trypanosoma evansi* VSG RhoTat 1.2 has been reported (Rogé *et al.*, 2013). A modified *P. pastoris* strain, M5, which prevents high production of mannose glycan structures, was used and good expression levels were attained (Rogé *et al.*, 2013). Cloning and expression of VSGs LiTat 1.3 and LiTat 1.5 fragments in the M5-strain of *P. pastoris* has also been carried out, where gene fusion technology was also employed by using ubiquitin-like molecules such as SUMO (small ubiquitin-related modifier) present in yeast (Phillipe Büsher, Unit of Parasite Diagnostics, Institute of Tropical Medicine, Antwerp, Belgium, Personal Communication). SUMO fusions have the advantages of enhancing protein expression and correct folding, reducing proteolytic degradation of target protein as well as increasing its solubility (Malakhov *et al.*, 2004; Butt *et al.*, 2005). Despite all these advantages, *P. pastoris* expression of VSGs LiTat 1.3 and LiTat 1.5 with SUMO fusions is expensive due to the Zeocin antibiotic required which acts as a selective antibiotic for vectors bearing a *Streptoalloteichus hindustanus ble* gene product causing cell death to those that do not confer resistance to it (Gatignol *et al.*, 1988).

An inexpensive and easy to use alternative system that can be employed is the bacterial expression system (Terpe, 2006). The most popular host cell is *Escherichia coli*, and expression is induced from a plasmid containing the target gene, as well as important elements necessary for recombinant expression such as origin of replication, transcriptional promoters, antibiotic resistance markers and fusion tags which assist in purification of proteins (Sørensen and Mortensen, 2005). Expression of glycoproteins in *E. coli* is possible as there are N-glycoproteins that have been described (Fisher 2011), thus favouring the expression of VSGs LiTat 1.3 and LiTat 1.5 in bacteria. In this study, the cloning and recombinant expression of VSGs LiTat 1.3 and LiTat 1.5 in the *E. coli* bacterial expression system is reported. Using the template DNA available in our laboratory, the two genes were amplified from either *T. b. brucei* genomic DNA or their respective complementary DNA and cloned into the pTZ57R/T vector, prior to sub-cloning into pGEX4T-1 and pET-28a expression vectors. Following expression, the recombinant VSG LiTat 1.3_{His} was affinity purified. *Pichia pastoris* expression was also carried out to compare the two expression systems.

2.2 Materials and methods

2.2.1 Materials

Molecular Biology: Restriction enzymes NotI, SalI, EcoRI [for nomenclature see Roberts *et al.* (2003)], 10 mM dNTPs mix, T4 DNA ligase, 10 x ligation buffer, shrimp alkaline phosphatase (SAP), pTZ57R/T vector, Buffer O, DNA high and middle range molecular weight markers, Taq polymerase, 10 x Taq buffer, 25 mM $MgCl_2$, GeneJet^{TM} Plasmid miniprep kit, TransformAid^{TM}, Transformation kit, IPTG, RNAse A were all obtained from Fermentas (Vilnius, Lithuania). The DNA clean and concentrator kit was obtained from ZymoResearch (Orange, CA, USA) and the peqGOLD Gel extraction kit was purchased from peQLab (Erlangen, Germany). *Escherichia coli* JM109 competent cells were obtained from New England Biolabs (Ipswitch, MA, USA). The pGEX4T-1 expression vector was purchased from GE Healthcare (Uppsala, Sweden). The pET28a expression vector, BL21 (DE3) and Rossetta2 (DE3) cells were purchased from Novagen (Madison, WI, USA). SeaKem® LE Agarose was purchased from WhiteSci (Roclland, ME, USA), ampicillin sodium salt from USB Corporation (Cleveland, OH, USA), Kanamycin sulfate from Gibco (Paisley, UK). Bacteriological agar, tryptone and yeast extract were purchased from Merk (Gauteng, SA). XGAL was purchased from Melford (Ipswitch, UK). The LiTat 1.3 and LiTat 1.5 cDNA were a kind gift from Professor Phillip Büscher, Unit of Parasite Diagnostics, Institute of Tropical Medicine, Antwerp, Belgium.

Trypanosome culture: Iscove's Modified Dubecco's Medium (IMDM), sodium bicarbonate, hypoxanthine, sodium pyruvate, thymidine, bathocuprone sulphate acid, β-mercaptoethanol, L-Cysteine were all purchased from Sigma (St Louis, MO, USA). Heat inactivated foetal calf serum (FCS) was obtained from Gibco (Paisley, UK). Filters (0.2 µm) were purchased from Pall Corporation (MI, USA) and 6 well plates were obtained from Nunc (Intermed, Denmark).

Yeast expression: VSG LiTat 1.5 11-11 (SUMO) in M5 and VSG LiTat 1.3 A6-16-19 (3) (SUMO) in M5 constructs were a gift from Prof Philippe Büscher, Unit of Parasite Diagnostics, Institute of Tropical Medicine, Antwerp, Belgium.

Purification and protein determination: His-select® nickel affinity resin and Imidazole were obtained from Sigma (St Louis, MO, USA). The bicinchoninic acid (BCA) Pierce protein determination kit was obtained from Thermo Scientific (Rockford, USA) and the Amicon Centripep® concentration filter was purchased from Milipore (Bedford, MA, USA) and GE Healthcare (Uppsala, Sweden) respectively.

Methods

2.2.2 Isolation of genomic DNA

Bloodstream form *T. b. brucei* Lister 427 strain trypanosome parasites were cultured in filter sterilised complete IMDM based HMI-9 medium [IMDM base powder, 3.6 mM sodium bicarbonate, 1 mM hypoxanthine, 1 mM sodium pyruvate, 0.16 mM thymidine, 0.05 mM bathocuprone sulphate acid, 10% (v/v) heat inactivated FCS, 1.5 mM L-cysteine, 0.2 mM β-mercaptoethanol] in non-adherent 6 well plates in a humidified chamber (37°C, 5% CO_2). Cells were grown over 5 days and sub cultured once they reached 70% confluence by removing half the contents of each well into a new well and replenishing each 'old' well with an equal volume of fresh medium.

Genomic DNA (gDNA) was isolated as described by Medina-Acosta and Cross (1993). Briefly, the cultured *T. b. brucei* parasites were collected by centrifugation (3 000 *g*, 10 min, RT) and the pellet washed with 10 ml PBS [100 mM $Na_2HPO_4.2H_2O$, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4] and centrifuged (3 000 *g*, 15 min, 4°C). The pellet was gently resuspended in 300 μl TELT buffer [50 mM Tris-HCl pH 8.0, 62.5 mM EDTA, 2.5 M LiCl] and incubated (5 min, RT). An equal amount of phenol:chloroform (1:1) was added and the suspension mixed by rotation in an end-over-end rotator (5 min, RT). The suspension was centrifuged (17 000 *g*, 5 min, 4°C) and the top supernatant layer was removed by aspiration and mixed with ice cold absolute ethanol (using a, 1 μl supernatant:2 μl ethanol ratio). The solution was left in ice for 15 min and centrifuged (17 000 *g*, 10 min, 4°C). The resulting DNA pellet was resuspended in 1 ml of 70% ethanol, centrifuged (17 000 *g*, 10 min, 4°C) and the ethanol was poured out while the remaining ethanol was evaporated by placing the DNA pellet at 37°C for 10 min. The pellet was dissolved in 100 μl TE buffer (10 mM Tris-HCL, 1mM EDTA, pH 8.0) containing 1 mg/ml RNAse A and incubated at 37°C for 45 min. The purity of the isolated gDNA was analysed on a 1% (w/v) agarose gel in 1 x Tris-acetate-EDTA (TAE) buffer [40 mM Tris-HCl buffer pH 7.4, 20 mM glacial acetic acid, 0.1 mM EDTA] at 80 V for 30 min. Spectrophotometric analysis was also carried out using the Thermo Scientific Nanodrop 2000 spectrophotometer, by reading absorbances at 280 and 260nm (A_{280}:A_{260} ratio) to determine the quality of the DNA, prior to storage at -20°C until further use.

2.2.3 Primer design and synthesis

Due to the absence of the gene sequences from *T. b. brucei* in the NCBI GenBank database, primer design was based on the *T. b. gambiense* gene sequences. *T. b. gambiense* mRNA for VSG protein LiTat 1.3 [Accession no. AJ304413 (Berberof *et al.*, 2001)] and *T. b. gambiense* variable surface glycoprotein LiTat 1.5 mRNA, complete cds [Accession no. HQ662603 (Van Nieuwenhove *et al.*, 2012b)]. Sequences were first analysed using the ExPASy translate tool (web.expasy.org/translate/) to select the appropriate frame and submitted to the webcutter 2.0 programme (http://rna.lundberg.gu.se/cutter2/ [accessed on 10.06.2012]) for restriction site or enzyme mapping. Primers were designed to include restriction sites so that cleavage of the product will create sticky ends for the subsequent sub-cloning into expression vectors (Marchuk *et al.*, 1991).

The primers for *T. b. gambiense* mRNA for VSG protein LiTat 1.3, (VSG LiTat 1.3), were: forward (35-mer), 5′-TC<u>GAATTC</u>**ATG**TCACCAGTAGGTGCGGCCTTTAGC-3′ and reverse (34-mer), 5′-GG<u>GCGGCCGC</u>AAAAAGCAAGGCCACAAATGCAGC-3′. The EcoRI and NotI restriction sites are underlined respectively. Primers designed for *T. b. gambiense* variable surface glycoprotein LiTat 1.5 mRNA, complete cds, (VSG LiTat 1.5) were: forward (32-mer), 5′-CT<u>GTCGAC</u>**ATG**ACCGGCAGAAAAGTCTCTATC-3′ and reverse (34-mer), 5′-GA<u>GCGGCCGC</u>AAAAAGCAAAAATGCAAGCCAAAG-3′. Underlined are the SalI and NotI sites respectively. Start codons in each of the forward primers is shown in bold. Primers were synthesized at the University of Cape Town MCB Synthetic DNA Unit. Forward and reverse expression vector (pGEX and pET) primers were available in the laboratory.

2.2.4 PCR amplification and T-vector cloning of VSGs LiTat 1.3 and LiTat 1.5

After setting up different conditions such as temperature and concentration of mixture components, suitable PCR conditions were achieved. Briefly, a master mix consisting of 50 ng template DNA (*T. b. brucei* gDNA or VSG LiTat 1.3/LiTat 1.5 cDNA), specific forward and reverse primer sets for VSG LiTat 1.3 or VSG LiTat 1.5 [0.25 µM each], 1 x Taq buffer, 0.25 µM dNTP mix, 1 U Taq polymerase, $MgCl_2$ [2.5 mM for VSG LiTat 1.3 and 3 mM for VSG LiTat 1.5], was made up to a reaction volume of 20 µl with dH_2O. The PCR reaction was carried out in the GeneAmp® PCR System 2700 thermocycler. The reaction was first heated up at 94°C for 5 min followed by 30 cycles of repeated

denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min and elongation at 72°C for 2 min. The final elongation step after the last PCR cycle was at 72°C for 7 min. The PCR products, gLiTat 1.3 and gLiTat 1.5 amplified from the gDNA template, or cLiTat 1.3 and cLiTat 1.5 amplified from the cDNA template, were first analysed on a 1% (w/v) agarose gel as described in section 2.2.2 before the 1 443 bp and 1 509 bp products for LiTat 1.3 and LiTat 1.5, respectively, were excised from the gel and purified using the peqGOLD Gel extraction kit according to the manufacturers' guide. The PCR products were cleaned so as to prevent cloning spurious bands, which may affect downstream applications (Marchuk *et al.*, 1991).

The Taq DNA polymerase enzyme has the ability to add a single deoxyriboadenosine (dA) nucleotide at the 3′ end of the DNA fragment, resulting in 3′ A overhangs, allowing direct ligation into the linearised T-vector (Zhou and Gomez-Sanchez, 2000; Kitabayashi *et al.*, 2003). The purified DNA was ligated into the pTZ57R/T vector (T-vector) using a vector: insert ratio of 1:4 in a reaction mix also containing 1 U of T 4 DNA ligase and 1 x ligation buffer at 4°C for 16 h. The ligation mix was then transformed into *E. coli* JM109 cells using the TransformAid™ transformation kit as described by the manufacturer, and the cells were plated on pre-warmed 2 xYT agar plates [1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 15 g/l bacteriological agar] containing ampicillin (50 µg/ml). The plates also contained X-gal (20 µg/ml) and IPTG (10 µg/ml) for blue and white colony screening. The plates were incubated at 37°C for 16 h. White positive colonies, were selected and screened for recombinancy by colony PCR using the specific insert primers. Overnight cultures of the recombinant colonies were prepared by growing colonies in 2 ml of 2 xYT liquid medium overnight at 37°C, after which a miniprep, to isolate plasmid DNA, was performed using the GeneJet™ Plasmid miniprep kit according to the manufacturer's guide. Recombinant plasmids were sequenced at Inqaba biotechnical Industries, (Hatfield, South Africa).

2.2.5 Subcloning into pGEX-4T-1 and pET-28a expression vectors

The chosen vectors used for expression were pGEX-4T-1 and pET-28a, which were readily available in the laboratory. The pGEX vector uses a *tac* promoter for chemically inducible high-level expression, while pET vectors use the T7*lac* promoter which tightly controls protein expression e.g. the supressal of basal expression of toxic proteins. These vectors also have fusion tags, which facilitate detection and purification of target proteins (Harper and Speicher, 2001; Novagen, 2003). Glycerol stocks of circular

pGEX-4T-1 and pET-28a expression vectors were streaked on 2xYT agar plates containing ampicillin (50 µg/ml) and kanamycin (34 µg/ml) respectively, for 16 h at 37°C. Single colonies were picked and cultured in 2 ml of 2xYT liquid medium for 16 h at 37°C, after which plasmid DNA, was isolated using the GeneJet™ Plasmid miniprep kit according to the manufacturer's guide.

The recombinant T-vector plasmids and expression vectors were digested by restriction enzymes prior to ligation as illustrated in the schematic Fig. 2.1. Briefly, T-vector-LiTat 1.3 constructs (25 µl) were digested with EcoRI and NotI restriction enzymes (1 µl each), while T-vector-LiTat 1.5 constructs (25 µl) were restricted with SalI and NotI (1 µl each) in the presence of 1 x Buffer O (3 µl), overnight at room temperature. Digestion was analysed by running 2 µl of each sample collected before and after digestion on a 1% (w/v) agarose gel in 1 x TAE buffer at 80 V for 45 min, prior to running the remaining 28 µl of the restriction digestion samples and gel purifying using the peqGOLD Gel extraction kit. Expression vectors, pGEX-4T-1 and pET-28a, were linearised by restricting with either enzyme combination: (EcoRI/NotI) or (SalI/NotI) in the presence of 1 x Buffer O. These restricted vectors were dephosphorylated by treating them with SAP (1 U) in the presence of 1 x SAP buffer before cleaning and concentrating using the ZymoResearch DNA clean and concentrator kit. The concentration of inserts and vectors was determined using the Nanodrop 2000 spectrophotometer and each insert was ligated into the MCS of an expression vector (Fig. 2.2), using a vector: insert ratio of 1:4 in a reaction mix also containing 1 U of T 4 DNA ligase and 1 x ligation buffer at 4°C o.n. The ligation mix was then transformed into *E. coli* JM109 cells using the TransformAid™ transformation kit as described by the manufacturer, and the cells were plated on pre-warmed 2 xYT agar plates, containing either ampicillin (50 µg/ml) for pGEX-4T-1 constructs or kanamycin (34 µg/ml) for pET-28a constructs and incubated at 37°C for 16 h.

Recombinant positive colonies were verified by colony PCR using either pGEX or pET forward and reverse primers under the following conditions: initial denaturation at 94°C for 5 min, 25 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 1 min and elongation at 72°C for 2 min. The final elongation step after the last PCR cycle was at 72°C for 7 min. Minipreps were prepared from selected colonies as previously described, and the recombinant plasmid DNA sequenced before the remaining samples were transformed into either *E. coli* BL21 DE3 or Rosetta 2 DE3 cells using the TransformAid™ transformation kit. The cells were plated on pre-warmed 2 xYT agar plates, containing either ampicillin (50 µg/ml) for pGEX4T-1 constructs or kanamycin (34 µg/ml) for pET-28a constructs in BL21 DE3 cells. An additional antibiotic,

chloramphenicol (34 µg/ml) was added for either construct in Rosetta 2 DE3 cells as this strain carries a chloramphenicol-resistant plasmid. Plates were incubated at 37 °C for 16 h.

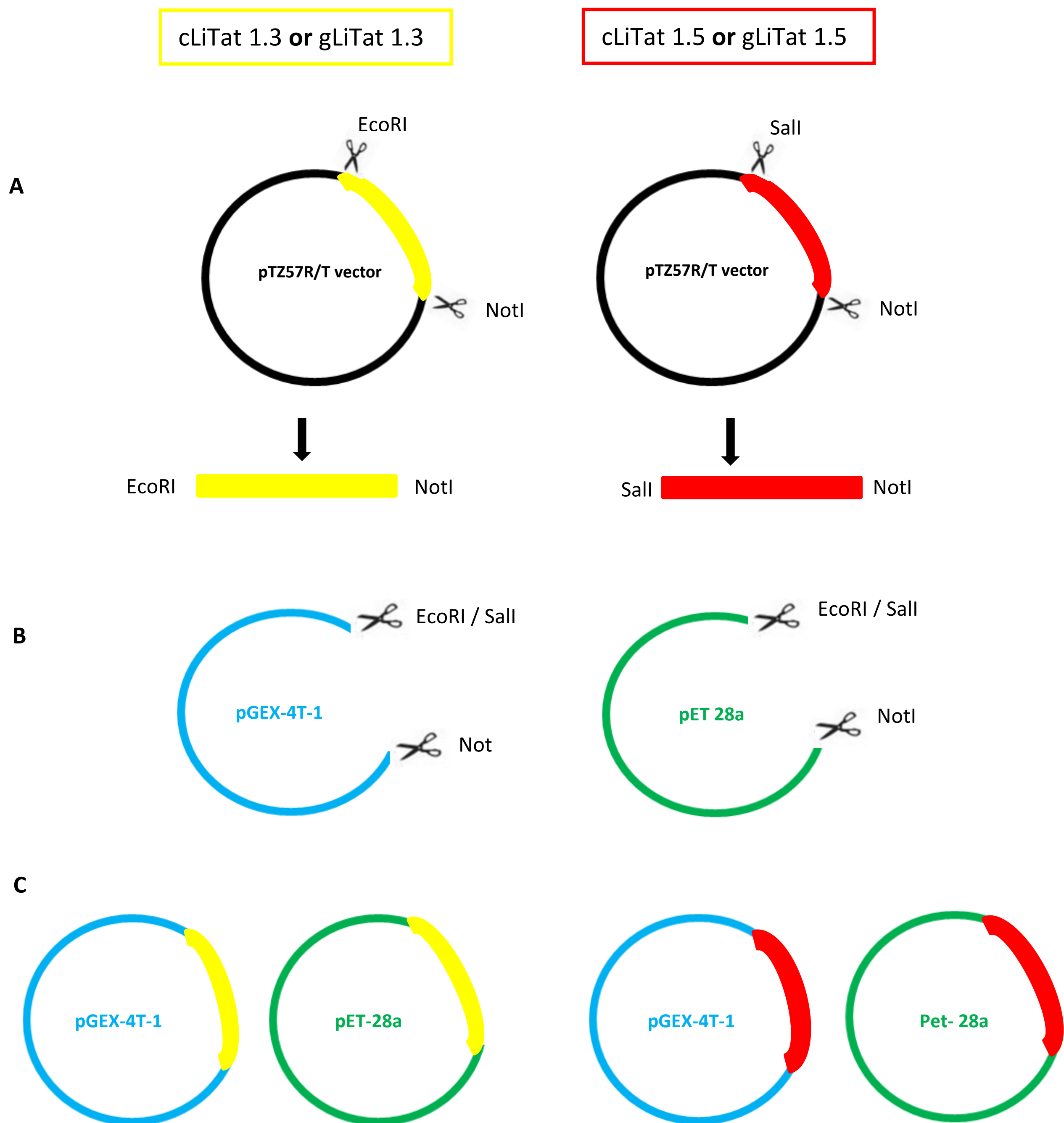


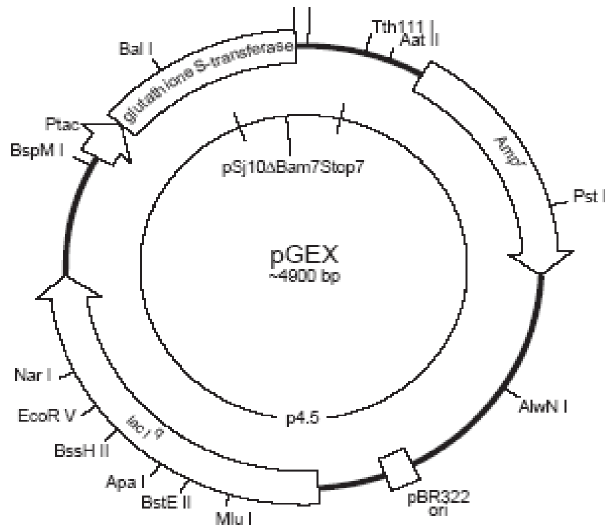
Fig. 2.1. Schematic representation of sub-cloning of cLiTat 1.3, gLiTat 1.3, cLiTat 1.5 and gLiTat 1.5 inserts into pGEX-4T-1 and pET-28a expression vectors. A) Restriction of the insert from the T-vector; **B)** Restricted expression vectors with their restriction sites and **C)** The inserts ligated into the respective expression vectors.

pGEX-4T-1 (27-4580-01)

Thrombin

Leu Val Pro Arg↓Gly Ser Pro Glu Phe Pro Gly Arg Leu Glu Arg Pro His Arg Asp
CTG GTT CCG CGT GGA TCC CCG GAA TTC CCG GGT CGA CTC GAG CGG CCG CAT CGT GAC TGA

BamH I EcoR I Sma I Sal I Xho I Not I Stop codons



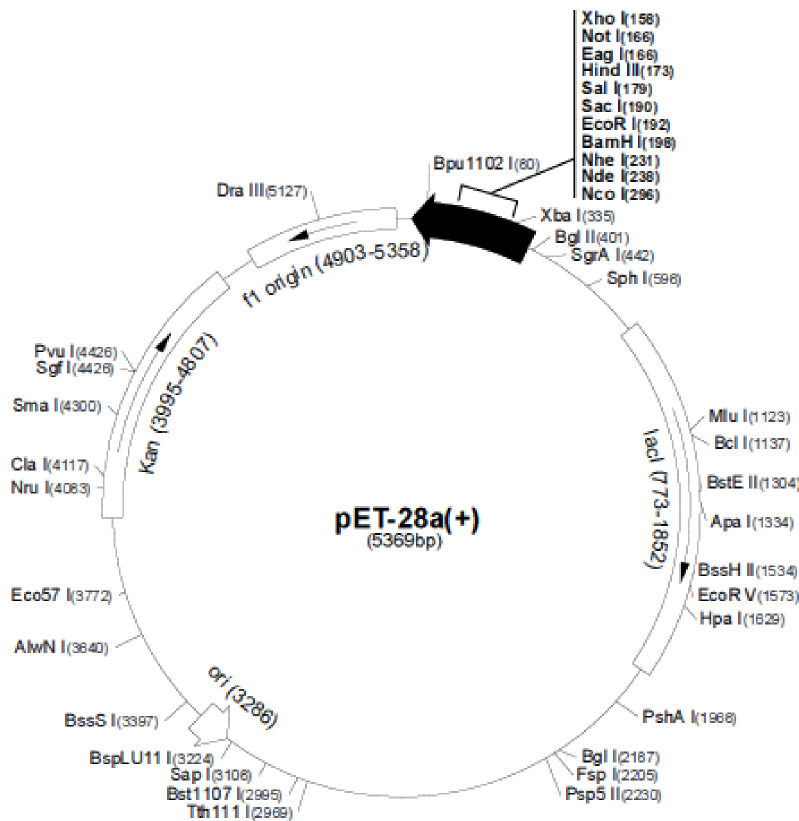


Fig. 2.2. Vector maps of pGEX-4T-1 and pET-28a expression vectors. http://pef.aibn.uq.edu.au/wordpress/wp-content/blogs.dir/1/files/Support/Bacteria/Manuals/pGEX_Vectors.pdf [accessed on 14.06.2012] and http://www.emdmillipore.com/life-science-research/pet-28a-plus-dna/EMD_BIO-69864/p_uuid?attachments=VSEQ [accessed on 14.06.2012].

2.2.6 Bacterial expression of VSGs LiTat 1.3 and LiTat 1.5

A small scale expression was performed with confirmed positive colonies (pGEX- 4T-1- cLiTat 1.3, pGEX- 4T-1- cLiTat 1.5, pGEX- 4T-1- gLiTat 1.3 and pGEX- 4T-1- gLiTat 1.5) in BL 21 DE3 cells. The pGEX-4T-1 expression vector, under the control of the *tac* promoter, contains the GST gene and induced expression results in a GST-tagged fusion protein (Smith and Johnson, 1988). Cells were grown in 10 ml 2 xYT medium containing ampicillin for 16 h at 37°C after which the culture was diluted with fresh medium (1:10) and cells grown to an optical density OD_{600} of 0.6. Expression was then induced with 1 mM IPTG and the cells grown over 4 h at 37°C. Having centrifuged (5 000 *g*, 15 min, 4°C), the bacterial pellet was resuspended in Lysis buffer [50 mM NaCl, 50 mM Tris, 1 mM EDTA, 0.5% (v/v) Triton-X-100, pH 8] containing lysozyme [1 mg/ml] and incubated at 37°C for 30 min. The cell lysate underwent two freeze-thaw cycles before being sonicated 3 x 30 s prior to analysis on a reducing 10% SDS-PAGE gel (section 2.2.8). A western blot, using anti-GST tag antibodies, was also carried out to confirm expression (section 2.2.9).

Solubility test of recombinant VSG cLiTat 1.3

To test for solubility, cell cultures were disrupted as described above, and centrifuged (7 000 *g*, 15 min, 4°C) and both supernatant and pellet fractions were assessed on a reducing 10% SDS-PAGE gel. Expression of recombinant proteins in *E. coli* may result in accumulation of the recombinant protein as insoluble aggregates known as inclusion bodies, hence the need to enhance solubility (Singh and Panda, 2005). To improve solubility, expression was carried out at lower temperatures and in addition to the pGEX system, the pET system was adopted. As described above, both pGEX-4T-1-cLiTat 1.3 and pET-28a-cLiTat 1.3 constructs in BL21 DE3 cells or Rosetta 2 DE3 cells were first grown overnight, diluted and induced with 1 mM IPTG in 2xYT medium containing the respective antibiotics at three different temperatures: 37°C, 30°C and 16°C, with shaking. Induction and expression at 16°C was carried out overnight, after cells were first allowed to reach OD_{600} of 0.6. The supernatant and pellet fractions of each culture were assessed on a reducing 10% SDS-PAGE gel.

Solubilisation and refolding of recombinant VSG cLiTat 1.3

Solubilising and refolding was carried out according to Sijwali *et al.* (2001) with modifications. A single colony of pET-28a-cLiTat 1.3 in BL21 DE3 cells was inoculated in 250 ml Terrific broth medium [1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4] with kanamycin (34 μg/ml) and expressed for 16 h at 37°C with shaking. The culture was centrifuged (5 000 *g*, 15 min, 4°C) and the bacterial pellet (inclusion bodies) was washed three times with pellet wash buffer [2 M urea, 0.02 M Tris-HCl, 2.5% (v/v) Triton X-100, pH 8] at 12 000 *g*, 30 min, 4°C. The protein pellet was then dissolved in 2.5 ml solubilisation buffer [6 M guanidine-HCl, 0.02 M Tris-HCl, 0.25 M NaCl, 0.02 M imidazole, pH 8] overnight at 4°C. The solubilised His-tagged protein (cLiTat 1.3_{His}) was dialysed against the following buffers: dialysis buffer 1 [4 M urea, 0.02 M Tris-HCl, 5% (w/v) glycerol, pH 8]; dialysis buffer 2 [2 M urea, 0.02 M Tris-HCl, 10 % (w/v) glycerol, pH 8]; dialysis buffer 3 [1 M urea, 0.02 M Tris-HCl, 15% (w/v) glycerol, pH 8]; dialysis buffer 4 [0.5 M urea, 0.02 M Tris-HCl, 20% (w/v) glycerol, pH 8]; refolding equilibration buffer [0.02 M Tris-HCl, 0.25 M L-arginine, 0.5 mM PMSF, 0.1% (w/v) NaN_3, 20% (w/v) glycerol, pH 8]. The refolding was assessed with SDS-PAGE as described in section 2.2.8.

Table 1. Dialysis buffers used for the refolding of cLiTat 1.3_{His}

Buffer [a]	Vol (ml)	Time (h)	Temp °C	[Urea] M
Dialysis buffer 1	250	6	RT	4.0
Dialysis buffer 2	1 000	18	RT	2.0
Dialysis buffer 3	500	6	RT	1.0
Dialysis buffer 4	1 000	6	4°C	0.5
Equilibration buffer	1 000	6	4°C	0.0

[a] See text for composition of buffers

Affinity purification of VSG cLiTat 1.3_{His}

His-select® nickel affinity resin (1 ml) was placed in a 10 ml chromatography column and allowed to settle before washing with two column volumes of deionised water and equilibrated with five column volumes of affinity equilibration buffer [50 mM NaH_2PO_4,

500 mM NaCl, 10 mM imidazole, pH 6.8]. The soluble VSG cLiTat 1.3_{His} protein (10 ml) was first filtered through 0.2 µm filters to remove any sediment present, prior to addition to the equilibrated resin and mixing using an end-over-end rotator at 4°C o.n. The unbound fractions were collected (1 ml) before the resin was washed with affinity equilibration buffer until an A_{280} absorbance of < 0.02 was attained. The bound fraction was recovered using 5 ml of elution buffer [50 mM NaH_2PO_4, 500 mM NaCl, 250 mM imidazole, pH 6.8]. The nickel resin was regenerated using two column volumes of deionised water, five column volumes of 6 M guanidine-HCl, another three column volumes of deionised water and finally, a three column volume wash with equilibration buffer before storage at 4°C in 0.05% (w/v) sodium azide. All the fractions, i.e. the unbound, washes and eluted fractions were analysed on a 10% SDS-PAGE gel (section 2.2.8) to assess purification of the recombinant protein. The purified fractions were pooled and concentrated with 10 kDa molecular weight cut-off (MWCO) Amicon Centripep centrifugal tubes to a final volume of 1 ml, and the final concentration determined as described in section 2.2.7. Samples were then analysed on a reducing 10% SDS-PAGE and visualised by silver staining.

2.2.7 Protein quantification

Protein concentration was determined using the BCA protein assay. The assay was established by Smith *et al.* (1985) and is based on two aspects, firstly, the biuret reaction where cupric ions (Cu^{2+}) are reduced to cuprous cations (Cu^{+}) as they form a violet coloured complex in alkaline conditions with peptides containing one or more peptide bonds and secondly, the reaction of the Cu^{+} with bicinchoninic acid, resulting in the formation of an intense purple colour. The assay was performed by first combining the working reagent (reagent A and B) in a 50:1 ratio, and then adding 200 µl to 25 µl duplicate samples of bovine serum albumin (BSA) [0.025-2 mg/ml] and protein sample in a Nunc® 96 well plate. The plate was incubated at 37°C for 30 min before the absorbance at 595 nm was read (A_{595}). A standard curve of the known concentrations of BSA vs A_{595} was plotted to determine the concentration of the protein sample (Fig. 2.3).

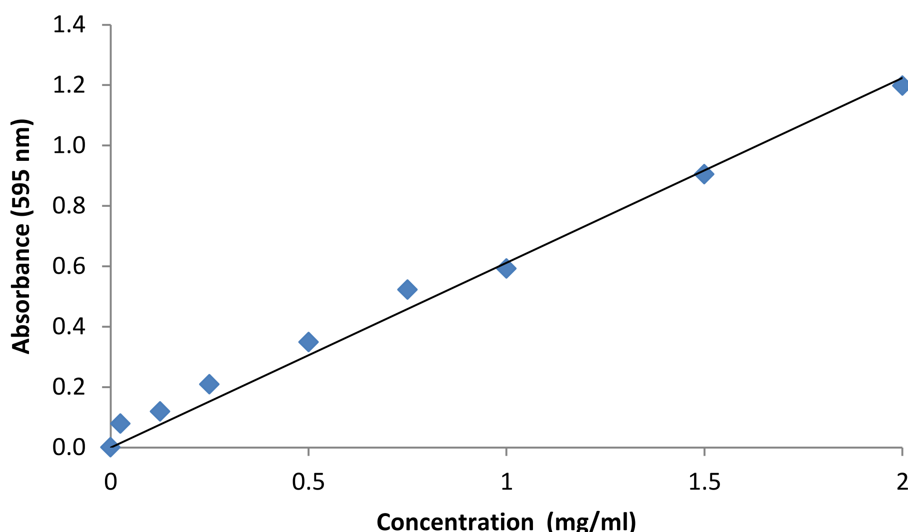


Fig. 2.3. Standard curve of BSA standards against absorbance at 595 nm. The equation of the line is y=0.6116x with a correlation coefficient (R^2) of 0.9879.

2.2.8 SDS-PAGE analysis of protein samples

Recombinantly expressed protein samples were assessed by reducing 10% SDS-PAGE as described by Laemmli (1970). Briefly, a discontinuous gel consisting of a stacking gel [500 mM Tris-HCl buffer pH 6.8] and running gel [1.5 M Tris-HCl buffer, pH 8.8] was set up to allow for the resolution and focus of the protein bands. The stacking gel has a large pore size, allowing the protein bands to collect as a thin layer at the interface between the stacking and running gel, migrating towards the running gel which has smaller pores. Samples were treated with an equal volume of reducing treatment buffer [125 mM Tris-HCl buffer, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol], where the SDS serves to denature the proteins, disrupting their secondary and tertiary structure as well as giving them a net negative charge allowing them to migrate based on size only. These samples were then boiled for 2 min prior to loading into the wells of the stacking gel and electrophoresed at 20 mA per gel in tank buffer [250 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1 % (m/v) SDS] for 2 h. Protein bands were visualised with Coomassie blue R-250 stain [0.125% (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid] and then destained in destaining solution [50% (v/v) methanol, 10% (v/v) acetic acid] and finally distilled water for complete destaining. The protein size was determined from a standard curve, relating the R_f value of each molecular weight marker to the log of molecular weight markers, as shown in the representative standard curves in Fig. 2.4. Panel A) shows the standard curve for the unstained markers and panel B) for the pre-

stained protein ladder used for western blots. The R_f value is calculated as the migration distance of the protein through the gel divided by the migration distance of the dye front.

For detection of very small amounts of protein in the gels, a more sensitive silver stain was used (Blum *et al.*, 1987). Briefly, the electrophoresed gel was placed in a scrupulously clean glass container to minimise background staining and soaked overnight in 50 ml fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.5% (v/v) 37% formaldehyde], prior to incubation in washing solution [50% (v/v) methanol] (3 x 20 min). The gel was soaked in pre-treatment solution [4 mg/ml $Na_2S_2O_3.5H_2O$] for 1 min, rinsed in distilled water (3 x 20 s) and soaked in impregnation solution [0.2% (m/v) $AgNO_3$, 0.75% (v/v) 37% formaldehyde] for 20 min. After rinsing in distilled water (2 x 20 s) the gel was incubated in developing solution [60 g/l Na_2CO_3, 0.5% (v/v) 37% formaldehyde, $Na_2S_2O_3.5H_2O$] until the first protein bands became visible. The developing solution was replaced with distilled water and the gel washed until colour was well developed. Development was stopped by soaking the gel in stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid] for 10 min.

2.2.9 Western blot analysis

Western blots were carried out as described by Towbin *et al.* (1979). Electrophoresed proteins in the gels were transferred onto a Bio Trace™ NT Pure nitrocellulose blotting membrane using the Sigma Semi-phor™ semi-dry blotting system in the presence of blotting buffer [45 mM Tris-HCl buffer pH 8.3, 173 mM glycine and 0.1% (w/v) SDS] at 17 volt with maximum current for 2 h. Unoccupied sites on the membrane were blocked with Elite non-fat milk powder [5% (w/v) in Tris-buffered saline (TBS) (20 mM Tris-HCl buffer, 200 mM NaCl pH 7.4)] for 1 h at room temperature. After washing with Tween-TBS (3 x 5 min), the membrane was incubated with the primary antibody (0.4 µg/ml anti-GST IgY or 1:1 000 mouse anti-His tag) diluted in 0.5% (w/v) BSA-TBS, for 2 h at room temperature or at 4°C over night. The membrane was washed with TBS (3 x 5 min, RT) and incubated in a secondary HRPO-conjugated rabbit anti-chicken IgY or goat anti-mouse antibody diluted in 0.5% (w/v) BSA-TBS (1:1 000 dilution) for 1 h at room temperature. Finally the membrane was washed with TBS (3 x 5 min) before being placed in chromogen-substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2, 0.1% (v/v) methanol, in TBS] and left to develop in the dark until the protein bands became visible.

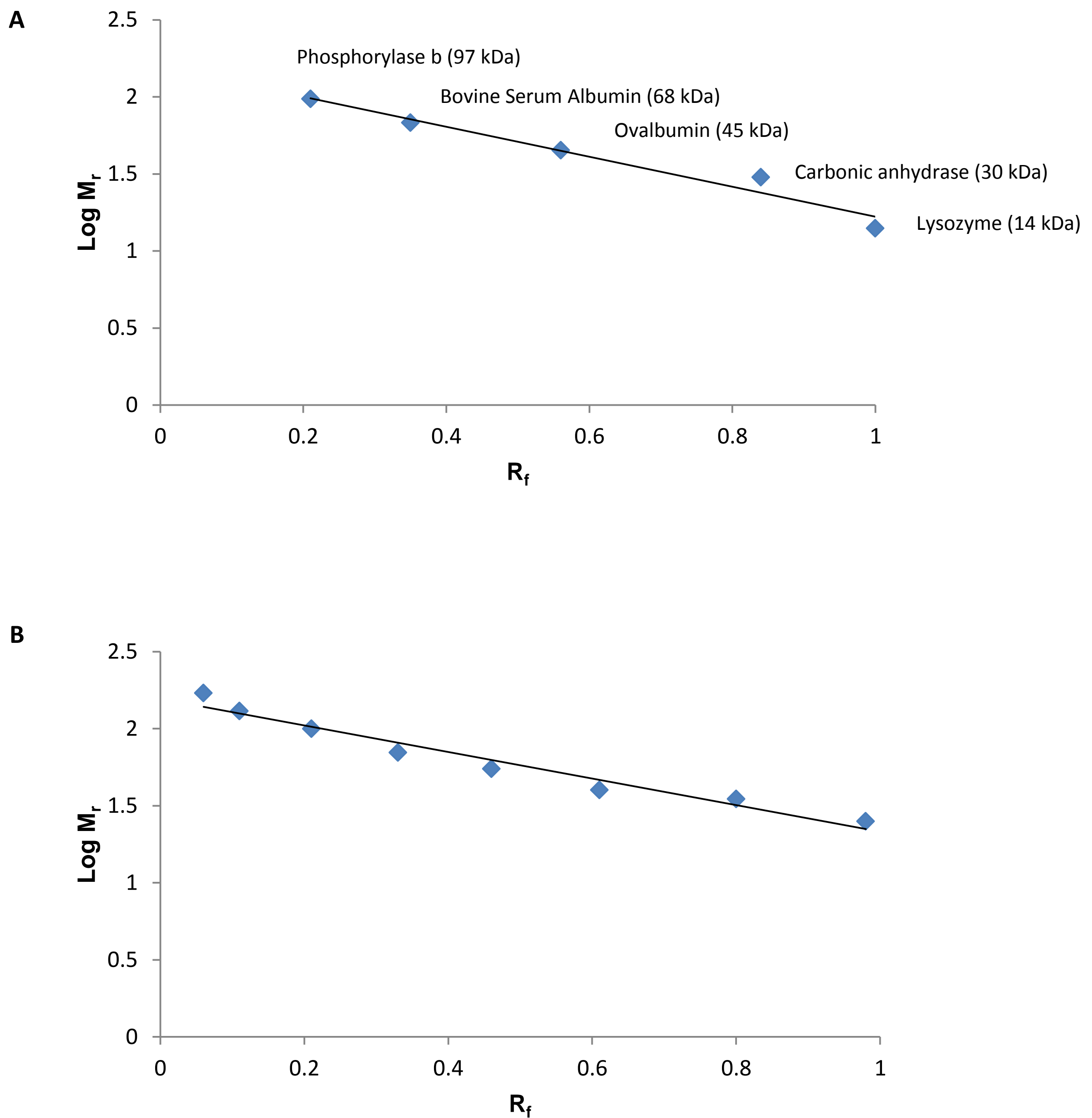


Fig. 2.4. Standard curve of log M_r against R_f for the estimation of protein M_r by SDS-PAGE. A: Molecular weight markers were made up from the components indicated on the curve. The equation of the trend line is y= -0.9721x + 2.1946, with a correlation coefficient (R^2) of 0.9621. **B:** Thermo Scientific PageRuler™ prestained protein ladder (170, 130, 100, 70, 55, 40, 35, 25, 15 kDa). The equation of the trend line is y=-0.8621x + 2.1929, with a correlation coefficient (R^2) of 0.9599.

2.2.10 Yeast expression and purification

Glycerol stocks of LiTat 1.5 11-11 SUMO in M5 and LiTat 1.3 A6-16-19 (3) SUMO were streaked on YPD agar plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 15 g/l bacteriological agar] containing zeocin antibiotic (100 µg/ml) and allowed to grow for 2-3 days at 30°C. Using a single colony of either of the constructs, 25 ml of BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6, 1.34% (w/v) yeast nitrogen base without amino acids [YNB], 0.00004% (w/v) Biotin, 1% (v/v) glycerol] containing 100 µg/ml zeocin, was inoculated in a 250 ml baffled flask and cultured at 29°C until an OD_{600} of 2.7 was reached. The cells were harvested by centrifugation (2000 *g*, 5 min, RT) and the pellet was gently resuspended in 100 ml BMMY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate pH 6, 1.34% (w/v) YNB, 0.00004% (w/v) Biotin, 0.5% (v/v) methanol] containing 100 µg/ml zeocin in a 500 ml baffled flask. The flask was covered with two layers of sterile cheesecloth and returned to incubator to continue growth. Expression was carried out over five days, and every 24 h, induction was maintained by adding 100% methanol to a final concentration of 0.5% methanol. Expression cultures (1 ml) were collected each day to analyse expression levels and determine the optimal time post-induction to harvest.

The samples were centrifuged at maximum speed in a tabletop microcentrifuge for 3 min and the supernatants were first concentrated by SDS / KCl precipitation prior to being analysed by SDS-PAGE. Briefly, 5% (w/v) SDS (10 µl) was added to the sample (100 µl) and mixed by inverting the tube and 3 M KCl (10 µl) was added. The mixture was again inverted and centrifuged (12 000 g, 2 min, RT). The supernatant was discarded and the precipitate was dissolved in stacking gel buffer (10 µl) and reducing treatment buffer (10 µl). Samples were analysed on a 10% reducing SDS-PAGE gel as described in section 2.2.8 and bands visualised with silver staining (section 2.2.8).

Three phase partitioning (TPP)

Through the use of *tert*-butanol (*t*-butanol) and ammonium sulfate, TPP precipitates proteins from aqueous solutions. The action of the ammonium sulfate salt and the *t*-butanol organic solvent results in the formation of three phases: the upper organic solvent phase, the lower water rich phase and the interfacial pellet. The protein of interest is found concentrated in the interfacial pellet, while most of the contaminants are present in the organic solvent phase (Dennison and Lovrien, 1997). TPP was

carried out as described by Pike and Dennison (1989). The yeast expression supernatant was first mixed with *t*-butanol, which constituted 30% (v/v) of the total mixture volume. Ammonium sulfate was then added in 10 % increments, starting with a concentration of 10% (w/v) to 40% (w/v) ammonium sulfate. With each increment the mixture was thoroughly mixed and centrifuged (6 000 *g*, 10 min, 4°C) in a swing out rotor. Three phases were formed, and the interphase (pellet) was collected making sure not to aspirate any *t*-butanol and resuspended in PBS, prior to SDS-PAGE analysis.

2.3 Results

2.3.1 Isolation of gDNA and PCR amplification

After the culturing of *T. b. brucei* trypanosomes, the parasites were harvested and the genomic DNA extracted using the phenol: chloroform method. The integrity of the extracted gDNA was analysed on a 1% agarose gel and was observed as a band >10 kb (Fig. 2.5). The smear running down the lane is an indication of possible degradation of the gDNA. The A_{280}:A_{260} ratio was found to be 1.74, 0.06 units below the accepted ratio of 1.8 for pure DNA (Wilfinger *et al.*, 1997). The extracted gDNA was therefore not of the highest quality but was acceptable for downstream applications i.e. PCR amplification.

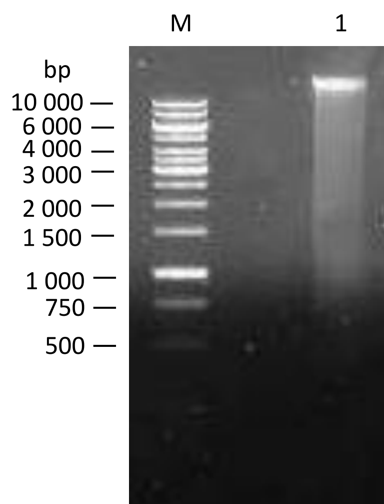


Fig. 2.5. Isolation of genomic DNA from *T. b. brucei*. Isolated DNA was run on a 1% agarose gel and stained with ethidium bromide. Lane M, O' GeneRuler 1kb DNA ladder; lane 1, *T .b. brucei* genomic DNA.

Using the designed primers, the genes encoding VSGs LiTat 1.3 and LiTat 1.5 were amplified by PCR using two different DNA templates: the cDNA as well as the extracted gDNA, and the expected sizes were 1.509 kb for LiTat 1.5 and 1.443 kb for LiTat 1.3. The PCR products were detected by agarose gel electrophoresis and the first analysed amplicons were from the gDNA template. The gLiTat 1.5 amplicon showed as a clear sharp band at ~1.5 kb as seen in Fig. 2.6, lane 1. The ~1.4 kb band for the gLiTat 1.3 amplicon (Fig. 2.6, lane 2) on the other hand, had a faint signal and other non-specific products were also present. Despite many attempts of varying PCR conditions such as $MgCl_2$ concentration, annealing temperature, as well as the number of PCR cycles, the non-specific products still formed, with the 0.4 kb product consistently maintaining a high intensity. As expected, the control sample, where no DNA template was used, did not show any PCR product (Fig. 2.6, lane 3).

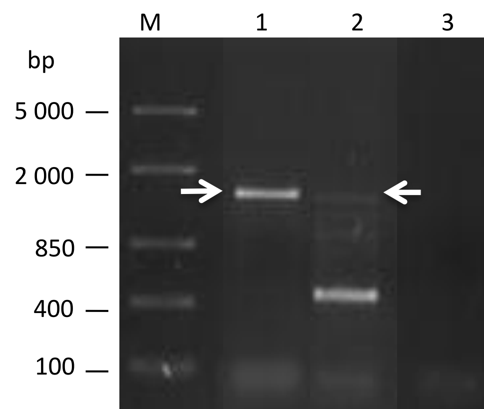


Fig. 2.6. Amplification of VSGs LiTat 1.5 and LiTat 1.3 from *T. b. brucei* gDNA analysed on a 1% agarose gel. Lane M, FastRuler DNA ladder Middle range; lane 1, gLiTat 1.5 PCR product; lane 2, gLiTat 1.3 PCR product; lane 3, no DNA template control. Left arrow indicates gLiTat 1.5 (~1.5 kb) and right arrow indicates gLiTat 1.3 (~1.4 kb). The gel was stained with ethidium bromide.

In comparison with amplification from a cDNA template, both cLiTat 1.5 and cLiTat 1.3 were seen at the expected sizes (Fig. 2.7). The cLiTat 1.5 products (Fig. 2.7, lanes 1 and 2) showed greater band intensity, as compared to cLiTat 1.3 products (Fig. 2.7, lanes 3 and 4), a trend similar to that of the gDNA PCR products. No non-specific PCR products were observed in cLiTat 1.3 PCR products, as compared to the gLiTat 1.3 product. This indicated that the PCR reactions with cDNA template were more specific.

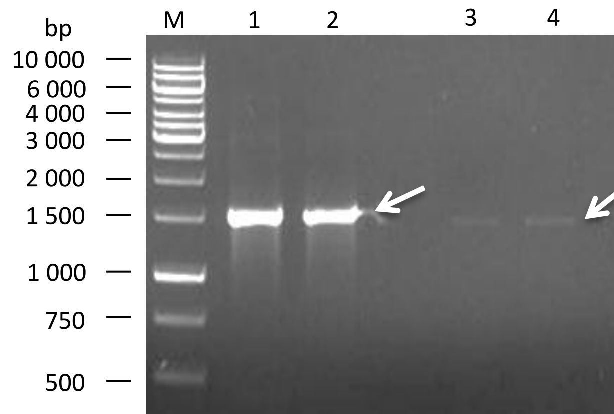


Fig. 2.7. Amplification of VSGs LiTat 1.3 and LiTat 1.5 from cDNA analysed on a 1% agarose gel. Lane M, O' GeneRuler 1kb DNA ladder; lanes 1 and 2, cLiTat 1.5 PCR product; lanes 3 and 4, cLiTat 1.3 PCR product. First arrow indicates cLiTat 1.5 (~1.5 kb), while second arrow indicates cLiTat 1.3 (~1.4 kb). The gel was stained with ethidium bromide.

The resultant PCR products were gel purified by excising the bands from the agarose gels under ultraviolet (UV) light, with extra caution for the gLiTat 1.3 band to avoid the excision of the non-specific bands.

2.3.2 T-vector cloning of VSGs LiTat 1.3 and LiTat 1.5

Having transformed competent *E. coli* JM109 cells with the ligation product of the gel purified amplicons and the pTZ57R/T-vector, the cells were plated on 2 xYT agar plates containing X-gal and IPTG, for blue vs white colour selection for clones containing insert.

The white colonies observed were screened for recombinancy by colony PCR, using the specific insert primers. Five out of six T-vector-gLiTat 1.3 colonies were recombinant as they showed the presence of the ~1.4 kb band (Fig. 2.8, lanes 1-4 and 6). However, a non-specific product of ~0.4 kb was also observed as was the case in the initial amplification from gDNA (Fig. 2.6, lane 2). Three out of five T-vector-gLiTat 1.5 colonies were recombinant, showing bands at ~1.5 kb (Fig. 2.8, lanes 8, 10 and 11). The positive recombinant colonies: T-vector-gLiTat 1.3 colonies 4 and 6 as well as T-vector-gLiTat 1.5 colonies 8 and 11 were selected for miniprep and sequenced.

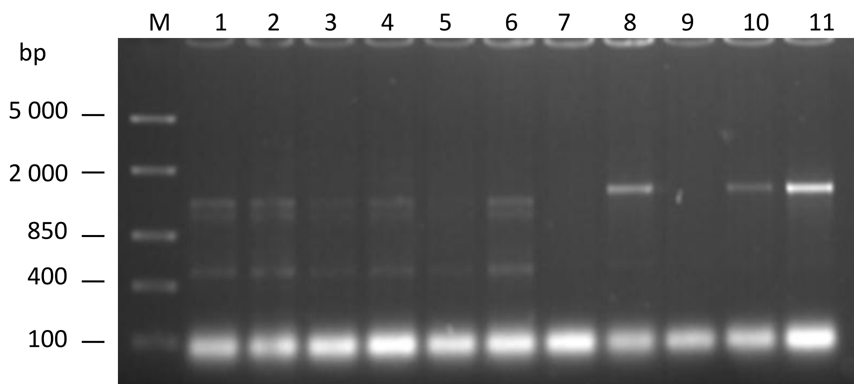


Fig. 2.8. Colony PCR screening of recombinant gLiTat 1.3 and gLiTat 1.5 T-vector clones. Following transformation into *E. coli* JM109 cells, PCR was performed on colonies using insert primers and analysed on a 1% agarose gel**.** Lane M, FastRuler DNA ladder Middle range; lanes 1- 6, PCR product for the screening of six T vector-gLiTat 1.3 colonies; lanes 7-11, PCR product for the screening of five T vector-gLiTat 1.5 colonies. The gel was stained with ethidium bromide.

With regards to T-vector-cLiTat 1.3 screening, three out of sixteen colonies were recombinant showing the ~1.4 kb band (Fig. 2.9 A, lanes 1, 2 and 8) with greater intensity in colony 8 as compared to colonies 1 and 2. Only two out of five colonies showed the 1.5 kb band for recombinant T-vector-cLiTat 1.5 (Fig. 2.9 B, lanes 4 and 5). Plasmid DNA, from the positive colonies: T-vector-cLiTat 1.3 colony 8 and the two T-vector-cLiTat 1.5 colonies, were isolated and sequenced.

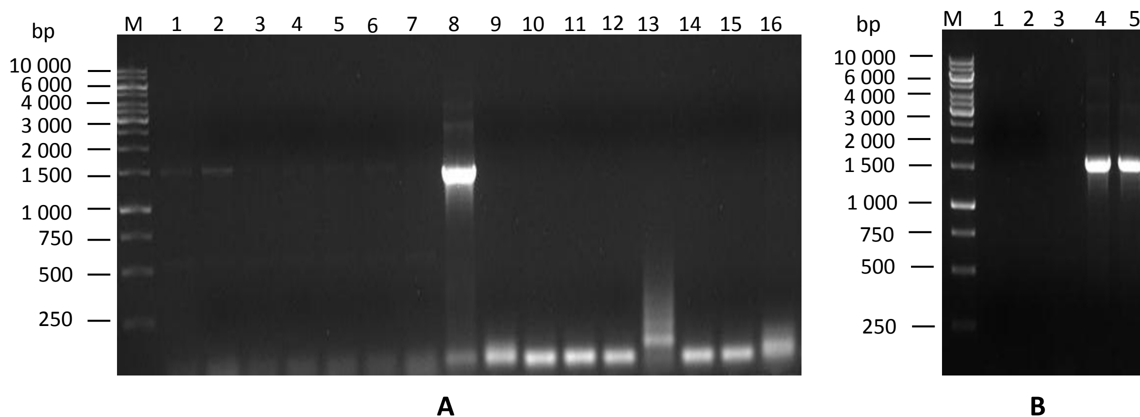


Fig. 2.9. Colony PCR screening of recombinant cLiTat 1.3 and cLiTat 1.5 T-vector clones. Following transformation into *E. coli* JM109 cells, PCR was performed on colonies using insert primers and analysed on a 1% agarose gel**. A:** Screening of T-vector-cLiTat 1.3 clones. Lane M, O' GeneRuler 1kb DNA ladder; lanes 1-16, PCR products for the screening of sixteen colonies. **B:** Screening of T-vector-cLiTat 1.5 clones. Lane M, O' GeneRuler 1kb DNA ladder; lanes 1-5, PCR product for the screening of five colonies. The gels were stained with ethidium bromide.

2.3.3 Subcloning into pGEX- 4T-1 and pET-28a expression vectors

Analysis of the sequencing results revealed that the cLiTat 1.3, cLiTat 1.5 and gLiTat 1.5 inserts were successfully ligated into T-vectors (Appendix A, B and D respectively) while analysis of the T-vector-gLiTat 1.3 clones revealed the presence of errors (Appendix C). The selected recombinant T-vector constructs were digested by restriction enzymes, to release the respective inserts for subcloning into expression vectors.

Despite the errors detected, restriction of T-vector-gLiTat 1.3 by EcoRI/NotI linearised the plasmid DNA and released the insert at ~1.4 kb (Fig. 2.10 A, lane 2). The double digestion of T-vector-gLiTat 1.5 by SalI/NotI released the ~1.5 kb insert (Fig. 2.10 B, lane 2) although there was incomplete digestion indicated by the middle band, almost the size of the uncut plasmid (Fig. 2.10 B, lane 1). The expression vectors pGEX-4T-1 (Fig. 2.10 C, lanes 2 and 3) and pET-28a (Fig. 2.10 C, lanes 5 and 6) were successfully linearised by digestion with either enzyme combination EcoRI/NotI or SalI/NotI.

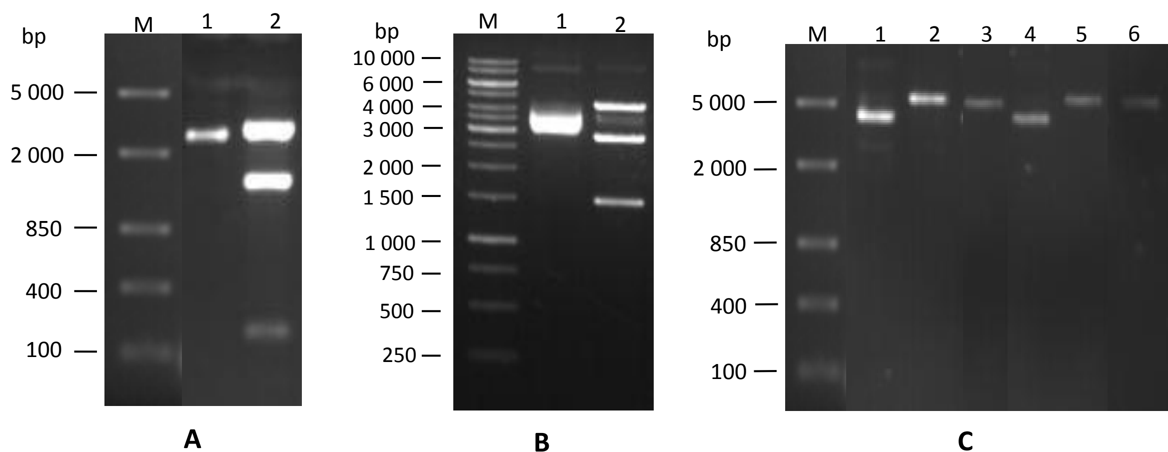


Fig. 2.10. Restriction digestion analysis of T-vector-gLiTat constructs and expression vectors on 1% agarose gels. A: Double digestion of T-vector-gLiTat 1.3. Lane M, FastRuler DNA ladder Middle range; lane 1, uncut T-vector-gLiTat 1.3 miniprep; lane 2, EcoRI/NotI restricted T-vector-gLiTat 1.3. **B:** Double digestion of T-vector-gLiTat 1.5. Lane M, O' GeneRuler 1kb DNA ladder; lane 1, uncut T-vector-gLiTat 1.5 miniprep; lane 2, SalI/NotI restricted T-vector-gLiTat 1.5. **C:** Linearised pGEX-4T-1 and pET-28a expression vectors following restriction digestion. Lane M, FastRuler DNA ladder Middle range; lane 1, uncut pGEX-4T-1 miniprep; lane 2, EcoRI/NotI restricted pGEX-4T-1; lane 3, SalI/NotI restricted pGEX-4T-1; lane 4, uncut pET-28a miniprep; lane 5, EcoRI/NotI restricted pET-28a; lane 6, SalI/NotI restricted pET-28a. The gels were stained with ethidium bromide.

The restriction of the T-vector-cLiTat 1.3 construct released the ~1.4 kb cLiTat 1.3 insert (Fig. 2.11, lane 2) while that of T-vector-cLiTat 1.5 released the ~1.5 kb insert

(Fig. 2.11, lane 4). The expression vectors were also linearised successfully, as before, by the EcoRI/NotI or SalI/NotI restriction enzyme combinations for pGEX-4T-1 (Fig. 2.11, lanes 6 and 7) and pET-28a (Fig. 2.11, lanes 9 and 10).

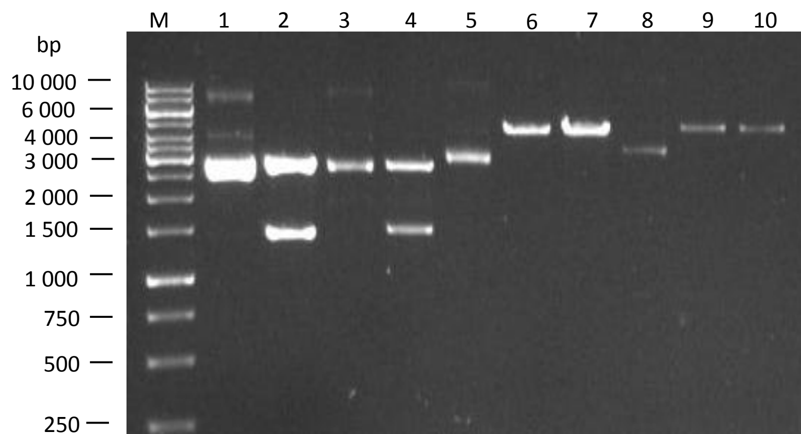


Fig. 2.11. Restriction digestion analysis of T-vector-cLiTat constructs and expression vectors on 1% agarose gel. Lane M, O' GeneRuler 1kb DNA ladder; lane 1, uncut T-vector-cLiTat 1.3 miniprep; lane 2, EcoRI/NotI restricted T-vector-cLiTat 1.3; lane 3, uncut T-vector-cLiTat 1.5 miniprep; lane 4, SalI/NotI restricted T-vector- cLiTat 1.5; lane 5, uncut pGEX-4T-1 miniprep; lane 6, EcoRI/NotI restricted pGEX-4T-1; lane 7, SalI/NotI restricted pGEX-4T-1; lane 8, uncut pET-28a miniprep; lane 9, EcoRI/Not I restricted pET-28a; lane 10, SalI/NotI restricted pET-28a. The gel was stained with ethidium bromide.

Once the inserts were gel purified, they were ligated to the cleaned and concentrated expression vectors and transformed into competent *E. coli* JM109 cells. To screen for the recombinant positive clones, colony PCR was carried out using pGEX-4T-1 or pET-28a expression vector forward and reverse sequencing primers. These primers flank the multiple cloning site of the vectors amplifying that region including the insert, resulting in a shift in size by ~0.145 kb for the recombinant pGEX-4T-1 constructs and ~0.150 kb for the recombinant pET-28a constructs.

Of the 5 colonies screened for recombinant pGEX-4T-1-gLiTat 1.3, three were positive at ~1.545 kb (Fig. 2.12 A, lanes 2-4), while all ten screened for recombinant pGEX-4T-1-gLiTat 1.5 showed recombinancy at ~1.645 kb (Fig. 2.12 B). The pET constructs had one pET-28a-gLiTat 1.3 recombinant colony at ~1.550 kb, out of four screened (Fig. 2.12 C, lane 4), while eight out of ten colonies were recombinant for pET-28a-gLiTat 1.5 at ~1.650 kb (Fig. 2.12 D, lanes 2-7, 9-10). The sequenced recombinant plasmids revealed the successful ligation of gLiTat 1.5 into expression vectors, while that of gLiTat 1.3 had errors present (Appendix H).

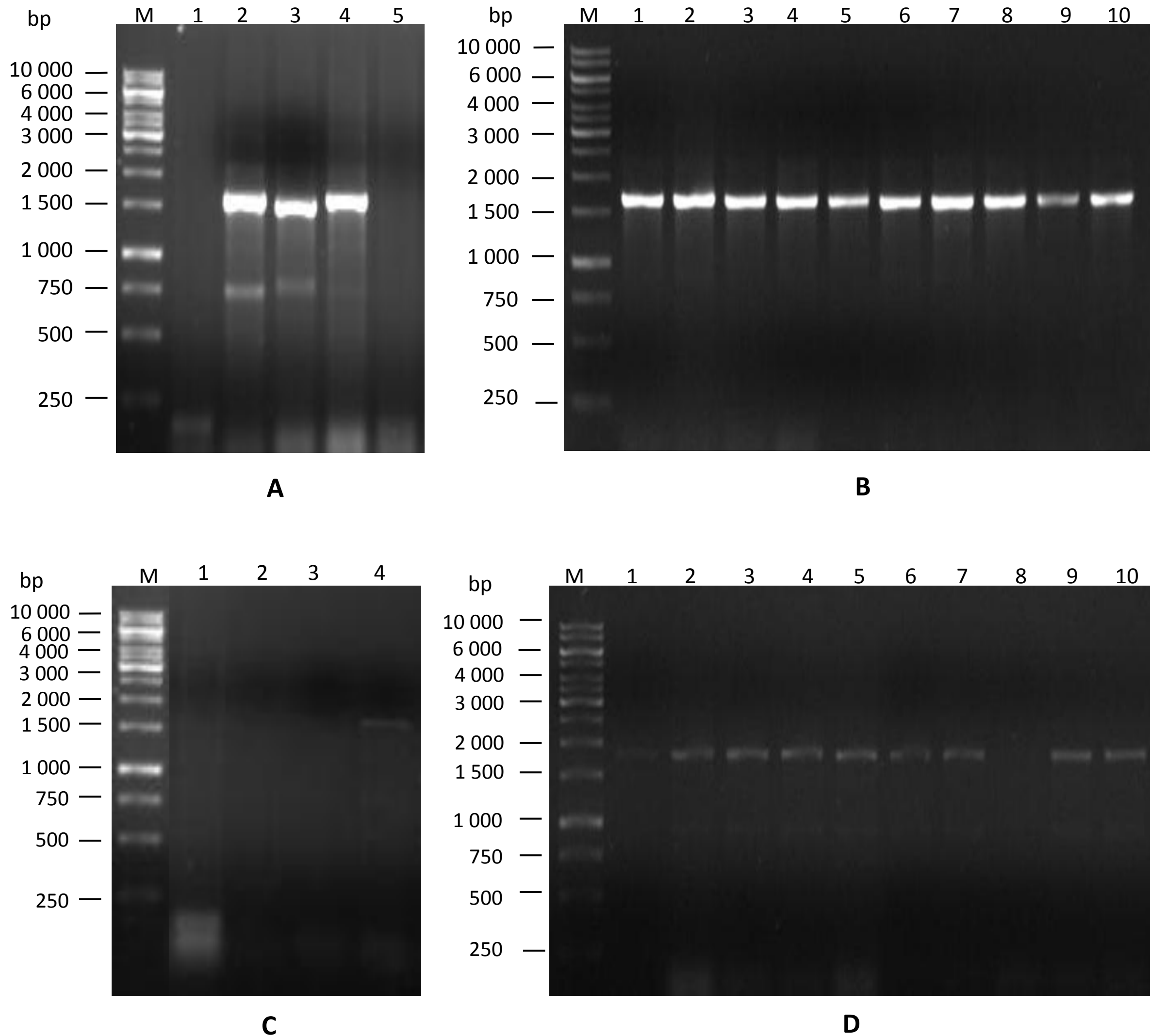


Fig. 2.12. Colony PCR screening of recombinant clones of gLiTat 1.3 and gLiTat 1.5 in pGEX-4T-1 and pET-28a expression vectors. Following transformation into *E. coli* JM109 cells, PCR was performed on colonies using vector (pGEX4T-1 or pET28a) primers and analysed on a 1% agarose gel. **A:** Lane M, O' GeneRuler 1kb DNA ladder; lanes 1-5, PCR product for the screening of five pGEX-4T-1-gLiTat 1.3 colonies. **B:** Lane M, O' GeneRuler 1kb DNA ladder; lanes 1-10, PCR product for the screening of ten pGEX-4T-1-gLiTat 1.5 colonies. **C:** Lane M, O' GeneRuler 1kb DNA Ladder; lanes 1-4, PCR product for the screening of four pET-28a-gLiTat 1.3 colonies. **D:** Lane M, O' GeneRuler 1kb DNA Ladder; lanes 1-10, PCR product for the screening of ten pET-28a-gLiTat 1.5 colonies. The gels were stained with ethidium bromide.

The screening of ten pGEX-4T-1-cLiTat 1.3 colonies revealed two recombinant clones at ~1.545 kb (Fig. 2.13 A, lanes 1 and 10) while three out of nine were recombinant for pGEX-4T-1-cLiTat 1.5 at ~1.645 kb (Fig. 2.13 A, lanes 12, 15 and 19). Five pET-28a-cLiTat 1.3 colonies screened showed three were recombinant at ~1.550 bp (Fig. 2.13 B, lanes 1-3) and the two screened for pET-28a-cLiTat 1.5 were both recombinant at ~1.650 kb (Fig. 2.13 C, lanes 1 and 2). Sequencing of the recombinants revealed successful ligation (Appendix E, F, G and I).

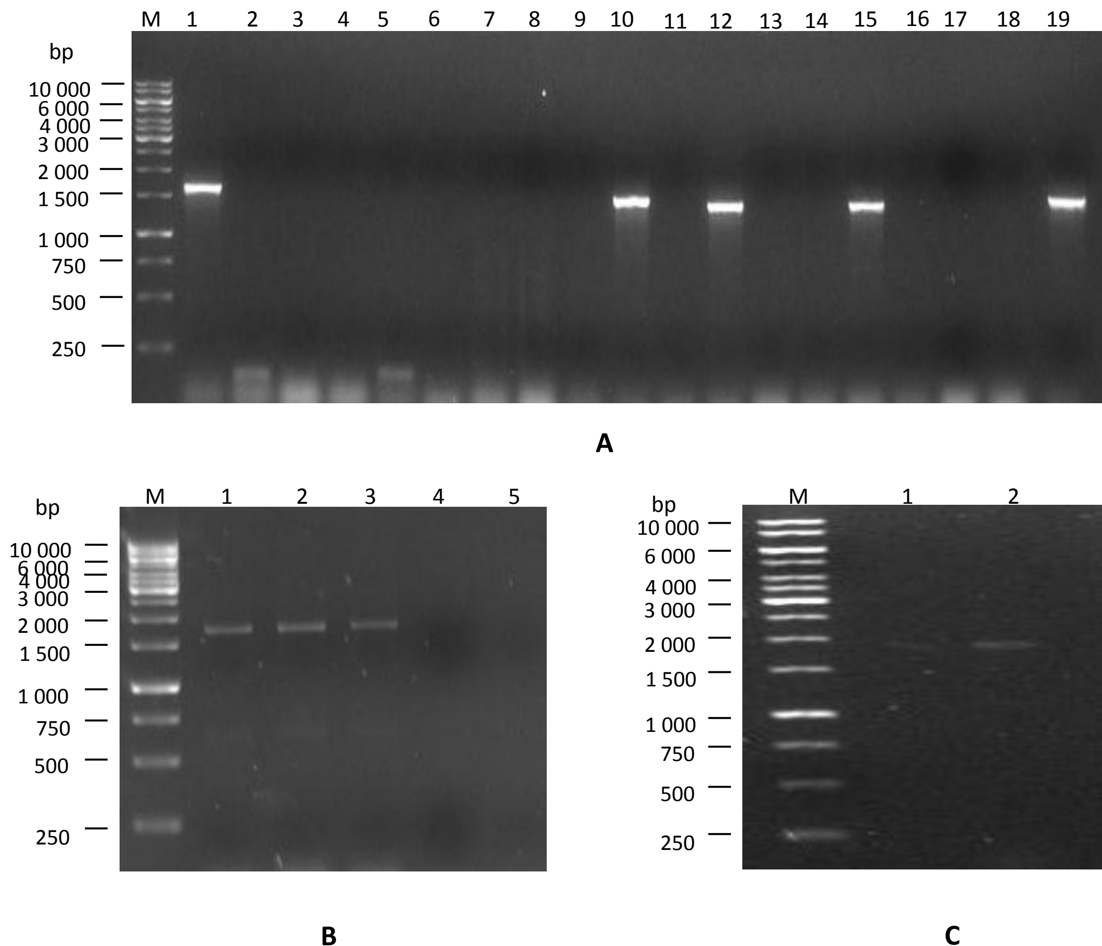


Fig. 2.13. Colony PCR screening of recombinant clones of cLiTat 1.3 and cLiTat 1.5 in pGEX-4T-1 and pET-28a expression vectors. Following transformation into *E. coli* JM109 cells, PCR was performed on colonies using vector (pGEX4T-1 or pET28a) primers and analysed on a 1% agarose gel. **A:** Lane M, O' GeneRuler 1kb DNA ladder; lanes 1-10, PCR product for the screening of ten pGEX-4T-1-cLiTat 1.3 colonies; lanes 11-19, PCR product for the screening of nine pGEX-4T-1-cLiTat 1.5 colonies. **B:** Lane M, O' GeneRuler 1kb DNA Ladder; lanes 1-5, PCR product for the screening of five pET-28a-cLiTat 1.3 colonies. **C:** Lane M, O' GeneRuler 1kb DNA Ladder; lanes 1 and 2, PCR product for the screening of two pET-28a-cLiTat 1.5 colonies. The gels were stained with ethidium bromide.

2.3.4 Bacterial expression of recombinant VSGs LiTat 1.3 and LiTat 1.5

Due to the errors present following sequencing analysis of pGEX4T-1-gLiTat 1.3, small scale expression was carried out only on the selected recombinant pGEX4T-1-cLiTat 1.3, pGEX4T-1-cLiTat 1.5 and pGEX4T-1-gLiTat 1.5 clones in *E.coli* BL21 DE3 cells using 1 mM IPTG induction at 37°C. Analysis of protein expression on a 10% SDS-PAGE gel showed the uncloned pGEX-4T-1 vector expressing the GST tag (control) at 26 kDa (Fig. 2.14 A, lane 1), while the cloned pGEX4T-1-cLiTat 1.3 expressed the cLiTat 1.3_{GST} protein at 94.9 kDa (Fig. 2.14 A, lane 3).

The expected size for cLiTat 1.5_{GST} was 96 kDa, however, both the induced pGEX-4T-1-cLiTat 1.5 and pGEX-4T-1-gLiTat 1.5 clones expressed the protein at an unexpected size of 28.8 kDa or 32 kDa (Fig. 2.14 A, lane 5 and 2.14 C, lane 2 respectively). The difference between the two sizes is due to the use of different molecular weight markers in each gel, which migrate differently. PageRuler pre-stained markers are prepared with dye molecules attached to them and this influences migration as compared to unstained markers (Novus-Biologicals). Optimising expression conditions for the cLiTat 1.5 protein such as expressing in different cell lines as well as at different temperatures, were futile, as the truncated protein was still expressed (results not shown). Neither the cLiTat 1.3_{GST} or the cLiTat 1.5_{GST} proteins were observed in the uninduced pGEX-4T-1 controls containing the respective inserts.

A western blot, probed with anti-GST tag antibodies confirmed the expression of the control GST tag at 26 kDa (Fig. 2.14 B, lane 1) as well as the recombinant cLiTat 1.3_{GST} at 94.9 kDa and cLiTat 1.5_{GST} at 28.8 kDa (Fig. 2.14 B, lanes 3 and 4 respectively). The gLiTat 1.5_{GST} band was confirmed at 32 kDa (Fig. 2.14 D, lane 2).

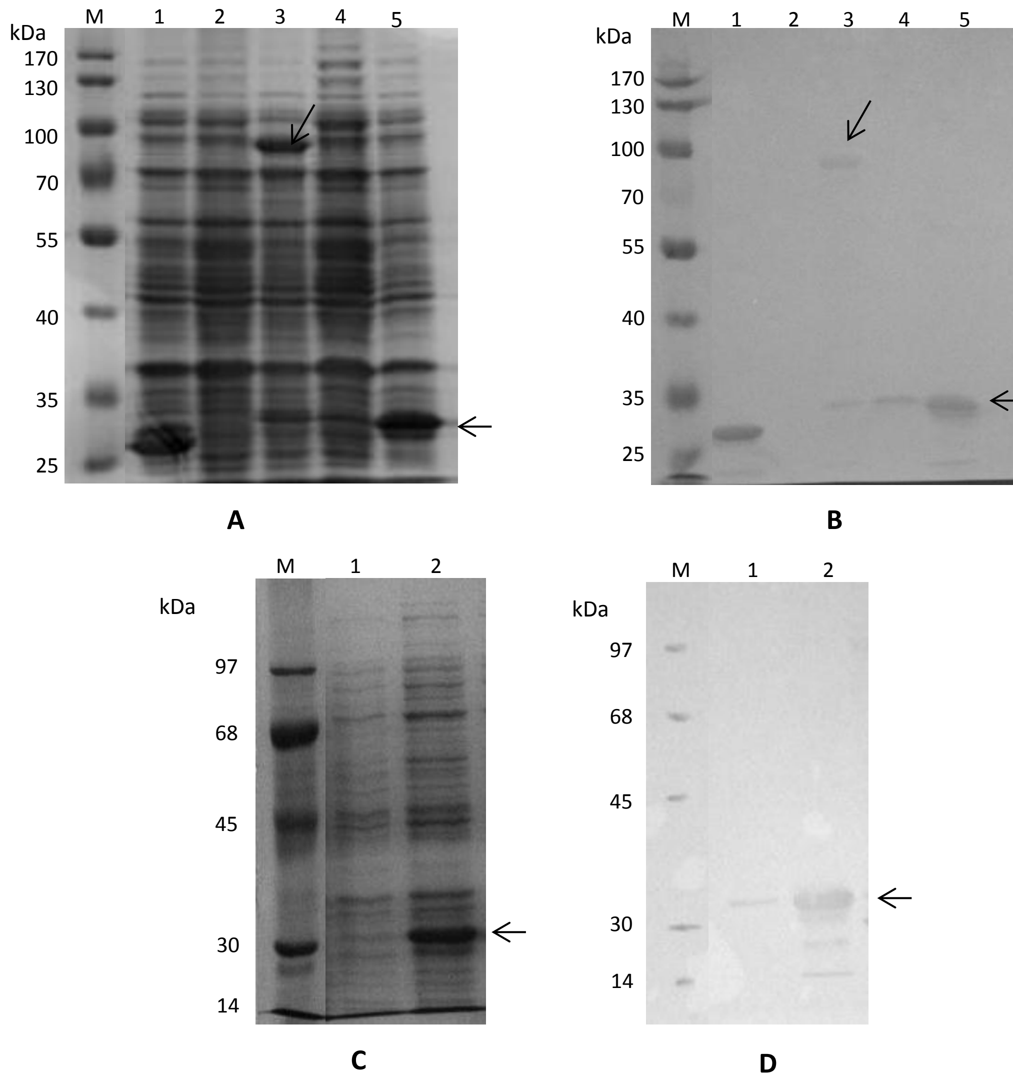


Fig. 2.14. Analysis by SDS-PAGE and western blot of the expression of recombinant VSG cLiTat 1.3, cLiTat 1.5 or gLiTat 1.5 in pGEX4T-1. A and C: Coomassie stained reducing 10% SDS-PAGE gel. **B and D:** Western blot probed with anti-GST antibody (0.4 µg/ml). (A and B) Lane M, PageRuler™ prestained protein ladder; lane 1, pGEX-4T-1 vector without insert, GST tag (control); lane 2, uninduced pGEX-4T-1-cLiTat 1.3_{GST}; lane 3, induced pGEX-4T-1-cLiTat 1.3_{GST}; lane 4, uninduced pGEX-4T-1-cLiTat 1.5_{GST}; lane 5, induced pGEX-4T-1-cLiTat 1.5_{GST}. (C and D) Lane M; lane 1 uninduced pGEX-4T-1-gLiTat 1.5_{GST}; lane 2, induced pGEX-4T-1-gLiTat 1.5_{GST}. Positions of target proteins are shown by arrows. Arrow in lane 3: cLiTat 1.3_{GST}, arrow in lane 5: cLiTat 1.5_{GST}, arrow in lane 2 gLiTat 1.5_{GST}.

2.3.5 Testing and optimising solubility of recombinant VSG cLiTat 1.3

Solubility was only assessed for recombinant cLiTat 1.3_{GST}, as the expression of both cLiTat 1.5_{GST} and gLiTat 1.5_{GST} gave unexpected results and was not pursued any further. Following expression, the cells were lysed and both the supernatant and pellet fractions obtained following centrifugation were assessed on a 10% SDS-PAGE gel. The recombinant cLiTat 1.3_{GST} was absent in the supernatant (Fig. 2.15, lane 2) but present in the pellet at 84.1 kDa (Fig. 2.15, lane 3) indicating that the protein was

insoluble and present in inclusion bodies. The protein was not present in the control uninduced clone containing the insert.

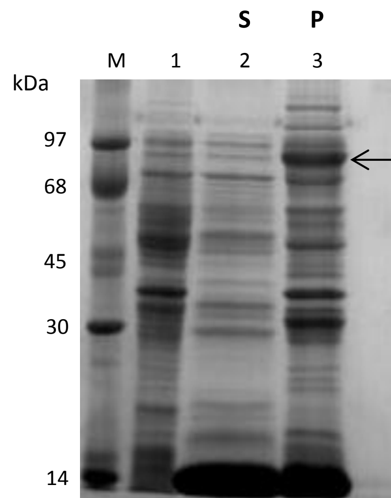


Fig. 2.15. Analysis by reducing 10% SDS-PAGE of the solubility of recombinantly expressed VSG cLiTat 1.3 in pGEX-4T-1. Lane M, molecular weight marker; lane 1, uninduced cLiTat 1.3_{GST} cell lysate control; lane 2, cLiTat 1.3_{GST} supernatant (S); lane 3, cLiTat 1.3_{GST} pellet (P). Target protein represented by arrow. The gel was stained with Coomassie blue.

Expression from the pET-28a-cLiTat 1.3 construct was performed and together with the pGEX-4T-1-cLiTat 1.3, they were expressed in either BL21 DE3 cells or Rossetta 2 DE 3 host cells at 37°C, 30°C and 16°C.

After lysing the cells, expression was assessed in the supernatant and pellet fractions by 10% SDS-PAGE. This revealed that expression of the pET-28a-cLiTat 1.3 construct in neither BL21 DE3 nor Rossetta 2 DE3 cells at 37°C improved solubility as the resultant 68.8 kDa cLiTat 1.3_{His} protein was present in the pellet (Fig. 2.16 A1, lanes 3 and 5). Under the same conditions, the same trend was observed in the expression of the pGEX-4T-1-cLiTat 1.3, where the 84.1 kDa cLiTat 1.3_{GST} protein was observed in the pellet (Fig. 2.16 A2, lanes 7 and 9).

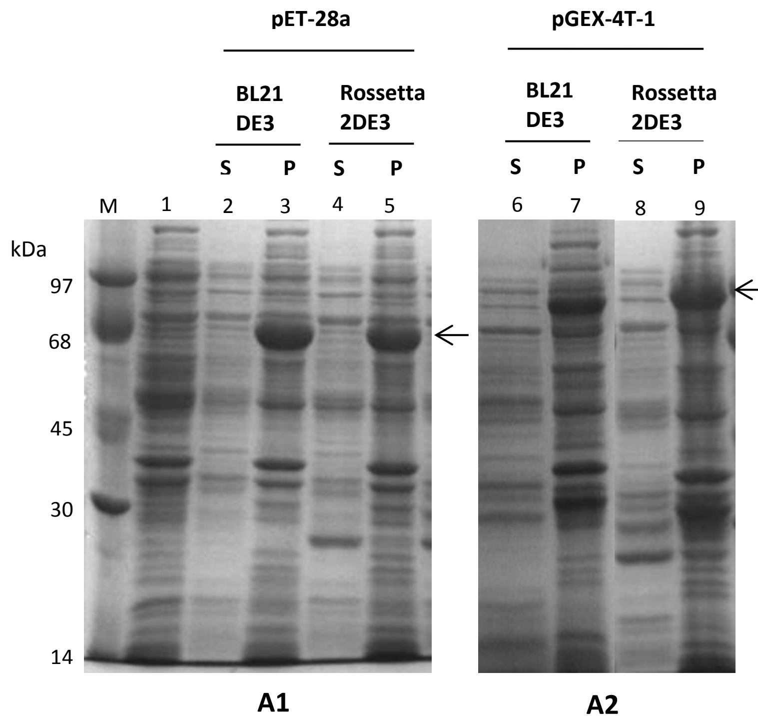


Fig. 2.16. Analysis by reducing 10% SDS-PAGE of the solubility of recombinantly expressed VSG cLiTat 1.3 at 37°C. Expression was carried out in both *E. coli* BL21 DE3 cells and Rosetta 2 DE3 cells, using the pET-28a system **(A1)** and the pGEX-4T-1 system **(A2)**. **A1:** Lane M, molecular weight marker; lane 1, un-induced cLiTat 1.3_{His} cell lysate (control); lane 2, cLiTat 1.3_{His} supernatant (S); lane 3, cLiTat 1.3_{His} pellet (P); lane 4, cLiTat 1.3_{His} supernatant (S); lane 5, cLiTat 1.3_{His} pellet (P). **A2**: Lane 6, cLiTat 1.3_{GST} supernatant (S); lane 7, cLiTat 1.3_{GST} pellet (P); lane 8, cLiTat 1.3_{GST} supernatant (S); lane 9, cLiTat 1.3_{GST} pellet (P). Target proteins represented by arrows. The gels were stained with Coomassie blue.

As presented in Fig. 2.17, expression in either BL 21 DE3 or Rossetta 2 DE3 cells at 30°C, revealed that the recombinant protein was still insoluble as it was present in the pellet fractions, cLiTat 1.3_{His} (Fig. 2.17 A1, lanes 2 and 4) and cLiTat 1.3_{GST} (Fig. 2.17 A2, lanes 6 and 8).

Fig. 2.17. Analysis by reducing SDS-PAGE (10%) of the solubility of recombinantly expressed VSG cLiTat 1.3 at 30 °C. Expression was carried out in both *E. coli* BL21 DE3 cells and Rosetta 2 DE3 cells, using the pET-28a system **(A1)** and the pGEX-4T-1 system **(A2)**. **A1:** Lane M, molecular weight marker; lane 1, cLiTat 1.3$_{His}$ supernatant (S); lane 2, cLiTat 1.3$_{His}$ pellet (P); lane 3, cLiTat 1.3$_{His}$ supernatant (S); lane 4, cLiTat 1.3$_{His}$ pellet (P). **A2**: Lane 5, cLiTat 1.3$_{GST}$ supernatant (S); lane 6, cLiTat 1.3$_{GST}$ pellet (P); lane 7, cLiTat 1.3$_{GST}$ supernatant (S); lane 8, cLiTat 1.3$_{GST}$ pellet (P). Target proteins represented by arrows. Gels were stained with Coomassie blue.

Solubility was finally achieved, though in low amounts, following expression of the pET-28a-cLiTat 1.3 construct at a much lower temperature of 16°C in both the BL 21 DE3 and Rossetta 2 DE3 cells. The amount of soluble cLiTat 1.3$_{His}$ present in the supernatant was very small (Fig. 2.18 A1, lanes 1 and 3) as compared to the corresponding pellet fractions (Fig. 2.18 A1, lanes 2 and 4). Expression of the pGEX-4T-1-cLiTat 1.3 construct under the same conditions did not result in soluble protein, as the protein was still present in the pellet (Fig. 2.18 A2, lanes 6 and 8).

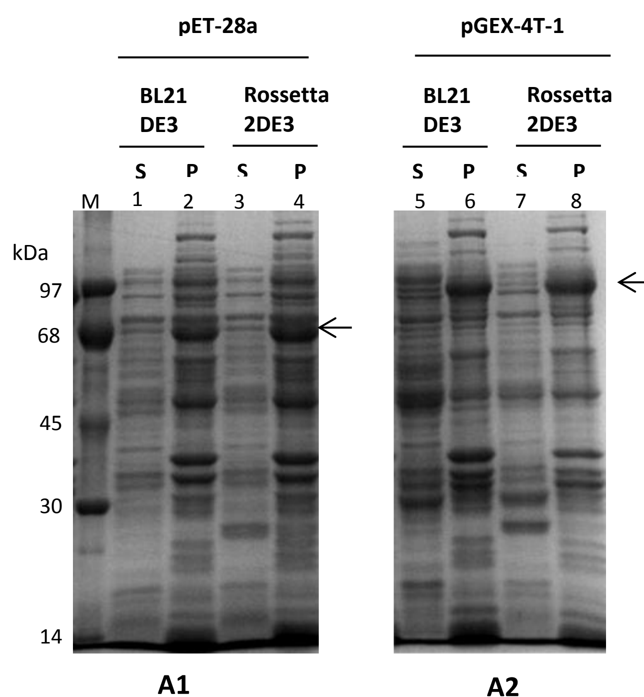


Fig. 2.18. Analysis by reducing SDS-PAGE (10%) of the solubility of recombinantly expressed VSG cLiTat 1.3 at 16°C. Expression was carried out in both *E. coli* BL21 DE3 cells and Rosetta 2 DE3 cells, using the pET-28a system **(A1)** and the pGEX-4T-1 system **(A2)**. **A1:** Lane M, molecular weight marker; lane 1, cLiTat 1.3 supernatant (S); lane 2, cLiTat 1.3 pellet (P); lane 3, cLiTat 1.3 supernatant (S); lane 4, cLiTat 1.3 pellet (P). **A2**: Lane 5, cLiTat 1.3 supernatant (S); lane 6, cLiTat 1.3 pellet (P); lane 7, cLiTat 1.3 supernatant (S); lane 8, cLiTat 1.3 pellet (P). Target protein represented by arrow. The gels were stained with Coomassie blue.

The results clearly demonstrate that solubility was enhanced by expressing at the low temperature of 16°C using the pET expression system. The Rossetta 2 DE3 cells did not show any significant improvement in solubility as there was no difference with expression in BL21 DE3 cells at lower temperatures.

2.3.6 Solubilising and refolding of cLiTat 1.3_{His}

Because of the low amount of soluble cLiTat 1.3_{His} expressed at 16°C, large scale expression and purification for possible crystallisation studies or the immunisation of chickens for antibody production, would have been a tedious procedure; hence it was necessary to recover the soluble protein from inclusion bodies. This was done by solubilising the inclusion bodies in the presence of a chaotrophic (denaturing) reagent and refolding by the removal of the denaturant.

The first approach involved solubilising the bacterial pellet containing inclusion bodies with 8 M urea and directly binding to a Ni-NTA affinity column for purification and refolding by gradient washing with buffer of decreasing concentrations of urea (8 M-0 M urea), prior to elution with 250 mM imidazole. This technique, however, did

not yield the desired outcome, as the cLiTat 1.3_{His} was released during the washes before elution with imidazole (results not shown). The second approach which was an improvement, involved solubilising the inclusion bodies with guanidine-HCl and refolding the cLiTat 1.3_{His} by dialysis against buffers with decreasing concentrations of urea, prior to purification by Ni-NTA affinity chromatography.

To ensure that any contaminants that may have adsorbed onto the hydrophobic inclusion bodies were not present (De Bernardez Clark, 1998), the insoluble protein pellet obtained from expression was first washed with pellet wash buffer containing 2 M urea. The SDS-PAGE analysis of the washes revealed that most of the contaminants were removed with each successive wash, with very little of the cLiTat 1.3_{His} protein being released, i.e. had become soluble (Fig. 2.19, lanes 2 and 3). Following treatment with the denaturant, the inclusion bodies were completely solubilised (Fig. 2.19, lane 4) and the solubilised protein was refolded by dialysis as described in the materials and methods section.

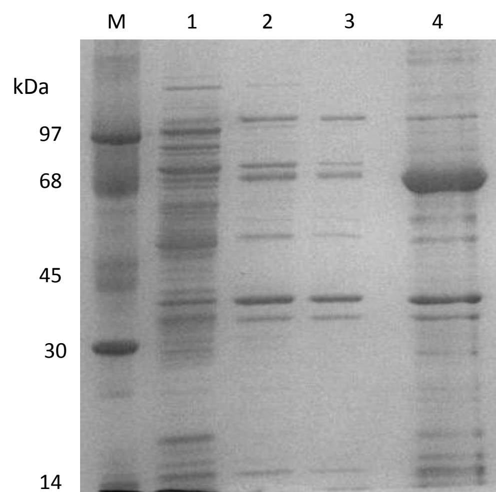


Fig. 2.19. Analysis of solubilised cLiTat 1.3_{His} by reducing 10% SDS-PAGE. Lane M, molecular weight marker; lane 1, first pellet wash; lane 2, second pellet wash; lane 3, third pellet wash; lane 4, solubilised protein pellet. Gel was stained with Coomassie blue.

2.3.7 Affinity purification of cLiTat 1.3_{His}

Following refolding by dialysis, the recombinant cLiTat 1.3 protein was subjected to Ni-affinity purification. The elution profile (Fig. 2.20 A, fractions 1-9) revealed the unbound fraction had an $A_{280} > 2.5$, which not only contained contaminating proteins, but some of the 68.8 kDa cLiTat 1.3_{His} protein, which may not have bound due to the

concentrated nature of the sample following dialysis (Fig. 2.20 B, lane 1). The first column wash contained some traces of the cLiTat 1.3 protein (Fig. 2.20 B, lane 2) but the subsequent washes did not reveal the presence of any proteins as indicated by the A_{280} of ~0 (Fig. 2.20 A, fractions 13-25) and the corresponding SDS-PAGE analysis (Fig. 2.20 B, lanes 3 and 4). Upon elution with imidazole, the bound His-tagged cLiTat 1.3 protein was released, shown by an absorbance of ~0.5 nm (Fig. 2.20 A, fraction 29) and a distinct band with no other contaminants (Fig. 2.20 B, lane 5).

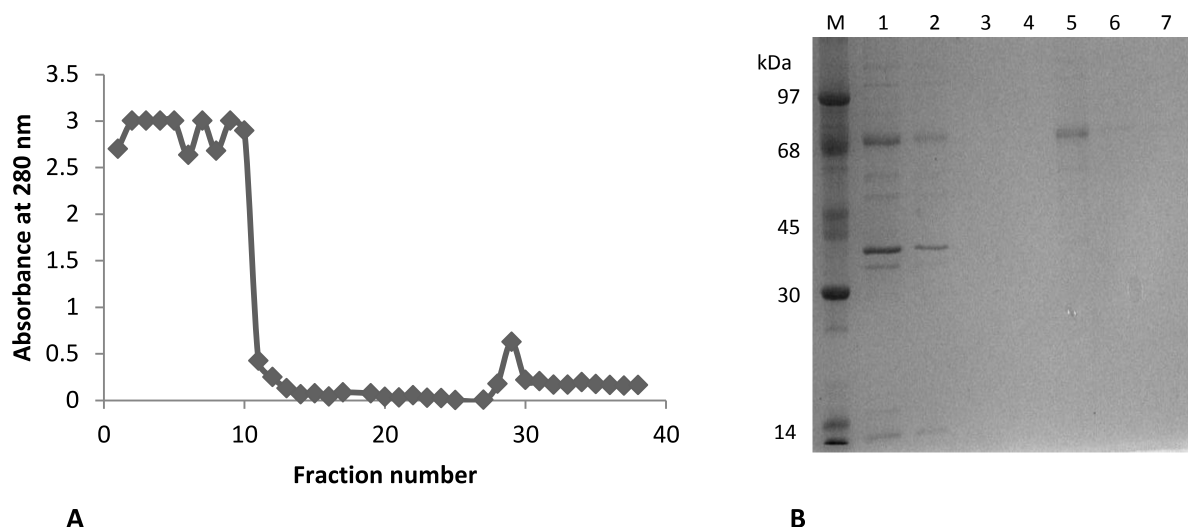


Fig. 2.20. Purification of cLiTat 1.3$_{His}$ on the His-select Nickel affinity column. Panel **A** shows the elution profile from the affinity column. Fractions collected were monitored by reading absorbances at 280 nm. Panel **B** shows Coomassie stained reducing 10% SDS-PAGE gel of the fractions collected. Lane M, molecular weight marker; lane 1, unbound fraction; lane 2, first column wash; lane 3, fifth column wash; lane 4, seventeenth column wash; lanes 5-7, eluted cLiTat 1.3 fractions.

The expression, solubilising and refolding as well as affinity purification of recombinant cLiTat 1.3$_{His}$ on a larger scale revealed the presence of contaminants in the elution fractions. These contaminants were more pronounced when all the elution fractions (15 ml) were pooled and concentrated down to 1 ml using ultrafiltration (Fig. 2.21, lane 2). Protein quantification of the concentrated protein revealed a surprisingly low concentration of 0.4 mg/ml of the cLiTat 1.3$_{His}$ protein. It was assumed that the protein may have eluted in the flow through while centrifuging the ultrafiltration device, but SDS-PAGE analysis of the filtrate did not reveal the presence of any protein. This loss, coupled with the degradation (precipitation) that appeared during storage at -20°C hindered further purification for possible crystallisation.

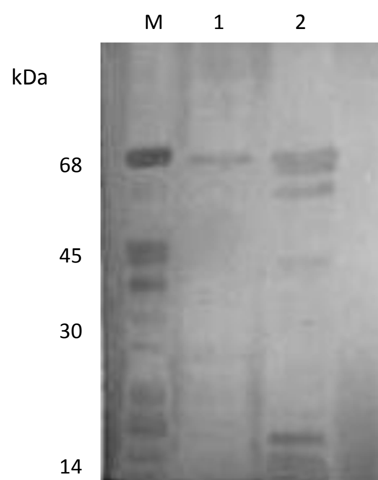


Fig. 2.21. Analysis of affinity purified and concentrated recombinant cLiTat 1.3$_{His}$ by 10% SDS-PAGE. Silver stained gel showing purified cLiTat 1.3$_{His}$ protein (10 μg/ml) before (lane 1) and after (lane 2) concentration by ultrafiltration.

2.3.8 Yeast expression and purification

Expression of the LiTat 1.5 11-11 SUMO construct and LiTat 1.3 A6-16-19 (3) SUMO construct in *P. pastoris* was carried out, and the samples collected over the five days of expression, showed the presence of the 66 kDa band of LiTat 1.3 A6-16-19 (3) SUMO which seemed to express as a doublet (arrow) during the first three days (Fig. 2.22, lanes 1-3), however, as expression progressed, the lower band disappeared (Fig. 2.22, lanes 4 and 5). The LiTat 1.5 11-11 SUMO in M5 construct did not express well, as the expected 68 kDa band was not observed throughout the five days of expression, but rather a 47.8 kDa band was more dominant (Fig. 2.22, lanes 6-10). This observation was consistent with that of the expression of the pGEX-4T-1-cLiTat 1.5 construct in *E. coli*, which expressed at an unexpected size of 28.8 kDa (Fig. 2.14 A, lane 5).

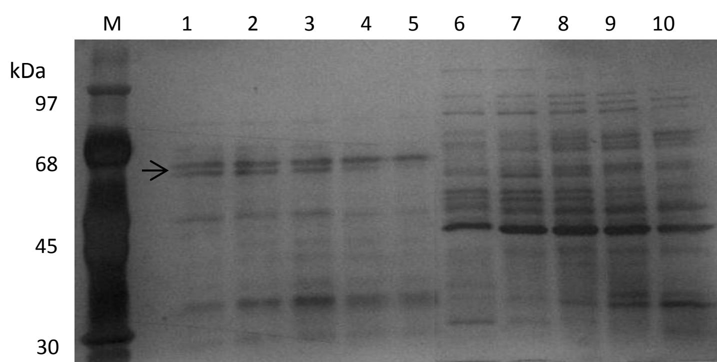


Fig. 2.22. Analysis by 10% SDS-PAGE of the expression of LiTat 1.3 A6-16-19 (3) SUMO and LiTat 1.5 11-11 SUMO in *Pichia pastoris*. Silver stained gel showing expression over a period of 5 days. Lane M, molecular weight marker; lanes (1-5), supernatant of LiTat 1.3 A6-16-19 (3) SUMO culture from day 1-5 expression (10 µl); lanes (6-10), supernatant of LiTat 1.5 11-11 SUMO culture from day 1-5 expression (10 µl). Arrow indicates LiTat 1.3 A6-16-19 (3) SUMO protein band.

Three phase partitioning was employed for purification, and it revealed that the 20% cut off ammonium sulfate precipitated the highest amount of LiTat 1.3 A6-16-19 (3) SUMO as the protein was most concentrated with fewer impurities (Fig. 2.23 A, lane 2), compared to the 10% fraction (Fig. 2.23 A, lane 1). The 30 and 40% ammonium sulfate fractions resulted in loss of some protein, however, most impurities were removed (Fig. 2.23 A, lanes 3 and 4). Probing with anti-His tag antibodies, the western blot confirmed that the protein was expressed and purified at 66 kDa in both the 10 and 20% TPP fractions (Fig. 2.23 B, lanes 1 and 2), but was faintly detected in the 30 and 40% fractions (Fig. 2.23 B, lanes 3 and 4). Three phase partitioning purification of LiTat 1.5 11-11 SUMO seemed to concentrate a 67.6 kDa protein at the 10% cut off ammonium sulfate (Fig. 2.23 A, lane 5), however, the western blot did not detect the His-tag in that and the other TPP fractions (Fig 2.23 B, lanes 5-8), suggesting that it may have been a *P. pastoris* protein expressed and concentrated at that size.

Following TPP, the LiTat 1.3 A6-16-19 (3) SUMO fusion protein was subjected to affinity purification using the Ni-chelate affinity column, and one of the eluted fractions revealed the purified 66 kDa protein (Fig. 2.24, lane 8), with some contaminants smaller than 35 kDa, that could be removed by molecular exclusion chromatography. Similar to the bacterial expression system, the yeast system gave a good yield of the LiTat 1.3 protein, however *P. pastoris* expression could not be further pursued due to the high cost of the zeocin antibiotic required.

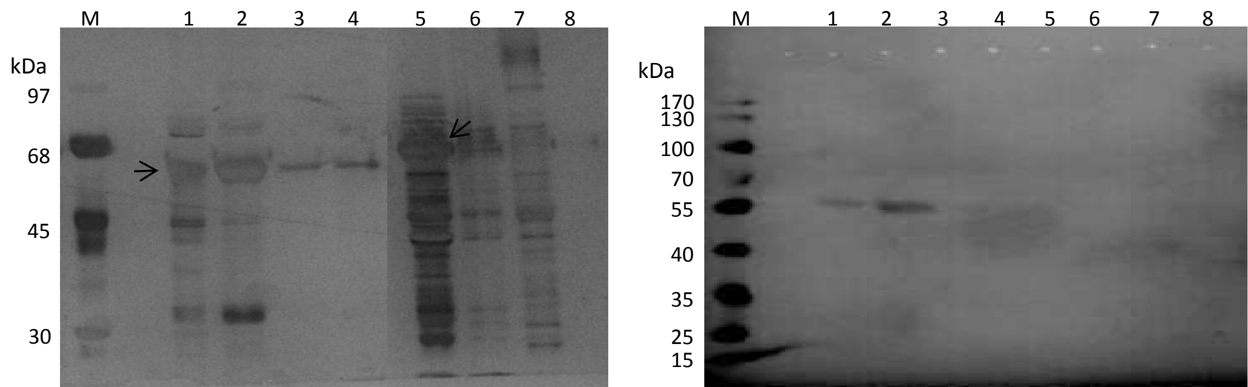


Fig. 2.23. Analysis by SDS-PAGE and western blot of the TPP fractions of LiTat 1.3 A6-16-19 (3) SUMO and LiTat 1.5 11-11 SUMO (A) Silver stained reducing 10% SDS-PAGE gel. **(B)** Western blot probed with anti-His tag antibody (1:1 000). Lane M, molecular weight markers; lanes 1-4, TPP with 10%; 20%; 30% and 40% ammonium sulfate cut for LiTat 1.3 A6-16-19 (3) SUMO purification. Lanes 5-8, TPP with 10%; 20%; 30%; and 40% ammonium sulfate cut for LiTat 1.5 11-11 SUMO purification. Arrow in lane 1 indicates LiTat 1.3 and arrow in lane 5 shows the suspected LiTat 1.5.

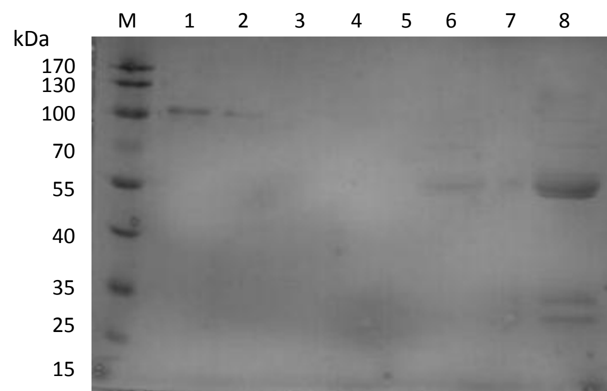


Fig. 2.24. Coomassie stained reducing 10% SDS-PAGE gel of the purification of LiTat 1.3. Lane M, molecular weight marker; lane 1, unbound fraction; lanes 2-5, column washes; lanes 6-8, eluted His tagged LiTat 1.3 A6-16-19 (3) SUMO fusion protein fractions (30 µg/ml).

2.4 Discussion

A number of serological tests used in the diagnosis of HAT are based on the VSGs of VATs LiTat 1.6, LiTat 1.5 and LiTat 1.3, obtained by culturing human infective *T. b. gambiense* bloodstream form clones, and extracting the VSGs (Büscher *et al.*, 1999; Van Nieuwenhove *et al.*, 2012a). This process is too risky for the staff handling the parasites, hence it would be worthwhile to find an alternative and safer method of acquiring these VSGs. The aim of this study therefore was to clone and recombinantly express the two VSGs LiTat 1.3 and LiTat 1.5 for possible use in HAT diagnosis.

The templates used for PCR were gDNA extracted from non-human infective *T. b. brucei* Lister 427 parasite strain which over the years has been subjected to in vitro culture (Brems *et al.*, 2005), as well as cDNA from collaborators in Belgium, synthesised from RNA extracted from the LiTat 1.3 and LiTat 1.5 trypanosome clone populations (Van Nieuwenhove *et al.*, 2012b). The PCR products amplified from either template were of the expected sizes: ~1.5 kb for both gLiTat 1.5 and cLiTat 1.5 while gLiTat 1.3 and cLiTat 1.3 were ~1.4 kb. Agarose gel analysis of both the cLiTat 1.3 and gLiTat 1.3 amplicons showed very faint band intensity, despite optimising both $MgCl_2$ concentration and annealing temperature, factors that affect product yield (Erlich *et al.*, 1991; Fermentas-handbook, 2010-2011). Despite the low yield, it was possible to progress to T-vector cloning, on the basis of the vector: insert ligation ratio (of 1:4) which allowed a high amount of insert DNA to be used, favouring ligation with vector molecule and increasing the number of possible recombinant plasmid molecules (Cranenburgh, 2004). The gLiTat 1.3 PCR product also had multiple bands present (non-specific products) which may have been a result of mispriming by one or both of the primers internal or external to the target protein (Don *et al.*, 1991). This could have been overcome by employing a different PCR method such as touchdown PCR, where the annealing temperature is gradually decreased during the first few cycles to ensure that priming occurs at sequences with perfect identity to the primers (Don *et al.*, 1991; Strachan and Read, 1999). Despite the presence of non-specific amplification bands, it was possible to continue with cloning by excising the band of interest.

Screening for recombinant T-vector clones following transformation of the ligation product of cleaned PCR products and pTZ57R/T-vector in *E.coli* JM109 cells, was initially done by blue vs. white screening. As stated by Padmanabhan *et al.* (2011), this procedure is based on discrimination i.e. visually identifying recombinants on the basis of colour, which may not be reliable in instances where the *lac*Z gene in the vector is

non-functional and not producing β-galactosidase. This results in the cells' inability to convert X-gal to the blue substance and the white colonies seen on the plate are false positives i.e. background vector. For more accurate confirmation of recombinant clones, colony PCR was employed (Azhahianambi *et al.*, 2008). It revealed the presence of both LiTat 1.3 and LiTat 1.5 inserts at the expected sizes for the positive colonies. The T-vector-gLiTat 1.3 positive colonies, similar to the initial amplification, showed the presence of multiple bands, further suggesting the possibility of mispriming. Restriction digestion of the recombinant clones with either EcoRI/NotI or SalI/NotI restriction enzyme combinations saw the release of the inserts at the expected sizes, further confirming recombinancy. Colony PCR Screening of the recombinant pGEX-4T-1 and pET-28a constructs using the vector primers resulted in a shift in size in the recombinant clones, as expected when using primers that flank the vector cloning sites i.e. a positive clone will be the size of insert plus the additional bases flanking the two primers on the plasmid (Zhou and Gomez-Sanchez, 2000).

The introduction of different restriction sites in the PCR primers ensures that the PCR products inserted into the vectors are in one orientation to ensure directional cloning (Scharf *et al.*, 1986; Gál *et al.*, 1999). Correct orientation is further confirmed by DNA sequencing, after which, an alignment is carried out with the published sequences present in the databases, for pairwise determination of similarity or identity between either DNA or protein sequences (Campanella *et al.*, 2003). The protein alignment of each of the cLiTat 1.3, cLiTat 1.5 or gLiTat 1.5 sequenced constructs, with their respective sequences in the NCBI database, revealed sequence identity and similarity ranging between 50-65% ensuring that the correct protein will be expressed.

Analysis of the T-vector-gLiTat 1.3 and pGEX4T-1-gLiTat 1.3 constructs on the other hand only revealed sequence identity and similarity of less than 5% and also present were many deletions and mutations. Repeated cloning and sequencing of the gLiTat 1.3 constructs did not result in any improvements, as the deletions and mutations were still present. The cause of this is unclear, and may be due to a number of factors. It may be due to the choice of template DNA used i.e. genomic DNA. Perhaps the region amplified for the LiTat 1.3 gene is part of the 66% of the VSG genes that are pseudogenes discussed in chapter 1, which contain frame shifts or in frame stop codons (Barry *et al.*, 2005; Weirather *et al.*, 2012). To corroborate this, however, an alignment between the *T. b. brucei* LiTat 1.3 gene (once the sequence has been identified) and the *T. b. gambiense* LiTat 1.3 gene would have to be done. The BLAST method as described by Weirather *et al.* (2012) would be ideal, as it performs similarity

mapping and the ability to identify any discontinuous regions of identity thus describing the relationship between genes. This might provide answers as to whether the sequencing results are due to the differences in the genes or are perhaps PCR errors. The PCR errors could have emanated from sample handling following initial PCR amplification where the PCR product was purified by excising it from the gel. The short wavelength UV light source (260 nm) used in this study is strongly absorbed by DNA and long exposure to it damages DNA, subsequently affecting cloning efficiency (Burton and Kaguni, 1997). In avoiding the excision of the non-specific bands present, the ethidium bromide stained gLiTat 1.3 PCR product may have been exposed to the UV light for too long, hence damaging the DNA. It is also possible that the PCR product itself was accidentally nicked.

While the sequencing of the LiTat 1.5 constructs confirmed successful orientation and cloning, recombinant protein expression was the opposite as the LiTat 1.5_{GST} protein was expressed at the unexpected size of 32 kDa instead of 96 kDa. It was initially assumed that the expression of the truncated protein was due to codon usage i.e. translation being limited due to the demand for rare codons lacking in the *E. coli* expression host (Brinkmann *et al.*, 1989). This led to expression being carried out in the *E. coli* Rosetta 2 DE3 strain, which contains a chloramphenicol-resistant pRARE plasmid which encodes tRNA genes for the rarely used codons, providing for universal translation (Novagen, 2003). This, however, did not remedy the problem, as the LiTat 1.5 protein was still expressed at 32 kDa. Interestingly, this anomaly was also observed in *P. pastoris* expression of the LiTat 1.5 11-11 SUMO construct, where the expected 68 kDa protein was not observed. In this instance, it was assumed that the expression levels may have been low, hence three phase partitioning was employed, to both concentrate and subsequently purify the protein (Dennison and Lovrien, 1997). This however did not yield the desired results, as the protein of the correct size could still not be isolated and its absence confirmed by the western blot probed with anti-His tag antibodies. These observations may suggest that recombinantly expressed LiTat 1.5 protein is unstable during recombinant expression in bacterial and yeast expression systems, and may require a different type of expression system.

Evidence of bacterial recombinant expression of VSGs has been reported only for VSG RhoTat 1.2 of *T. evansi* (Sengupta *et al.*, 2012). In their report, induction with 1mM IPTG resulted in a good yield of the expressed protein, and the same was observed in this study as the pGEX-4T-1-cLiTat 1.3 construct yielded the cLiTat 1.3_{GST} protein, which was confirmed by the western blot probed with anti-GST tag antibodies. While

the cLiTat 1.3$_{GST}$ protein was expressed at the expected size at both 37°C and 30°C, the protein accumulated as insoluble aggregates i.e. inclusion bodies (San-Miguel *et al.*, 2013). Expression at the lower temperature of 16°C resulted in the soluble cell fraction (supernatant) containing protein, though very little, as compared to the insoluble cell fraction (pellet). This corresponded with the findings by Vera *et al.* (2007) who showed how sub-optimal growth temperatures have a positive influence on solubility. This solubility was observed with the pET-28a system, despite lacking the factors present in other pET vectors that enhance solubility such as provision for fusion to a highly soluble polypeptide e.g. thioredoxin or provision of a signal sequence for translocation into the periplasmic space (Novagen, 2003). Looking at the expression hosts used, the expression patterns were similar throughout. The *lon* and *omp* T protease deficient *E. coli* host strain BL 21 (DE3) (Berrow *et al.*, 2006) did not show any effect on solubility, similar to the Rosetta 2 DE3 derivative - despite previous evidence that showed that the use of codon enhanced strains (Rosetta) contributes positively to enhancing solubility (Berrow *et al.*, 2006).

Despite attaining soluble cLiTat 1.3$_{His}$ protein at 16°C, its low yield led to the route of solubilising and refolding the inclusion bodies for a higher protein yield. Complete cell lysis by sonication and lysozyme treatment was important to ensure release of inclusion bodies as well as prevent contamination of the preparation by intact cells, which sediment together with inclusion bodies (Lilie *et al.*, 1998). Following the washing of the pellet, the 6 M guanidine-HCl denaturant was added to solubilise the protein. The gradual removal of the denaturant by dialysis ensured proper refolding and the presence of additives such as L-arginine in the refolding equilibration buffer helped reduce protein aggregation, as it is an aggregation suppressor (Tsumoto *et al.*, 2004). Glycerol, another component present in the buffers, maintained the stability of the conformation of the protein, as it enhances hydrophobic interactions 'as a result of an increase in the solvent ordering around proteins' (Alibolandi and Mirzahoseini, 2011).

The renatured cLiTat 1.3$_{His}$ was purified to homogeneity using Ni-NTA affinity chromatography. On a larger scale, however, purity of the eluted protein was compromised, due to the presence of some contaminant proteins in the concentrated elution fractions that may have contained multiple histidine residues which allowed them to bind to the Ni column. This warranted further purification for their removal (Gräslund *et al.*, 2008). Further purification was however not possible, as the protein seemed to have 'disappeared' following ultrafiltration concentration. Retention of proteins in the ultrafiltration membranes depends on the molecular weight of the protein

and for high retention; the MWCO should be three to six times smaller than the molecular weight of the protein being retained (Pall-Corporation, 2013). In this work, the 10 MWCO used which is six times smaller than the 68.8 kDa cLiTat 1.3_{His} protein was appropriate for concentration and would not allow the loss of the protein in the filtrate. This suggests that the 'loss' of the protein may have been due to strong adsorption on the membrane such that the gentle rinsing of the membrane with PBS buffer after centrifugation, may have been inadequate for recovering the protein. Perhaps allowing the column to stand in PBS for a length of time (e.g. 1 h) may have improved recovery, as the protein would fully desorb from the membrane.

While *E. coli* is the preferred host system for recombinant protein expression, yeasts as hosts combine the advantages of prokaryotic and eukaryotic expression systems (Boettner *et al.*, 2002). The greatest advantages of *P. pastoris* expression are the ability to produce high levels of soluble protein as well as performing post translational modifications e.g. glycosylation and disulfide bond formation (Sudbery, 1996). Unlike the expression of LiTat 1.5 11-11 SUMO construct which did not yield protein, expression of LiTat 1.3 A6-16-19 (3) SUMO fusion protein over the five days gave the desired results, as seen by the expression of the 66 kDa protein. Concentration and purification by TPP revealed the 20% ammonium sulfate cut to be optimal, as most of the contaminants were removed and the LiTat 1.3 protein concentrated. Further purification on the Ni-NTA column revealed that the His-tagged protein bound well and eluted with few contaminants which could have been removed by molecular exclusion chromatography.

The study therefore showed the successful cloning of VSGs LiTat 1.3 and LiTat 1.5 from cDNA and LiTat 1.5 from gDNA as well as the successful expression of LiTat 1.3_{His} in both the *E. coli* bacterial expression system and *Pichia pastoris* yeast expression, giving a high yield of protein. Expression of VSG LiTat 1.5 in either expression system did not yield the desired results, hence was not successful. With the lack of purified recombinant VSGs LiTat 1.3 and LiTat 1.5, these proteins could not be used for crystallisation studies or antibody production for immunofluorescence studies presented in the next chapter.

CHAPTER 3:

PRODUCTION OF ANTI-VSG LITAT 1.3, ANTI-VSG LITAT 1.5 AND ANTI-VSG RHOTAT 1.2 ANTIBODIES FOR IMMUNOCYTOCHEMISTRY

3.1 Introduction

Human African trypanosomiasis caused by *T. b. gambiense and T. b. rhodesiense* is transmitted to humans through the bite of an infected tsetse fly (Brun *et al.*, 2009; Kristensson *et al.*, 2010). The disease is known to progress within two stages. Firstly, the haemolymphatic phase, where the trypanosomes multiply in the blood, lymph and subcutaneous tissues and secondly, the meningoencephalitic phase, where the trypanosomes cross the blood brain barrier invading the central nervous system (Geiger *et al.*, 2011). Accurate diagnosis is of great importance prior to administering treatment and follows a three-step pathway: serological screening for potential infection, confirming the presence of parasites in blood samples using microscopy and examining the cerebrospinal fluid following a lumbar puncture to identify the stage of the disease (Koffi *et al.*, 2006).

The serological tests used in identifying infected individuals are based either on antibody detection (Ab-detection) or antigen detection (Ag-detection) (Davison *et al.*, 1999). More commonly used in the field are the Ab-detection tests, which consist of a lyophilised suspension of fixed and stained bloodstream form trypanosomes of different VATs, which react with the specific antibodies present in the blood, plasma or serum of infected individuals. The tests include Latex/*T. b. gambiense* (VATs LiTat 1.3, LiTat 1.5 and LiTat 1.6) and CATT/*T. b. gambiense* (VAT LiTat 1.3) (Büscher *et al.*, 1999). Efforts to improve specificity and heat stability of these tests were recently made through the development of a dipstick and a lateral flow device for *T. b. gambiense* infection, namely the HAT Sero-Strip and HAT Sero-K-SeT respectively which detect antibodies against VSGs of VAT LiTat 1.3 and LiTat 1.5 (Büscher *et al.*, 2013). These tests have been reported to be under field evaluation in the Democratic Republic of Congo (Tiberti *et al.*, 2013). Such lateral flow tests also have the advantage of detecting very little (nanogram) amounts of antibody or antigens in blood samples, which is convenient for *T. b. gambiense* infections known to have low parasitaemia (Sullivan *et al.*, 2013).

While antibody detection tests have been extensively used as first line screening tests, they are unable to distinguish between active and cured forms of HAT, as antibodies

still remain in the bloodstream even after a person has been cured. Based on this, Ag-detection tests are deemed more desirable as they can discriminate between current and past infections (Hutchinson *et al.*, 2004; Eyford *et al.*, 2013). One approach that has been used to identify targets/markers in the Ag-detection tests is the analysis of the parasite proteome and sub-proteomes, which provide an understanding of the interaction between the host and pathogens as well as identifying the differentially expressed trypanosome proteins (Holzmuller *et al.*, 2010; Tiberti *et al.*, 2013). The focus however has only been on *T. b. brucei* and more investigations need to be done for the *T. b. gambiense* and *T. b. rhodesiense* sub-species (Tiberti *et al.*, 2013). Antibodies on the other hand, have been widely used in Ag-detection tests, identifying trypanosomal proteins released into the circulation following lysis by the host immune system as the parasites undergo antigenic variation (Manful *et al.*, 2010). Examples of such tests include the card indirect agglutination trypanosomiasis test (TrypTect CIATT) which consists of latex particles coated with a monoclonal antibody specific to a trypanosome antigen which detects circulating antigens from both *T. b. gambiense* and *T. b. rhodesiense* infection (Nantulya, 1997), as well as the Ag-ELISA (Enzyme-linked immunosorbent assay) for *T. evansi* infection which causes surra in camels and buffaloes (Davison *et al.*, 1999).

The antibodies used in Ag-detection tests can be easily produced by immunising animals such as chickens with the target antigen/protein, after which the antibodies produced will be transferred to the egg yolk and easily isolated by polyethylene glycol (PEG) precipitation (Patterson *et al.*, 1962; Polson *et al.*, 1985). This relatively cheap and safe mode of antibody production makes it attractive to pursue antigen based tests such as Ag-ELISA using antibodies against the *T. b. gambiense* VSGs of VATs LiTat 1.3 and LiTat 1.5 for the serological screening of infected individuals. This might also eliminate the risk of false negatives observed in the Ab-detection tests (e.g. CATT/*T. b. gambiense*), which result from the absence of antibodies during early weeks of infection.

In this study antibodies against the native VSGs LiTat 1.3 and LiTat 1.5 were raised in chickens, isolated and characterised. Antibody production was evaluated by ELISA and antibody specificity was confirmed by dot and western blots. The antibodies were used in the immunofluorescence labeling of *T. b. brucei* parasites. The same was done with purified recombinant VSG RhoTat 1.2, a VSG expressed in *Trypanosoma evansi*.

3.2 Materials and methods

3.2.1 Materials

Antibodies: The proteins used for antibody production: *T. b. gambiense* native VSG T. b. g LiTat 1.3-conA Piek 2-VSG-2mg (nLiTat 1.3), *T. b. gambiense* native VSG LiTat 1.5 VSG-1mg-OT04E1B2 (nLiTat 1.5) and recombinant *T. evansi* RhoTat 1.2 (rRhoTat 1.2) were a gift from Prof Philippe Büscher, Unit of Parasite Diagnostics, Institute of Tropical Medicine, Antwerp, Belgium. Freund's complete and incomplete adjuvants were purchased from Sigma (Munich, Germany). Rabbit anti-chicken HRPO and - FITC conjugated donkey anti-IgY were purchased from Jackson Immunoresearch (PA, USA). The Nunc Maxisorb™ 96 well plates were purchased from Nunc (Intermed, Denmark). PEG (M_r 6000) was purchased from Merk (Hohenbrunn, Germany).

3.2.2 Immunisation of chickens

All immunisations were administered in complete or incomplete Freund's adjuvant. Freund's adjuvant consists of mineral oil, either containing heat killed mycobacteria (complete) or lacking the mycobacteria component (incomplete) (Billiau and Matthys, 2001). Once mixed with the suspension of antigen, a stable water-in-oil emulsion is formed and the adjuvant serves to generate a depot that traps antigens at the site of injection, allowing a slow release of antigen, for continual stimulation of the immune system (Leroux-Roels, 2010). Two chickens each received 1 ml antigen solution mixed in adjuvant [1:1 (v/v) ratio], so that each chicken received 50 µg of antigen per immunisation. The antigens used were VSG proteins nLiTat 1.3, nLiTat 1.5 or rRhoTat 1.2. Injections were made intramuscularly into each breast and the first immunisation (week 0) was in Freund's complete adjuvant, while the subsequent immunisations (weeks 2, 4, 6, 8) were in Freund's incomplete adjuvant at the same dose. Eggs were collected each day and stored at 4°C.

3.2.3 Isolation of IgY

All IgY isolations were performed by PEG precipitation (Polson *et al.*, 1985). PEG is a water-soluble synthetic polymer, which acts as a fractional precipitating agent in protein purification. Precipitation is achieved by the steric exclusion of the proteins, from

regions of the solvent occupied by PEG, which concentrates them until they exceed their solubility limit resulting in precipitation (Atha and Ingham, 1981).

Briefly, egg yolks were separated from the egg white and washed gently under running water to remove albumin. The yolk sack was punctured and the yolk volume determined in a measuring cylinder. Two volumes of 100 mM Na-phosphate buffer [100 mM Na-phosphate, 0.02% (w/v) NaN_3, pH 7.6] were added and mixed in thoroughly before dissolving solid PEG 3.5% (w/v) by gentle stirring. The precipitated vitellin fraction was removed by centrifugation (4 420 *g*, 30 min, RT), and the supernatant was filtered through absorbent cotton wool to remove the lipid fraction. The PEG concentration was increased to 12% (w/v) by adding 8.5% (w/v) PEG, mixed and centrifuged (12 000 *g*, 10 min, RT). The supernatant was removed and the resultant pellet was dissolved in 100 mM Na-phosphate buffer in a volume equal to that obtained after filtration. The final concentration of PEG was brought to 12% (w/v), stirred thoroughly and centrifuged (12 000 *g*, 10 min, RT). The supernatant was discarded and the antibody pellet dissolved in 1/6 of the original egg yolk volume using 100 mM Na-phosphate buffer, 0.1% (w/v) NaN_3, pH 7.6. The IgY concentration was determined by reading the A_{280} absorbance of a 1 in 50 dilution of IgY in 100 mM Na-phosphate buffer and calculating the concentration of the undiluted solution. The extinction coefficient of IgY is $E^{1\ mg/ml}_{280\ nm} = 1.25$ (Goldring and Coetzer, 2003).

3.2.4 Enzyme Linked Immunosorbent Assay (ELISA)

To evaluate antibody production following immunisation, ELISAs were conducted. The ELISA technique provides qualitative information about antibody specificity. The mechanism involves the adsorption of the antigen to a surface, followed by the application of the IgY antibody which binds to the antigen. An enzyme labelled detection antibody which binds to the IgY is then added, and acts on a chromogenic substrate, producing a color reaction (Crowther, 1995). Briefly, ELISA plates were coated with 1 µg/ml antigen in PBS (150 µl per well) overnight at 4°C. The coating antigen was removed and wells were blocked for non-specific binding with 0.5% (w/v) BSA-PBS (200 µl per well) for 1 h at 37°C. After blocking the wells were washed two times with 0.1% (v/v) Tween-20 in PBS (PBS-Tween). Primary IgY antibodies isolated from eggs collected during immunisation were used at 50 µg/ml diluted in 0.5% (w/v) BSA-PBS (100 µl per well) for 2 h at 37°C. Wells were washed three times with PBS-Tween. Secondary antibody, rabbit anti-chicken HRPO diluted (1:15 000) in 0.5% (w/v) BSA-PBS (100 µl per well) was added and incubated for 1 h at 37°C. Wells were

washed three times with 0.1% (v/v) PBS-Tween followed by the addition of substrate solution [0.05% (w/v) ABTS, 0.015% (v/v) H_2O_2 in 0.15 M citrate phosphate buffer, pH 5] (145 µl per well). Plates were left in the dark and readings at 405 nm were measured in the FLUOStar Optima microplate reader (BMG Labtech,Offenburg, Germany), every 15 min until an A_{405} of 1 was reached. For titration ELISAs, serial 1/10 dilutions of the primary antibody were prepared on the plate with a starting concentration of 100 µg/ml of antibody.

3.2.5 Antibody characterisation

To determine the optimum antibody concentration for use in western blots, a dot blot was performed. The dot blot technique is similar to the western blot, the only difference being that the proteins detected are not first separated by electrophoresis, but rather applied directly to the nitrocellulose membrane as a dot. Briefly, on nitrocellulose strips, grids were drawn in pencil. Respective antigen solutions prepared at three different concentrations (100 µg/ml, 50 µg/ml and 10 µg/ml) were applied to the smallest possible dot to the nitrocellulose and allowed to dry. Unoccupied sites on the membrane were blocked with Elite non-fat milk [5% (w/v) in Tris-buffered saline (TBS) (20 mM Tris-HCl buffer, 200 mM NaCl pH 7.4)] for 30 min at room temperature. Each strip was then washed with TBS (3 x 2 min) and incubated in the appropriate primary antibody prepared at three different concentrations (100 µg/ml, 50 µg/ml and 10 µg/ml) diluted in 0.5% (w/v) BSA-TBS for 2 h at room temperature. The strips were washed in TBS (3 x 2 min) and incubated in secondary HRPO-conjugated rabbit anti-chicken IgY or goat anti-mouse antibody diluted in 0.5% (w/v) BSA-TBS (1:1 000 dilution) for 1 h at room temperature. The nitrocellulose strips were washed with TBS (3 x 2 min) before incubation in the chromogen-substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2, 0.1% (v/v) methanol, in TBS] until blue/purple dots were observed. The controls used were omission of primary or secondary antibody and use of pre-immune chicken IgY. Western blots were performed as previously described in section 2.2.9, using the optimised antibody concentrations.

3.2.6 Fluorescence immunolabelling of VSGs LiTat 1.3, LiTat 1.5 and RhoTat 1.2

Bloodstream form *T. b. brucei* Lister 427 strain trypanosome parasites were cultured as described in section 2.2.2. The parasites were harvested by centrifugation (2 000 *g*, 5

min) and the medium discarded before washing two times with ice-cold PBS (2 000 *g*, 5 min). The resulting pellet was resuspended in PBS and 1 x 10^6 cells were placed on slides coated with 0.01% (w/v) poly-L-lysine, which facilitates the attachment of cells to surfaces (Mazier *et al.*, 1975). After the slides had dried, the adhered cells were fixed with ice-cold 50% (v/v) methanol-PBS and left to dry prior to incubation overnight at 4°C. The slides were then washed with cold PBS (3 x 5 min), permeabilised with 0.5% (v/v) Triton X-100-PBS for 2 min and washed again with ice-cold PBS (3 x 5 min). To prevent non-specific binding, the slides were blocked with 2% (w/v) BSA-PBS for 45 min and the blocking agent washed off with 0.2% (v/v) Triton X-100-PBS (3 x 5 min). Prior to probing with the primary antibody, the IgY antibody concentration was first optimised, probing with 10, 7 and 5 µg/ml of the appropriate antibody. The 5 µg/ml concentration produced a better signal.

The cells were then probed with 5 µg/ml of pre-immune or appropriate primary antibody diluted in 0.2% BSA-PBS for 2 h at room temperature. The slides were washed with 0.2% (v/v) Triton X-100-PBS (4 x 5 min) and incubated with an FITC conjugated donkey anti-IgY secondary antibody (1:200 dilution in 0.2% BSA-PBS) for 1 h in the dark. From this point all remaining procedures were carried out in the dark to prevent bleaching of the fluorescence. The slides were washed thoroughly with 0.2% (v/v) Triton X-100-PBS (6 x 3 min) to help minimize any background staining, and the nuclei were stained by incubation in Hoechst stain (2 µg/ml) for 5 min. Slides were washed in PBS (5 min) prior to mounting in moviol and viewed using the Zeiss LSM 710 confocal microscope.

3.3 Results

3.3.1 Antibody production and ELISA assays of immune response

Following immunisations, eggs were collected each day from chickens over the 12 week period. Single eggs per week from each chicken had IgY isolated from them to assess antibody production by ELISA. Using the optimum antigen coating and antibody concentration determined by checkerboard ELISA (results not shown), the ELISA plates were coated with 1 µg/ml of antigen and probed with 50 µg/ml of primary antibody.

Chicken nLiTat1.3-1 immunised with nLiTat 1.3 showed a good immune response with peaks at weeks 4, 6 and 11 and week 4 having the highest A_{405nm} of ~1 (Fig. 3.1). Antibody production in chicken nLiTat1.3-2 on the other hand seemed to peak only at

week 2 and remained at a constant level from weeks 4 to 12 with an A_{405nm} ranging between 0.6–0.8 (Fig. 3.1). Chickens nLiTat1.5-1 and nLiTat1.5-2 immunised with nLiTat 1.5 showed a comparable trend in antibody production with A_{405nm} absorbances falling between a narrow range of 0.5–0.7 throughout the 12 week period (Fig. 3.2). Anti-rRhoTat 1.2 antibodies from both rRhoTat 1.2-1 and rRhoTat 1.2-2 chickens peaked at week 2 and also showed uniform antibody production from weeks 7-12 (Fig. 3.3).

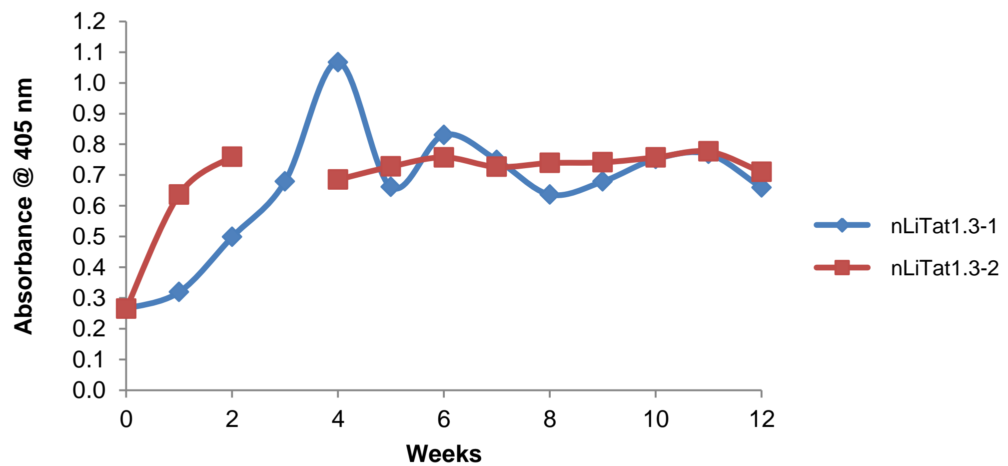


Fig. 3.1. ELISA showing antibody production from chickens immunised with nLiTat 1.3. ELISA plates were coated with 1 µg/ml nLiTat 1.3 and probed with IgY isolated from chickens nLiTat1.3-1 and nLiTat1.3-2 from weeks 0 to 12. Values at 405 nm are the average of duplicate wells. Gap indicates no egg production during week 3 from chicken nLiTat1.3-2.

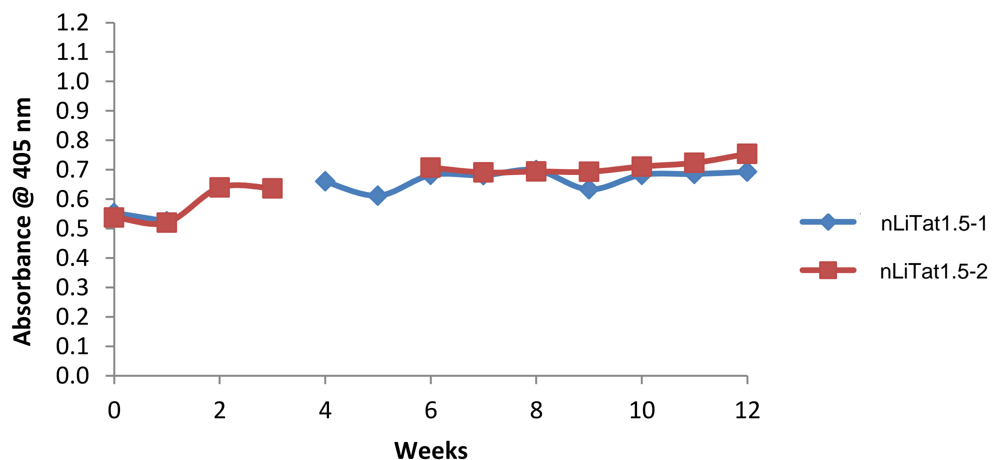


Fig. 3.2. ELISA showing antibody production from chickens immunised with nLiTat 1.5. ELISA plates were coated with 1 µg/ml nLiTat 1.5 and probed with IgY isolated from chickens nLiTat1.5-1 and nLiTat1.5-2 from weeks 0 to 12. Values at 405 nm are the average of duplicate wells. Gap indicates no egg production during weeks 2 and 3 from chicken nLiTat1.5-1, as well as weeks 4 and 5 from chicken nLiTat1.5-2.

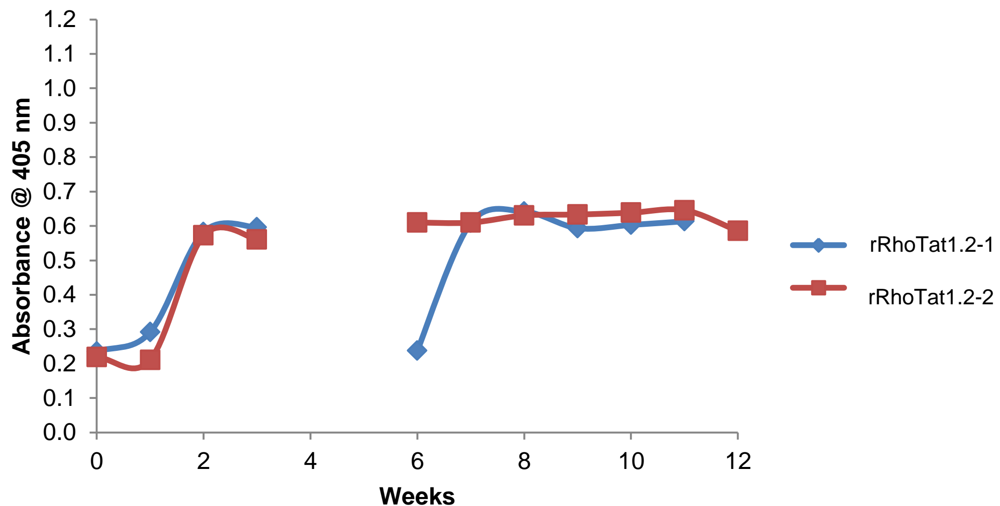


Fig. 3.3. ELISA showing antibody production from chickens immunised with rRhoTat 1.2. ELISA plates were coated with 1 µg/ml rRhoTat 1.2 and probed with IgY isolated from chickens rRhoTat 1.2-1 and rRhoTat 1.2-2 from weeks 0 to 12. Values at 405 nm are the average of duplicate wells. Gap indicates no egg production during weeks 4, 5 and 11 from chicken rRhoTat 1.2-1, as well as weeks 4 and 5 from chicken rRhoTat 1.2-2.

The IgY from weeks showing similar levels of antibody production were pooled to measure antibody titres, expressed as the greatest dilution that still gives an A_{405} value above that of corresponding non-immune control. Despite the generally low A_{405} readings, the nLiTat1.3-1 week 6 and nLiTat1.3-2 weeks 7-12 pools showed higher antibody titers as compared to the rest of the pools (Fig. 3.4 A) and a closer analysis at the higher IgY dilutions, the two pools still peaked, with an A_{405} of ~0.5 nm at concentrations as low as 3 µg/ml (Fig. 3.4 B). The anti-rRhoTat 1.2 IgY pools from both chickens rRhoTat1.2-1 and rRhoTat1.2-2 did not clearly show which of them had the best titer, as they more or less had the same A_{405} of ~0.45 nm across the IgY dilutions (Fig. 3.5 A and B) but substantially higher than that of the non-immune samples.

Due to the limited amount of antigen, the titration ELISA for anti-nLiTat 1.5 IgY was only performed on the week 12 pools from both chickens nLiTat1.5-1 and nLiTat1.5-2. The two both showed higher titers than those with non-immune control antibodies, the highest being nLiTat1.5-1 week 12 pool, with an A_{405} of ~0.6 nm (Fig. 3.6 A). At higher IgY dilutions the nLiTat1.5-1 week 12 pool titer remained high, and similar to nLiTat1.3-1 week 6 and nLiTat1.3-2 weeks 7-12 pools, the A_{405} peaked at concentrations as low as 3 µg/ml (Fig. 3.6 B). In all the assays, the pre-immune control remained low across the antibody dilutions, as expected.

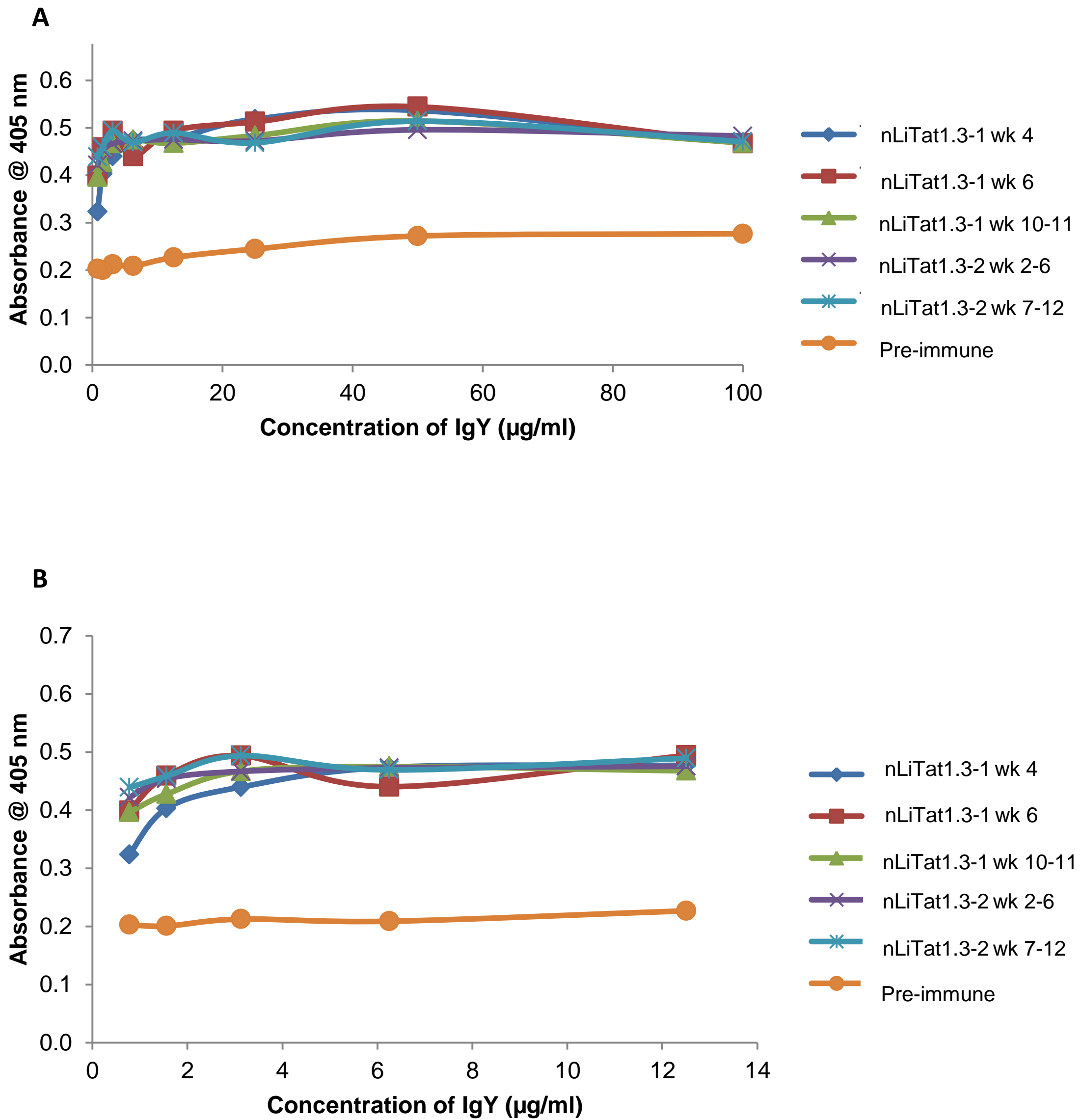


Fig. 3.4. ELISAs showing titration of antibodies from chickens immunised with nLiTat 1.3. ELISA plates were coated with 1 µg/ml nLiTat 1.3 and probed with IgY pools from weeks 4, 6, 10-11 from chicken nLiTat 1.3-1 and weeks 2-6, 7-12 from chicken nLiTat 1.3-2. **A)** plot of whole IgY dilution range (0.87-100 µg/ml) and **B)** plot of higher IgY dilution range (0.87-12.5 µg/ml). Values at 405 nm are the average of duplicate wells. Pre-immune served as a control.

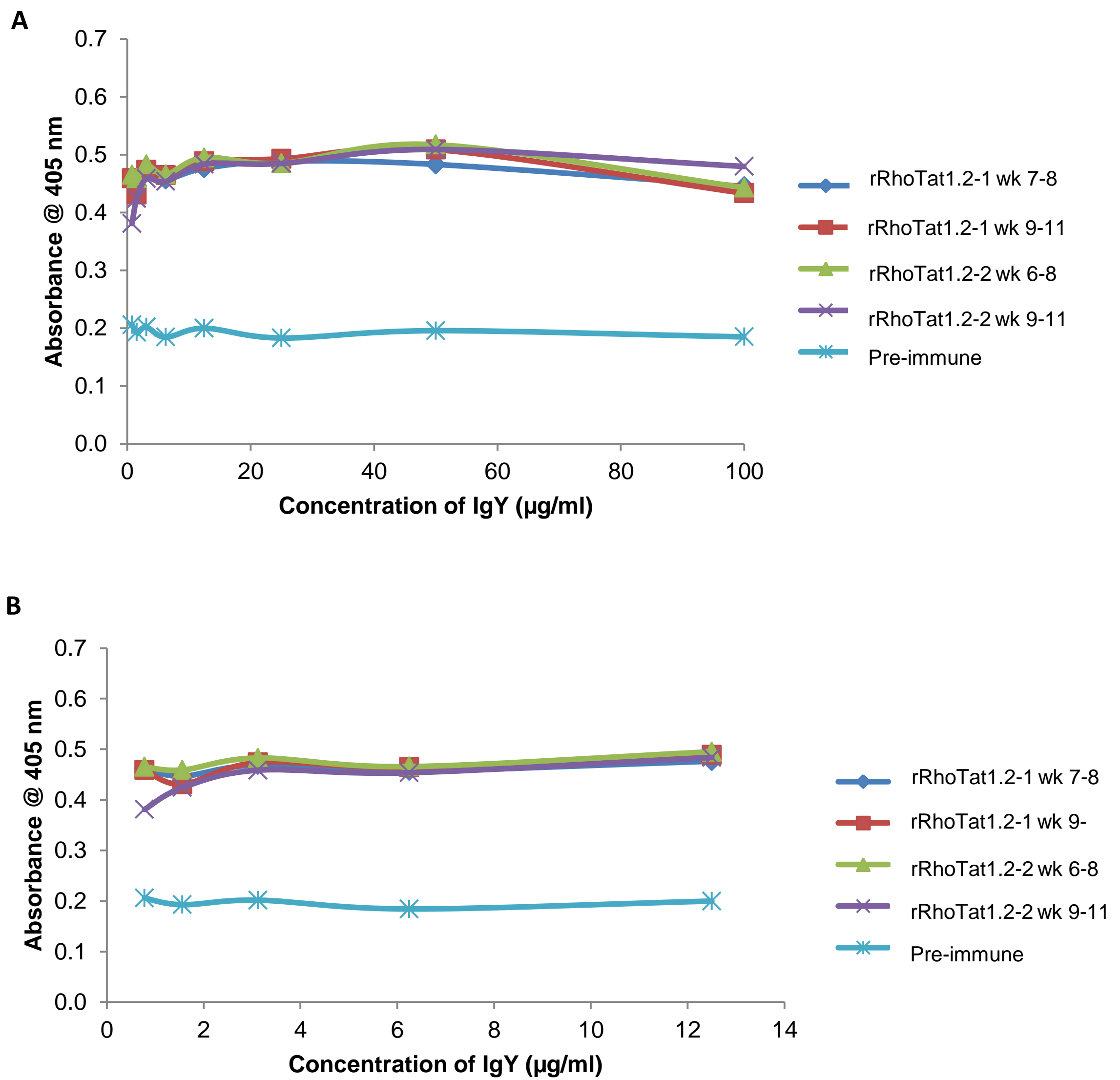


Fig. 3.5. ELISAs showing titration of antibodies from chickens immunised with rRhoTat 1.2. ELISA plates were coated with 1 µg/ml rRhoTat 1.2 and probed with IgY pools from weeks 7-8, 9-11 from chicken rRhoTat1.2-1 and weeks 6-8, 9-11 from chicken rRhoTat1.2-2. **A)** plot of whole IgY dilution range (0.87-100 µg/ml) and **B)** plot of higher IgY dilution range (0.87-12.5 µg/ml). Values at 405 nm are the average of duplicate wells. Pre-immune served as a control.

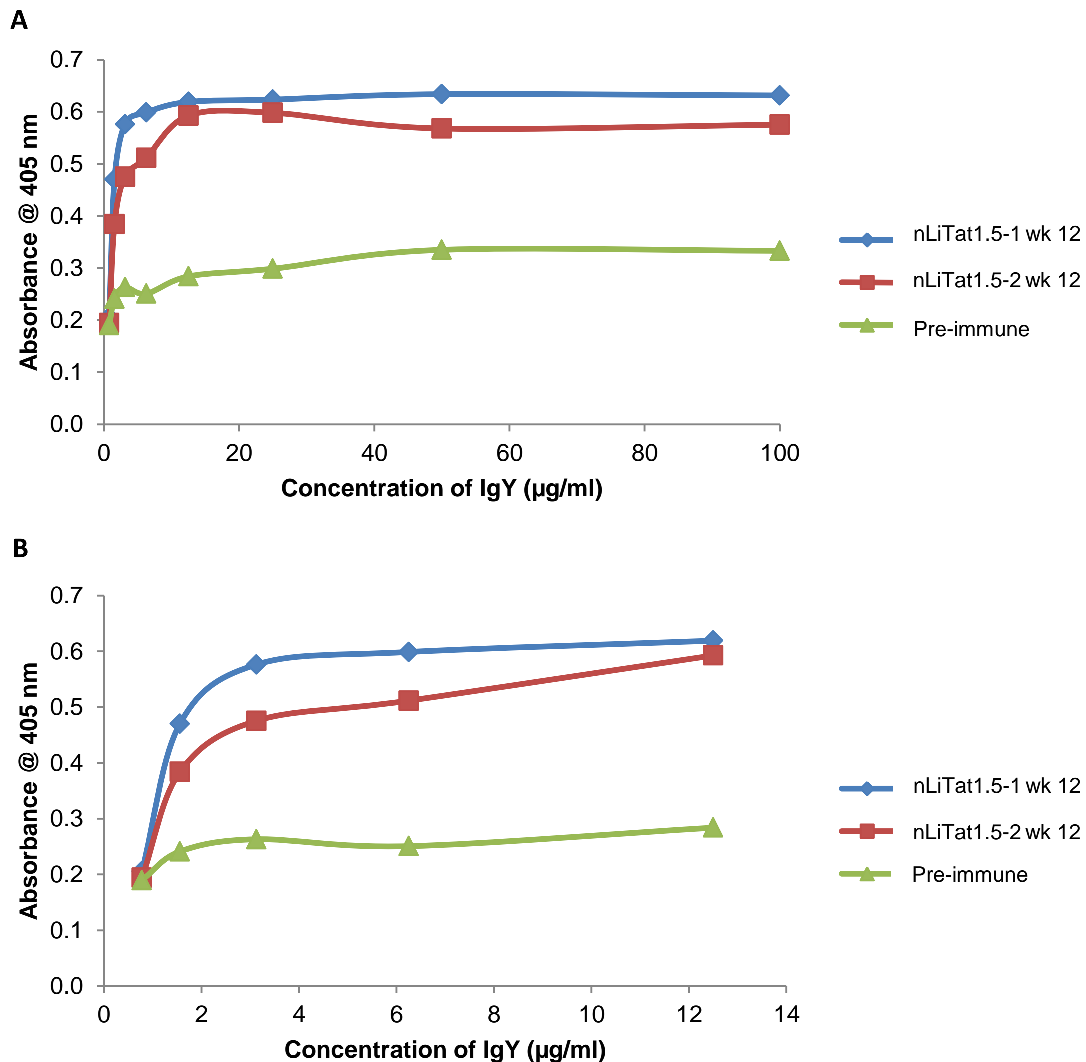


Fig. 3.6. ELISAs showing titration of antibodies from chickens immunised with nLiTat 1.5. ELISA plates were coated with 1 µg/ml nLiTat 1.5 and probed with IgY pools from week 12 from both chicken nLiTat 1.5-1 and nLiTat 1.5-2. **A)** plot of whole IgY dilution range (0.87-100 µg/ml) and **B)** plot of higher IgY dilution range (0.87-12.5 µg/ml). Values at 405 nm are the average of duplicate wells. Pre-immune served as a control.

3.3.2 Antibody characterisation by western blot

Using the antibody pools that gave the best titres in ELISA, the antibodies were characterised by western blot. A dot blot was performed first, to optimise the best antibody concentration for use in the western blot detection. A uniform trend was observed in the antibody detection of both nLiTat 1.5 and rRhoTat 1.2, where there was less detection at 100 µg/ml and 50 µg/ml of primary antibody and notably greater detection at the lower concentration of 10 µg/ml (Fig. 3.7), showing a “hook” or prozone effect. The intensity of the dots also appeared to be similar at each antigen concentration. The anti-nLiTat 1.3 antibodies exhibited a different pattern, as they gave a strong signal at all three concentrations, with an increase in intensity at 50 and 10

µg/ml (Fig. 3.7). Ten µg/ml was therefore the best for use in western blot analysis, and this corresponds with the titration ELISA findings where higher IgY dilutions (lower concentrations) were able to give a high/good signal. The controls i.e. omission of primary antibody (first grid), omission of secondary antibody (second grid) and pre-immune IgY (third grid) of each antigen panel were negative for each test, as expected (Fig. 3.7).

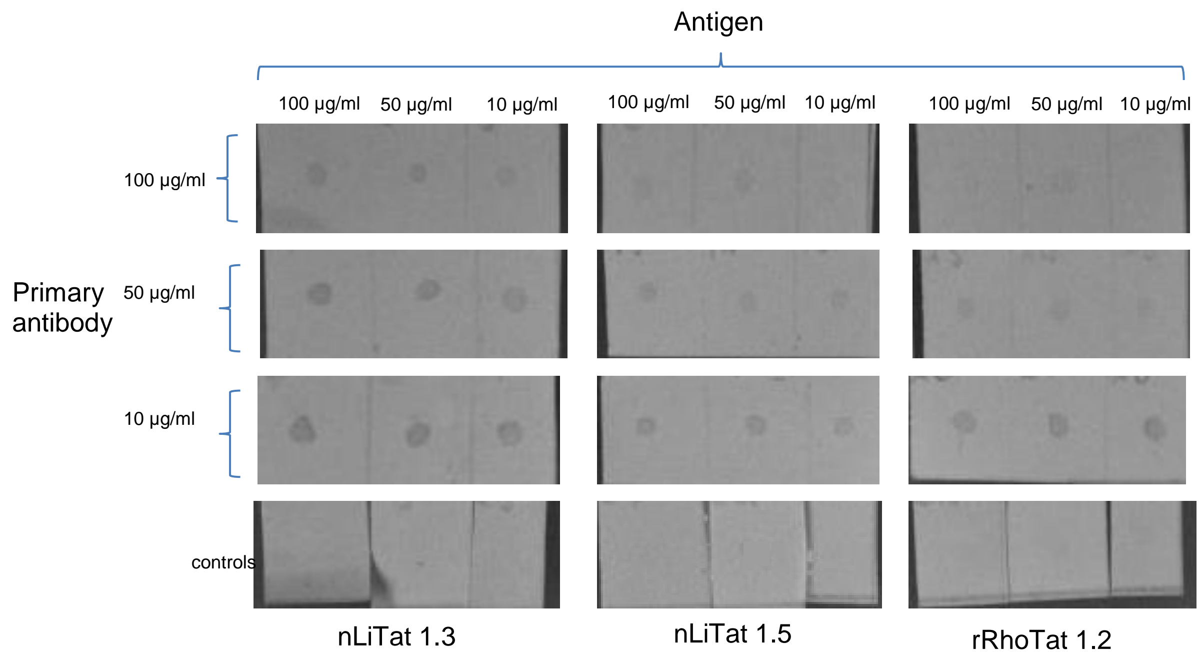


Fig. 3.7. Dot blot of anti-VSGs nLiTat 1.3, nLiTat 1.5 and rRhoTat 1.2 antibodies. Each nitrocellulose strip had antigen placed at three concentrations and probed with the respective antibody at either 100 µg/ml, 50 µg/ml or 10 µg/ml. The controls for each antigen panel were: omission of primary antibody (first grid), omission of secondary antibody (second grid) and pre-immune IgY (third grid).

The VSG nLiTat 1.3, nLiTat 1.5 and rRhoTat 1.2 proteins used to immunise the chickens were run on a reducing SDS-PAGE gel and electro blotted onto a nitrocellulose membrane prior to being probed by the antibodies. Each antibody was able to detect its respective antigen at the apparent sizes (Fig. 3.8: 63.2 kDa for nLiTat 1.3, 59.6 kDa for nLiTat 1.5 and 49 kDa for rRhoTat 1.2). The additional band seen in the rRhoTat 1.2 western blot may be the result of a denatured epitope that was recognised by the antibodies. The non-immune antibodies at the same concentration did not show any binding (results not shown).

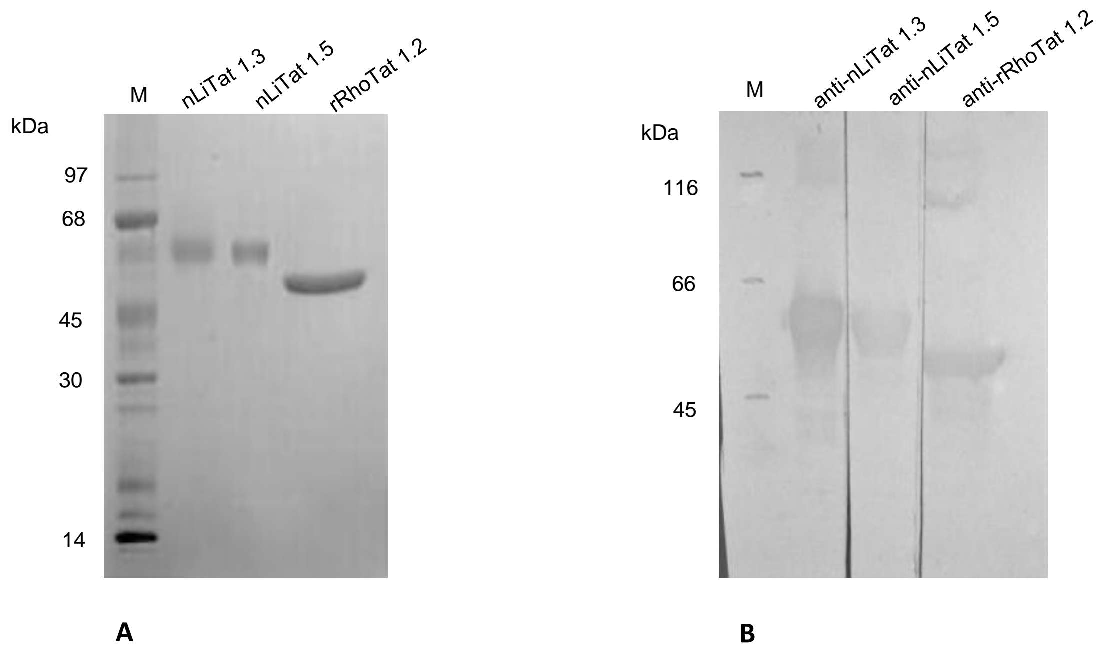


Fig. 3.8. Analysis by SDS-PAGE and western blot of VSGs nLiTat 1.3, nLiTat 1.5 and rRhoTat 1.2. (A) Coomasie stained reducing 10% SDS-PAGE gel. **(B)** Western blot probed with anti-nLiTat 1.3, anti-nLiTat 1.5 or anti-rRhoTat 1.2 antibodies (10 µg/ml). Lane M, molecular weight markers; lane 1, nLiTat 1.3; lane 2, nLiTat 1.5; lane 3, rRhoTat 1.2 (10 µg of each protein was loaded).

3.3.3 Fluorescence immunolabelling of VSGs LiTat 1.3, LiTat 1.5 and RhoTat 1.2

Bloodstream form *T. b. brucei* Lister 427 strain trypanosome parasites were cultured and probed with the antibodies for immunolabelling. The DIC image revealed the morphology of the parasites as elongated flagellates, while the Hoechst stained each cell's nucleus and kinetoplast. Immunolabelling with anti-nLiTat 1.5 antibodies revealed detection of VSG LiTat 1.5 which had a higher amount of fluorescence (Fig. 3.9, G, H) as compared to the detection of VSG LiTat 1.3 using the anti-nLiTat 1.3 antibodies (Fig. 3.9, K, L). The anti-RhoTat 1.2 antibodies on the hand revealed extremely faint detection of VSG RhoTat 1.2 (Fig. 3.9, G, H).

The results therefore show the cross-reactivity of the *T. b. gambiense* VSG antibodies (anti-nLiTat 1.3, anti-nLiTat 1.5) and *T. evansi* VSG antibodies (anti-rRhoTat 1.2) with the VSG proteins expressed in *T. b. brucei*. As expected, the pre-immune chicken antibodies did not show as labelling (Fig. 3.9, C, D).

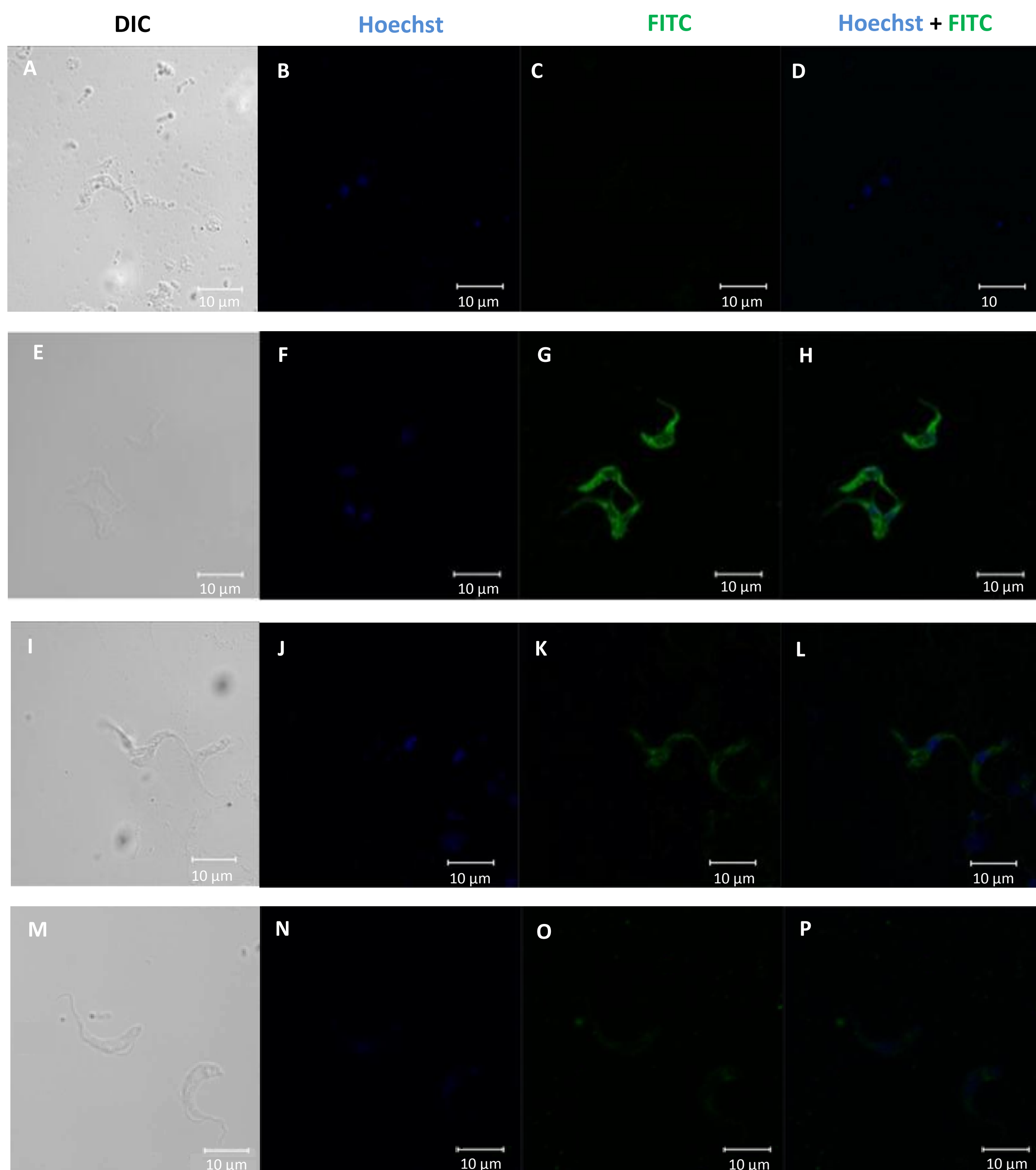


Fig. 3.9. Detection of native VSGs LiTat 1.3, LiTat 1.5 and RhoTat 1.2 on cultured *T. b. brucei* Lister 427 strain parasites. Trypanosomes were fixed on slides and probed with 5 µg/ml: pre-immune (control) antibodies (A-D); antibodies produced against nLiTat 1.5 (E-H); antibodies against nLiTat 1.3 (I-L); and anti-rRhoTat 1.2 antibodies (M-P). The **DIC** represents the light image; nuclei were stained with **Hoechst**; **FITC** is the filter for the respective antibodies used.

3.4 Discussion

Variable surface glycoproteins are present on the cell surface as a thick coat, and the VSGs LiTat 1.3 and LiTat 1.5 are known to appear early during infection and are also predominantly expressed in *T. b. gambiense* infections (Van Nieuwenhove *et al.*, 2011). Due to their predominance, they qualify to be used as antigens for diagnosis hence, individuals carrying the trypanosome specific antibodies are selected for screening based on the antibody and antigen agglutination reaction in the serological tests such as CATT/*T. b. gambiense* and LATEX/*T. b. gambiense* (Koffi *et al.*, 2006; Van Nieuwenhove *et al.*, 2012a). Alternatively, trypanosome antigens in tissue fluids of infected individuals may be detected, using antibodies raised against that antigen, as discussed by Komba *et al.* (1992). The aim of the present study therefore was to raise antibodies against the three VSGs: *T. b. gambiense* native LiTat 1.3 and LiTat 1.5, as well as recombinant *T. evansi* RhoTat 1.2 and characterise them for possible use in antigen detection tests.

A number of studies have been carried out involving anti-VSG antibody production. Various animal hosts are used e. g. mice as reported by Van Nieuwenhove *et al.* (2011) where anti-VSG LiTat 1.5 and anti-VSG LiTat 1.3 monoclonal antibodies were raised and used in a peptide phage display library to identify *T. b. gambiense* VSG LiTat 1.3 and LiTat 1.5 mimotopes that could potentially replace the native VSG proteins in serological tests for sleeping sickness. Rabbits are also used, as seen in the studies where anti-VSG antibodies raised in rabbits were used for analysing the cycle of aggregation-disaggregation of the bloodstream form *T. brucei* parasites in mice (O'Beirne *et al.*, 1998). Rabbits are said to have the advantages of convenient size, ease in handling as well as the ability to produce high-titer antibodies (Suckow *et al.*, 2012).

Another commonly used host is the chicken which has several advantages, one of them being its ability to transfer IgY to the egg yolk. This allows harvesting of the antibodies by collecting the eggs containing the antibodies in the egg yolk- a process which eliminates the invasive blood collection method performed on e. g. rabbits (Tini *et al.*, 2002; Narat, 2003). Another important advantage of chicken IgY antibodies, is their inability to cross-react with rheumatoid factor (anti-mammalian IgG antibody) present in the serum of patients. This circumvents the challenge of false positives in human serum diagnostic assays such as the sandwich ELISA, if IgY is used as capture antibody unlike the normally used antibody of mammalian origin. The false positives result from the binding of the detection antibody to the mammalian capture antibody, a

process stimulated by the rheumatoid factor present in the test serum, resulting in the undetection of the antigen of interest (Larsson *et al.*, 1991).

Antibody production against other trypanosomal antigens has been reported. The evaluation of the antibodies produced against a mutant inactive full length form of the *T. viva*x cysteine protease as well as the *T. congolense* oligopeptidase B by Vather (2010) and Kangethe *et al.* (2012) respectively, showed a general trend of antibody production with high antibody levels five weeks after the initial immunisation. Contrary to their findings, the highest antibody level was observed with anti-nLiTat 1.3 antibodies from chicken nLiTat 1.3-2, two weeks after the first immunisation. The specific week at which antibody production peaked for the remainder of the chickens could not be accurately pin pointed since the chickens did not lay eggs in some weeks, however there was increased antibody production as the weeks progressed.

The titres of the IgY pools were higher than that of the pre-immune antibodies, indicating good antibody production, with strong signals observed at lower concentrations similar to findings by Huson (2006) who analysed purified trypanosomal anti-oligopeptidase B antibodies. Affinity purification of the antibodies could have improved the A_{405} values which were rather low, possibly due to the presence of impurities that were not removed during PEG precipitation, hence interfering with the effective binding of the antibody to the respective antigens. It may also have been a case of the antigen not binding optimally to the ELISA plates, resulting in a decrease in detection limit.

Affinity purification using resins such as the Aminolink coupling resin which through its aldehyde groups, forms covalent bonds with the primary amines in the protein, resulting in an affinity matrix that effectively purifies the respective antibodies. This ensures higher purity, enhanced reactivity and specificity which results in greater recognition of antigen in the ELISA (Domen *et al.*, 1990; Fassina *et al.*, 2001) as observed in the studies by Kangethe *et al.* (2012) and Huson (2006). This unfortunately could not be done, due to the limited amount of antigen available.

The antibodies also showed immunoreactivity with their respective antigens, targeting the appropriate proteins in both dot and western blot analyses. The dot blot, however, seemed to exhibit the "hook" or prozone effect which is an artefact of high tite antibodies where higher antibody levels show lower reactivity than lower levels of antibody. This is the result of excessive amounts of antibody that bind non-specifically to constant low levels of antigen, and are washed off in the washing step, leading to lower amounts of primary antibody bound for labelled secondary antibody to recognise

(Butch, 2000). This was clearly seen when the highest IgY dilution of 10 µg/ml had the strongest dot intensity, compared to the lower dilutions. While the antibodies in the western blot targeted their respective proteins, their specificity could have been confirmed by probing a parasite lysate separated on SDS-PAGE with the antibodies. To determine whether these antibodies would not only detect the purified VSG proteins, as in the western blot, they were used in immunolabelling of fixed trypanosome parasites. Immunofluorescence studies carried out by Figueiredo *et al.* (2008) revealed a uniform distribution of VSG 221 and VSG 13 on *T. brucei* Lister 427 strain VAT MiTat 1.2 parasites. Interestingly in the current study, the anti-nLiTat 1.3 and anti-nLiTat 1.5 antibodies were able to detect their respective VSGs, localised over the whole cell surface, also showing uniform distribution, yet the trypanosome strain used was neither of VAT LiTat 1.5 or LiTat 1.3. Based on DNA fingerprinting and isoenzyme electrophoresis data, MacLeod *et al.* (2001) discovered that *T. b. gambiense* isolates of type 2 are closely related to West African *T. b. brucei* isolates, which therefore possibly explains the cross reactivity of the *T. b. gambiense* anti-nLiTat 1.3 and anti-nLiTat 1.5 antibodies with the respective antigens on the surface of the *T. b. brucei* parasites.

Studies carried out by Lai *et al.* (2008) revealed the close relation between *T. brucei* and *T. evansi*, stating that *T. evansi* was a strain of *T. brucei* which over time lost its kinetoplast DNA. This explained the findings by Jia *et al.* (2012), of the conservative presence of VSG 117 in *T. evansi*, a VSG which was originally identified in *T. brucei* strain 427. In the present study, the detection of VSG RhoTat 1.2, though very faint, suggests that the RhoTat 1.2 gene as well, is conserved between the two species, *T. brucei* and *T. evansi*.

The current study showed the specificity of the raised anti-VSG antibodies, as they were able to recognise their respective proteins adsorbed to ELISA plates as well as in the western blot analysis. The anti-VSG nLiTat 1.3 and nLiTat 1.5 antibodies significantly recognised their respective VSG proteins on fixed trypanosome parasites, while VSG RhoTat 1.2 was faintly detected by the anti-RhoTat 1.2 antibodies. As the antibodies have some level of specificity to their respective VSGs, it would be worthwhile to pursue their use in antigen detection based tests for screening HAT infected individuals.

CHAPTER 4:

GENERAL DISCUSSION

Human African trypanosomiasis (HAT) is one of the neglected tropical diseases, endemic in the low-income/poor rural parts of sub-Saharan Africa (Stich *et al.*, 2002). This parasitic disease caused by *Trypanosoma brucei* is transmitted by a bite from a tsetse fly that has acquired the infection from a human host or zoonotic animals (Simarro *et al.*, 2008; Kristensson *et al.*, 2010).

The disease is prevalent in populations that depend on agriculture, hunting or animal husbandry for their livelihoods. The situation is worsened in instances where the animal form of African trypanosomiasis which affects cattle co-exists with HAT. This is seen when the human infections decrease the work force while the animal infection deprives the farmer of meat and milk, altogether contributing to decreased productivity and development (Connor, 1994; Brun *et al.*, 2009). In an attempt to improve the situation, various control measures have been implemented which include vector control using insecticides, insect traps and sterile insect technique, as well as treatment of HAT infected hosts with drugs such as pentamidine, melarsoprol, eflornithine as well as nifurtimox (Welburn *et al.*, 2006; Baral, 2010; Steverding, 2010).

The two forms of HAT that exist are caused by *T. b. gambiense* which is prevalent in West and Central Africa, and by *T. b. rhodesiense* which is dominant in the Eastern and Southern Africa (Cecchi *et al.*, 2009). The life cycle of trypanosomes is shared between the tsetse fly vector and the mammalian host bloodstream (Donelson, 2003). Before the trypanosomes enter the mammalian host via a bloodmeal of an infected tsetse fly, the parasites undergo numerous changes during the cycle in the vector and acquire a surface coat composed of variable surface glycoprotein (VSG) (Borst and Fairlamb, 1998; Roditi and Lehane, 2008; Sharma *et al.*, 2009). Once in the host, the trypanosomes express a VSG of a particular variant antigenic type (VAT) and through the process of antigenic variation, a different VAT is expressed intermittently (David and McCulloch, 2001). As a result of the constant VAT switching, the host immune system can not effectively carry out antibody mediated lysis, thus prolonging infection and continuation of the life cycle (Carrington *et al.*, 1998).

As addressed in the earlier chapters, VSGs of VATs LiTat 1.3, LiTat 1.5 and LiTat 1.6 are predominantly expressed in *T. b. gambiense* infection (Van Nieuwenhove *et al.*,

2011). These VATs therefore form the basis of the serological tests used to screen infected patients. They serve as antigens in the CATT/*T. b. gambiense* and LATEX/*T. b. gambiense* tests, or the latest Sero-Strip and HAT Sero-K-SeT dipstick and lateral flow tests, targeting the trypanosome specific antibodies in sera from infected hosts (Büscher *et al.*, 1999; Büscher *et al.*, 2013). These tests have shown to be fast and simple to perform, however, they have one major obstacle, which is the maintenance of the cloned *T. b. gambiense* trypanosomes for the extraction of the VSGs, a procedure that poses the risk of infection to laboratory staff (Mitashi *et al.*, 2012; Van Nieuwenhove *et al.*, 2012a). While efforts such as identifying VSG LiTat 1.3 and LiTat 1.5 peptide mimotopes, that could potentially replace the native VSG proteins used in the serological tests, have been made (Van Nieuwenhove *et al.*, 2012a), the first part of the present study was aimed at alleviating the problem of antigen supply through recombinant cloning and expression of VSGs LiTat 1.3 and LiTat 1.5.

A number of studies have been carried out on the recombinant production of trypanosomal antigens for use in serological diagnosis. Tran *et al.* (2009) reported the cloning and recombinant expression of the *T. b. gambiense* invariant surface glycoprotein ISG75, found on the surface of bloodstream form trypanosomes, while Lejon *et al.* (2005) recombinantly expressed the *T. evansi* VSG RhoTat 1.2. In both these studies, PCR amplification was from a complementary DNA (cDNA) template which yielded the respective products at the expected sizes, without any non-specific amplification observed. Similarly in the present study, amplification of both VSGs LiTat 1.3 and LiTat 1.5 from their respective cDNA templates was specific with products observed at the expected sizes. Successful amplification from genomic DNA has been reported in the cloning and expression of a *T. congolense* non-variant gene (Mochabo *et al.*, 2013), which was also clearly observed in this study following the amplification of VSG LiTat 1.5 from *T. b. brucei* Lister 427 strain genomic DNA. Amplification of LiTat 1.3 on the other hand, revealed some non-specific products.

Following amplification, the PCR products were isolated and cloned into a T-vector, restricted by the appropriate restriction enzymes and the inserts sub-cloned into the pGEX-4T-1 and pET-28a expression vectors. Studies by Marcipar *et al.* (2004) showed the importance of choosing the right expression vector for producing recombinant antigenic proteins for diagnostic purposes in the *E. coli* bacterial expression systems. They compared the pMALc2 and pET32 expression vectors in the recombinant expression of the *T. cruzi* ribosomal P2β protein and their findings revealed that the diagnostic performance of the recombinant protein was improved with the pET expressed protein as seen by a high antibody detection limit in infected sera, as

compared to the pMALc2 vector. In the present study, cloning of the VSGs LiTat 1.5 and LiTat 1.3 inserts into the pET-32 vector was unsuccessful, hence the use of the pET-28a vector. It is difficult to explain why expression in a particular expression vector is not successful. This was also observed in previous studies where a gene would be expressed when cloned into pET32a but not into pET28a or pGEX-4T-1. Hence it is common practice in our laboratory to use different expression vectors simultaneously. Perhaps with successful recombinant expression of VSGs LiTat 1.5 and LiTat 1.3 using the pET-28a system, there too, could be high antibody detection limits when the VSGs are used in the serological assays in the screening of *T. b. gambiense* infected sera

Following successful cloning and recombinant expression of VSG LiTat 1.3, solubility testing was undertaken. In an attempt to optimise solubility by expressing at lower temperatures and in two different expression hosts [*E. coli* BL21 (DE3) and Rosetta 2DE3] very little soluble protein was obtained. Perhaps successful cloning in the pET-32 vector, which produces a thioredoxin fusion protein that increases the yield of soluble protein in the cytoplasm, may have improved solubility, as observed for the recombinant expression of VSG RhoTat 1.2 (Novagen, 2003; Sengupta *et al.*, 2012). The VSG LiTat 1.3 protein was solubilised and refolded, however, the shift in sizes between the renatured and the non-renatured forms that is indicative of correct folding (Sijwali *et al.*, 2001), was not clearly seen. The same issue was encountered in the studies carried out by Vather (2010) who expressed and solubilised a *T. vivax* cysteine protease, but perhaps assessing renaturation using other techniques such as circular dichroism (near and far UV CD) which provides valuable information not only about protein structure, but the extent of any structural changes, would provide more conclusive results than the visual observation on the SDS-PAGE gels (Motta *et al.*, 2012).

Optimal purification of proteins used in serological tests ensures that test specificity is maintained without any errors introduced by impure antigens (Ohyama *et al.*, 1985; Büscher *et al.*, 1999). The recombinant VSG LiTat 1.3 protein was purified by Ni-NTA affinity chromatography, however, there were some contaminants observed. Further purification as performed in the studies by Tran *et al.* (2009) and Lejon *et al.* (2005), who employed a number of purification methods to ensure thorough purification of the recombinant antigens resulting in high specificity and sensitivity of diagnostic assays, would be necessary. With successful purification, the recombinant VSG LiTat 1.3 may be assessed in serological assays for screening *T. b. gambiense* infected patient's sera.

The unsuccessful recombinant expression of VSG LiTat 1.5 which expressed as a 'truncated' protein was assumed to be due to rare-codon usage. When codon usage of the target protein differs significantly from the average codon usage of the expression host, this could cause the following problems during expression: decreased mRNA stability (by slowing down translation); premature termination of transcription and/or translation, which leads to a variety of truncated products; frameshifts, deletions and misincorporations (e.g. lysine for arginine); inhibition of protein synthesis and cell growth. As a concequence, the observed levels of expression are often low or there will be no expression at all (Gustafsson *et al.*, 2004; Chen and Texada, 2006). There are several commercial *E. coli* strains available such as BL21 (DE3) CodonPlus-RIL, BL21 (DE3) CodonPlus-RP and the Rosetta that encode for a number of the rare codons (http://www.med.unc.edu/pharm/sondeklab/Lab%20Resources/manuals/codon_plus_manual.pdf [Accessed 4 March 2014]).

While there have not been any reports of rare codon usage in the recombinant expression of trypanosomal proteins, the expression pattern observed with the VSG LiTat 1.5 protein in this study may be due to codon usage. Despite the use of the *E. coli* Rosetta 2DE3 cells which did not seem to improve/resolve the outcome, the other strains listed above would still need to be tried, before codon usage can be ruled out. On the other hand, expression in *P. pastoris* yeast which proved to be a good expression system for trypanosomal proteins such as VSG RhoTat 1.2 (Rogé *et al.*, 2013), was only observed for the protein expressed from the LiTat 1.3-SUMO construct, as the LiTat 1.5-SUMO construct did not reveal expression but rather degradation, thus suggesting instability of the protein.

The next part of the present study involved the production of antibodies that could potentially be used in antigen detection tests for screening *T. b. gambiense* infection. The screening method discussed thus far is based on the use of antigens to detect the presence of antibodies against the VSGs LiTat 1.3 and LiTat 1.5 after which the individuals with positive serology undergo parasitological examination, to confirm the presence of trypanosomes in the blood (Garcia *et al.*, 2006). Antigen-detection tests on the other hand represent an alternative screening method, which involve the use of assays to detect trypanosome specific antigen in infected sera and is advantageous in that it is synonymous with parasitological examination (Chappuis *et al.*, 2005).

The chicken hosts were immunised with the native forms of *T. b. gambiense* VSGs LiTat 1.3 and LiTat 1.5 proteins, as well as a purified recombinant *T. evansi* VSG RhoTat 1.2 obtained from Prof Philippe Büscher of the Unit of Parasite Diagnostics,

Institute of Tropical Medicine, Antwerp, Belgium. The resulting polyclonal antibodies were isolated and characterised by ELISA, dot and western blot. The chickens showed substantial levels of antibody production, and the reactivity of the antibodies against their respective proteins evaluated by titration ELISA showed an increase in detection at higher dilutions, similar to findings by Lomo *et al.* (1997) in the evaluation of the *T. b. brucei* anti-multicatalytic proteinase complex antibodies, albeit at much lower A_{405} values. The low readings may have been due to the presence of non-specific antibodies which reduced detection as the antibodies were not affinity purified. According to Uhlen and Ponten (2005), while polyclonal antibodies have an advantage of multiple epitope binding, the antibodies specific to the antigen used to immunise comprise only 1% of the total antibody population, hence the importance of antibody purification to remove the 99% non-specific and unwanted antibodies.

Comparing the detection of antigen in the ELISA assay and the dot blot, there was greater detection or stronger signals observed in the dot blot, using nitrocellulose as the binding surface, compared to the ELISA microtitre plates, similar to findings by Gerthoffer and Ba (http://biolibrary.licor.com/docs/Gerthoffer.pdf [Accessed on 16 August 2013]), who compared the dot blot and ELISA titrations of antibodies against human heat shock proteins. The authors concluded that dot blots are more sensitive and have a superior working range of antibody dilutions.

The immunofluorescence assay used in the present study successfully showed the cross-reactivity of the anti-*T. b. gambiense* antibodies with the *T. b. brucei* antigens as they localised the VSG LiTat 1.3 and LiTat 1.5 proteins on the surface of the *T. b. brucei* parasites. The close relation between the two species observed by MacLeod *et al.* (2001) may also suggest that they both express VSGs with epitopes that are serologicaly similar, as observed in the Claes *et al.* (2002) studies, where the CATT/*T. evansi* serological tests assessing anti-RhoTat 1.2 antibody production following infection with *T. evansi* and *T. equiperdum* revealed the presence of anti-RhoTat 1.2 antibodies from both infections. The findings therefore suggest that the two VSGs LiTat 1.3 and LiTat 1.5 are not restricted to *T. b. gambiense*

In the present study, the VSGs LiTat 1.3 and LiTat 1.5 were cloned and recombinantly expressed, however, with greater success achieved with VSG LiTat 1.3. For future work, recombinant expression of VSG LiTat 1.5 would require further optimisation and possible analysis by N-terminal sequencing to provide more information and understanding of its amino acid composition. This would help in identifying the possible cause for the truncated expression of VSG LiTat 1.5 observed in the present study.

Optimal conditions for soluble expression would also need to be explored to avoid the solubilising and refolding process which depends on several variables, which also need to be optimised. With successful purification of both VSG LiTat 1.5 and LiTat 1.3, any of the following can be pursued with the recombinantly expressed proteins: to be tested as antigens on the CATT/*T. b. gambiense* and LATEX/*T. b. gambiense* serological tests as well as to be subjected to crystallisation studies, to visualise their structure and enhance the understanding of their function. Also, antibodies against the recombinant proteins may be raised and sensitivity and specificity in the antigen detection tests, compared with the antibodies raised in this study.

The success of recombinant technology in this regard, would be a stepping stone towards safe, cheap and simple antigen and/or antibody based serological tests for use in the diagnosis of Human African trypanosomiasis.

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APPENDIX

Appendix A: Protein alignment of *T. b. gambiense* VSG LiTat 1.3 (database accession no. AJ304413) with the sequenced T-vector-cLiTat 1.3 clone.

Key: (01LiTat 1.3= *T. b. gambiense* VSG LiTat 1.3), (02LiTat 1.3= T-vector-cLiTat 1.3 clone).
(I = identical), (. = very similar), (: = similar).
(Yellow highlight = vector sequence).

```
Length: 514
# Identity:     314/514 (61.1%)
# Similarity:   329/514 (64.0%)
# Gaps:         136/514 (26.5%)
# Score: 1489.0
#

01LiTat1.3          1 ------------------MSPVGAAFSLLLLAAMDLKIVSAAVNGNVRAF     32
                                        ||||||||||||||||||||||||||||||||
02LiTat1.3          1 LRXLIRARYLANASRFEFMSPVGAAFSLLLLAAMDLKIVSAAVNGNVRAF     50

01LiTat1.3         33 NTLCSVIHIVKGEPQVDGTNIVTNVDEDIDAIRRLNLSLADDAMFETDFK     82
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.3         51 NTLCSVIHIVKGEPQVDGTNIVTNVDEDIDAIRRLNLSLADDAMFETDFK    100

01LiTat1.3         83 ATADDNKKPQEYQENREKWEADKKLIVAGKTDINGIKLKRIGSSHARQVA    132
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.3        101 ATADDNKKPQEYQENREKWEADKKLIVAGKTDINGIKLKRIGSSHARQVA    150

01LiTat1.3        133 SAVVNRSLKIAEELRKQLKSTATASAVIAELNKALYGAKGKETTVAGETY    182
                      ||||||||||||||||||||||.|||||||||||||||||||||||||||
02LiTat1.3        151 SAVVNRSLKIAEELRKQLKSTAAASAVIAELNKALYGAKGKETTVAGETY    200

01LiTat1.3        183 ASSGQQGCGNNNPTSQKPGLSLLSDLVCLCATTLGTDTTCLGKAGTNLKY    232
                      ||||.|||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.3        201 ASSGTQGCGNNNPTSQKPGLSLLSDLVCLCATTLGTDTTCLGKAGTNLKY    250

01LiTat1.3        233 DSPTEAKKAAAELIKACPKHGKAAVTPGALTTAKALFYAALKDNGGSTQA    282
                      ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.3        251 DSPTEAKKAAAELIKACPKHGKAAVTPGALTTAKALFYAALKDNGGSTQA    300

01LiTat1.3        283 ENIILGSKNAATCDGGGNGDCIFYKP--------------ANADGMLDIP    318
                      ||||||||.               :|              .|||||||.|
02LiTat1.3        301 ENIILGSKT---------------QPRVTEEERRLHLLQTRNADGMLDXP    335

01LiTat1.3        319 WLKAIDTAMELIKTAAAETKVNAKIAAQVSALRSTALAAYIQAEAGDKQD    368
                      |||.:.....  ..:..|||...:...:.|.|||.:| .:..:::.|.|:
02LiTat1.3        336 WLKQLXPPWS--SNSGGETKSTPRXXHRXSXLRSXSL-QHTFSQSSDXQN    382

01LiTat1.3        369 TQLADTPATVPTQTTTE--GDCSQHQTSDKCKDPCKWDENNADKNKKKCS    416
                      .      :..|..:|..  |..:..:....|.....
02LiTat1.3        383 X------SRXPCTSTXHXVGXINYQRKXXFCNXXRI--------------    412

01LiTat1.3        417 LDPKKAVEKAGQDGITDSKCTGKEQKACKDGCKWDGTECKDSSILINKKF    466

02LiTat1.3        413 --------------------------------------------------    412

01LiTat1.3        467 ALSMVSAAFVALLF    480

02LiTat1.3        413 --------------    412
```

Appendix B: Protein alignment of *T. b. gambiense* VSG LiTat 1.5 (database accession no. HQ662603) with the sequenced T-vector-cLiTat 1.5 clone.

Key: (01LiTat 1.5= *T. b. gambiense* VSG LiTat 1.5), (02LiTat 1.5= T-vector-cLiTat 1.5 clone),
(**I** = identical), (. = very similar), (: = similar).
(Yellow highlight = vector sequence).

```
Length: 517
# Identity:     311/517 (60.2%)
# Similarity:   324/517 (62.7%)
# Gaps:         110/517 (21.3%)
# Score: 1468.0
#

01LiTat1.5         1 --------------MTGRKVSITSKSLLAAVAALLTLVYATKSASTAAIT     36
                                   ||||||||||||||||||||||||||||||||||||
02LiTat1.5         1 XXXXXXGLHLDSVDMTGRKVSITSKSLLAAVAALLTLVYATKSASTAAIT     50

01LiTat1.5        37 DADTGPAQITDVCKEEFYLSELRKELAAGITRRRTQRQGLLKIQKKYRLA     86
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5        51 DADTGPAQITDVCKEEFYLSELRKELAAGITRRRTQRQGLLKIQKKYRLA    100

01LiTat1.5        87 ADLASSTEQRCLYSALAAKLEEKAESVQQQADKADDTATTAMELIDEHVG    136
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5       101 ADLASSTEQRCLYSALAAKLEEKAESVQQQADKADDTATTAMELIDEHVG    150

01LiTat1.5       137 KLKYAQKLLKTKPTVDGSGYSRASSGGNIHLELTRETIASGGCAAIDSWA    186
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5       151 KLKYAQKLLKTKPTVDGSGYSRASSGGNIHLELTRETIASGGCAAIDSWA    200

01LiTat1.5       187 KFSNTHTAINTAKLTEIKITPDTELTTKIFKDKITIGGFSTCTGSAVAKT    236
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5       201 KFSNTHTAINTAKLTEIKITPDTELTTKIFKDKITIGGFSTCTGSAVAKT    250

01LiTat1.5       237 PTFSSVLNTCTVSNSETVTYARNAPDYIYGGTAQAVYKDHDPD-QGCKDA    285
                     ||||||||||||||||||||||||||||||||||||||||||| :..|..
02LiTat1.5       251 PTFSSVLNTCTVSNSETVTYARNAPDYIYGGTAQAVYKDHDPDXRLAKXR    300

01LiTat1.5       286 AAAAEPGATDDIKLRYAVCEALKIIQTDGGKVPPLNGKALKGDKLVTNIL    335
                     .....|.........||||||||||||.|.:..||..|....::......
02LiTat1.5       301 XRRXXPAQRMTSNXXYAVCEALKIIQTXGVRXXPLMAKXRATNQXYXRNC    350

01LiTat1.5       336 RNCLPAYQAVSKPWDSVEAKNLNDFIESAYGADDGKFKDIFDTPLDSRQI    385
                     ..|:|:    .||.||||.|..|.:.:......:........|....|
02LiTat1.5       351 XTCIPS----XKPXDSVEXKISNFYDQXXVXXXEIXXFSXXXTXTSGR--    394

01LiTat1.5       386 TVKLNDKSEDKALTALSTASERNAATSHSAGQRNKKEIETSKKQPAGAPV    435
                     .:.|:..:....|..........:.....
02LiTat1.5       395 ILXLDXIAXQDXLXXXCXXXXMXSXXXXX---------------------    422

01LiTat1.5       436 ASKESEEKCKDKPQGDCKEENGCEFKEGKCQVKVTATTGKDGKTTNTTGS    485

02LiTat1.5       423 --------------------------------------------------    422

01LiTat1.5       486 NSFVINKTPLWLAFLLF    502

02LiTat1.5       423 -----------------    422
```

Appendix C: Protein alignment of *T. b. gambiense* VSG LiTat 1.3 (database accession no. AJ304413) with the sequenced T-vector-gLiTat 1.3 clone.

Key: (01LiTat 1.3= *T. b. gambiense* VSG LiTat 1.3), (02LiTat 1.3= T-vector-gLiTat 1.3 clone).
(I = identical), (. = very similar), (: = similar).

```
Length: 817
# Identity:      12/817 ( 1.5%)
# Similarity:    16/817 ( 2.0%)
# Gaps:         786/817 (96.2%)
# Score: 15.0
#

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3         1 XAYXSXLERPQKAKMQAKEYALIETKFLSVFPVDPVVVVTSAWHFLSLNS     50

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3        51 QPSSSLHSFLWLVLAYFFFFGSDWCIGLLFFCFNFFFMPLASAMASRCVA    100

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3       101 FASCGSCFFFAFVIQFDGYLSISGIKNLFEFSVVCTICFDKIVILRFGCV    150

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3       151 PRLTYCLVCGAACQYICYFVSFQYFAIGWQLAPVFLYSLERFAYCMAELE    200

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3       201 VVSCSGSRNCYRIFATLFMVMVCVERCFCCGVAVGRAVLRIGYSFAAYGA    250

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3       251 AVHIRERLCLAYSEAFAAGERANGYFVFEYFCGVVGVLNLNFCLCRLYCV    300

01LiTat1.3         1 ------------------------MSPVGAAFS-------------LLLL     13
                                             :||:.|..|             ...|
02LiTat1.3       301 SAPKSFPALNFCASLVVVCLPRLVILSPLFARLSRCRLRCLGFSSFCAYL    350

01LiTat1.3        14 AAMDLKIVSAAVNGNVRAFNTLCSVIHIVKGEPQVDGTNIVTNVDEDIDA     63
                     ||...:|.|.|  |.:..|.
02LiTat1.3       351 AAWLARISSIA--GGIGIFV------------------------------    368

01LiTat1.3        64 IRRLNLSLADDAMFETDFKATADDNKKPQEYQENREKWEADKKLIVAGKT    113

02LiTat1.3       369 --------------------------------------------------    368

01LiTat1.3       114 DINGIKLKRIGSSHARQVASAVVNRSLKIAEELRKQLKSTATASAVIAEL    163

02LiTat1.3       369 --------------------------------------------------    368

01LiTat1.3       164 NKALYGAKGKETTVAGETYASSGQQGCGNNNPTSQKPGLSLLSDLVCLCA    213

02LiTat1.3       369 --------------------------------------------------    368

01LiTat1.3       214 TTLGTDTTCLGKAGTNLKYDSPTEAKKAAAELIKACPKHGKAAVTPGALT    263

02LiTat1.3       369 --------------------------------------------------    368

01LiTat1.3       264 TAKALFYAALKDNGGSTQAENIILGSKNAATCDGGGNGDCIFYKPANADG    313

02LiTat1.3       369 --------------------------------------------------    368

01LiTat1.3       314 MLDIPWLKAIDTAMELIKTAAAETKVNAKIAAQVSALRSTALAAYIQAEA    363
```

```
02LiTat1.3        369 --------------------------------------------------   368

01LiTat1.3        364 GDKQDTQLADTPATVPTQTTTEGDCSQHQTSDKCKDPCKWDENNADKNKK   413

02LiTat1.3        369 --------------------------------------------------   368

01LiTat1.3        414 KCSLDPKKAVEKAGQDGITDSKCTGKEQKACKDGCKWDGTECKDSSILIN   463

02LiTat1.3        369 --------------------------------------------------   368

01LiTat1.3        464 KKFALSMVSAAFVALLF    480

02LiTat1.3        369 -----------------    368
```

Appendix D: Protein alignment of *T. b. gambiense* VSG LiTat 1.5 (database accession no. HQ662603) with the sequenced T-vector-gLiTat 1.5 clone.

Key: (01LiTat 1.5= *T. b. gambiense* VSG LiTat 1.5), (02LiTat 1.5= T-vector-gLiTat 1.5 clone).
(I = identical), (. = very similar), (: = similar).
(Yellow highlight = vector sequence).

```
Length: 530
# Identity:     306/530 (57.7%)
# Similarity:   323/530 (60.9%)
# Gaps:         150/530 (28.3%)
# Score: 1430.5
#

01LiTat1.5         1 ------------------MTGRKVSITSKSLLAAVAALLTLVYATKSAST     32
                                       ||||||||||||||||||||||||||||||||
02LiTat1.5         1 XGXXFELGTSRMHLDSVDMTGRKVSITSKSLLAAVAALLTLVYATKSAST     50

01LiTat1.5        33 AAITDADTGPAQITDVCKEEFYLSELRKELAAGITRRRTQRQGLLKIQKK     82
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5        51 AAITDADTGPAQITDVCKEEFYLSELRKELAAGITRRRTQRQGLLKIQKK    100

01LiTat1.5        83 YRLAADLASSTEQRCLYSALAAKLEEKAESVQQQADKADDTATTAMELID    132
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5       101 YRLAADLASSTEQRCLYSALAAKLEEKAESVQQQADKADDTATTAMELID    150

01LiTat1.5       133 EHVGKLKYAQKLLKTKPTVDGSGYSRASSGGNIHLELTRETIASGGCAAI    182
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5       151 EHVGKLKYAQKLLKTKPTVDGSGYSRASSGGNIHLELTRETIASGGCAAI    200

01LiTat1.5       183 DSWAKFSNTHTAINTAKLTEIKITPDTELTTKIFKDKITIGGFSTCTGSA    232
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5       201 DSWAKFSNTHTAINTAKLTEIKITPDTELTTKIFKDKITIGGFSTCTGSA    250

01LiTat1.5       233 VAKTPTFSSVLNTCTVSNSETVTYARNAPDYIYGGTAQAVYKDHDPDQGC    282
                     ||||||||||||||||||||||||||||||||||||||||||...|.:..
02LiTat1.5       251 VAKTPTFSSVLNTCTVSNSETVTYARNAPDYIYGGTAQAVYKIMTPTKAA    300

01LiTat1.5       283 KDAAAAAEPG---ATDDIKLRYAVCEALKIIQTDGGKVPPLNGKALK---    326
                     |.......|.   .|.....|.|     :|||||||||||||||.||
02LiTat1.5       301 KTRQRRPSPAHGHQTPXCSXRSA-----QIIQTDGGKVPPLNGKXLKATT    345

01LiTat1.5       327 GDKLVTNILRNCLPAYQAVSKP----WDSVEAKNLNDFIESAYGADDGKF    372
                     .:|....:|.:...:...:|:.    .|...|:.:.:|...       :.
02LiTat1.5       346 SNKYTKKLLLHTSSSSVGLSRSEITNXDQXCARRIKNFXYX-------RL    388

01LiTat1.5       373 KDIFDTPLDSRQITVKLNDKSEDKALTALSTASERNAATSHSAGQRNKKE    422
                     :.|.....:|..:..:|.:.
02LiTat1.5       389 RRIRHCKANSITVYSQLAEX------------------------------    408

01LiTat1.5       423 IETSKKQPAGAPVASKESEEKCKDKPQGDCKEENGCEFKEGKCQVKVTAT    472

02LiTat1.5       409 --------------------------------------------------    408

01LiTat1.5       473 TGKDGKTTNTTGSNSFVINKTPLWLAFLLF    502

02LiTat1.5       409 ------------------------------    408
```

Appendix E: Protein alignment of *T. b. gambiense* VSG LiTat 1.3 (database accession no. AJ304413) with the sequenced pGEX-4T-1-cLiTat 1.3 clone.

Key: (01LiTat 1.3= *T. b. gambiense* VSG LiTat 1.3), (02LiTat 1.3= pGEX4T-1-cLiTat 1.3 clone).
(I = identical), (. = very similar), (: = similar).
(Yellow highlight = vector sequence).

```
Length: 523
# Identity:     307/523 (58.7%)
# Similarity:   318/523 (60.8%)
# Gaps:         159/523 (30.4%)
# Score: 1453.0
#

01LiTat1.3         1 ---------------------------MSPVGAAFSLLLLAAMDLKIVSA     23
                                                :||......||||||||||||||
02LiTat1.3         1 WTRXDQGXTXLGXIXXXXRXXVRGIPXISPKRCGLCLLLLAAMDLKIVSA     50

01LiTat1.3        24 AVNGNVRAFNTLCSVIHIVKGEPQVDGTNIVTNVDEDIDAIRRLNLSLAD     73
                     |||||||||||||||||||||||||:||||||||||||||||||||||||
02LiTat1.3        51 AVNGNVRAFNTLCSVIHIVKGEPQVNGTNIVTNVDEDIDAIRRLNLSLAD    100

01LiTat1.3        74 DAMFETDFKATADDNKKPQEYQENREKWEADKKLIVAGKTDINGIKLKRI    123
                     |||||||||||||||||||||.||||||||||||||||||||||||||||
02LiTat1.3       101 DAMFETDFKATADDNKKPQEYXENREKWEADKKLIVAGKTDINGIKLKRI    150

01LiTat1.3       124 GSSHARQVASAVVNRSLKIAEELRKQLKSTATASAVIAELNKALYGAKGK    173
                     |||||||||||||||||||||||||||||||.||||||||||||||||||
02LiTat1.3       151 GSSHARQVASAVVNRSLKIAEELRKQLKSTAAASAVIAELNKALYGAKGK    200

01LiTat1.3       174 ETTVAGETYASSGQQGCGNNNPTSQKPGLSLLSDLVCLCATTLGTDTTCL    223
                     |||||||||||||.||||||||||||||||||||||||||||||||||||
02LiTat1.3       201 ETTVAGETYASSGTQGCGNNNPTSQKPGLSLLSDLVCLCATTLGTDTTCL    250

01LiTat1.3       224 GKAGTNLKYDSPTEAKKAAAELIKACPKHGKAAVTPGALTTAKALFYAAL    273
                     ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.3       251 GKAGTNLKYDSPTEAKKAAAELIKACPKHGKAAVTPGALTTAKALFYAAL    300

01LiTat1.3       274 KDNGGSTQAENIILGSKNAATCDGGGNGDCIFYKPANADGMLDIPWLKAI    323
                     |||||||||||||||||.......||||||.|||||..
02LiTat1.3       301 KDNGGSTQAENIILGSKTQPRVTXGGNGDCXFYKPATL------------    338

01LiTat1.3       324 DTAMELI---------KTAAAETKVNAKIAAQVSALRSTALAAYIQAEAG    364
                      ||....         :.:..||| :...:..||...||||.:.:|
02LiTat1.3       339 -TACXYXXXNYXHGANQXSGGETK-STXDSGXVSGXXSTALCSILQ----    382

01LiTat1.3       365 DKQDTQLADTPAT-VPT------QTTTEGDCSQHQTSDKCKDPCKWDENN    407
                       |..:.||...| .|:      :...
02LiTat1.3       383 --QSRRQADXARTXAPSXQNHYRRXLX-----------------------    407

01LiTat1.3       408 ADKNKKKCSLDPKKAVEKAGQDGITDSKCTGKEQKACKDGCKWDGTECKD    457

02LiTat1.3       408 --------------------------------------------------    407

01LiTat1.3       458 SSILINKKFALSMVSAAFVALLF    480

02LiTat1.3       408 -----------------------    407
```

Appendix F: Protein alignment of *T. b. gambiense* VSG LiTat 1.5 (database accession no. HQ662603) with the sequenced pGEX-4T-1-cLiTat 1.5 clone.

Key: (01LiTat 1.5= *T. b. gambiense* VSG LiTat 1.5), (02LiTat 1.5= pGEX4T-1-cLiTat 1.5 clone).
(I = identical), (. = very similar), (: = similar).
(Yellow highlight = vector sequence).

```
Length: 534
# Identity:     327/534 (61.2%)
# Similarity:   334/534 (62.5%)
# Gaps:         142/534 (26.6%)
# Score: 1548.5
#

01LiTat1.5           1 --------------------MTGRKVSITSKSLLAAVAALLTLVYATKSA     30
                                           ||||||||||||||||||||||||||||||
02LiTat1.5           1 XXXXXXRIWFRVDPRNSRVDMTGRKVSITSKSLLAAVAALLTLVYATKSA     50

01LiTat1.5          31 STAAITDADTGPAQITDVCKEEFYLSELRKELAAGITRRRTQRQGLLKIQ     80
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5          51 STAAITDADTGPAQITDVCKEEFYLSELRKELAAGITRRRTQRQGLLKIQ    100

01LiTat1.5          81 KKYRLAADLASSTEQRCLYSALAAKLEEKAESVQQQADKADDTATTAMEL    130
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5         101 KKYRLAADLASSTEQRCLYSALAAKLEEKAESVQQQADKADDTATTAMEL    150

01LiTat1.5         131 IDEHVGKLKYAQKLLKTKPTVDGSGYSRASSGGNIHLELTRETIASGGCA    180
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5         151 IDEHVGKLKYAQKLLKTKPTVDGSGYSRASSGGNIHLELTRETIASGGCA    200

01LiTat1.5         181 AIDSWAKFSNTHTAINTAKLTEIKITPDTELTTKIFKDKITIGGFSTCTG    230
                       ||||||||||||||||||||||||||||||||||||||||||||||||||
02LiTat1.5         201 AIDSWAKFSNTHTAINTAKLTEIKITPDTELTTKIFKDKITIGGFSTCTG    250

01LiTat1.5         231 SAVAKTPTFSSVLNTCTVSNSETVTYARNAPDYIYGGTAQAVYKDHDPDQ    280
                       ||||||||||||||||||||||||||||||||||||||||.||||||||.
02LiTat1.5         251 SAVAKTPTFSSVLNTCTVSNSETVTYARNAPDYIYGGTAQXVYKDHDPDX    300

01LiTat1.5         281 GCKDAAAAAEPGATDDIKLRYAVCEALKIIQTDGGKVPPLNGKALKGDKL    330
                       ||||||||||.||||||.......:..||||..|    .:...|......
02LiTat1.5         301 GCKDAAAAAEXGATDDINSAMQYAKRFKIIQXTG----QVXXMAKHRXXT    346

01LiTat1.5         331 VTNILRNCLPAYQAVSKPWDSVEAK------------NLNDFIESAYGAD    368
                       ..|||||||||||||.....|||.:            ..|..|......|
02LiTat1.5         347 SXNILRNCLPAYQAVVSRGTSVEXELXNFIXIXXWVXRWNQXISXHXXXD    396

01LiTat1.5         369 DGKFKDIFDTPLDSRQITVKLNDKSEDKALTALSTASERNAATSHSAGQR    418
                       |....:      .:||.||.|       .:...:.|...|.
02LiTat1.5         397 DNXXXE------QARQXTVNL-------XVXXXNXAXXXNX---------    424

01LiTat1.5         419 NKKEIETSKKQPAGAPVASKESEEKCKDKPQGDCKEENGCEFKEGKCQVK    468

02LiTat1.5         425 --------------------------------------------------    424

01LiTat1.5         469 VTATTGKDGKTTNTTGSNSFVINKTPLWLAFLLF    502

02LiTat1.5         425 ----------------------------------    424
```

Appendix G: Protein alignment of *T. b. gambiense* VSG LiTat 1.5 (database accession no. HQ662603) with the sequenced pGEX-4T-1-gLiTat 1.5 clone.

Key: (01LiTat 1.5= *T. b. gambiense* VSG LiTat 1.5), (02LiTat 1.5= pGEX4T-1-gLiTat 1.5 clone).
(**I** = identical), (. = very similar), (: = similar).
(Yellow highlight = vector sequence).

```
Length: 531
# Identity:     228/531 (42.9%)
# Similarity:   269/531 (50.7%)
# Gaps:         178/531 (33.5%)
# Score: 1049.0
#

01LiTat1.5         1 --------------------MTGRKVSITSKSLLAAVAALLTLVYATKSA     30
                                         |||||||||||:||||||||||||||||||
02LiTat1.5         1 XXHXPNRIWFRVDPRNSRVDMTGRKVSITSKNLLAAVAALLTLVYATKSA     50

01LiTat1.5        31 STAAITDADTGPAQITDVCKEEFYLSELRKELAAGITRRRTQRQGLLKIQ     80
                     ||||||||||||||||.|||..||||||||||||.|||.||:||.|||::
02LiTat1.5        51 STAAITDADTGPAQITGVCKGAFYLSELRKELAAEITRCRTRRQELLKLE    100

01LiTat1.5        81 KKYRLAADLASSTEQRCLYSALAAKLEEKAESVQQQADKADDTATTAMEL    130
                     :|||:||.||:....||||:||||||||:||||||||||||.|||||:||
02LiTat1.5       101 RKYRMAATLATQANDRCLYAALAAKLEEQAESVQQQADKADKTATTAIEL    150

01LiTat1.5       131 IDEHVGKLKYAQKLLKTKPTVDGSGYSRASSGGNIHLELTRETIASGGCA    180
                     ||:|.||||||||||||||.|||:|.|||:||.||||||||:|..:.||.
02LiTat1.5       151 IDQHAGKLKYAQKLLKTKPAVDGNGDSRANSGDNIHLELTRQTHNNKGCT    200

01LiTat1.5       181 AIDSWAKFSNTHTAINTAKLTEIKITPDTELTTKIFKDKITIGGFSTCTG    230
                     .| ||.:|...|:|:..||||||||..|.:.|||||:||||||.|:.|.|
02LiTat1.5       201 EI-SWERFRGAHSAVQPAKLTEIKIKSDADHTTKIFEDKITIGAFTGCKG    249

01LiTat1.5       231 SAVAKTPTFSSVLNTCTVSNSETVTYARNAPDYIYGGTAQAVYKDHDPDQ    280
                     ..|::|.|||:|||:||||||||||||:|:|.|.|.....|.|.|||.:|
02LiTat1.5       250 FGVSQTQTFSNVLNSCTVSNSETVTYAQNSPAYRYTTAKTAFYTDHDHEQ    299

01LiTat1.5       281 GCKDAAAAAEPGATDDIKLRYAVCEALKIIQTDGGKVPPLNGKALK---G    327
                     ||:||........|  .:.|:|||||| :.:.||. ..|:|....:   .
02LiTat1.5       300 GCEDAQFRLPEQLT--TQARHAVCEAL-LYKKDGA-AAPINASTERRQXS    345

01LiTat1.5       328 DKLVTNILRNCLPAYQAVSKP-----WD-SVEAKNLNDFIESAYGADDGK    371
                     :|.:|    :|.|...|....     |: ..:..|....:|
02LiTat1.5       346 NKXLT----SCXPXQHAGXASXEITIWNRXXQHGNQTIXLE---------    382

01LiTat1.5       372 FKDIFDTPLDSRQITVKLNDKSEDKALTALSTASERNAATSHSAGQRNKK    421

02LiTat1.5       383 --------------------------------------------------    382

01LiTat1.5       422 EIETSKKQPAGAPVASKESEEKCKDKPQGDCKEENGCEFKEGKCQVKVTA    471

02LiTat1.5       383 --------------------------------------------------    382

01LiTat1.5       472 TTGKDGKTTNTTGSNSFVINKTPLWLAFLLF    502

02LiTat1.5       383 -------------------------------    382
```

Appendix H: Protein alignment of *T. b. gambiense* VSG LiTat 1.3 (database accession no. AJ304413) with the sequenced pGEX-4T-1-gLiTat 1.3 clone.

Key: (01LiTat = *T. b. gambiense* VSG LiTat 1.3), (02LiTat = pGEX4T-1-gLiTat 1.3 clone).
(I = identical), (. = very similar), (: = similar).

```
Length: 746
# Identity:      10/746 ( 1.3%)
# Similarity:    13/746 ( 1.7%)
# Gaps:         711/746 (95.3%)
# Score: 2.5
#

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3         1 XXXGNARGRSSVSHDAAXKSXAXNAXETMLRANFLLMXIEESLXSVPSHL     50

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3        51 QPSLXALCSFPXXLEXDIPSCPAFSTAFLGXNEHFFVLAXLFXSHLXGSL    100

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3       101 HLXLVWXWLXSPSVXXCVRTVAXVXASXVSXLXXASAMYAAXAXLXXXNT    150

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3       151 CAAILXLTFVSXAAVLISSXAXSIAFXHXISSMPSXXXGLXKMXXPXPPX    200

01LiTat1.3         1 --------------------------------------------------      0

02LiTat1.3       201 SHXXAXLLPRNMFTAXVDPLXSLXAAYXXALXXVAPGVXAAXTXXYRIXS    250

01LiTat1.3         1 ------------MSPVGAAFSLLLLAAMDLKIV----SAAVNGNVRAFNT     34
                                 ...||:.    :...|.|.||    ..|.:.....|
02LiTat1.3       251 XXQLXXXXRLVXYFXVGSX----IXXXMXLSIVDXTXXXAXHXXXXGF--    294

01LiTat1.3        35 LCSVIHIVKGEPQVDGTNIVTNVDEDIDAIRRLNLSLADDAMFETDFKAT     84
                     .|.|...
02LiTat1.3       295 XCXVWXA-------------------------------------------    301

01LiTat1.3        85 ADDNKKPQEYQENREKWEADKKLIVAGKTDINGIKLKRIGSSHARQVASA    134

02LiTat1.3       302 --------------------------------------------------    301

01LiTat1.3       135 VVNRSLKIAEELRKQLKSTATASAVIAELNKALYGAKGKETTVAGETYAS    184

02LiTat1.3       302 --------------------------------------------------    301

01LiTat1.3       185 SGQQGCGNNNPTSQKPGLSLLSDLVCLCATTLGTDTTCLGKAGTNLKYDS    234

02LiTat1.3       302 --------------------------------------------------    301

01LiTat1.3       235 PTEAKKAAAELIKACPKHGKAAVTPGALTTAKALFYAALKDNGGSTQAEN    284

02LiTat1.3       302 --------------------------------------------------    301

01LiTat1.3       285 IILGSKNAATCDGGGNGDCIFYKPANADGMLDIPWLKAIDTAMELIKTAA    334

02LiTat1.3       302 --------------------------------------------------    301

01LiTat1.3       335 AETKVNAKIAAQVSALRSTALAAYIQAEAGDKQDTQLADTPATVPTQTTT    384

02LiTat1.3       302 --------------------------------------------------    301
```

```
01LiTat1.3       385 EGDCSQHQTSDKCKDPCKWDENNADKNKKKCSLDPKKAVEKAGQDGITDS    434

02LiTat1.3       302 --------------------------------------------------    301

01LiTat1.3       435 KCTGKEQKACKDGCKWDGTECKDSSILINKKFALSMVSAAFVALLF    480

02LiTat1.3       302 ----------------------------------------------    301
```

Appendix I: Protein alignment of *T. b. gambiense* VSG LiTat 1.3 (database accession no. AJ304413) with the sequenced pET-28a-cLiTat 1.3 clone.

Key: (01LiTat 1.3 = *T. b. gambiense* VSG LiTat 1.3), (02LiTat 1.3 = pET-28a-cLiTat 1.3 clone).
(**I** = identical), (. = very similar), (: = similar).
(Yellow highlight = vector sequence).

```
# Length: 525
# Identity:     190/525 (36.2%)
# Similarity:   204/525 (38.9%)
# Gaps:         162/525 (30.9%)
# Score: 769.0
#

01LiTat1.3           1 ---------------------------------------MSPVGAAFSLL     11
                                                              :|...||||||
02LiTat1.3           1 RFXMGRTXPHHHHSSGLVPRXSHLASMTGGQXMGRGSXFLSXXXAAFSLL     50

01LiTat1.3          12 LLAAMDLKIVSAAVNGNVRAFNTLCSVIHIVKGEPQVDGTNIVTNVDEDI     61
                       |||||||..|.|.|||.|.||.|||||||..|||.|.|.|||||||||||
02LiTat1.3          51 LLAAMDLXXVXAXVNGXVXAFXTLCSVIHXXKGEXQXDXTNIVTNVDEDI    100

01LiTat1.3          62 DAIRRLNLSLADDAMFETDFKATADDNKKPQEYQENREKWEADKKLIVAG    111
                       |.||||||||..||.|.|||||..||.||||||.|||||||||||||.||
02LiTat1.3         101 DXIRRLNLSLXXDAXFXTDFKAXXDDXKKPQEYXENREKWEADKKLIXAG    150

01LiTat1.3         112 KTDINGIKLKRIGSSHARQVASAVVNRSLKIAEELRKQLKSTATASAVIA    161
                       ||.|||..||||||||||||||||||||||||..|..||.|||.||||||
02LiTat1.3         151 KTXINGXXLKRIGSSHARQVASAVVNRSLKIAXXLXMQLXSTAAASAVIA    200

01LiTat1.3         162 ELNKALYGAKGKETTVAGETYASSGQQGCGNNNPTSQKPGLSLLSDLVCL    211
                       |||||||..||||||||||||||.|.|.||..||.........:|||.|.
02LiTat1.3         201 ELNKALYXXKGKETTVAGETYASIGTQCCGXXNPXKPXTWALAVSDLXCX    250

01LiTat1.3         212 CATTLGTDTTCLGKAGTNLKYDSPTE-AKKAAAELIKACPKHGKAAVTPG    260
                       |.|. ........|...:||||.|.. .....|:  :|||.||.......
02LiTat1.3         251 CXTR-SNRXXLXRKXXNHLKYDXPXXLXXSCXAD--QACPXHGXXXYARC    297

01LiTat1.3         261 ALTTAKALFYAALKDNGGSTQAENIILGSKNAATCDGGGNGDC-----IF    305
                       ..|..:  |:..|||..... ..|..|.||...         |     :.
02LiTat1.3         298 XDTXKR--FFXXLKDXVDHX-XXNXFLXSKRXX---------CXXEPRLH    335

01LiTat1.3         306 YKPANADGMLDIPWLKAIDTAMELIKTAAAETKVNAKIAAQVSALRSTAL    355
                       ........:.....|.|||..........     .|:......:.....
02LiTat1.3         336 SSXTXXXXRMXXYRXKXIDTXXXXXXAXXV-----XKVPXXYXVMXXPXS    380

01LiTat1.3         356 AAYIQAEAGDKQDTQLADTPATVPTQTTTEGDCSQHQTSDKCKDPCKWDE    405
                       .::.......|.. |.......:|...|.
02LiTat1.3         381 XSFXXXXRSXKXQ-QXXXPXXXLPHPNTY---------------------    408

01LiTat1.3         406 NNADKNKKKCSLDPKKAVEKAGQDGITDSKCTGKEQKACKDGCKWDGTEC    455

02LiTat1.3         409 --------------------------------------------------    408

01LiTat1.3         456 KDSSILINKKFALSMVSAAFVALLF    480

02LiTat1.3         409 -------------------------    408
```