Heterologous expression of invariant surface glycoproteins, ISG65 and ISG75 of *Trypanosoma brucei brucei* and *T. b. gambiense*, for antibody production and diagnosis of African trypanosomiasis

By

Omolara O. Baiyegunhi

BSc (Hons) Biochemistry

Submitted in fulfilment of the academic requirements for the degree of Master of Science in the Discipline of Biochemistry

School of Life Sciences

University of KwaZulu-Natal

Pietermaritzburg

September 2013

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, from August 2011 to September 2013, under the supervision of Professor Theresa. H. T. Coetzer

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.

Omolara Baiyegunhi (candidate)

Professor Theresa H. T. Coetzer (supervisor)

DECLARATION–PLAGIARISM

I, Omolara Baiyegunhi declare that

1. The research reported in this dissertation, except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Their words have been re-written but the general information attributed to them has been referenced.
    b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.
5. This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

Signed: ……

DEDICATION

I dedicate this work to my dearest departed daddy (Dr S.O. Adekunle, 1941-2012), thank you for teaching me the value of education. I am sure that you would have been very proud of me if you were still here.

ABSTRACT

Accurate diagnosis of the presence of an infectious organism is very important for therapeutic interventions and consequently the recovery of the individual. There is a need for identifying new diagnostic antigens for the serological diagnosis of trypanosomiasis, a disease of humans and animals in Africa caused by protozoa belonging to the genus *Trypanosoma*. Invariant surface glycoproteins (ISGs) are present in most strains of the parasite and have the potential to replace the variable surface glycoproteins as diagnostic antigens. In order to avoid the challenges of *in vivo* culturing of bloodstream form (BSF) trypanosomes in laboratory animals, ISG65 and ISG75, the two most common ISGs were heterologously expressed in *Escherichia coli* and *Pichia pastoris* expression systems.

The extracellular domains of *ISG65* and *ISG75* of both *T. b. brucei* and *T. b. gambiense* were amplified by PCR from genomic DNA using appropriate primers to give inserts of 1121 bp and 1342 bp sizes. These were sub-cloned into the pGEX-4T1 and pET28a expression vectors. Chemically competent *E. coli* BL21 (DE3) were transformed using the resultant plasmids and the transformed *E. coli* cells were used for heterologous protein expression.

The expressed proteins were purified by three phase partitioning (TPP), nickel or glutathione affinity and molecular exclusion chromatography and analysed by reducing SDS-PAGE. The glycosylation status of ISG65 and ISG75 expressed in the M5 strain of *P. pastoris*, which has an engineered N-glycosylation pathway that produces glycosylated proteins similar to what is obtained in trypanosomes, was determined. The enzymatic action of Endoglycosidase H resulted in a shift in the electrophoretic migration of ISG65 but not ISG75 on SDS-PAGE, confirming N-glycosylation.

Anti-ISG65 and anti-ISG75 antibodies were produced in chickens and affinity purified using the respective recombinant proteins immobilised on affinity matrices. The antibodies recognised native ISG65 and ISG75 respectively in western blots of lysates of *T. b. brucei* parasites cultured *in vitro*. Similar recognition of the native ISGs by the anti-recombinant ISG antibodies was also obtained using immunofluorescence microscopy of fixed

T. b. brucei parasites. The results obtained demonstrate the potential of application of the recombinant ISG65 and ISG75 and their respective antibodies in the diagnosis of African trypanosomiasis.

ACKNOWLEDGEMENTS

All glory to God the Father in heaven. Lord, it was all by your grace.

Success is never a sole accomplishment, so I would like to acknowledge all those that helped to make this study possible.

My heartfelt thanks go to my supervisor Prof. THT Coetzer, thank you for investing in me and shaping me into a worthy scientist. Thank you also for all the lab meetings, both the serious and relaxed ones, they all contributed to the success of my research.

I would also like to thank all the members of staff of the Biochemistry department for their help: Dr Elliot, Dr Niesler, Prof Goldring (thank you for believing in me), and my dear friend Dr Vukea (for your invaluable friendship and support both personal and professional, may God bless you richly).

A big thank you to all the members of staff of the School of Life Sciences: Charmaine (always efficient), Tanya, Natalie, Mrs Flockhart, Shirley (EM Unit), Yegan, Jess, Megan, Ma Agnes, Richard, Khetiwe and all the other cleaners. You all helped to make things flow smoothly. Also, I would like to thank the College of Agriculture, Engineering and Science for granting me a bursary.

Special thanks to all the postgrads of SLS, Celia, Kyle, Rob, Wanani, Milly, Laurelle, Trish, Rhys, Nick, Scott, Kelvin, Faiaz, Richard (my first lab 44 mentor), Phumzile, my dear friend Kayleen (for your loving and caring demeanour, always willing to listen and assist), my very special lab 44 ‘peeps’, Sanele (little sis), Phindile (Prof to be), Faith (energetic Faith’o’), Kwanele, Mbali, Lauren, Jax and the baby of the lab, ‘Wonder boy’.

A big thank you to the special man in my life, my darling husband Lloyd, for your patience, love, support and for inspiring me to undertake this Master’s study. I love you big. I’m also grateful to my mum, step mum, father in-law, siblings and my whole family back home in Nigeria. I appreciate all your prayers and words of encouragement.

Lastly, I want to appreciate Pastor and Mrs Habile, my dear MCC family, and my darling friends, Funmi, Dotty, Nothando, Ellen, Tosin, Grany, Unathi Kolanisi, Sandra, Unathi Majova, Rofiah, Seun, Drs Adeyemi, Awolesi and Sarumi for all the love and support. I appreciate you all and I pray that all your dreams will come to pass.

"Better is the end of a thing than the beginning thereof".
Ecclesiastes 7:8

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

ABBREVIATIONS

AAT	animal African trypanosomiasis
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
BCA	bicinchoninic acid
BMGY	buffered glycerol complex medium
BMMY	buffered methanol-complex medium
bp	base-pair
BSA	bovine serum albumin
CATT	card agglutination test for trypanosomiasis
CNS	central nervous system
CSF	cerebrospinal fluid
C-terminal	carboxy terminal
dH_2O	distilled water
DIC	differential interference contrast
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ESCRT	endosomal sorting complex required for transport
Fc	fragment crystallisable
FITC	fluorescein isothiocyanate
g	relative centrifugal force

GPI	glycophosphatidyl inositol
GST	glutathione *S*-transferase
h	hour(s)
HAT	human African trypanosomiasis
HRPO	horseradish peroxidase
IFA	immunofluorescence assay
IFAT	immunofluorescent antibody test
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	immunoglobulin Y
IPTG	isopropyl β-D-1-thiogalactopyranoside
ISG	invariant surface glycoprotein
ISG65*Pp*	ISG65 fusion protein expressed in *Pichia pastoris*
kb	Kilobase
kDa	kilo-Dalton
kDNA	kinetoplast DNA
Mbp	mega base pairs
MEC	molecular exclusion chromatography
min	minute(s)
M_r	relative molecular weight
MWCO	molecular weight cut off
N-terminal	amino terminal

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pol	DNA-dependent RNA polymerase
RNA	ribonucleic acid
RT	room temperature (22 ± 2 ^{o}C)
s	second(s)
SDS	sodium dodecyl sulfate
Tbb_{GST}ISG65	*T. b. brucei* ISG65 fusion protein with GST tag
Tbb_{GST}ISG75	*T. b. brucei* ISG75 fusion protein with GST tag
Tbb_{His}ISG65	*T. b. brucei* ISG65 fusion protein with His tag
Tbb_{His}ISG75	*T. b. brucei* ISG75 fusion protein with His tag
Tbg_{GST}ISG65	*T. b. gambiense* ISG65 fusion protein with GST tag
Tbg_{His}ISG65	*T. b. gambiense* ISG65 fusion protein with His tag
Tbg_{His}ISG75	*T. b. gambiense* ISG75 fusion protein with His tag
Tbg_{Pp}ISG75	*T. b. gambiense* ISG75 fusion protein expressed in *Pichia pastoris*
TBS	tris buffered saline
TEMED	N,N,N´,N´-tetramethyl ethylene diamine
TPP	three phase partitioning
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
VSG	variant surface glycoprotein

wks	Weeks
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YNB	yeast nitrogen base
YP	yeast extract, peptone
YPD	yeast extract, peptone, dextrose
2x YT	2 × yeast extract, tryptone

CHAPTER 1
Literature Review

1.1 Introduction

African trypanosomiasis is a disease caused by protozoa from the genus *Trypanosoma* of the Kinetoplastida order (Barrett *et al.*, 2003). This genus contains a number of species and sub-species which infect both animals and humans in Africa and other continents of the world (Haag *et al.*, 1998). Human African trypanosomiasis (HAT), commonly referred to as sleeping sickness, is caused by two sub-species of *Trypanosoma brucei: T. b. gambiense* and *T. b. rhodesiense* (El-Sayed *et al.*, 2000). Other *Trypanosoma* species, *T. b. brucei, T. congolense, T. evansi, T. equiperdum* and *T. vivax* cause the animal form of the disease, animal African trypanosomiasis (AAT) locally referred to as nagana (Losos and Ikede, 1972). Flies from *Glossina* species commonly referred to as tsetse fly, act as vectors for these trypanosomes with the exception of the species *T. evansi* and *T. equiperdum* (Steverding, 2008). The vectors for *T. evansi* are blood sucking insects of the genera *Tabanus, Stomoxys, Atylotus* and *Lyperosia* (Brun *et al.*, 1998). However, *T. equiperdum* is transmitted without an invertebrate vector via the sexual route during coitus (Claes *et al.*, 2005).

1.1.1 Economic impact of African trypanosomiasis

Trypanosomiasis in Africa has impacted significantly on the continent's economic activities directly or indirectly as a result of its impact on human and livestock health on the continent. The presence of tsetse flies has rendered a large proportion of Africa's arable land mass unsuitable for profitable livestock breeding and farming (Baral, 2010; Kuzoe, 1993; Figure 1.1). It is estimated that 50 million cattle and 10 million small ruminants in Africa are at risk of AAT in tsetse prevalent regions (Mattioli *et al.*, 2004). The annual loss to Africa due to animal deaths from trypanosomiasis infections has been estimated at over one billion US dollars (Kristjanson *et al.*, 1999). The human form of the disease has a mortality rate which is ranked ninth out of 25 among the human infections and parasitic

diseases in Africa (Fèvre *et al.*, 2004). In assessing the overall burden of this disease, it has been approximated that up to 1.35 million DALYs (disability-adjusted life year) are lost yearly (Fèvre *et al.*, 2004).

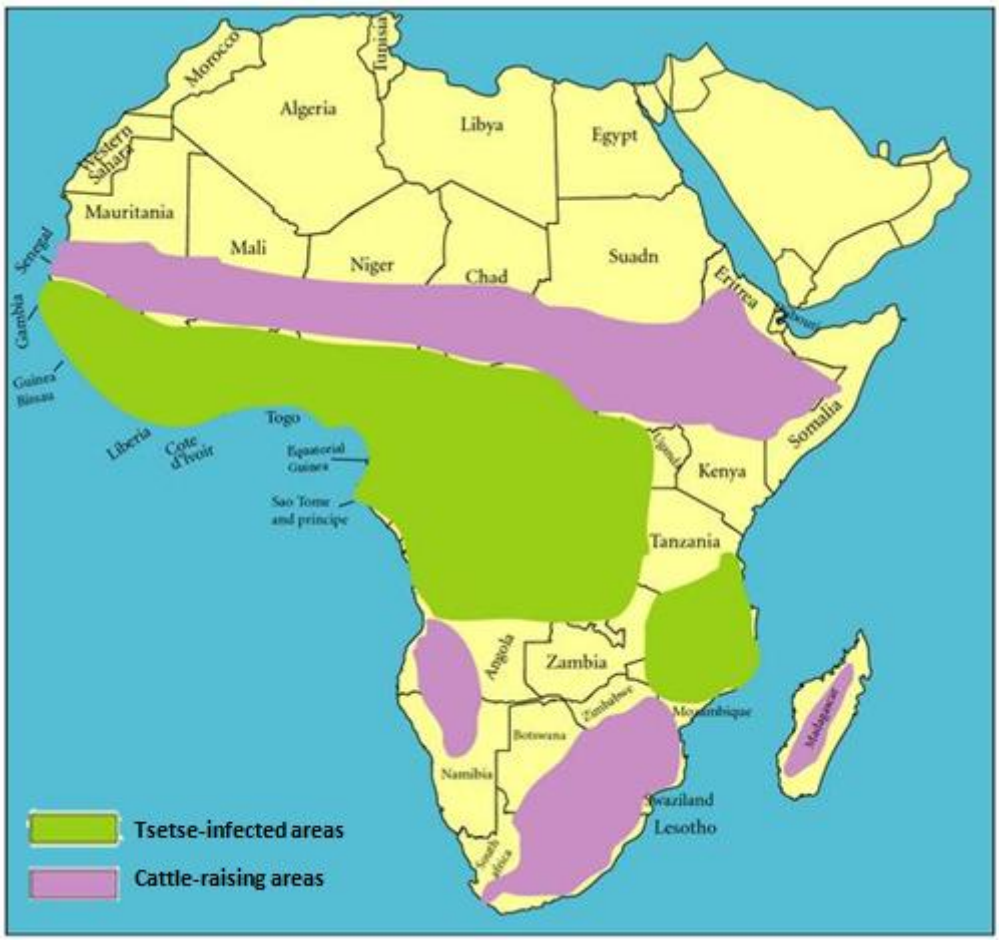


Figure 1.1 Distribution of tsetse and cattle raising areas in Africa (Baral, 2010).

1.1.2 Historical information

Occurrence of trypanosomiasis in Africa dates as far back as the 17th century (Steverding, 2008). Reports of the disease were made by colonial doctors in Western Africa, mostly making reference to the symptoms and effects of the disease on sufferers. Empirical information on the disease was not obtained until the early 19th century (1902) when trypanosomes were identified in the blood of a sick Englishman working on the Gambia

River. The parasites were named *Trypanosoma gambiense* (Maudlin, 2006). In 1910, *T. b. rhodesiense* was discovered in Zambia by J.W.W Stephens and H.B. Fanthan (Williams, 1996). Since then, substantial research has been dedicated to gain an understanding of the disease and combating its effects on humans and animals, in Africa and in other places in the world where the disease has been identified.

1.1.3 Epidemiology of human and animal trypanosomiasis

Trypanosomiasis, although prevalent in sub-Saharan Africa (WHO, 2013), also occurs in humans and animals in Asia, Central and South America, Europe and the Middle East (Baral, 2010). Chagas disease is a form of trypanosomiasis endemic in some parts of Latin America and is caused by *T. cruzi* (Castro *et al.*, 2006). In Africa, sleeping sickness has been identified as two distinct diseases, Gambian and Rhodesian sleeping sickness (Welburn *et al.*, 2001a). Both diseases are geographically separated along the line of the Great Rift valley with *T. b. gambiense* on the west and *T. b. rhodesiense* on the east (Hutchinson *et al.*, 2004; Figure 1.2). The Gambian HAT caused by *T. b. gambiense* is localised in the countries of West and Central African, including: Angola, Congo, Cote d'Ivoire, Gambia, Guinea, Nigeria and Sudan (Simarro *et al.*, 2010). Sleeping sickness in Eastern and Southern Africa predominantly in Tanzania, Uganda and Zambia, is, however, caused by *T. b. rhodesiense* (Hoare, 1972; Radwanska *et al.*, 2002). Uganda is the only country in which both diseases occur albeit in different regions of the country. In the North-West region of the country, *T. b. gambiense* is prevalent while *T. b. rhodesiense* is endemic in the South Eastern region (Fèvre *et al.*, 2001).

Although the two protozoans are morphologically indistinguishable (Welburn *et al.*, 2001a), the symptoms and clinical manifestations, pathogenesis and treatment are different for the two diseases (Maclean *et al.*, 2012). Rhodesian sleeping sickness is a virulent and generally acute infection which could be fatal in three to six months if left untreated (Odiit *et al.*, 1997) cited by (Fèvre *et al.*, 2001). However, *T. b. gambiense* sleeping sickness is a chronic infection that could persist for years in the sufferer (Giroud *et al.*, 2009). The primary reservoir of *T. b. gambiense* is humans (Jannin and Cattand, 2004), whereas wild and domestic animals act as reservoirs for Rhodesian sleeping sickness (Fèvre *et al.*, 2008;

Welburn *et al.*, 2001b). Pigs and some wild animals are minor reservoirs for *T. b. gambiense* (Malvy and Chappuis, 2011).

Animal African trypanosomiasis is a collection of diseases with the same or similar symptoms in animals caused by a number of different trypanosome species (Uzcanga *et al.*, 2001). It is predominant in regions where there is an overlap of the tsetse fly belt and the cattle grazing land. The reservoirs for AAT are wild and domestic animals. However, in areas where the domestic animals are trypanotolerant, they are excluded from being reservoirs of the disease (Van Den Bossche, 2001).

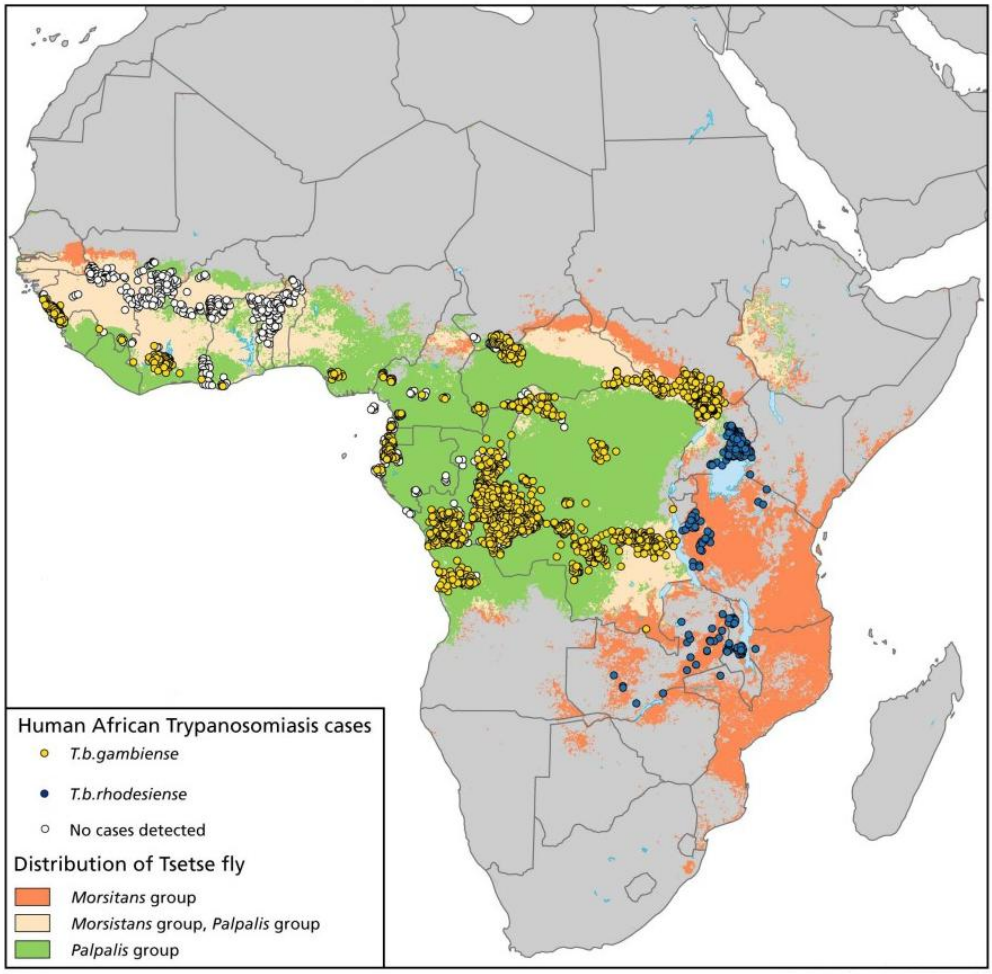


Figure 1.2 Map showing cases of human African trypanosomiasis, species of parasite and distribution of tsetse flies (Wertheim *et al.*, 2012).

1.1.4 Vectors of trypanosomiasis

Since the early 19th century, tsetse flies have been identified as vectors of African trypanosomiasis (Maudlin, 2006). These flies are classified under the order *Diptera* and family *Glossinidae*. There are three sub-genera under the genus *Glossina*, these include *Mortisans, Palpalis* and *Fusca* groups. Flies from these three groups are generally adapted to different habitats and ecozones (Leak, 1999).

The *Glossina morsitans* sub-species is the primary vector for human and animal trypanosomiasis in Eastern and Southern Africa, from Mozambique to Tanzania to Zambia and Zimbabwe (Rogers *et al.*, 1996; Figure 1.2). *Glossina pallidipes* are also important vectors in this region (Van Den Bossche, 2001). *Glossina longipalpis* and *G. palpalis*, which are vectors for *T. b. gambiense* occur in the savannah belt of West Africa, from Guinea to Cameroon (Tanowitz *et al.*, 2000; Figure 1.2). Other species like *G. pallidipes* are highly mobile and occur over a wide range of climatic vegetation conditions.

1.1.5 Innate immunity and trypanotolerance

Humans generally exhibit some degree of innate protection to trypanosomiasis. This immune protection is mediated by a minor class of high density lipoproteins present in human sera called trypanosome lytic factor-1 [(TLF)-1] (Widener *et al.*, 2007). The (TLF)-1 comprises three major proteins: apolipoprotein A-I, haptoglobin-related protein and apolipoprotein L-1 (Kieft *et al.*, 2010). Some trypanosome species, *T. b. gambiense* and *T. b. rhodesiense*, however, have evolved mechanisms that enable them to escape lysis by human serum, thus enabling them to infect humans. The possession of a gene known as *serum-resistance-associated* (*SRA*) by *T. b. rhodesiense* is responsible for its ability to resist lysis by human sera. This gene is found exclusively in *T. b. rhodesiense* and its sequence is conserved across isolates (Gibson, 2005). In humans, SRA binds to TLF-1 leading to the formation of an SRA/TLF-1 complex that allows the parasite to continue to proliferate. On the other hand, *T. b. gambiense* lacks the *SRA* gene. Its mechanism of inducing resistance is due to a loss of expression of a functional haptoglobin/haemoglobin

(Hp/Hb) receptor, which is necessary for the uptake of TLF-1 by the parasite (Kieft *et al.*, 2010).

Trypanotolerance describes the ability of certain breeds of cattle to remain productive and healthy after infection with trypanosomes (Naessens, 2006). Indigenous taurine (*Bos taurus*) breeds of cattle such as the N'Dama and West African short horn are naturally resistant to trypanosomiasis and are termed trypanotolerant. These animals exhibit, to varying degrees, the capacity to limit and control parasitaemia and anaemia which are the main pathogenic effects of trypanosomiasis (Black *et al.*, 2001; Guirnalda *et al.*, 2007). Trypanotolerance is also common in game animals in Africa which act as reservoirs of the disease (Brown, 2008). The boran (*Bos indicus*) cattle breeds, for example the Zebu cattle, which have humps, are susceptible to trypanosomiasis and are termed trypanosusceptible. Trypanotolerant breeds are exploited in the humid and sub-humid zones of West and Central Africa for sustainable livestock production (D'Leteren *et al.*, 1998).

1.2 Biology of African trypanosomes

1.2.1 Classification of African trypanosomes

Trypanosomes are eukaryotes belonging to the Protista kingdom in the Eukarya domain. They are classified under the phylum Sarcomastigophora, sub-phylum Mastigophora and class Zoomastigophora (Maudlin *et al.*, 2004). Like other members of this sub-phylum, trypanosomes possess whip-like flagella which they use for locomotion. The Trypanosomatidae family is placed under the order Kinetoplastida because they possess a unique organelle at the base of the flagellum, the kinetoplast (Baral, 2010), which is an extension of the mitochondrion. There are different groups under the *Trypanosoma* genus namely, salivaria and stercoraria (Figure 1.3). This classification is based on their mode of transmission by their vectors (Haag *et al.*, 1998). The salivarian parasites are transmitted through the saliva of their invertebrate vector while the stercoraria trypanosomes are transmitted to their animal hosts through faeces (Baral, 2010). The infectious trypanosomes for HAT and AAT i.e. *T. brucei, T. congolense, T. equiperdium, T. evansi, T. simiae, T. suis and T. vivax* all fall under the salivaria group (Figure 1.3). On the other

hand, *T. cruzi*, *T. rangeli*, *T. theileri* all fall under the stercoraria group (Mitashi *et al.*, 2012).

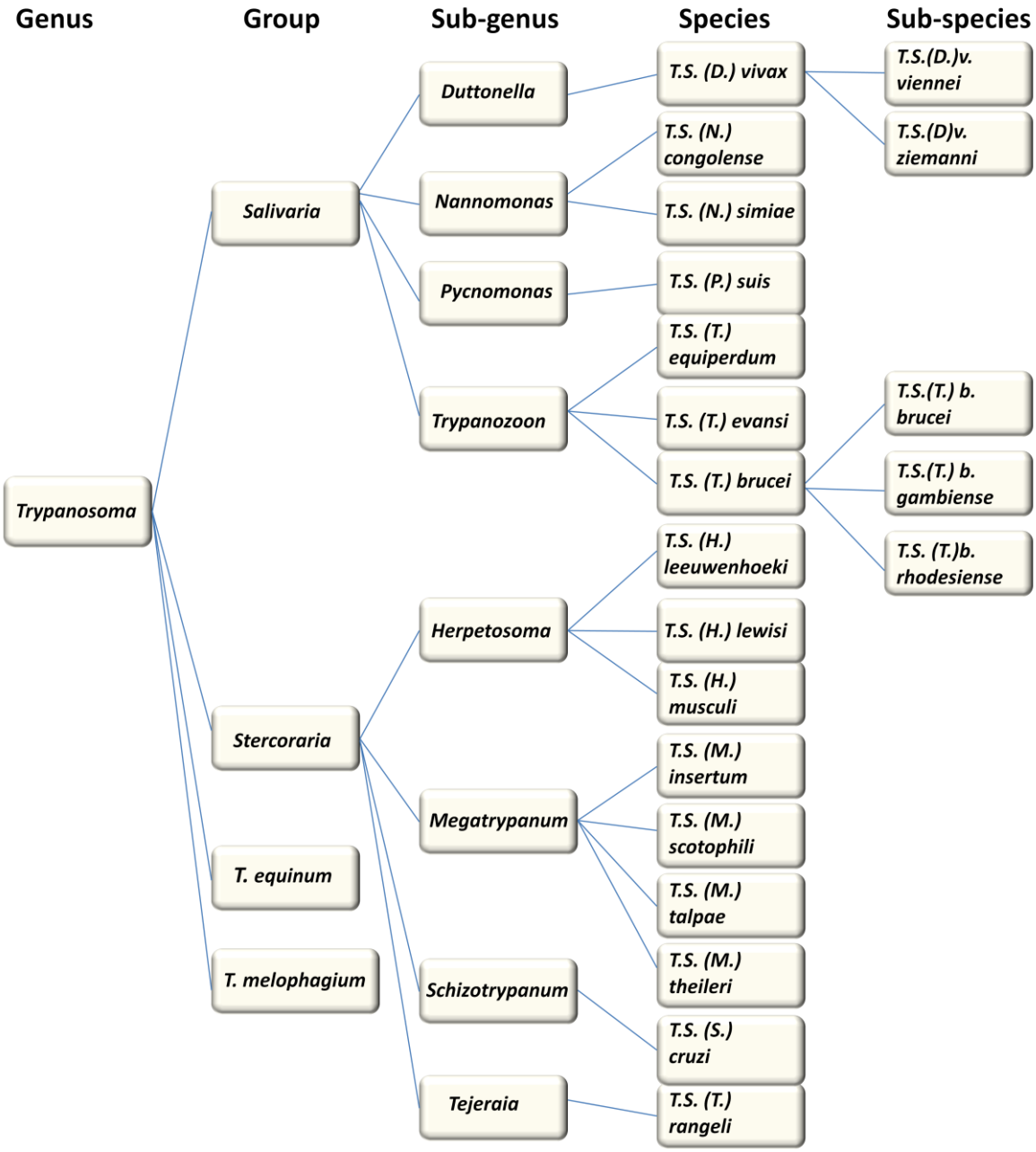


Figure 1.3 Classification of trypanosomes [Adapted from Vickerman (2000)].

1.2.2 The trypanosome genome

Trypanosomes are diploid organisms with both strands of the megabase chromosomes containing long overlapping gene clusters (Berriman, 2005; Daniels *et al.*, 2010). The trypanosome genome is organised into large directional gene clusters, containing protein-coding genes in head-to-tail orientation. In the *T. brucei* genome, there are 388 clusters of two or more unidirectional genes (Barry and Mcculloch, 2001). The genetic information of *T. brucei* totals approximately 35 Mb per haploid genome, and is enclosed in a nucleus with a diameter of approximately 2.5 µm. In the trypanosome nucleus, nuclear functions such as transcription and replication are compartmentalised. The most distinctive nuclear compartment is the nucleolus and it is sub-structured into fibrillar and granular components. The genes are generally not distributed according to function or transcript abundance. Transcription is polycistronic so the majority of gene expression takes place post transcriptionally (Daniels *et al.*, 2010). Three classes of eukaryotic DNA-dependent RNA polymerase (pol) are found in trypanosomes: pol I, pol II and pol III (Daniels *et al.*, 2010). Each enzyme class is responsible for the transcription of different types of RNA (Boeger *et al.*, 2005).

1.2.3 Morphology of the trypanosome

African trypanosomes are a large group of unicellular protozoan parasitic organisms. They are generally spindle shaped cells of 20-30 µm in length and 1.5-3.5 µm in width (Figure 1.4) with a single flagellum (Chappuis *et al.*, 2005). The flagellum, which can be free or attached, emerges from a specialised invagination of the plasma membrane called the flagellar pocket. The flagellar pocket is involved in the endocytosis and exocytosis pathways of the cell (Nolan *et al.*, 1997). It is also part of a multi-organelle complex involved in cell polarity and division (Field and Carrington, 2009). The flagellum runs along the entire length of the cell from its point of emergence at the posterior, to the anterior part of the cell.

The distinguishing feature of the trypanosome is its kinetoplast, a self replicating DNA-containing organelle, which is a specialised portion of the mitochondrion. The

mitochondrial DNA, which is different from the nuclear DNA, is organised in an interlocked and super coiled network in the kinetoplast (Gehrig and Efferth, 2008). It is also called kinetoplast DNA and exists in two forms: mini and maxi circles (Ryan *et al.*, 1988). Trypanosomes also possess other cellular organelles including the nucleus, Golgi apparatus, glycosomes, rough and smooth endoplasmic reticulum, and various vesicles (Brun *et al.*, 1998). The glycosome is a special organelle in trypanosomes which is compartmentalised for glycolysis (Michels, 1988).

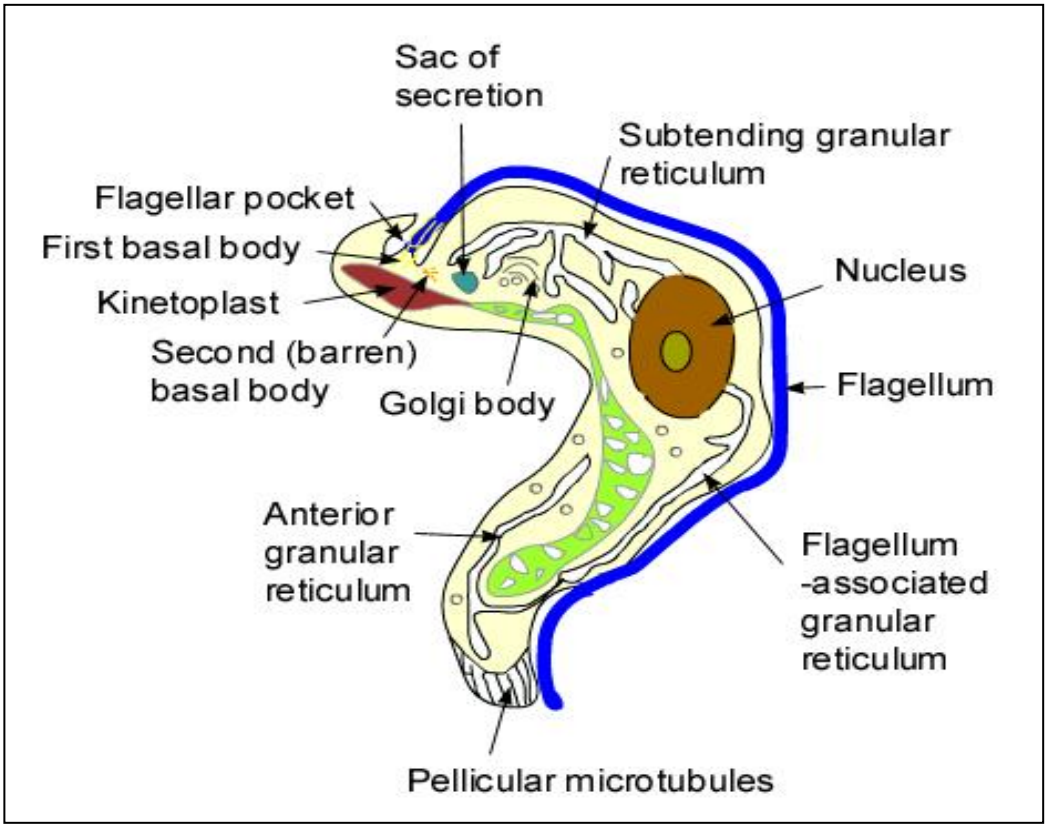


Figure 1.4 Diagrammatic representation of *Trypanosoma brucei* indicating its morphological features (Vickerman, 1969).

The morphology of *T. evansi* and *T. equiperdum* is not vastly different from that of *T. brucei*. They possess a free flagellum and a small sub-terminal kinetoplast (Chappuis *et al.*, 2005). Dyskinetoplastic forms, in which the kDNA is absent, have been reported in both species. This occurs in wild strains due to mutation or treatment with trypanocides such as

diminazene as well as dyes like ethidium bromide. Dyskinetoplastic forms have also been observed in long term *in vitro* cultivation of the parasites (Brun *et al.*, 1998).

1.2.4 Life-cycle of trypanosomes

The life-cycle of trypanosomes is digenetic (Gehrig and Efferth, 2008), alternating between its mammalian host and the invertebrate vector (Figure 1.5). In the midgut of the tsetse fly, the trypanosome exists in its procyclic form. It multiplies by binary fission, migrates to the salivary glands and is transformed into the short epimastigote form. These epimastigotes attach to the epithelial cells in the fly's salivary glands, proliferate, and differentiate into the free, non dividing metacyclic forms (Savage *et al.*, 2012).

The life-cycle stages of the trypanosomes in the mammalian host begin when the tsetse takes a blood meal from the host (Gehrig and Efferth, 2008). The metacyclic trypomastigotes in the tsetse's saliva are inoculated into the mammal (Figure 1.5, stage 1). The parasites multiply locally at the site of the bite and migrate into the bloodstream, lymphatic system and extracellular spaces, where they differentiate into slender BSFs (Figure 1.5, stages 2-4). Two different trypomastigote forms, the long, slender proliferative BSFs and a short, stumpy non-dividing form exist in the mammalian host (Nolan *et al.*, 1997). The slender forms continue proliferating and then differentiate into the short stumpy form, which is re-introduced into the tsetse fly during a bloodmeal (Matthews, 1997). The stumpy form differentiates into the procyclic form in the tsetse's midgut (Savage *et al.*, 2012) and the cycle continues (Figure 1.5, stage 6).

Generally, African trypanosomes, *T. brucei, T, congolense and T. vivax* have essentially the same life-cycles apart from a few modifications. After establishing an infection in the midgut of the fly, *T. congolense* migrates to the proboscis (mouth parts) rather than the salivary glands (Urwyler *et al.*, 2007), where it attaches as the epimastigote form. Unlike other African trypanosomes which are extracellular (Chappuis *et al.*, 2005), *T. congolense* is a strictly intravascular parasite. It also does not invade the tissues of its mammalian host like the *T. brucei* parasites (Coustou *et al.*, 2010). *Trypanosoma vivax* does not have a midgut stage at all, but develops in the proboscis. This has made it easier for it to adapt to

different vectors. However, for *T. cruzi*, the metacyclic trypomastigote form exists in the hindgut of its vector the reduviid bug (De Souza, 1984). It is then transmitted to humans by faecal contamination on the skin or in mucous membranes. It exists in amastigote and trypomastigote forms in its human host (De Souza, 2002).

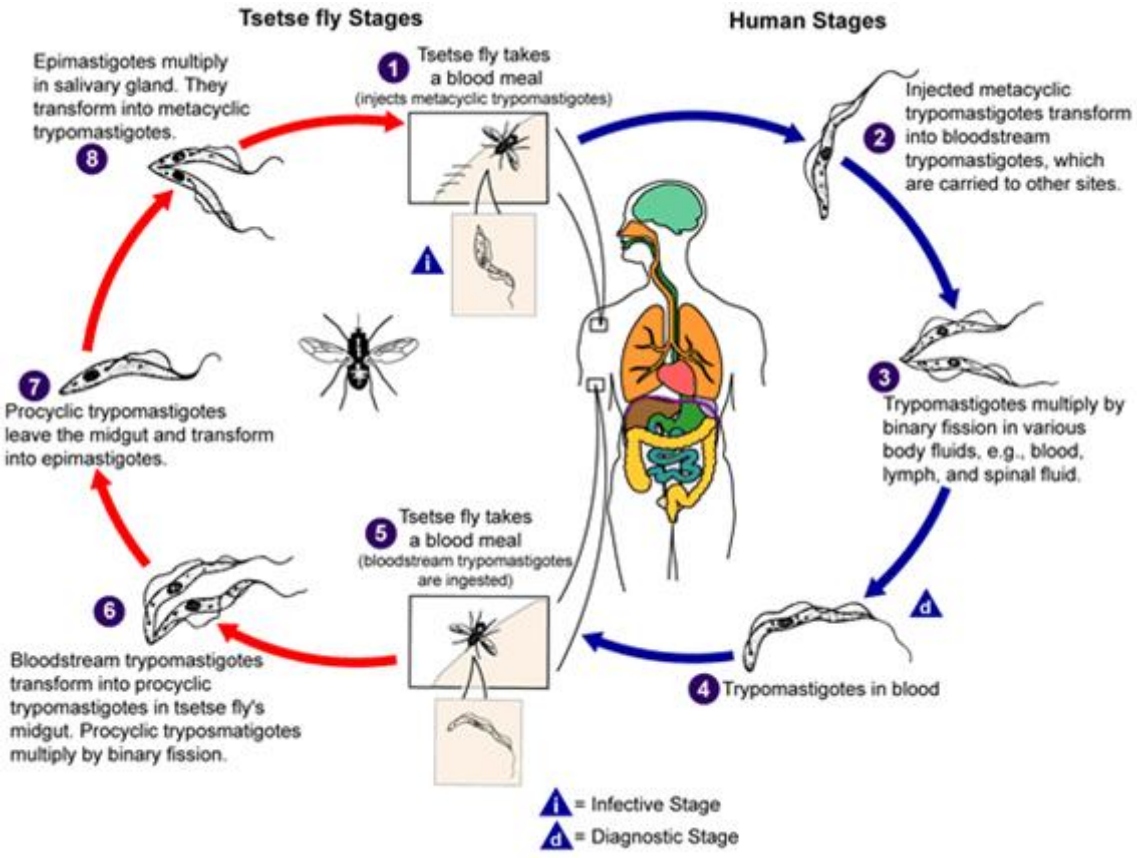


Figure 1.5 Diagrammatic representation of the life cycle of *Trypanosoma brucei* taken from (Chappuis *et al.*, 2005).

1.2.5 Surface proteins of trypanosomes

The generic life-cycle changes of the trypanosome between its vector and human host are characterised by the expression of different surface proteins. These proteins are involved in a variety of functions including immune evasion, attachment or invasion of the host's extra vascular system (Savage *et al.*, 2012). The surface area of trypanosomes consists of approximately 10^7 variable surface glycoprotein (VSG) molecules which occupy most of

the surface membrane, forming a densely packed monolayer (Mehlert *et al.*, 1998; Stockdale *et al.*, 2008). It is a highly fluid surface barrier with rapid lateral diffusion of glycophosphatidyl inositol (GPI)-anchored VSG molecules within the coat (Bulow *et al.*, 1988). Variable surface glycoproteins, the most abundant surface proteins in the BSFs of these parasites are very important for their evasion of mammalian host's immune system (Donelson *et al.*, 1998). The VSGs are replaced by GPI-anchored procyclins upon differentiation from BSFs to procyclic forms in the tsetse's midgut (Walrad *et al.*, 2009).

Other trypanosome surface proteins include: adenylate cyclase, a cysteine rich acidic integral membrane protein (CRAM), the flagellum adhesion protein, a glucose transporter, the transferrin receptor, GPI anchored *brucei* alanine rich protein (BARP) and ISGs (Lee *et al.*, 1990; Mehlert *et al.*, 2012a; Nolan *et al.*, 1997; Nolan *et al.*, 2000; Urwyler *et al.*, 2007; Yang *et al.*, 2000; Ziegelbauer and Overath, 1992). The latter will be described further in section 1.3.

1.2.6 Antigenic variation in trypanosomes

Trypanosomes are pre-adapted to evade their host's immune system by their ability to switch expression of their surface coat of VSG in a process called antigenic variation (Graham and Barry, 1995; Hall *et al.*, 2013). With increasing parasitaemia in an infected host, antibodies produced by the host's complement system actually "kill" most of the parasites. However, a small parasite population survives and these are able to switch their VSG coat expression to an alternate and immunologically distinct VSG (Lythgoe *et al.*, 2007; Marcello and Barry, 2007). These parasites proliferate and start a new wave of parasitaemia in the host. Through this mechanism, trypanosomes are able to maintain chronic infection in an untreated subject (Barry and Mcculloch, 2001).

The genome of trypanosomes is estimated to contain over 1000 transcriptionally silent VSG genes, which are activated clonally during an infection (Agur *et al.*, 1989; Graham and Barry, 1995; Weirather *et al.*, 2012). The N-terminal domain of the VSG is variable due to its exposure to the host's immune system (Schwede *et al.*, 2011). It consists of 350-400 amino acid residues, while the C-terminal domain consists of 40-80 residues and is

more conserved (Carrington *et al.*, 1991). The C-terminal residue links the VSG to the parasite membrane via the GPI-anchor (Overath *et al.*, 1994b). Differential VSG gene expression in trypanosomes is achieved by the use of programmed gene rearrangements in conjunction with specialised transcription units (Graham and Barry, 1995) or alternate activation, without DNA rearrangement of different telomeric expression sites (Pays, 1991).

1.3 Invariant surface glycoproteins (ISGs) of trypanosomes

Invariant surface glycoproteins (ISGs) are a family of stage specific invariant proteins identified only in the BSFs of trypanosomes. These proteins were identified in the early 1990s through surface labelling of BSF trypanosomes isolated from the blood of rodents infected with *T. brucei* (Ziegelbauer and Overath, 1992; Ziegelbauer *et al.*, 1992). The ISGs identified include ISG64, ISG65, ISG70, ISG75 and ISG100 (Jackson *et al.*, 1993; Nolan *et al.*, 1997; Ziegelbauer and Overath, 1992). They were named based on their apparent size from migration on SDS-PAGE gels (Ziegelbauer and Overath, 1992). These proteins are N-glycosylated and are distributed over the entire surface of the trypanosome. Invariant surface glycoprotein-65 and ISG75 are the major non-VSG glycoproteins distributed on the surface of BSF trypanosomes (Jackson *et al.*, 1993; Ziegelbauer and Overath, 1992). There is an endosomal pool of ISGs in addition to the surface localisation (Chung *et al.*, 2004). Unlike the other ISGs distributed on the surface of the parasite, ISG100 is localised in the flagellar pocket, in a perinuclear lysosomal-like vacuole, as well as in small endosomal-like vesicles in the parasite (Baral, 2010; Nolan *et al.*, 1997).

1.3.1 The genomic organisation of *invariant surface glycoprotein* (*ISG)* genes

In the trypanosome genome, the genes for ISG65 and ISG75 exist in multiple gene copies in the range of 4-16 copies per genome. These genes are clustered at two loci (Ziegelbauer *et al.*, 1995) and are arranged in tandem repeats (Overath *et al.*, 1994a). The *ISG65* and *ISG75* gene families are conserved across the members of the *Trypanozoon* sub-genus: *T. b. brucei, T. b. gambiense, T. b. rhodesiense, T. evansi and T. equiperdum* (Tran *et al.*, 2006; Tran *et al.*, 2008). Their presence in other sub-genera is variable. The ISG65 and

ISG75 polypeptides have a large extracellular domain, a single trans-membrane domain and a small C-terminal intracellular domain. Both the trans-membrane and C-terminal domain of these glycoproteins are highly conserved in contrast to the N-terminal domains which is heterogenous (Chung *et al.*, 2004). The cytoplasmic domain of both ISG65 and ISG75 has three conserved lysine residues.

Upon maturation of the ISG polypeptide, the N-terminal signal peptide is cleaved off before targeting to the trypanosome surface. This signal sequence comprises of a few amino acids; 21 in the case of ISG65 and 29 for ISG75 (Ziegelbauer *et al.*, 1992). The *ISG100* gene has a different topology to *ISG65* and *ISG75*. It is a single copy gene that codes for 100 kDa protein with a large internal domain composed of a serine-rich repeat motif representing over 90% of the coding frame (Nolan *et al.*, 1997).

1.3.2 Structure and function of invariant surface glycoproteins (ISGs)

There are about 70,000 and 50,000 molecules of ISG65 and ISG75 respectively per cell (Leung *et al.*, 2011; Ziegelbauer *et al.*, 1992). These trans-membrane proteins span the trypanosome's membrane in an alpha-helix. The organisation of the ISGs in the surface coat is not fully understood but it has been proposed that they adopt a compact globular structure that is hidden in niches formed by the extended rod-like VSG molecules (Freymann *et al.*, 1990; Ziegelbauer *et al.*, 1992; Figure 1.6). This shielding of ISGs by VSGs is thought to prevent them from being exposed to the host's antibodies in the course of an infection (Ziegelbauer *et al.*, 1992). More recent work has, however, demonstrated that ISGs are actually recognised by the host's immune system (Radwanska *et al.*, 2000b) and are exposed to the host's antibodies (Chung *et al.*, 2004; Giroud *et al.*, 2009). It was explained that the coiled conformation assumed by the highly conserved C-terminal domains of ISGs in the coat (Figure 1.6, H) make them accessible to the host's immune system despite shielding by VSGs (Leung *et al.*, 2011).

Given that trypanosomes are not cleared by their host's anti-ISG antibodies, the possibility that ISGs also have mechanisms in place for evading the host's immune responses has been raised (Leung *et al.*, 2011). Their mechanism of immune evasion as hypothesised is a

combination of an efficient capping system via hydrodynamic flow (Engstler *et al.*, 2007), rapid endocytosis and their efficient turnover (Chung *et al.*, 2004).

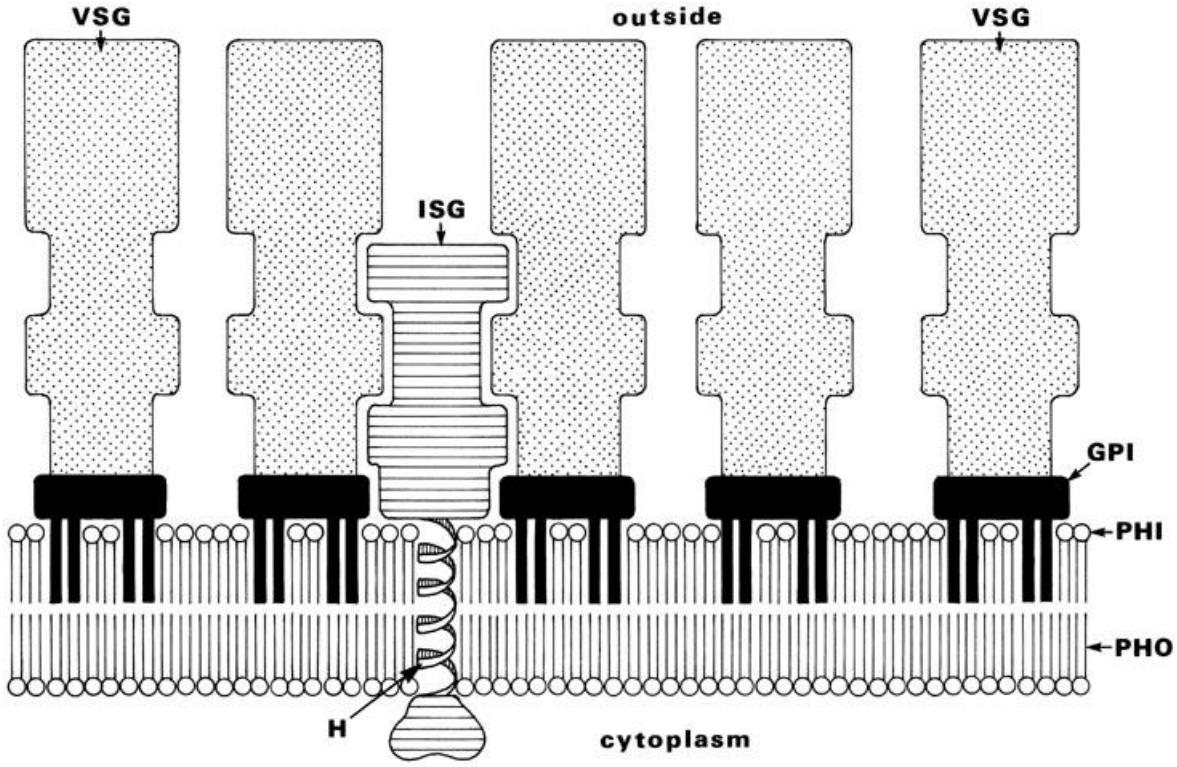


Figure 1.6 The trypanosome's surface coat (Mehlhorn, 2008).

In BSF trypanosomes, endocytosis is an important process for the turnover of surface ligands as well as for the recycling of important surface glycoproteins like the VSGs and ISGs (Natesan *et al.*, 2011). All endocytosis is mediated via uptake at the flagellar pocket (Figure 1.7). The upregulation of endocytic activity in *T. brucei* BSFs compared with the procylic forms, infers a direct involvement of endocytosis in pathogenicity and immune evasion processes of trypanosomes (Carlander *et al.*, 1999). In contrast to VSGs which are highly stable with a half life of 72 hours (Field *et al.*, 2010), ISGs are rapidly recycled and have a very high turnover rate, with a half life of approximately three to six hours (Hädge and Ambrosius, 1984). ISGs intercalate with VSG on the surface, but are rapidly turned over by an ubiquitylation-dependent mechanism (Chung *et al.*, 2008). Invariant surface glycoproteins are able to endocytose and deliver bound antibody from the surface, into the

endosomal system in a manner similar to VSGs (Chung *et al.*, 2004; Kelley *et al.*, 1999; Leung *et al.*, 2011). These findings have led to the postulations that ISGs perform a role in pathogenicity of the parasite (Koumandou *et al.*, 2013; Chung *et al.*, 2004).

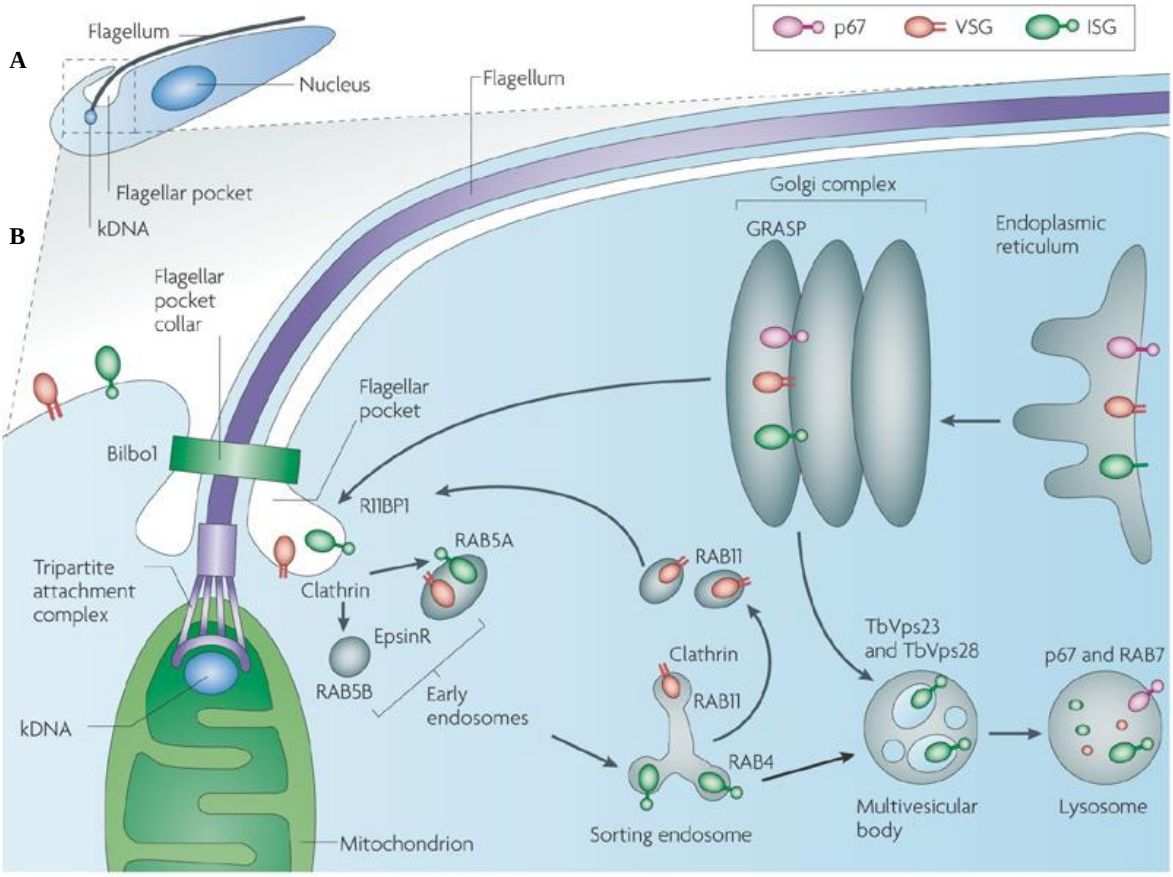


Figure 1.7 Endocytosis and the trypanosome flagellar pocket. Schematic showing the trafficking of ISGs and VSGs through the endosomal system. **(A)** A trypanosome. The flagellar pocket is the site of endocytosis in trypansosomes. **(B)** Enlarged section of the flagellar pocket. Endocytosis of ISGs and VSGs through the flagellar pocket is clathrin dependent. Internalised ISGs and VSGs are delivered to the early and sorting endosomes via the RAB proteins. Some population of the ISGs and VSGs is recycled back to the trypanosome surface and the rest is sorted via the multivesicular body to the lysosome for degradation. After synthesis in the endoplasmic reticulum, ISGs and VSGs are transported through the Golgi complex to the trypanosome surface via the flagellar pocket (Field and Carrington, 2009).

Experiments to determine the trafficking route of ISGs showed that they are co-localised with the endosomal trafficking protein "required for receptor-mediated endocytosis-8" (RME-8). This highly conserved protein is broadly distributed across multiple endocytic compartments and is a mediator of recycling pathways (Koumandou *et al.*, 2013). Invariant

surface glycoprotein-65 and ISG75 also co-localise with RAB4, RAB5A and RAB11 (Leung *et al.*, 2011; Figure 1.7) while ISG100 co-localises with RAB5B (Hall *et al.*, 2004). The RAB proteins are highly compartmentalised and have been described as ideal candidates for determining organelle identity and transport specificity in cells (Zerial and Mcbride, 2001). The identification of ISGs in the recycling pathways mediated by the RAB proteins as well as their association with RME-8 seems to accentuate the fact that ISGs are recycled and RME-8 is required to support their recycling. Also, the short half life of ISGs is consistent with a recycling role in the trypanosomes.

There is evidence for the involvement of ISG75 in the uptake of suramin, a drug used for treating HAT. It is proposed that ISG75 binds the drug at the cell surface and the suramin-ISG75 complex is delivered via the flagellar pocket to the endosomal system, leading to accumulation in the lysosome where the drug is liberated by proteases (Alsford *et al.*, 2012). An in depth understanding of ISG trafficking could shed more light on immune evasion and drug interactions in trypanosomes (Koumandou *et al.*, 2013). The presence of ISG100 in the flagellar pocket suggests that it might play a role in the pathway for the endocytosis or it may have a structural role in the compartments involved in intracellular trafficking of trypanosomes (Nolan *et al.*, 1997).

1.4 Diagnosis and treatment of African trypanosomiasis

1.4.1 Pathogenesis of African trypanosomiasis

Trypanosomiasis infection in humans is generally divided into two stages: the haemo-lymphatic stage and the neurological or meningo-encephalitic stage (Amin *et al.*, 2010). The first stage is characterised by waves of parasitaemia which is sustained by multiplication of the parasites, immune responses by the host, as well as antigenic variation by the trypanosomes (Duszenko *et al.*, 2006). The parasites are confined to the tissues and body fluids in the first stage and subsequently cross the blood-brain barrier to invade the central nervous system (CNS) in the second or neurological stage (Kristensson *et al.*, 2010).

Symptoms typically experienced in first stage HAT include: headache, fever, joint pains, facial oedema, pruritus or itching, moderate to severe anaemia, general malaise and mental changes. Some patients experience swelling of lymph nodes in the posterior triangle of the neck called Winterbottom's sign (Barrett *et al.*, 2003) The Winterbottom's sign, named after Thomas Winterbottom who first noticed this swelling in the 1800s, is typical in *T. b. gambiense* HAT. However, chancres, inflammatory lesions at the location of the tsetse fly's bite on the skin, are typical of *T. b. rhodesiense* infections (Barrett *et al.*, 2003). Mental changes associated with *T. b. gambiense* infections include: mental confusion, emotional instability, manic episodes, melancholia, delirium or dementia. In patients suffering from *T. b. rhodesiense* HAT, mental slowness, dullness, mania, or hallucinatory states may be observed (Büscher and Lejon, 2004). Second stage patients experience confusion, endocrine dysfunction, reversal of the diurnal wake/sleep rhythm leading to sensory sleep disturbances, mental changes, tremor, paraesthesia and poor coordination.

The clinical manifestations of trypanosomiasis in cattle and domestic animals include: cachexia, a chronic wasting characterised by anaemia, weight loss and immuno suppression, chronic weakness, recurrent fever, reproductive problems, such as still births, abortions, amongst others (Nantulya, 1990). Anaemia is the major cause of death in infected cattle (Naessens, 2006). Generally, in susceptible animals, trypanosomiasis is a chronic disease, although acute cases have been reported for *T. vivax* infections in cattle and small ruminants (Bode *et al.*, 2011). The mortality rate is high if these animals are left untreated. Treated animals could also die due to relapses in conditions of high stress. Factors like poor nutrition, also contribute to the morbidity of the infected animals.

1.4.2 Diagnosis of African trypanosomiasis

The diagnosis of HAT is done in three phases which are; mass population screening, diagnostic confirmation and staging. The first phase of *T. b. gambiense* HAT diagnosis is achieved through the use of rapid population screening tools like the card agglutination test for trypanosomiasis (CATT), immunofluorescent antibody test (IFAT), latex card agglutination test (LATEX) and enzyme-linked immunosorbent assays (ELISAs) (Chappuis *et al.*, 2005).

The CATT/*T. b. gambiense* is a fast and simple agglutination assay for detecting *T. b. gambiense* specific antibodies in blood, plasma or serum (Brun *et al.*, 2010; Magnus *et al.*, 1978). The antigen consists of whole, stained BSF trypanosomes expressing LiTat1.3 VSGs (Hutchinson *et al.*, 2004). The major challenge with this test is the fact that it relies on a single VSG, which might not be expressed in the course of that infection (Asoganyi *et al.*, 1994; Dukes *et al.*, 1992). Also, CATT could give false positive tests because antibodies could persist for years in a cured HAT patient (Paquet *et al.*, 1992). The LATEX/*T. b. gambiense* test procedure is similar to CATT and it shows higher specificity and similar sensitivity to CATT (Chappuis *et al.*, 2005). The test is based on the detection of antibodies that agglutinate three VSGs: LiTat1.3, LiTat1.5 and LiTat1.6 immobilised on latex beads (Penchenier *et al.*, 2003; Truc *et al.*, 2002).

Molecular amplification techniques are also applicable and sensitive for the diagnosis of trypanosomiaisis (Radwanska *et al.*, 2002; Mugasa *et al.*, 2012; Mwandiringana *et al.*, 2012). Some of the available techniques include: conventional PCR, real-time PCR, oligochromatography-PCR (PCR-OC), loop-mediated isothermal amplification (LAMP) and real-time nucleic sequence-based amplification (NASBA) (Matovu *et al.*, 2012; Mitashi *et al.*, 2012). The LAMP relies on the amplification of a multicopy transposon-like sequence for the detection of the parasite (Kuboki *et al.*, 2003; Mitashi *et al.*, 2012; Njiru *et al.*, 2008). It can amplify specific DNA sequences under constant temperature thus eliminating the need for a thermocycler. It is applicable to the diagnosis of both *T. b. gambiense* and *T. b. rhodesiense* HAT using blood, serum or CSF samples (Mitashi *et al.*, 2012). The LAMP does not require post-amplification handling and the equipment required is less expensive than that for PCR, thus it is a promising molecular diagnostic tool for rural areas (Matovu *et al.*, 2012; Mugasa *et al.*, 2012). Real-time nucleic sequence-based amplification is a real-time PCR involving the amplification of RNA. The major short coming is the need for RNA purification procedures however, it is more sensitive than conventional PCR (Matovu *et al.*, 2012).

Enzyme-linked immunosorbent assays for the diagnosis of HAT use the same antigens as LATEX/*T. b. gambiense* but in this case, they are fixed on ELISA plates. High sensitivity

of the test has been reported using sera, plasma or saliva as test samples. Immunofluorescent antibody test (IFAT) developed in the 1970s is also used for the diagnosis of *T. b. gambiense* HAT. It involves the detection of *T. b. gambiense* specific IgG using IgG-specific fluorescent conjugates. This test is applicable to samples like sera, dried blood on filter paper and cerebrospinal fluid (CSF). The requirement for expensive and sophisticated equipment, however, prohibits their use for field tests and mass population screening (Mitashi *et al.*, 2012). New lateral flow immunochromatographic rapid diagnostic tests for serodiagnosis of *T. b. gambiense* HAT are in advanced stages of development. These tests have improved sensitivity over CATT and also are packaged in a single patient reagent format that prevents waste unlike the multi patient format of CATT (Yansouni *et al.*, 2013). One of such new lateral flow rapid diagnostic tests for the diagnosis of HAT is the SD BIOLINE HAT designed by Standard diagnostics incorporated and Foundation for Innovative New Diagnostics (FIND). It was launched in December 2012 in Kinshasha, Democratic Republic of Congo (DRC) after undergoing successful clinical trials in Angola, the DRC and Central African Republic (FIND, 2012b).

The second phase of diagnosis involves the confirmation of the presence of the parasites in bodily fluids like blood, CSF, chancre or lymph node aspirates. This is necessary due to the occurrence of false positive results from mass population screening. It can be achieved by direct application of the blood sample onto a microscope slide prior to examination under the microscope. The microscope slides can be prepared as wet blood films or thick blood films. The sensitivity reported for these methods is variable (Mitashi *et al.*, 2012). Other parasitological methods that offer improved sensitivity include: the mini haematocrit centrifugation technique (mHCT), mini anion exchange centrifugation technique (mAECT) and quantitative buffy coat (QBC) method (Brun *et al.*, 2010). These methods involve the concentration of the parasites via centrifugation. The samples are placed in special capillary tubes designed for this purpose. The QBC, however, involves staining of parasites with acridine orange to facilitate visualisation by fluorescence microscopy. This method has been reported to be 100% sensitive. It, however, requires, expensive specialised equipment (Chappuis *et al.*, 2005; Mitashi *et al.*, 2012).

One of the major challenges to parasitological detection of *T. b. gambiense* HAT is the characteristically low parasitaemia observed, with as few as 100 parasites/ml in some cases (Chappuis *et al.*, 2005; Matovu *et al.*, 2012). The presence of fewer parasites in circulation makes it more difficult to make a positive identification. Also most parasitological methods are laborious and time consuming.

The third phase of trypanosomiasis diagnosis is stage determination. Staging of the disease is a critical step that guides the choice of treatment, due to the fact that the drugs used to treat first and second stage HAT are different. Moreover, the drugs for second stage HAT are very toxic and care is taken to avoid the prescription of such drugs for first stage sufferers (Matovu *et al.*, 2012). Confirmation of the presence of the trypanosomes in the CSF is conclusive for diagnosis of second stage HAT (Hutchinson *et al.*, 2004). Elevation of certain biomarkers like CSF white blood cell count, CSF protein concentration, IgM and neopterin can also be indicative of second stage HAT (Matovu *et al.*, 2012; Tiberti *et al.*, 2013). The cut-off value for white blood cell count in patients with HAT is five cells/µl in CSF (WHO, 1998) and patients with higher CSF values than these are classified to be in second stage HAT (Lejon and Büscher, 2005). More recent studies, however, recommend a cut-off value above 20 cells/µl especially for patients suffering from *T. b. gambiense* HAT (Maclean *et al.*, 2012). The increase in CSF protein level is accounted for by the elevation of immunoglobulin expression (Maclean *et al.*, 2012). The cut-off values prescribed varies according to the method used for protein quantification. The values include: 250 mg/L for trichloroacetic acid precipitation, 370 mg/L for colorimetric methods and 450 mg/L for sulfosalicylic acid precipitation (Lejon and Büscher, 2005; WHO, 1983). In practice, however, protein determination is rarely performed for stage determination in rural areas due to the unavailability of the reagents (Lejon and Büscher, 2005).

There are no equivalents to the CATT and LATEX tests for the diagnosis of early stage *T. b. rhodesiense* infection. Proteomic screening methods to identify specific and sensitive diagnostic antigens have not been successful so far (Manful *et al.*, 2010). Immunofluorescence and ELISA-based tests are available but expensive. Screening is usually done by direct observation of trypanosomes in peripheral blood of patients. Second

and third phase diagnosis of the *rhodesiense* HAT is carried out by the same techniques used for *gambiense* HAT.

In general, the diagnosis of animal trypanosomiasis infections is carried out by direct observation of the parasites in blood or buffy-coat samples (Hutchinson *et al.*, 2004). A positive test result is taken as conclusive proof of the disease. Sero-diagnostic tests like CATT/*T. evansi* which is similar to the *T. b. gambiense* CATT are also used to detect *T. evansi* (OIE, 2012). Another serological diagnostic assay for AAT is the complement fixation test (CFT) used for the diagnosis of dourine caused by *T. equiperdum* (Williamson *et al.*, 1988; Zablotskij *et al.*, 2003). Enzyme-linked immunosorbent assays based on the detection of anti-trypanosome antibodies have also been developed for *T. evansi* infections (Reyna-Bello *et al.*, 1998; Tran *et al.*, 2009). There are also PCR tests available for diagnosis of AAT (Aradaib and Majid, 2006; Giroud *et al.*, 2009; Masake *et al.*, 1997). These PCR tests allow for the precise identification of the infecting trypanosome taxa (Solano *et al.*, 2002). A number of trypanosmal genes have been targeted successfully for molecular diagnosis of trypanosomiaisis. Some of which include the *VSG* genes, repetitive nuclear DNA sequences, ribososmal DNA, satellite DNA, amongst others. One of the most recent genes targeted is the *ISG75* gene for the diagnosis of surra caused by *T. evansi* (Mugasa et al., 2012; Rudramurthy et al., 2013). The primers used were designed based on the conserved regions of the *ISG75* gene. Given the similarity of ISG75 sequences across the trypanosoma genus, this test can be applicable for the diagnosis of other animal infectious trypanosomes like *T. equiperdum* and *T. brucei* (Rudramurthy *et al.*, 2013). These PCR techniques are not only useful for the diagnosis of trypanosomiasis but also as follow up tests to assess the success of treatment and cure from the disease. This is because killed trypanosomal DNA does not remain in the blood for more than 24-48 h, consequently the chances of false positive tests are reduced (OIE, 2010).

Currently available diagnostic tools for HAT and AAT are limited in specificity and sensitivity, therefore novel molecular or serological methods are required (Deborggraeve *et al.*, 2006; Welburn *et al.*, 2009). Also, new tools are required for the assessment of

treatment outcomes in order to fulfil the WHO requirement for assessment of treatment success or failure (Tiberti *et al.*, 2013).

1.4.3 Control and treatment of human and animal African trypanosomiasis

A multi-faceted approach is adopted for the control of African trypanosomiasis in animal and human populations. Vector control and the use of trypanocides are the control measures for bovine and other animal forms of African trypanosomiasis (Baral, 2010; Van Den Bossche, 2001). Vector control strategies include: chemical control through use of insecticides (Allsopp, 1984), the use of fly baits and traps as well as the adoption of the sterile insect technique (Vreysen *et al.*, 2000). For controlled cattle grazing areas, vector control can also be achieved by extensive clearing of vegetations which sustain tsetse populations. Insecticide-treated cattle are protected from tsetse bites and tsetse populations are also reduced due to death of flies that take blood meals from insecticide treated cattle. In *T. b. rhodesiense* endemic areas, where cattle serve as reservoirs for the disease in the absence of wild animals, treatment of cattle with insecticides, could also help control the human disease.

Vector control is also the major focus in the eradication of *T. b. rhodesiense* HAT (Welburn *et al.*, 2001b). However, for the control of *T. b. gambiense* HAT, thorough active case finding is sufficient. This is achieved through mass population screenings for the disease in endemic regions and subsequent treatment of active cases detected (Radwanska *et al.*, 2002; Welburn *et al.*, 2001a). Given that these are primarily remote rural areas with weak health systems, there is low compliance to treatment. Moreover, the limited infrastructural development in these areas makes it challenging to reach the target population.

Treatment of stage one HAT, involves the use of drugs like suramin and pentamidine (Chretien *et al.*, 2005; Seke Etet and Mahomoodally, 2012). These drugs do not cross the blood-brain barrier, so they are not effective in treating the meningo-encephalitic stage of the disease (Kristensson *et al.*, 2010). Melarsoprol is used for the treatment of second stage HAT. It is a very toxic arsenic compound associated with numerous severe side effects like

encephalopathy, hypertension, peripheral neuropathy, renal and hepatic dysfunction, amongst others (Kristensson *et al.*, 2010). A further stage two drug currently used is eflornithine (α-difluoromethylornithine/DFMO) (Priotto *et al.*, 2008). It also has severe side effects, is expensive and requires daily intravenous doses (Kristensson *et al.*, 2010). The combination of nifurtimox and eflornithine is a treatment strategy being adopted for the treatment of *T. b. gambiense* HAT (Priotto *et al.*, 2009; WHO, 2010). Drug combination therapy has the potential of reducing overall toxicity by allowing reduction in the dosage of each drug in the combination. Also, the emergence of drug resistant trypanosome strains is delayed due to protection offered by the two partner drugs against the selection of resistant strains (Priotto *et al.*, 2006).

A class of diamidine pro-drugs are currently being evaluated for the treatment of HAT (Thuita *et al.*, 2012). Pafuramidine (DB289) was the first oral drug to enter phase III clinical trials for first stage HAT (Steverding, 2008; Thuita *et al.*, 2012). The development program for the drug was, however, terminated due to severe kidney injury that occurred in the patients (Paine *et al.*, 2010; Thuita *et al.*, 2013). N-methoxy-6-{5-[4-(N-methoxyamidino) phenyl]-furan-2-yl}-nicotinamidine (DB844) was also evaluated given its effectiveness in treating second stage HAT in mice (Thuita *et al.*, 2012; Ansede *et al.*, 2005). Nevertheless, further development of the drug was discontinued, due to its moderate cure rate in monkey models of second stage HAT (Thuita *et al.*, 2012). The most recent oral diamidine pro-drug evaluated is DB829. This drug was effective for mice and monkey models of first stage HAT and has been recommended as a preclinical candidate for oral treatment of the disease (Thuita *et al.*, 2013).

Chemotherapy of AAT relies on the use of trypanocidal drugs like isometamidium chloride, homidium, quinapyramine and diminazine aceturate (Van Den Bossche *et al.*, 2000; Figure 1.8). Another drug used in treating infected livestock is Berenil (Bacchi, 1993). Suramin, which is used for treatment of the human disease is used to treat "surra" caused by *T. evansi* (Baral, 2010). Another drug used for prophylaxis and treatment of "surra" is melarsen oxide cysteamine or cymelarsan (Ndoutamia *et al.*, 1993).

Drug resistance is a major challenge facing chemoprophylaxis and chemotherapy of HAT and AAT (Anene *et al.*, 2001; Gehrig and Efferth, 2008). To combat this, new drug discovery is at the forefront of trypanosomiasis research, as well as developing efficient drug targeting strategies to deliver the drug directly to the parasite, reducing the need for high doses that lead to toxicity (Zucca and Savoia, 2011).

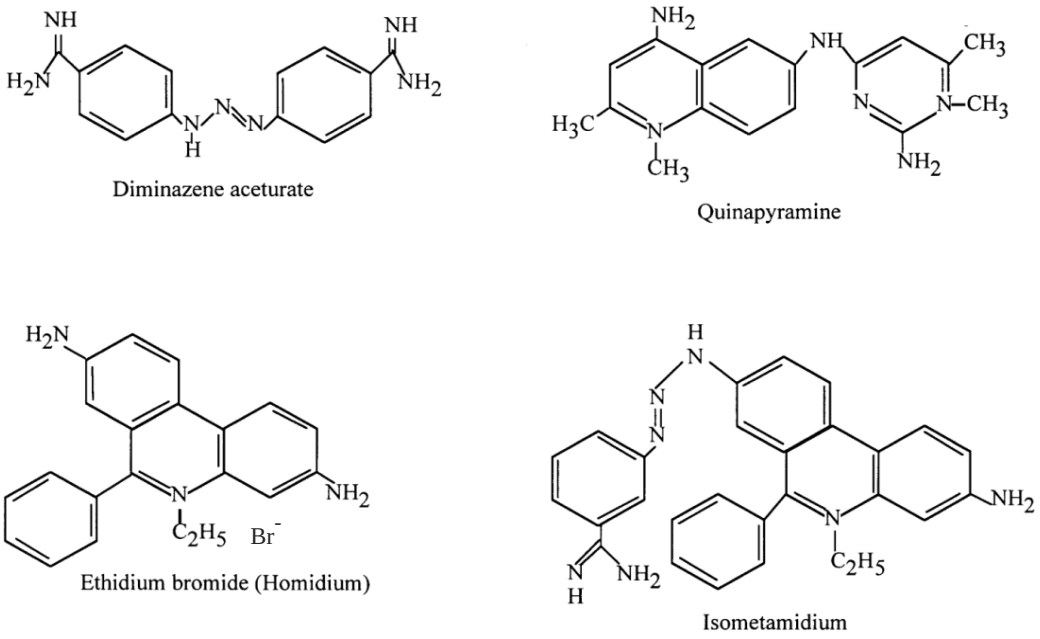


Figure 1.8 Molecular structures of four common trypanocides.

Vaccination is a viable alternative to chemotherapy in the control of African trypanosomiasis. However, there are currently no vaccines available against trypanosomiasis in Africa and the rest of the world (Agbo *et al.*, 2003). Vaccine development has been challenging due to antigenic variation by the parasites (Lipsitch and O'Hagan, 2007). To combat this challenge, a new vaccine approach involving the use of DNA vaccines is being explored. DNA vaccines have been shown to be successful in inducing immune protection against many protozoan caused diseases (Handman *et al.*, 2000; Laddy and Weiner, 2006). Trans-sialidase from *T. brucei* (Silva *et al.*, 2009) as well

as ISGs (Lanca *et al.*, 2011) have been identified as potential candidates for DNA vaccination of the disease.

1.5 Objectives and outline of the present study

The overall aim of the present study was to explore the potential of using ISG65 and ISG75 of *T. b. brucei* and *T. b. gambiense* as diagnostic antigens for African trypanosomiasis. The initial requirement therefore, was the production of these invariant antigens in sufficient quantities for the immunochemical assays. A secondary objective for the present study hence, was to optimise standardised protocols for the production of ISG65 and ISG75. The method of choice was recombinant production in heterologous systems: *Escherichia coli* and *Pichia pastoris*. Since ISGs are present in most strains of trypanosomes, this study focused on the expression of ISGs from one strain that infects animals, *T. b. brucei* and another strain pathogenic to humans, *T. b. gambiense*.

The heterologous expression of ISG65 is reported in Chapter 2. The gene coding for the extracellular domains of *ISG65* of *T. b. brucei* and *T. b. gambiense* were amplified by PCR. This was cloned into the pTZ57R/T-vector and sub-cloned into pGEX-4T1, pET28a and pP-αhSUMO3 expression vectors. The expressed fusion proteins were purified using chromatographic techniques and analysed by SDS-PAGE and western blotting techniques.

A further ISG, ISG75, was also recombinantly expressed in both eukaryotic (*P. pastoris*) and prokaryotic (*E. coli*) heterologous sytems. This process is detailed in Chapter 3 of the present study. The *ISG75 T. b. brucei* amplicon obtained by PCR was sub-cloned into pGEX-4T1 and pET28a expression vectors. In order to determine the optimum system for the production of these antigens, the expression profile of this fusion protein was compared to that of ISG75 from the *T. b. gambiense* strain. The growth profile and the production capacity of the *E. coli* cells expressing both proteins were also investigated. The recombinantly expressed soluble proteins were analysed by SDS-PAGE and western blotting. Expression of the protein in a eukaryotic system was facilitated by a construct engineered using pP-αhSUMO3 expression vector in M5-strain of *P. pastoris*. The glycosylation status of this fusion protein from *P. pastoris* was investigated.

The antigenicity of the heterologous proteins, ISG65 and ISG75 is reported in Chapter 4. Chickens were immunised using the recombinant proteins and the antibodies were isolated from the eggs. Affinity columns prepared using the recombinant ISG65 and ISG75 proteins were used to purify the antibodies. The titres of the antibodies were determined using enzyme-linked immunosorbent assays. Native ISG65 and ISG75 from *in vitro* culture of trypanosomes were detected by these antibodies in western blots as well as immunofluorescence microscopic analysis. Finally, the principal findings of the present study are summarised and discussed in Chapter 5.

CHAPTER 2

Heterologous expression and purification of invariant surface glycoprotein-65 (ISG65) of two sub-species of *Trypanosoma brucei*

2.1 Introduction

African trypanosomiasis, a parasitic disease of humans and animals is caused by protozoans of the *Trypanosoma* genus (Barrett *et al.*, 2003). This disease commonly referred to as sleeping sickness in humans and nagana in cattle is economically important in Africa (Steverding, 2008). The disease in humans is caused by *T. b. gambiense* and *T. b. rhodesiense*, while *T. b. brucei*, *T. congolense* and *T. vivax* infect animals (Steverding, 2008). Trypanosomes are transmitted to their mammalian hosts by tsetse flies. The metacyclic form trypanosomes present in the tsetse fly's proboscis are injected into the mammalian host during a blood meal and subsequently differentiate into BSF parasites (Matthews *et al.*, 2004). The life-cycle changes of the trypanosome between its vector and mammalian hosts are characterised by changes in its expression of surface proteins (Pays and Nolan, 1998). Variable surface glycoproteins (VSGs) are the most abundant surface proteins expressed by the BSF trypanosomes. These proteins, which are attached to the surface membrane by a glycophosphatidyl inositol (GPI) anchor are very important for the protozoan's evasion of their mammalian host's immune system (Larsson and Mellstedt, 1992). The VSGs are replaced by GPI anchored procyclins upon differentiation from BSFs to procyclic forms in the tsetse's midgut (Mehlert *et al.*, 1998).

Other surface proteins that have been identified in *T. brucei* include: the transferrin receptor, adenylate cyclase, a glucose transporter (Nolan *et al.*, 1997), a cysteine rich acidic integral membrane protein (CRAM) which is localised in the flagellar pocket (Lee *et al.*, 1990) as well as brucei alanine rich protein (BARP) (Urwyler *et al.*, 2007), which is also a GPI anchored protein (Nolan *et al.*, 2000). Invariant surface glycoproteins (ISGs) are a group of type-1 trans-membrane proteins identified in most strains of trypanosomes. These glycoproteins are arranged randomly in the VSG coat (Mehlert *et al.*, 2012b). Next

to the VSGs, ISGs are the most abundant proteins on the plasma membrane of BSF trypanosomes. The ratio of ISGs to VSGs is approximately one to 150 (Ziegelbauer *et al.*, 1992). The two most commonly known ISGs, i.e. ISG65 and ISG75, are estimated at 70,000 and 50,000 molecules per cell compared to 10^7 molecules of VSGs (Ziegelbauer *et al.*, 1992). While the VSGs are anchored to the parasite's membrane by the GPI anchor, the ISGs span the membrane by forming a trans-membrane α-helix (Carlander *et al.*, 1999). An endosomal pool of ISGs was identified in addition to surface localisation (Chung *et al.*, 2004). Invariant surface glycoprotein-65 is present in most species and sub-species in the *Trypanosoma* genus; including *T. b. brucei*, *T. congolense*, *T. equiperdum T. evansi*, *T. b. gambiense* and *T. b. rhodesiense* (Ziegelbauer *et al*., 1992).

The roles and functions of ISG65 in trypanosomes are not yet fully understood. Invariant surface glycoprotein-65 has a short half-life of less than four hours and it is endocytosed and trafficked through the parasite's endosomal system (Chung *et al.*, 2004; Chung *et al.*, 2008). Some of the endosomal ISG65 population is ubiquitylated and targeted for degradation while some portions are recycled back to the parasite's surface (Leung *et al.*, 2011). Endocytosis, intracellular trafficking and recycling are vital components of virulence, immune evasion and survival mechanisms of trypanosomes (Carlander *et al.*, 1999; Natesan *et al.*, 2007; Natesan *et al.*, 2011). Endocytosis is up-regulated in the BSF parasites compared to procyclic forms (Natesan *et al.*, 2007) and ISG65 is specific to this stage of the parasite's life-cycle. ISG65 experiences multiple rounds of endocytosis and re-exportation prior to being turned over by a ubiquitylation-dependent mechanism (Koumandou *et al.*, 2013). The involvement of ISG65 in the endosomal system and trafficking pathways of trypanosomes has led to speculations about its involvement in the pathogenesis and immune evasion process of trypanosomes. Further characterisation of ISG65 trafficking and endocytosis is of importance in order to properly understand its role in immune evasion (Koumandou *et al.*, 2013).

The ISG65 polypeptide is synthesised with an amino terminal signal sequence of 20 residues, which is cleaved upon protein maturation. The extracellular domain of ISG65 has two hydrophobic regions, between residues 1 and 20 and residues 386 and 410

(Figure 2.1). The first hydrophobic region denotes the signal sequence and the second, a membrane spanning alpha-helix. This is the trans-membrane domain of the protein. C-terminal to this is the intracellular domain from amino acid residues 411 to 436 (Ziegelbauer *et al.*, 1992). This domain, which is highly conserved, is responsible for targeting ISG65 to the surface of the trypanosome (Chung *et al.*, 2004). Domain predictions for *T. b. brucei* 927/4 GUTat10.1 strain ISG65 (Accession XM_946493) carried out in this study using the web-based TMHMM server v. 2.0 (http://www.cbs.dtu.dk/ services/TMHMM-2.0/ accessed on 12-06-11) gave similar results to this (Appendix 1).

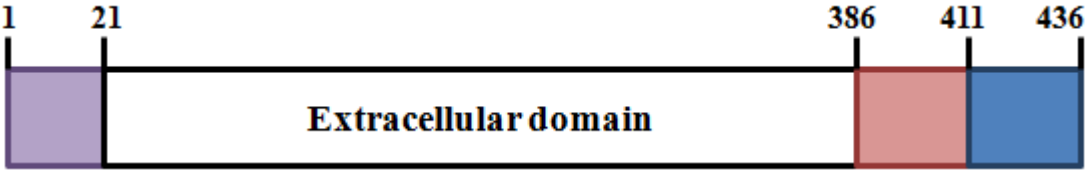


Figure 2.1 Schematic diagram of the mature ISG65 polypeptide with predicted domains. From the N-terminus, the purple box indicates the signal peptide, the white box refers to the extracellular domain, the pink box depicts the trans-membrane region and the blue box indicates the intracellular domain.

Here, the cloning, recombinant expression, purification and partial characterisation of the extracellular domain of ISG65 from *T. b. brucei* and *T. b. gambiense* are reported. The extracellular domain of *ISG65* amplified by PCR was cloned into a pTZ57R/T-vector and sub-cloned into pET28a and pGEX-4T1 expression vectors. The resultant constructs with His- and GST-tags were expressed in an *E. coli* BL21 (DE3) expression system. A construct containing the *ISG65* gene in pP-αhSUMO3 vector was also expressed in a *Pichia pastoris* expression system and the glycosylation status of the expressed protein was determined. The expression of recombinant ISG65 fusion proteins was confirmed by western blotting analysis using anti-His tag and anti-GST tag antibodies. The fusion proteins were purified using three phase partitioning, affinity chromatography and molecular exclusion chromatography.

2.2 Materials and Methods

2.2.1 Materials

Common laboratory chemicals and reagents used for buffer preparation were obtained from Fermentas (Vilnus, Lithuania), Roche Diagnostics (Germany), Sigma-Aldrich (Munich, Germany), and Thermo Scientific (Lithuania).

Cloning: EcoR1 and Not1 restriction endonucleases, *Taq* polymerase, FastRuler® middle range DNA ladder, Instaclone PCR cloning kit, Zymo DNA clean and concentrator kit, pEq Gold gel extraction kit, TransformAid® kit and GeneJET® miniprep kit were purchased from Fermentas (Vilnus, Lithuania). BL21 (DE3) and JM109 *E. coli* competent cells were purchased from New England Biolabs (Ipswich, MA, USA). The pET28a expression vector was obtained from Novagen (San Diego, CA, USA), shrimp alkaline phosphatase (SAP) was from Roche Diagnostics (Germany) and O'GeneRuler 1kb DNA ladder was from Thermo Scientific (Lithuania) .

Protein purification and quantification: Analytical grade thrombin protease and Ni-NTA affinity resin were purchased from Novagen (San Diego, CA, USA). Gluthatione-agarose was purchased from Sigma-Aldrich (Munich, Germany), HisTrap® FF Crude, GSTrap® FF and HiPrep® 16/60 Sephacryl® S-200 HR pre-packed columns were from GE Healthcare (Sweden). BCA Protein Assay Kit and 10 kDa cut off dialysis tubing were purchased from Pierce (Rockford, IL, USA). Nunc-Immuno 96-well plates were purchased from Nunc Intermed (Denmark) and PageRuler prestained protein ladder was from Thermo Scientific (Lithuania) .

Antibodies: Mouse anti-His antibody, rabbit anti-chicken IgY horseradish peroxidase (HRPO) conjugate and goat anti-mouse IgG HRPO conjugate were purchased from Jackson ImmunoResearch (USA). Chicken anti-GST tag antibody was an in-house preparation.

2.2.2 Trypanosome culture and genomic DNA isolation

Bloodstream forms of *T. b. brucei* Lister 427 strain were cultured in HMI-9 based Iscove's Modified Dulbecco's Medium (IMDM) at 37^{o}C with 5% CO_2 in a humid atmosphere in non-adherent culture flasks with vented caps (Hirumi and Hirumi, 1989). Briefly, 16.66 g of IMDM was dissolved in 1 L of HMI-9 (3.6 mM $NaHCO_3$, 1 mM hypoxanthine, 1 mM sodium pyruvate, 0.16 mM thymidine, 0.05 mM bathocuproine disulfonic acid disodium salt, 1.5 mM L-cysteine and 0.2 mM 2-mercaptoethanol). Heat inactivated foetal calf serum [10% (v/v)] was added and medium filtered using a 0.2 µm filter before use. Trypanosomes were cultured until they reached the stationary phase, after which the suspension was centrifuged (2 000 x *g*, 5 min, RT), and the pellet used for DNA isolation.

The genomic DNA of *T. b. brucei* Lister 427 strain was extracted from *in vitro* culture as previously described (Medina-Acosta, 1993). Briefly, *T. b. brucei* cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) before resuspending in 150 µl TELT buffer (50 mM Tris-HCl pH 8.0, 62.5 mM EDTA, 2.5 M LiCl) and incubated at RT for 5 min. Phenol-chloroform (1:1) was added to the lysate and the emulsion centrifuged (17 000 x *g*, 5 min, RT). The pellet was washed twice with absolute ethanol before drying at 37^{o}C for 10 min. The pellet was resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1 mg/ml RNAse and incubated at 37^{o}C for 45 min prior to DNA visualisation on a 1% (w/v) agarose gel.

2.2.3 Cloning and sub-cloning of *ISG65*

2.2.3.1 Cloning of *ISG65* into a T-vector

The GUTat10.1 strain *T. b. brucei* 927/4 (Accession XM_946493) nucleotide sequence for ISG65 was obtained from GenBank (http://www.ncbi.nlm.nih.gov accessed on 09-06-11) and used in the design of primers. The forward (5´-TTGGAATTCACTAATGGTGGA GATAAACA-3´) and reverse (3´-GCCGCGGCCGCCATTGCTGTTCTCTGATG-5´) primers containing EcoR1 and Not1 [according the nomenclature of Roberts *et al* (2003)] restriction sites (underlined) were designed to amplify the extracellular domain of the *ISG65* gene (Figure 2.1). For the amplification of *T. b. brucei ISG65*, 50.2 ng of the

genomic DNA isolated (Section 2.2.2) was used as template, whereas for *T. b. gambiense ISG65*, the genomic DNA of *T. b. gambiense* LiTat 1.3 (a gift from Prof. Philippe Büscher, Unit of Parasite Diagnostics, Institute of Tropical Medicine, Antwerp, Belgium) was used as template for PCR. In addition to DNA and 0.25 µM of each primer, the PCR mix consisted of 1 U of *Taq* polymerase, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 10 x *Taq* buffer and sterile dH_2O. The PCR with a reaction volume of 20 µl was carried out in a GeneAmp 2400 thermocycler (Applied Biosystems, Singapore), using the following program: initial denaturation at 94^{o}C for 5 min, denaturation at 94^{o}C for 1 min, annealing at 50^{o}C for 1 min, extension at 72^{o}C for 2 min, 30 cycles and final extension at 72^{o}C for 7 min.

The PCR products were cleaned using the Zymo DNA clean and concentrator kit (Fermentas, Lithuania) as specified in the product manual and ligated into the pTZ57R/T vector using T4 DNA ligase (Fermentas, Lithuania) using standardised protocols (Sambrook *et al.*, 1989). Briefly, the PCR product was mixed with 2 volumes of DNA binding buffer and transferred to a Zymo-spin® column in a collection tube. The column was centrifuged (12 000 x *g*, 30 s) and the flow-through was discarded. It was washed twice by adding 200 µl of DNA wash buffer and centrifuging (12 000 x *g*, 30 s). Six µl of DNA elution buffer was placed in the column matrix and incubated for 1 min at RT. Pure DNA was eluted from the column by centrifugation (12 000 x *g*, 30 s). Ligation was carried out using 1.4 µg of cleaned PCR product in a 10 µl reaction volume containing 27.5 ng of pTZ57R/T vector DNA, 2.5 U of T4 DNA ligase, 1 µl of 10 x buffer for T4 ligase and sterile dH_2O. The ligation mix was incubated overnight at 4^{o}C, heated at 65^{o}C for 10 min to deactivate T4 DNA ligase, and transformed into competent JM109 *E. coli* cells by chemical transformation using the TransformAid® kit (Fermentas, Lithuania) as follows: a freshly streaked JM109 *E. coli* culture (4 x 4 mm) was inoculated in C-medium (1.5 ml) and incubated at 37^{o}C for 2 h. The cells were pelleted by centrifugation (13 000 x *g*, 1 min, RT), resuspended in T-solution and incubated on ice for 5 min before centrifugation (13 000 x *g*, 1 min, RT). The supernatant was discarded, the pellet resuspended in T-solution and incubated on ice for 5 min. The prepared cells (50 µl) were mixed with ligation mix (3 µl), incubated on ice for 5 min and plated immediately on 2x YT agar plates [1.6% (w/v) peptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5%

(w/v) agar, 50 µg/ml ampicillin, 20 mg/ml X-gal, 100 mM IPTG]. Recombinant clones were selected by blue and white colony screening and confirmed by colony PCR using *ISG65* and vector specific primers.

2.2.3.2 Sub-cloning of *ISG65* into expression vectors

Recombinant plasmids were isolated using the Gene JET® miniprep kit (Fermentas, Lithuania) as per the product manual. Briefly, 1.5 ml of culture was centrifuged (6 800 x *g*, 2 min, RT) and the pelleted cells resuspended in resuspension solution (250 µl). The cell suspension was transferred to another tube and lysis solution (250 µl) was added and mixed (15 s), followed by neutralisation solution (350 µl). The preparation was mixed and centrifuged (13 000 x *g*, 5 min, RT). The supernatant was transferred to the GeneJET® spin column and centrifuged (13 000 x *g*, 1 min, RT). The flow-through was discarded, and the spin column was washed twice by adding wash solution (500 µl) and centrifuging (13 000 x *g*, 1 min, RT). The column was transferred to a fresh 1.5 ml microcentrifuge tube, elution buffer added (50 µl), incubated at RT for 2 min and the plasmid DNA was eluted by centrifugation (13 000 x *g*, 2 min, RT).

The *TbbISG65* and *TbgISG65* inserts were digested from the recombinant pTZ57R/T plasmid DNA using restriction endonucleases: EcoR1 and Not1 in a 50 µl reaction volume containing 11 µg of plasmid DNA, 5 µl of buffer O, 10 U of each enzyme and sterile distilled water. The pET28a and pGEX-4T1 expression vectors were similarly restricted and dephosphorylated using shrimp alkaline phosphatase in a 40 µl reaction volume containing 35 µl of vector DNA, 4 µl of 10 x reaction buffer and 1 U of shrimp alkaline phosphatase. The reaction mixture was incubated at 37^{o}C for 2 h and heated at 65^{o}C (15 min) to inactivate the enzyme (Sorensen and Mortensen, 2005). The vectors were cleaned using the Zymo DNA clean and concentrator kit (Fermentas, Lithuania) as described (Section 2.2.3.1). The *TbbISG65* and *TbgISG65* inserts were ligated as described (Section 2.2.3.1) into the cleaned pET28a and pGEX-4T1 expression vectors. The ligation products were transformed into *E. coli* JM109 and subsequently into *E. coli* BL21 (DE3) competent cells by chemical transformation using the TransformAid® kit (Fermentas, Lithuania) as previously described (Section 2.2.3.1).

For transformation into *E. coli* JM109 cells, 3 µl of ligation mix was mixed with the prepared cells and plated onto 2x YT agar plates containing appropriate antibiotic (50 µg/ml ampicillin and 34 µg/ml kanamycin sulfate for ligation products using pGEX-4T1 and pET28a vectors respectively). Transformed colonies were confirmed by PCR using vector and insert specific primers. Plasmid DNA from positive colonies was isolated as described previously. One µl of recombinant plasmid DNA was mixed with chemically competent *E. coli* BL21 (DE3) cells prepared as described before. This was plated onto 2x YT plates containing the appropriate antibiotic. Transformed colonies were confirmed by PCR using vector and insert specific primers. Tags were supplied to the inserts by the vectors: a His-tag was from the pET28a and the glutathione-*S*-transferase (GST) tag from the pGEX-4T1 vector. Glycerol stocks were prepared from overnight cultures of recombinant colonies and stored at -80^{o}C. The plasmid DNA of each construct was sequenced at Inqaba Biotechnical Industries (Pretoria, South Africa) using vector and insert specific primers.

A summary of all the *ISG65* constructs designed in this study is shown in Table 2.1.

Table 2.1 Description of recombinant *T. b. brucei* and *T. b. gambiense* plasmids designed.

Fusion protein	Trypanosome strain	Amino acid range	Expression Vector	Fusion tag
***Tbb$_{His}$*ISG65**	*T. brucei brucei*	AA21-387	pET28a	Poly histidine
***Tbb$_{GST}$*ISG65**	*T. brucei brucei*	AA21-387	pGEX-4T1	Glutathione *S*-transferase (GST)
***Tbg$_{His}$*ISG65**	*T. b. gambiense*	AA21-387	pET28a	Poly histidine
***Tbg$_{GST}$*ISG65**	*T. b. gambiense*	AA21-387	pGEX-4T1	Glutathione *S*-transferase (GST)
ISG65*Pp**	*T. b. gambiense*	AA1-344	pP-αhSUMO3	hSUMO3 with Poly histidine

* (Gift from Prof. Philippe Büscher, Unit of Parasite Diagnostics, Institute of Tropical Medicine, Antwerp, Belgium.)

2.2.4 Expression of ISG65 using different expression vectors and hosts

2.2.4.1 Recombinant expression of ISG65 in bacteria

Three-way streaks were made from glycerol stocks of the *ISG65* constructs (see Table 2.1), onto 2x YT [1.6% (w/v) peptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar] plates containing the appropriate antibiotic. Ampicillin (50 µg/ml) was used for the constructs in pGEX-4T1 and kanamycin sulfate (34 µg/ml) was used for the constructs in pET28a. Colonies were picked from the overnight agar plates and inoculated into 2x YT broth containing the appropriate antibiotic and incubated for 16 h at 37^{o}C. This culture was diluted 1 in 10 with fresh medium and incubated at 37^{o}C until an optical density value at 600 nm of 0.5 to 1.0 was reached. Expression was induced with 0.3 to 1 mM IPTG and incubated at various temperatures: 25^{o}C to 37^{o}C for 4 h.

2.2.4.2 Preparation of cell lysates and solubility test for expressed proteins

The bacteria cells were harvested from culture by centrifugation (6 500 x *g*, 10 min, 4^{o}C), resuspended in lysis buffer [1% (v/v) PBS-Triton X-100, pH 7.4, containing 1 mg/ml lysozyme], incubated at 37^{o}C for 15 min and put through several freeze-thaw and sonication (on ice, 4 x 30 s, with 10 s intervals) cycles to facilitate cell lysis. The lysed cells were centrifuged (6 500 x *g*, 10 min, 4^{o}C) to separate the soluble fraction from insoluble cellular organelles. The supernatant (soluble fraction) and pellets (insoluble fraction) were analysed by 10% reducing SDS-PAGE (Laemmli, 1970) for the presence of expressed fusion protein.

2.2.4.3 Recombinant expression of ISG65 in yeast (*P. pastoris*)

A single colony of transformed *P. pastoris* cells from a yeast extract peptone dextrose (YPD) [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar] plate containing 100 µg/ml Zeocin® was inoculated in buffered glycerol-complex medium (BMGY) [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, 0.00004% (w/v) biotin, 1% (v/v) glycerol, 100 µg/ml Zeocin®], to create a biomass by incubating at 29^{o}C with agitation

(usually for 48-72 h). Expression was induced by resuspending the harvested cells (centrifugation at 4^{o}C, 2 000 x *g*, 5 min) in buffered methanol-complex medium (BMMY) [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, 0.00004 % (w/v) biotin, 0.5% (v/v) methanol, 100 µg/ml Zeocin®] and incubating at 29^{o}C with agitation. Induction was maintained by daily additions of methanol to a final concentration of 0.5% (v/v) for the entire duration of the expression (5 days).

Intracellular and secreted expression was monitored by reducing SDS PAGE analysis of 1 ml samples of culture collected daily as follows: 1 ml of the culture was centrifuged (17 000 x *g*, RT, 3 min) and the supernatant was separated from the pellet. To analyse secreted expression, 200 µl of the supernatant was concentrated by adding equal volumes of 5% (w/v) SDS and 3 M KCl (20 µl of each), mixing and centrifuging (12 000 x *g*, RT, 2 min) to obtain a pellet. This was resusupended in equal volumes of resuspension buffer (500 mM Tris-HCl, pH 6.8) and reducing treatment buffer (Section 2.2.5), boiled and loaded onto the wells of an SDS-PAGE gel. To analyse intracellular protein production, the pellet was washed twice with dH_2O and resuspended in 200 µl of 0.1 M NaCl and incubated for 10 min at RT. Cells were pelleted (17 000 x *g*, RT, 3 min), NaOH was aspirated and cells resuspended in SDS-PAGE reducing treatment buffer. Samples were boiled for 5 min and centrifuged (17 000 x *g*, RT, 3 min) prior to loading onto the wells of a 10% reducing SDS-PAGE gel.

2.2.5 SDS-PAGE analysis of ISG65

The SDS-PAGE method, described by Laemmli (1970), was used to analyse protein samples. Sodium dodecyl sulfate, an anionic detergent binds to most proteins, converting the native conformations into negatively charged rod-like complexes (Reynolds and Tanford, 1970). Similar charge-to-mass ratios acquired by the protein samples will ensure a size dependent migration in an electrical field. A discontinuous buffer system consisting of a stacking gel buffer (500 mM Tris-HCl, pH 6.8) and a separating gel buffer (1.5 M Tris-HCl, pH 8.8) was used. The protein samples were treated with an equal volume of a reducing treatment buffer [125 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol,

10% (v/v) 2-mercaptoethanol] prior to boiling at 100^{o}C for 5 min and loading onto the SDS-PAGE gel. Electrophoresis was conducted at 18 mA per gel for 2 h in tank buffer [250 mM Tris-HCl buffer, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS] using the BioRad Mini Protean III electrophoresis equipment (BioRad, CA, USA).

A calibration curve was constructed by plotting the log M_r of molecular mass standard proteins against their distance travelled in the gel (Figure 2.2). Visualisation of electrophoresed proteins was achieved by staining with Coomassie Blue [0.125% (w/v) Coomassie Blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid] for 4 h. The Coomassie dye binds via physical adsorption to the basic and aromatic amino acid side chains and not to the polyacrylamide matrix (Sambrook and Gething, 1989). Detection limit of Coomassie Blue is usually 50-100 ng of protein (Silva *et al.*, 2004).

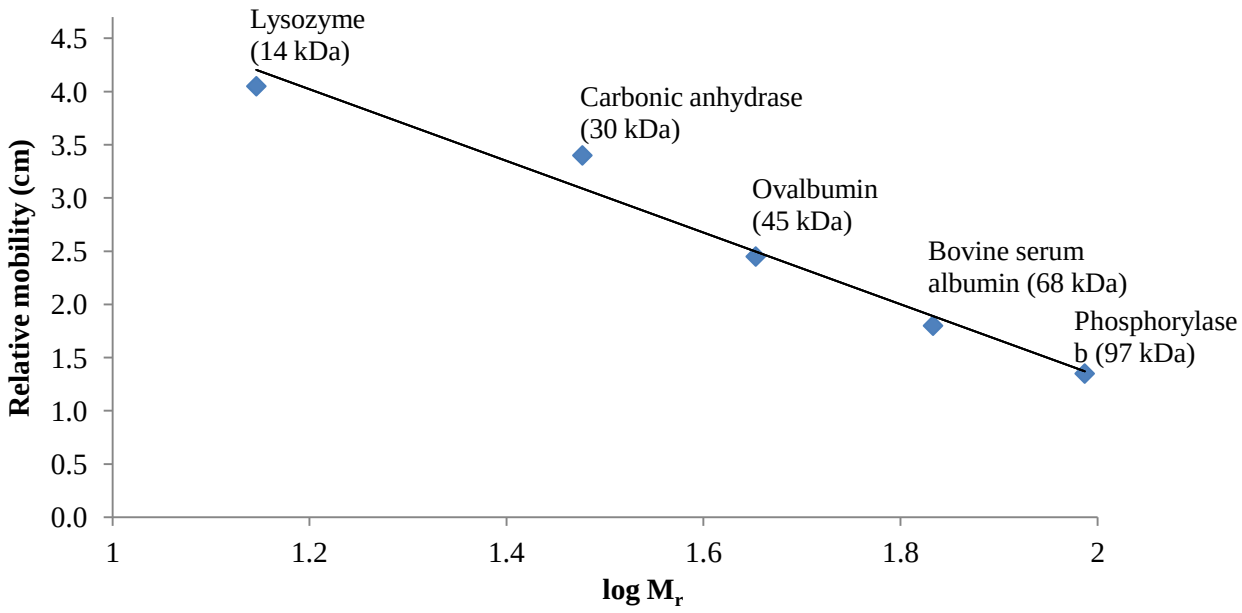


Figure 2.2 Reducing SDS-PAGE calibration curve. To determine the M_r of protein samples of unknown size, protein markers of known size were separated alongside samples to be analysed. The relative mobility of the protein markers were measured and plotted against log M_r. The equation of the trendline was $y = -3.4x + 8.1$, with a correlation coefficient of 0.97.

More sensitive protein detection (1-10 ng of protein) was achieved by using the silver staining method of Blum (1987). The proteins become visible when the Ag^{+} ions are

reduced to elementary Ag and the stained proteins develop the typical brownish-grey-black colour (Heukeshoven and Dernick, 1985). In this method, on completion of electrophoresis, the gel was placed in a scrupulously clean glass dish containing fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.5% (v/v) formaldehyde] for 1 h, washed with washing solution [50% (v/v) methanol; 3 x 20 min]. The gel was subsequently soaked in pre-treatment solution [0.2% (w/v) $Na_2S_2O_3.5H_2O$] which prevents the precipitation of silver complexes on the gel's surface and consequently reduces the intensity of background staining. This was followed by rinsing in dH_2O (3 x 20 s) and soaking in impregnation solution [0.2% (w/v) $AgNO_3$, 0.028% (v/v) formaldehyde] for 20 min. The gel was rinsed again in dH_2O (3 x 20 s), immersed in developing solution [6% (v/v) Na_2CO_3, 0.0004% (w/v) $Na_2S_2O_3.5H_20$, 0.0185% (v/v) formaldehyde] until protein bands were visible. Development of protein bands was stopped by immersion in stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid] for 10 min.

2.2.6 Western blotting analysis of ISG65

Lysates containing ISG65 fusion proteins were separated on a 10% reducing SDS-PAGE gel at 20 mA per gel and blotted onto nitrocellulose by placing the gel on the membrane, in a sandwich of four sheets of blotting paper. Protein transfer was achieved using a western blotting apparatus (Mighty small transphor, Hoefer, USA) filled with blotting buffer [50 mM Tris-HCl, 20 mM glycine, 20% (v/v) methanol, 10% (w/v) SDS] at 40 mA for 16 h. Protein transfer was confirmed by staining the nitrocellulose strip transiently with Ponceau-S [0.1% (w/v) Ponceau-S, 1% (v/v) glacial acetic acid]. The unoccupied sites on the nitrocellulose strip were blocked with 5% (w/v) low fat milk in TBS (20 mM Tris, 200 mM NaCl, pH 7.4) for 1 h, washed (3 x 5 min) with TBS before incubation in primary antibody in 0.5% (w/v) BSA in TBS. The primary antibodies used were: mouse anti-His antibody (1:1 000), chicken anti-GST IgY (0.5 µg/ml), and chicken anti-ISG65 IgY (7.5 µg/ml). Horseradish peroxidase (HRPO) conjugated goat anti-mouse IgG (1:1 000), and rabbit anti-IgY (1:5 000); in 0.5% (w/v) BSA-TBS, were used as secondary antibodies. The blot was developed using either 0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v)

H_2O_2 in TBS or an enhanced chemiluminescence detection kit (Pierce, Rockford, USA) and imaging using the Sys Gene® imaging system (Syngene®, USA).

2.2.7 Purification of recombinant ISG65

2.2.7.1 Fractionation of *Tbb*$_{His}$ISG65 and ISG65*Pp* by Three phase partitioning (TPP)

The first step for the purification of the bacteria expression lysates and yeast expression supernatants was three phase partitioning (TPP) using the method described by Pike and Dennison (1989). The lysates (bacteria) and supernatants (yeast) were clarified by filtering through Whatman 1 filter paper. The recombinant protein was precipitated from clarified samples by mixing with tertiary-butanol [30% (v/v) of the total mixture volume] and increasing concentrations of ammonium sulfate from 10% - 40% (w/v) of the final volume. The proteins in the sample were excluded from the soluble and organic phases to give a precipitate as an intermediate layer between the two phases. This was achieved by centrifugation (6 000 x *g*, 10 min, 4^{o}C) using a swing out rotor. The precipitate was resuspended in PBS and an aliquot analysed using reducing SDS-PAGE to assess purification.

2.2.7.2 Affinity purification of ISG65 fusion proteins using nickel chelate chromatography

The TPP concentrated *Tbb*$_{His}$ISG65, *Tbg*$_{His}$ISG65 and ISG65*Pp* fusion protein samples were purified by affinity chromatography using pre-packed nickel chelate resins: HisTrap® FF crude on an ÄKTA purifier (GE Healthcare, Sweden). Each sample was filtered and injected into a HisTrap® FF crude affinity column (GE Healthcare, Sweden) containing 1 mL of nickel chelate resin at a flow rate of 0.5 ml/min. The packed column was washed with a 10-fold excess volume of binding buffer (0.5 M NaCl, 20 mM imidazole, 20 mM Na_2HPO_4, pH 7.4) and the bound proteins were eluted with elution buffer (0.5 M NaCl, 0.5 M imidazole, 20 mM Na_2HPO_4, pH 7.4) at 1 ml/min. The A_{280} of fractions (1 ml) was monitored (ÄKTA prime plus, GE Healthcare, Sweden). The purity of the protein sample was assessed by analysing aliquots of the fractions using 10% reducing SDS-PAGE (Section 2.2.5) and western blotting (Section 2.2.6) with appropriate primary antibodies.

2.2.7.3 Purification of ISG65 fusion proteins using GST affinity chromatography

The GST-tagged proteins *Tbb*$_{GST}$ISG65 and *Tbg*$_{GST}$ISG65 were purified using glutathione-agarose (GST) affinity resins. The purification was carried out as per the manufacturer's instructions. Briefly, lyophilised glutathione-agarose was swollen in dH_2O (14 ml per mg) overnight at 4^oC. Swollen resin (1 ml) was placed in a Poly-Prep® chromatography column (Bio-Rad, CA, USA) and equilibrated with 20 column volumes of PBS. The lysate (9 ml) prepared as per Section 2.2.4.2, was cycled over the glutathione-agarose column (1 ml) overnight at 4^oC. The resin was washed with 1 % (v/v) PBS-Triton X-100 (20 ml), and bound proteins were eluted using elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl buffer, pH 8). One ml fractions were collected and analysed by 10% reducing SDS-PAGE (Laemmli, 1970; Section 2.2.5) for the presence of purified ISG65 fusion protein with a GST-tag.

On-column cleavage of ISG65 from the GST tag while it was adsorbed to the GST-resin was done with 2 U of thrombin (1 U/µl, Novagen, USA) as follows: the lysate (9 ml), prepared as per Section 2.2.4.2, was cycled over the glutathione-agarose column (1 ml) overnight at 4^oC. The resin was washed with 1 % (v/v) PBS-Triton X-100 (20 ml), and equilibrated with thrombin cleavage buffer (20 mM Tris-HCl buffer, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$). Thrombin cleavage buffer (1 ml) was used to gently resuspend the resin into a 50% slurry to which thrombin was added (2 U). The column was incubated overnight at RT with gentle rocking. Cleaved ISG65 present in the buffer was directly collected from the column. A 3 ml wash fraction using 1 % (v/v) PBS-Triton X-100 was also collected. Bound GST and any uncleaved fusion protein were eluted from the resin with elution buffer (10 mM reduced glutathione, 50 mM Tris-HCl buffer, pH 8). The eluted protein samples were analysed using 10% reducing SDS-PAGE (Laemmli, 1970; Section 2.2.5).

2.2.7.4 Molecular exclusion chromatography (MEC)

A HiPrep® 16/60 Sephacryl® S-200 HR column (16 x 600 mm, flow rate of 0.5 ml/min, 120 ml, GE Healthcare, Sweden) was used to further purify *Tbb*$_{His}$ISG65 using the ÄKTA

purification system (GE Healthcare, Sweden). The column was equilibrated with 2 column volumes of MEC buffer (50 mM NaH_2PO_4, 300 mM NaCl, 0.02% (w/v) NaN_3, pH 8), and calibrated using 1 ml of calibration solution [3 mg/ml blue dextran (2000 kDa), 5 mg/ml each of sheep IgG (120 kDa), BSA (68 kDa), ovalbumin (45 kDa) and myoglobin (16.6 kDa)]. The availability constant (K_{av}) was determined for each protein using the equation: $K_{av} = (V_e-V_o) / (V_t-V_o)$. Where the elution volume (V_e) of blue dextran denotes the void volume (V_o) and the total volume is denoted by V_t. K_{av} is related to log M_r in a Fisher's plot (Figure 2.3B).

2.2.7.5 Concentration of purified ISG65

Concentration of the purified ISG65 samples was necessary before use in subsequent applications. This was done using the Centripep® centrifugal device of M_r 10 000 (Merck Millipore, Bedford, USA) cut-off as per the manufacturer's instructions. Dialysis against polyethylene glycol M_r 20 000 was also employed using a dialysis membrane with M_r 10 000 cut-off.

2.2.8 Quantification of recombinant ISG65

The purified ISG65 recombinant proteins were quantified using the BCA® Protein Assay Kit (Pierce, Rockford, USA). The working reagent of the BCA® Protein Assay Kit contains bicinchoninic acid (BCA) which offers a sensitive colorimetric detection of Cu^+. The alkaline medium of this assay allows the protein sample to reduce Cu^{2+} present in the working reagent, to Cu^+. The absorbance of the BCA/copper complex at 562 nm is nearly linear with increasing protein concentrations over a broad working range (20-2 000 µg/ml). A wavelength of 595 nm was used successfully in this assay. A standard curve (Figure 2.4) was constructed by plotting an average of the absorbance of triplicate samples of known concentrations of bovine serum albumin (BSA) [25 µl of 0.025-2 mg/ml] mixed with the BCA working solution (200 µl) in a Nunc® 96 well microtitre plate. The plate was covered and incubated at 37^{o}C for 30 min prior to absorbance measurement using the FLUORStar Spectrophotometer (BMG Labtech, Offenburg, Germany). The standard curve was used to determine the concentration of purified ISG65.

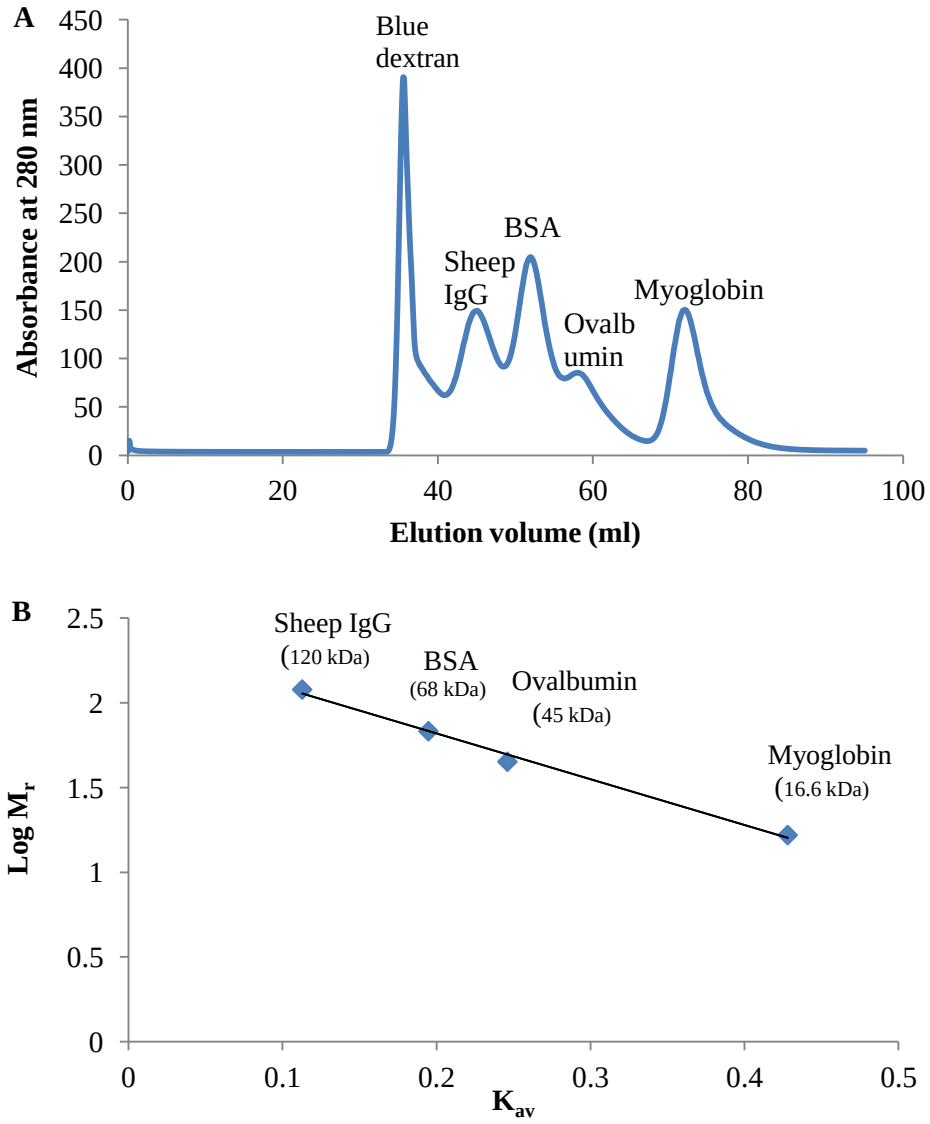


Figure 2.3 Elution profile of calibration of MEC column and Fischer's plot for estimation of protein M_r using MEC data. (A) Elution profile of calibration of S-200 column. The calibration solution [3 mg/ml blue dextran (2000 kDa), 5 mg/ml each of sheep IgG (120 kDa), BSA (68 kDa), ovalbumin (45 kDa) and myoglobin (16.6 kDa)] was applied to a Sephacryl® S-200 HR column (16 x 600 mm, flow rate of 0.5 ml/min) in MEC buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8). **(B)** Fischer's plot for estimation of protein M_r using data from calibration of S-200 column. The availability constant (K_{av}) for each standard protein was determined and plotted against the Log M_r. The equation of the trendline is y = -2.7x + 2.4 with a correlation coefficient of 0.99.

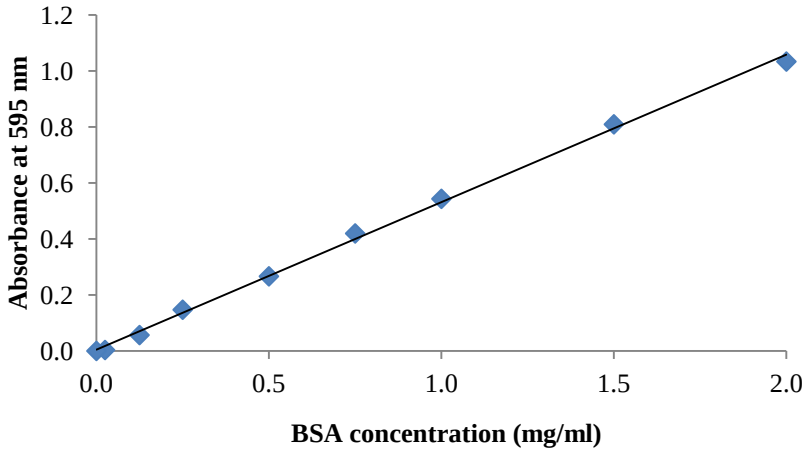


Figure 2.4 Standard curve for BCA protein quantification assay. Bovine serum albumin (BSA) samples of known concentrations (0.025-2 mg/ml) were mixed with BCA working reagent and absorbance measured at 595 nm in order to construct a standard curve. The equation of the trendline is y = 0.54x + 0.004 with a correlation coefficient of 1.

2.2.9 Glycosylation assessment

In order to check if the recombinant ISG65 was N-glycosylated, the protein sample was treated with Endoglycosidase H (Sigma, USA) as previously described (Caffrey *et al.*, 2001). Endoglycosidase H cleaves between the two N-acetyl glucosamine residues in the diacetyl chitobiose core of the oligosaccharide, generating a truncated sugar molecule with one N-acetyl glucosamine residue remaining on the asparagine (Tarentino and Maley, 1974; Hsieh *et al.*, 1983). In the assay, the sample was reduced by treatment with SDS [5% (w/v)] and 2-mercaptoethanol [10% (w/v)] with boiling at 100^{o}C for 5 min. The optimum pH for Endoglycosidase H is pH 5-5.6; thus, sodium citrate buffer (0.5 M sodium citrate, pH 5.5) was added to the reaction mixture before the addition of the enzyme. The mixture was incubated at 37^{o}C for 3 h. The resultant samples were analysed by 10% reducing SDS PAGE alongside untreated samples to compare electrophoretic mobilities in order to deduce if there was a reduction in size due to the Endoglycosidase H treatment.

2.3 Results

2.3.1 Cloning and sub-cloning of *TbbISG65* and *TbgISG65*

2.3.1.1 Cloning of *TbbISG65* and *TbgISG65* into pTZ57R/T-vector

The genomic DNA of *T. b. brucei* Lister 427 strain was successfully isolated (Figure 2.5). A spectrophotometric analysis of the sample, gave a value of 1.9 as the A_{260}/A_{280} ratio. This was consistent for a DNA sample without protein or RNA contamination (Popa *et al.*, 2007). A distinct band of very high molecular weight was observed as a result of its low electrophoretic mobility in a 1% agarose gel (Figure 2.5A). The extracellular domains of *TbbISG65* and *TbgISG65* were amplified successfully by PCR. The expected size of approximately 1121 bp was obtained for the PCR products (Figure 2.5B). This was determined by comparing the migration of the PCR products to those of the components of the DNA ladder in a 1% agarose gel.

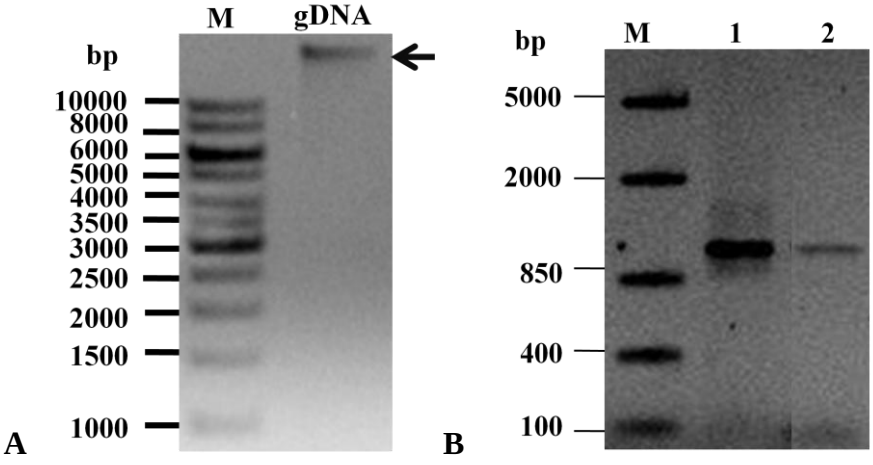


Figure 2.5 Agarose gel (1%) analysis of *T. b. brucei* genomic DNA and amplification of *ISG65* gene. (A) Isolated genomic DNA. Lane M, O'GeneRuler 1kb DNA ladder; **(B)** Amplified *ISG65* fragments. Lane M, FastRuler® middle range DNA ladder; lane 1, *TbbISG65* PCR product amplified by *ISG65* primers; lane 2, *TbgISG65* PCR product amplified by *ISG65* primers.

The PCR product with sticky ends, due to the action of *Taq* DNA polymerase was ligated into the pTZ57R/T vector prior to transformation into *E. coli* JM109 competent cells.

Recombinant white colonies were confirmed by colony PCR using *ISG65* primers. Products for colonies containing *TbbISG65* are shown (Figure 2.6A, lanes 2-7) with an approximate size of 1121 bp as expected. The PCR products for colonies containing *TbgISG65* visualised by 1% agarose gel also showed the expected size of 1121 bp (Figure 2.6B, lane 2). No bands were observed for PCR products of non-recombinant colonies (Figure 2.6A, lanes 1 and 8 and Figure 2.6B, lanes 1 and 3).

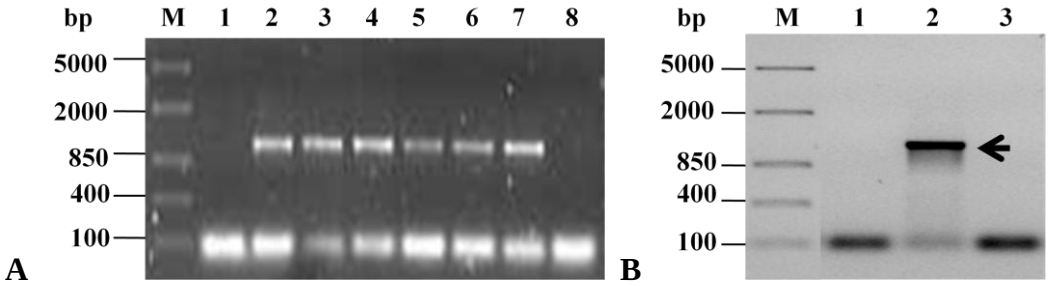


Figure 2.6 Agarose gel (1%) analysis of colony PCR for screening of recombinant pTZ57R/T after transformation. (A) PCR products for colonies containing the *TbbISG65* gene. Lane M, FastRuler® middle range DNA ladder; lanes 2-7, PCR products amplified using *ISG65* specific primers; lanes 1 and 8, PCR products of non-recombinant colonies. **(B)** PCR products for colonies containing the *TbgISG65* gene. Lane M, FastRuler® middle range DNA ladder; lane 2, PCR products amplified using *ISG65* specific primers; lanes 1 and 3, PCR products of non-recombinant colonies. Arrow indicates band of expected size.

The plasmid DNA of the non-recombinant (lanes 1-2, Figure 2.7) and recombinant (lanes 3-9, Figure 2.7) pTZ57R/T vector isolated by Gene JET® miniprep kit (Fermentas, Lithuania) were analysed by 1% agarose as shown by bands of 2886 bp (Figure 2.7, circled) and 4007 bp (Figure 2.7, indicated by arrow). The recombinant pTZ57R/T vector DNA had a larger size (4007 bp) than the non recombinant (2886 bp) due to the presence of the *ISG65* insert of 1121 bp.

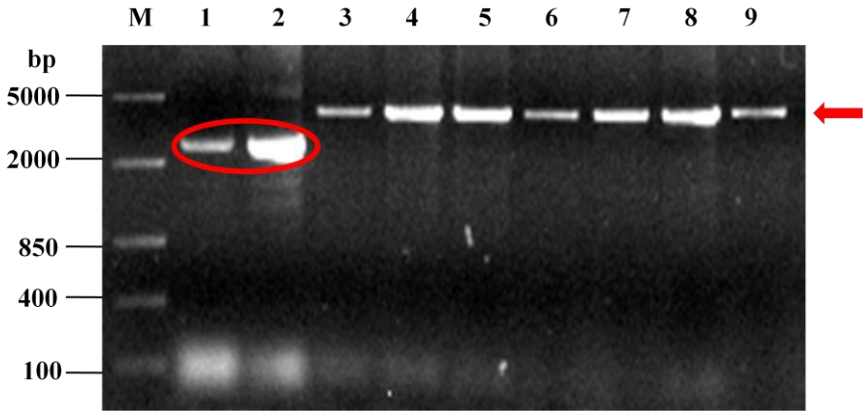


Figure 2.7 Agarose gel (1%) analysis of pTZ57R/T vector DNA. Isolated plasmid mini prep of recombinant and non-recombinant pTZ57R/T vector. Lane M, FastRuler® middle range DNA ladder; lanes 1 and 2, non-recombinant pTZ57R/T vector DNA (circled); lanes 3-8, recombinant pTZ57R/T vector DNA containing *Tbb*ISG65; lane 9, recombinant pTZ57R/T vector DNA containing *TbgISG65*.

Double restriction of the recombinant pTZ57R/T vector with EcoR1 and Not1 released fragments of estimated sizes of 2886 bp and 1121 bp, corresponding to pTZ57R/T and *ISG65* fragments, respectively (Figure 2.8).

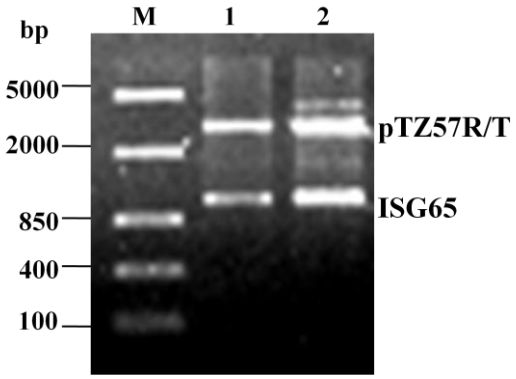


Figure 2.8 Agarose gel (1%) analysis of double restriction digest of recombinant pTZ57R/T vector DNA. Lane M, FastRuler® middle range DNA ladder; lane 1, plasmid DNA of *Tbb*ISG65 in pTZ57R/T vector*;* lane 2, plasmid DNA of *Tbg*ISG65 in pTZ57R/T vector. Upper band, pTZ57R/T vector DNA, lower band, *ISG65* gene.

2.3.1.2 Sub-cloning of *TbbISG65* and *TbgISG65* into expression vectors

In order to optimise the production and purification of recombinant ISG65, the *TbbISG65* and *TbgISG65* inserts were sub-cloned into different expression system vectors. The expression vectors pET28a and pGEX-4T1 were linearised using EcoR1 and Not1 endonucleases prior to sub-cloning. Agarose gel analysis showed differences in the sizes of the linearised (lanes 1 and 3) and non-linearised (lanes 2 and 4) pET28a and pGEX-4T1 expression vectors (Figure 2.9).

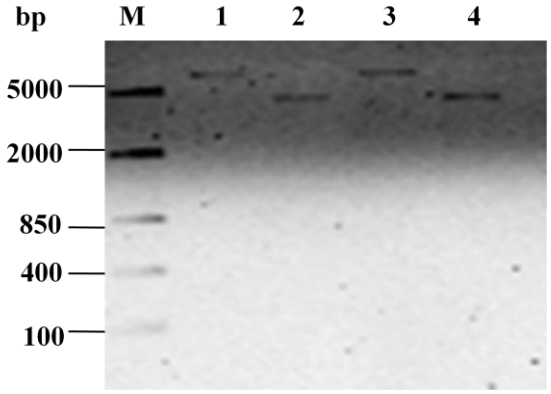


Figure 2.9 Agarose gel (1%) analysis of linearised pGEX-4T1 and pET28a expression vectors. EcoR1 and Not1 linearised expression vector DNA. Lane M, FastRuler® middle range DNA ladder. Lanes 1 and 2, linearised and non-linearised pGEX-4T1 vector DNA; lanes 3 and 4; linearised and non-linearised pET28a vector DNA.

Recombinant colonies were screened by colony PCR using *ISG65* and vector primers (pET and pGEX) and the products are shown in Figures 2.10 and 2.11. Recombinant colonies containing pET28a-*TbbISG65* screened using ISG65 primers gave a product of the expected size of 1121 bp (Figure 2.10A, lane 2). A size of 1121 bp was also observed for pGEX-4T1-*TbbISG65* transformed colonies screened using *ISG65* primers (Figure 2.10B, lanes 4-5). The control PCR product, for which the *T. b. brucei* gDNA was used as template instead of colonies, was observed at a size of 1121bp (Figure 2.10B, lane 7). Colonies that gave no PCR products were identified as non-recombinant (Figure 2.10A, lane 1 and Figure 2.10B, lanes 1, 2, 3 and 6).

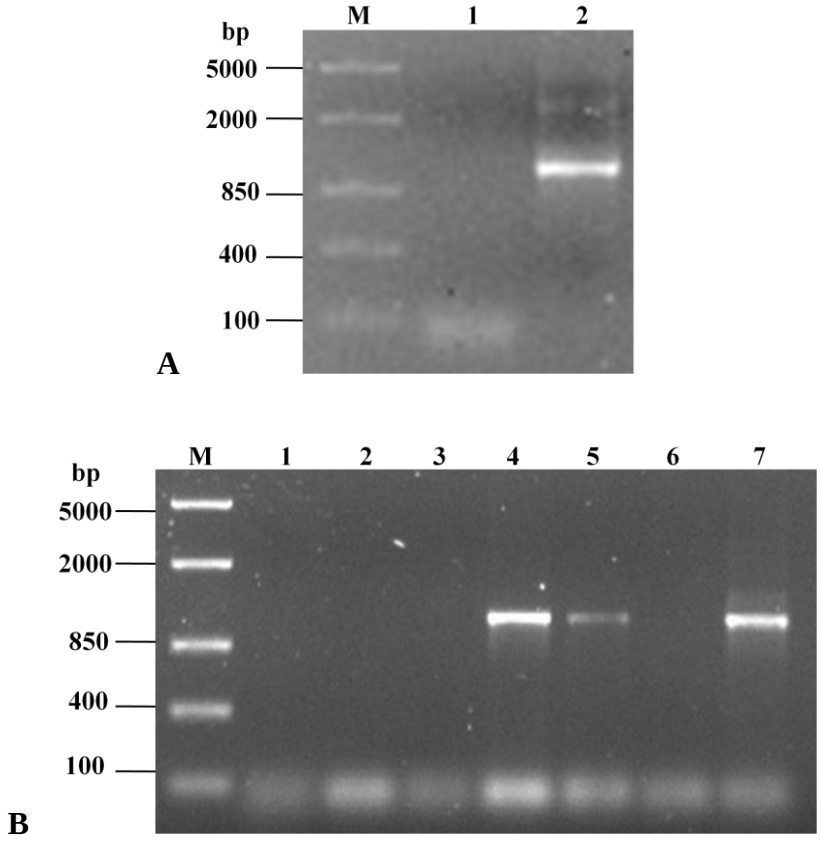


Figure 2.10 Agarose gel (1%) analysis of colony PCR for screening of *TbbISG65* in expression vectors. (A) pET28a-*TbbISG65* transformed colonies. Lane M, FastRuler® middle range DNA ladder; PCR products of non-recombinant (lane 1) and recombinant colonies (lane 2). **(B)** pGEX-4T1-*TbbISG65* transformed colonies. Lane M, FastRuler® middle range DNA ladder; lanes 1-3 and 6, PCR products of non-recombinant colonies; lanes 4-5, PCR products of recombinant colonies; lane 7, PCR product using *T. b. brucei* gDNA as colony PCR control.

The recombinant colonies containing pET28a-*TbgISG65* plasmids screened using pET primers gave a product of approximately 1300 bp (Figure 2.11A). This corresponds to the *TbgISG65* fragment (1121 bp) plus the coding region of the pET28a vector amplified by the vector primer. The products for recombinant pGEX-4T1-*TbgISG65* colonies screened using both pGEX and *ISG65* primers are shown in Figure 2.11B. PCR products equivalent to 1121 bp were obtained when ISG65 primers were used with recombinant colonies (Figure 2.11B, lanes 10-15) and with *T. b. brucei* gDNA (Figure 2.11B, lane 9). However the products obtained using pGEX primers were approximately 1300 bp because the

coding region of the pGEX-4T1 vector was amplified as well as the *ISG65* gene (Figure 2.11B, lanes 2-3). No bands were observed for PCR products of non-recombinant colonies (Figure 2.11B, lanes 1, 4-8 and 13).

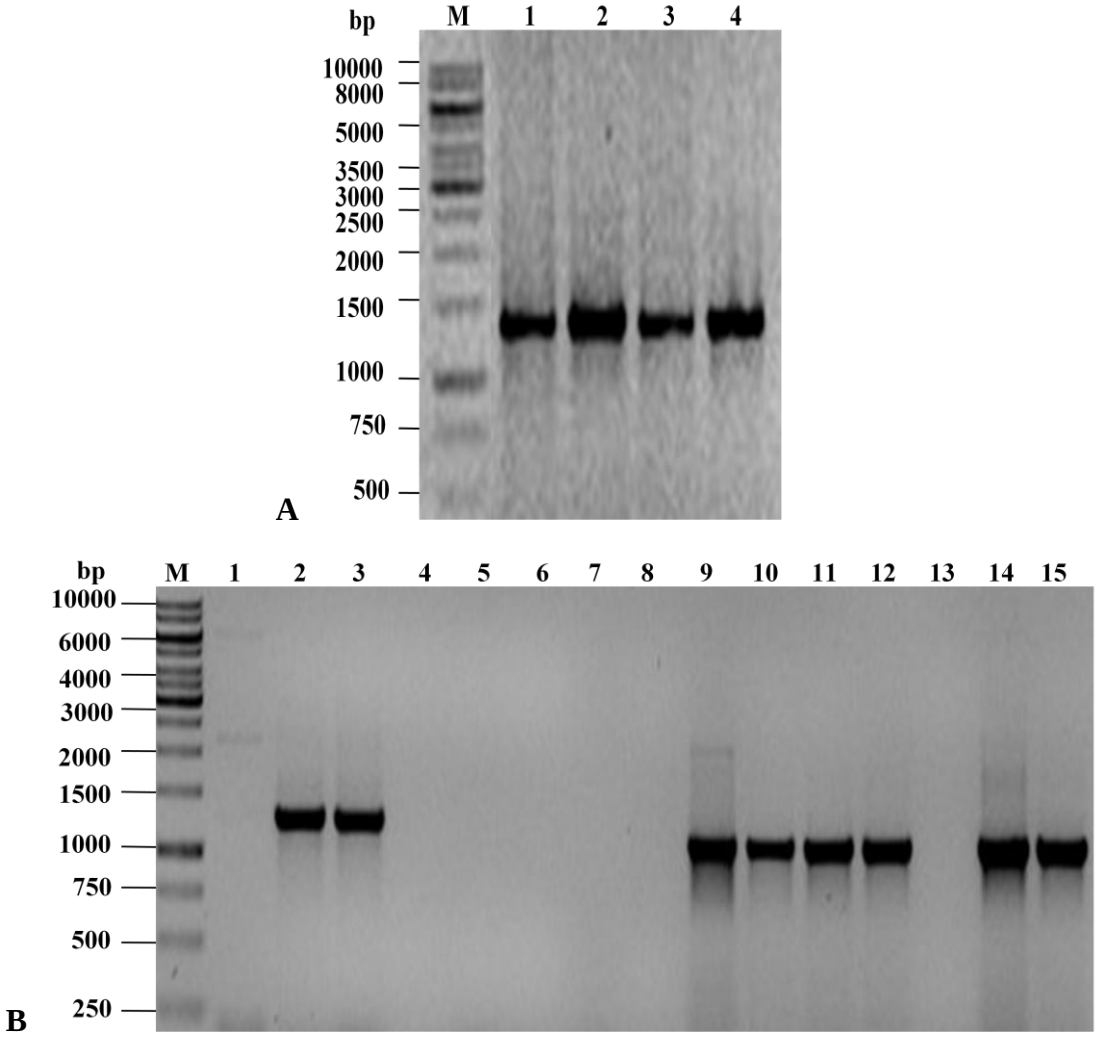


Figure 2.11 Agarose gel (1%) analysis of colony PCR for screening of *TbgISG65* in expression vectors. (A) pET28a-*TbgISG65* transformed colonies. Lane M, O'GeneRuler 1kb DNA ladder; lanes 1-4, PCR products using pET primers. **(B)** pGEX-4T1-*TbgISG65* transformed colonies. Lane M, O'GeneRuler 1kb DNA ladder; lanes 2-3, PCR products using pGEX primers; lane 9, PCR product using *T. b. brucei* gDNA as colony PCR control; lanes 10-15, PCR products using *ISG65* primers; lanes 1, 4-8 and 13, PCR products of non-recombinant colonies.

2.3.2 Heterologous expression of ISG65 in *E. coli*

2.3.2.1 Heterologous expression of Tbb_{His}ISG65 and Tbb_{GST}ISG65

The His-tagged fusion protein Tbb_{His}ISG65 was observed at a size of 55 kDa on a 10% reducing SDS-PAGE gel (Figure 2.12A). Monitoring of Tbb_{His}ISG65 expression in *E. coli* over a four hour period post IPTG induction showed a clear expression band of 55 kDa in the samples collected at the first and second hours of expression, this band was not present in the pre-induction sample (Figure 2.12A, lanes 0-2). A decrease in band intensity was observed for samples from the third and fourth hour of expression which could signify a decrease in expression levels (Figure 2.12A, lanes 3-4). The Tbb_{GST}ISG65 fusion protein was observed at a larger size of 79 kDa due to the presence of the GST fusion tag of 25 kDa (Figure 2.12B).

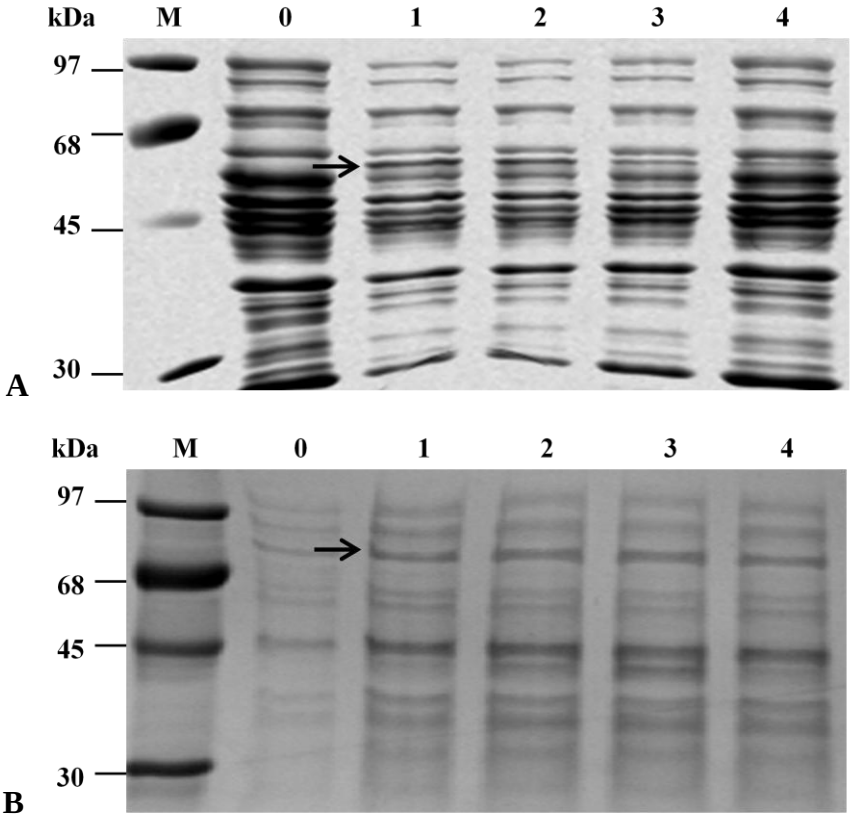


Figure 2.12 Heterologous expression analysis of Tbb_{His}ISG65 in *E. coli* over time after IPTG induction. Coomassie-stained 10% reducing SDS-PAGE gel at different induction time intervals. Target proteins are identified by arrows. Lane M, molecular weight markers; lanes 0-4, expression lysates at 0-4 h post IPTG induction; **(A)** total cell lysate for Tbb_{His}ISG65; **(B)** total cell lysate for Tbb_{GST}ISG65.

2.3.2.2 Heterologous expression of *Tbg*$_{His}$ISG65 and *Tbg*$_{GST}$ISG65

Heterologous expression of *Tbg*$_{His}$ISG65 in *E. coli* monitored over a four hour period was observed from the first hour and increased progressively in subsequent samples (Figure 2.13A). The His-tagged fusion protein was observed at a size of 55 kDa (see arrow, Figure 2.13A); a similar size as the *Tbb*$_{His}$ISG65 homologue (Figure 2.12A). Reducing SDS-PAGE analysis of *Tbg*$_{GST}$ISG65 expression lysate, showed a prominent 79 kDa band which increased progressively in intensity from the first to fourth hour of expression (Figure 2.13B, lanes 1-4). The differences in the sizes of the *Tbg*$_{His}$ISG65 (Figure 2.13A) and *Tbg*$_{GST}$ISG65 (Figure 2.13B) fusion proteins are due to the differences in the sizes of the fusion tags: His-tag (6 kDa) and the larger GST tag (25 kDa).

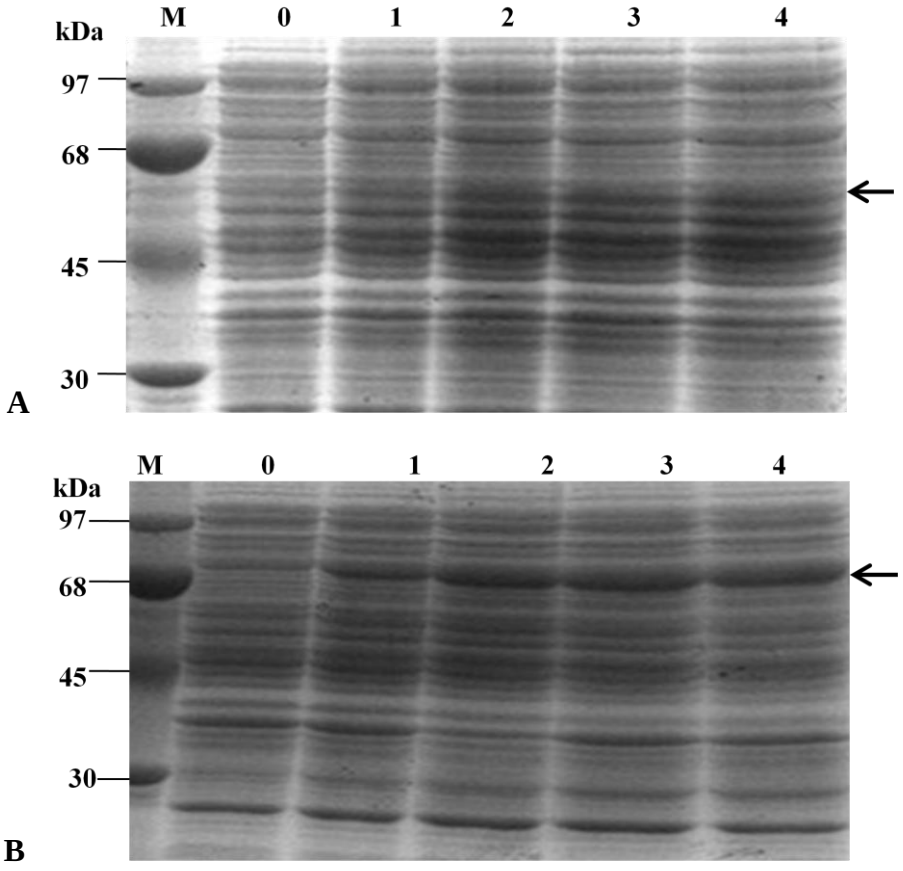


Figure 2.13 Heterologous expression analysis of *Tbg*ISG65 in *E. coli* over time after IPTG induction. Coomassie-stained 10% reducing SDS-PAGE gel at different induction time intervals. Target proteins are identified by arrows. Lane M, molecular weight markers; lanes 0-4, expression lysates at 0-4 h post IPTG induction; **(A)** total cell lysate for *Tbg*$_{His}$ISG65; **(B)** total cell lysate for *Tbg*$_{GST}$ISG65.

2.3.2.3 Assessment of the solubility of ISG65 fusion proteins

Reducing SDS-PAGE analysis of the enzymatically lysed *E. coli* cells expressing *Tbb*$_{His}$ISG65 (Figure 2.14A) and *Tbb*$_{GST}$ISG65 (Figure 2.14C) fusion proteins showed that the proteins were expressed as both soluble and insoluble proteins at 37^{o}C. The fusion protein *Tbb*$_{His}$ ISG65 was identified as a 55 kDa band in both the insoluble (Figure 2.14A, lanes 1 and 3) and soluble (Figure 2.14A, lanes 2 and 4) fractions of the cell lysates. Analysis of the samples of *Tbb*$_{His}$ISG65 expressed at 25^{o}C showed very minimal levels of the fusion protein in the insoluble fraction; a higher proportion was present in the soluble cell fraction (Figure 2.14B, lanes 1 and 2). The assessment of the solubility status of the GST-tagged fusion protein *Tbb*$_{GST}$ISG65 also showed its accumulation both in inclusion bodies and in the soluble fractions of the expressing *E. coli* cells (Figure 2.14C).

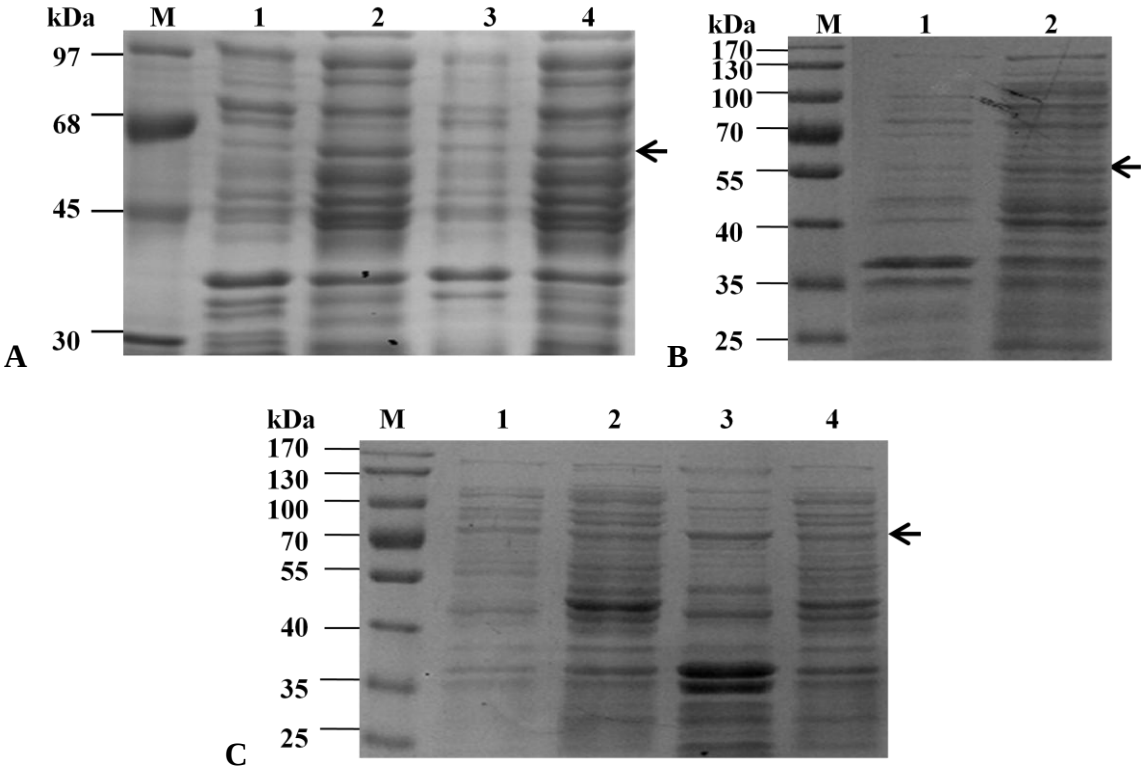


Figure 2.14 Assessment of the solubility of recombinant *Tbb*$_{His}$ISG65 and *Tbb*$_{GST}$ISG65. Coomassie-stained 10% reducing SDS-PAGE of soluble and insoluble cell fractions. Lane M, molecular weight markers; **(A)** soluble (lanes 2 and 4) and insoluble (lanes 1 and 3) fractions of *Tbb*$_{His}$ISG65 expressed at 37^{o}C; **(B)** soluble (lane 2) and insoluble (lane 1) fractions of *Tbb*$_{His}$ISG65 expressed at 25^{o}C; **(C)** soluble (lanes 2 and 4) and insoluble (lanes 1 and 3) fractions of *Tbb*$_{GST}$ISG65 expressed at 37^{o}C. Target proteins are identified by arrows.

The solubility status of the fusion proteins, *Tbg*$_{His}$ISG65 and *Tbg*$_{GST}$ISG65 was also assessed by reducing SDS-PAGE analysis of the insoluble and soluble cell fractions of the expression lysates. Both fusion proteins, *Tbg*$_{His}$ISG65 (Figure 2.15A) and *Tbg*$_{GST}$ISG65 (Figure 2.15B) were accumulated as both soluble and insoluble proteins in the expressing *E. coli* cells. However, a greater percentage of the expressed proteins were accumulated as inclusion bodies in the insoluble cell fractions (lane 2, Figures 2.15A and B)

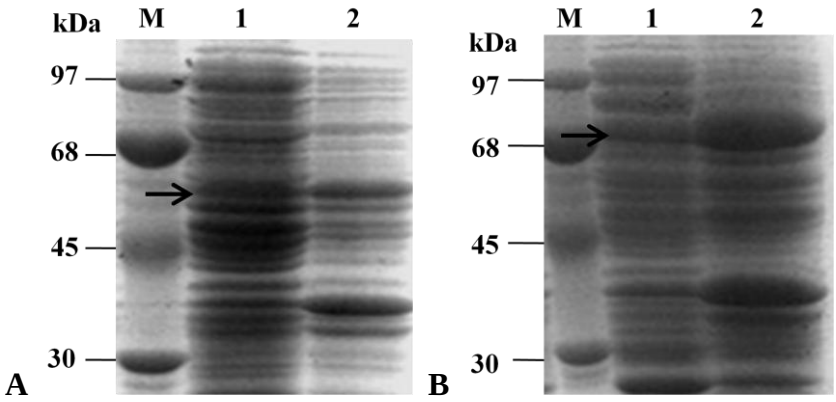


Figure 2.15 Assessment of the solubility of recombinant *Tbg*$_{His}$ISG65 and *Tbg*$_{GST}$ISG65. Coomassie-stained 10% reducing SDS-PAGE gel of soluble and insoluble cell fractions. Target proteins are identified by arrows. Lane M, molecular weight markers; **(A)** soluble (lane 1) and insoluble (lane 2) fractions of *Tbg*$_{His}$ISG65; **(B)** soluble (lane 1) and insoluble (lane 2) fractions of *Tbg*$_{GST}$ISG65.

2.3.3 Heterologous expression of ISG65 in *P. pastoris*

In order to take advantage of another eukaryotic expression system, ISG65 was expressed in the yeast *P. pastoris*. Reducing SDS-PAGE analysis revealed an 82 kDa protein in both the expression supernatant (Figure 2.16, lanes 1-3) and cell pellet (Figure 2.16, lanes 4-6), showing that the expressed ISG65*Pp* was not targeted only to the secretory pathway of the yeast cells, some of the expressed protein was also accumulated in the cells. The 82 kDa size is indicative of the ISG65 fusion protein with a hSUMO3 tag of approximately 23 kDa.

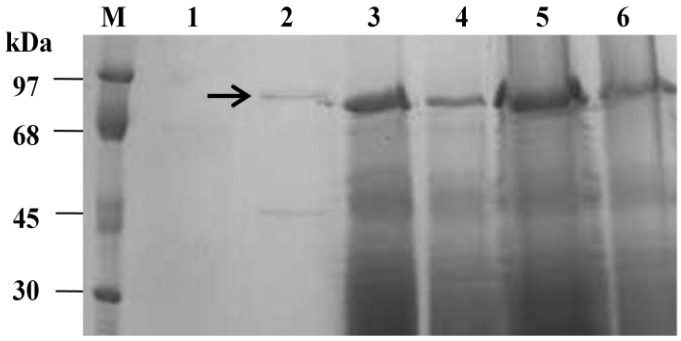


Figure 2.16 Reducing SDS-PAGE analysis of intracellular and secreted expression of ISG65 in *P. pastoris*. The gel was silver stained. Lane M, molecular weight markers; lanes 1-3, expression supernatants for days 1, 3 and 5; lanes 4-6, cell pellets from days 1, 3, and 5. ISG65*Pp* identified by arrow.

2.3.4 Western blotting analysis of ISG65 expression

There was a need to confirm that the bands identified in Figures 2.12 to 2.16 were indeed representative of the expressed ISG65 fusion proteins. Also, an additional expression band of 40 kDa was identified in all the ISG65 expression lysates and these needed to be analysed as well. Western blotting analysis using mouse anti-His tag antibody identified Tbb_{His}ISG65 as two bands at 55 kDa and 40 kDa (lanes 2-5, Figure 2.17A). Interestingly, Tbb_{GST}ISG65 was also identified as two bands at 79 kDa and 40 kDa using anti-ISG65 antibodies (lanes 2-5, Figure 2.17B).

Expression of ISG65*Pp* in yeast was confirmed using mouse anti-His tag antibody. The antibody recognised two bands for ISG65*Pp* at 82 kDa and 40 kDa (Figure 2.18). The 82 kDa band was representative of the ISG65*Pp* fusion protein with a hSUMO tag that is approximately 23 kDa in size. Interestingly, the antibodies recognised a lower 40 kDa band for ISG65 fusion proteins in all the expression systems analysed. An explanation is required for this second band consistently observed for ISG65 in the present study.

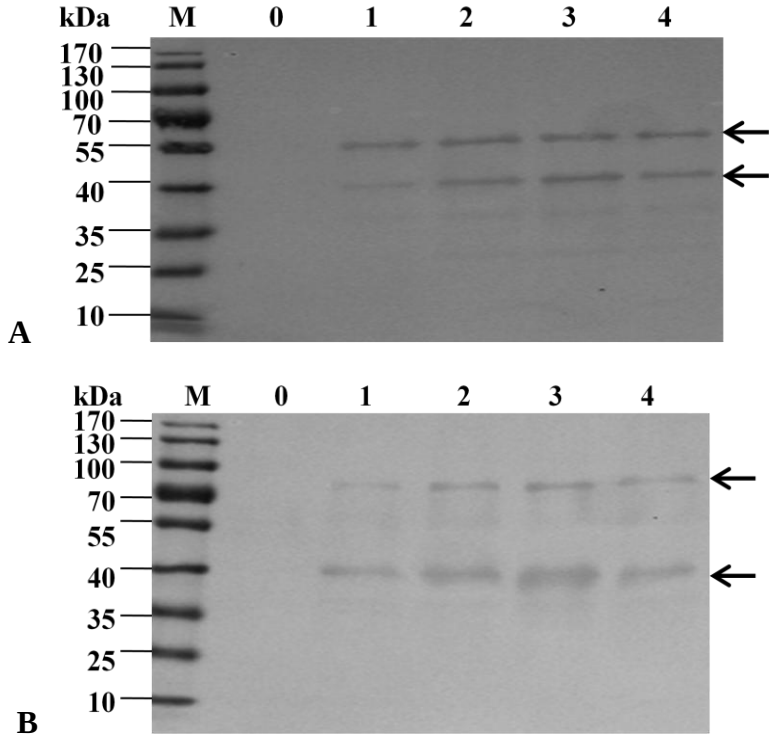


Figure 2.17 Western blot analysis of ISG65 expression. Samples were separated by 10% reducing SDS-PAGE, transfered onto nitrocellulose membranes and probed with antibodies to confirm expression of ISG65 fusion proteins (see arrows). (**A**) *Tbb*$_{His}$ISG65 lysate probed with mouse anti-His tag antibody. Lane M, pre-stained MWM; lanes 0-4, expression lysates for 0, 1, 2, 3 and 4 h post IPTG induction. **(B)** *Tbb*$_{GST}$ISG65 lysate probed with chicken anti-ISG65 IgY. Lane M, pre-stained MWM; lanes 0-4, expression lysates for 0, 1, 2, 3 and 4 h post IPTG induction.

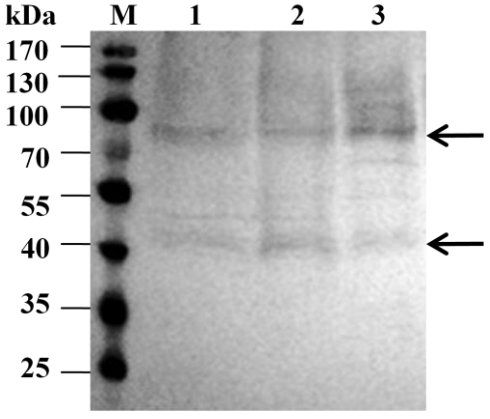


Figure 2.18 Western blot analysis of ISG65*Pp* expression. Samples were separated by 10% reducing SDS-PAGE, transfered onto nitrocellulose membranes and probed with mouse anti-His tag antibody to confirm expression of ISG65*Pp* fusion protein. Lane M, pre-stained MWM; lanes 1-3, ISG65*Pp* supernatants from days 1, 3 and 5 of expression. (ISG65*Pp* identified by arrows).

2.3.5 Purification of ISG65 expressed from different constructs

2.3.5.1 Fractionation of *Tbb$_{His}$*ISG65 and ISG65*Pp* using TPP

The secretion of the expressed proteins in yeast systems into the medium poses the challenge of the expressed protein contained in a large volume, and a concentration step is thus required before purification. The technique of three-phase partitioning (TPP) is effective for the purification of proteins directly from large volumes of crude suspension (Pillay *et al.*, 2010). Resuspension of the precipitate obtained in a small volume effected concentration of the sample. ISG65*Pp*, though present in all the different pellets, was most abundant in the 20% ammonium sulfate pellet (Figure 2.19A, arrow). The preferential precipitation of ISG65 at this ammonium sulfate concentration not only served to concentrate the protein, but also assisted in the purification of ISG65 from the crude sample by reducing the number of contaminating proteins. *Tbb$_{His}$*ISG65 was also abundant in the 20% ammonium sulfate fraction (Figure 2.19B, arrow).

2.3.5.2 Purification of ISG65 fusion proteins using nickel affinity chromatography

ISG65*Pp*, *Tbb$_{His}$*ISG65 and *Tbg$_{His}$*ISG65 were expressed as fusion proteins with poly histidine tags to facilitate purification by nickel affinity chromatography. *Tbb$_{His}$*ISG65 was purified as a fusion protein of two bands at 55 kDa and 40 kDa (Figure 2.20A) as confirmed by a western blot of the expression lysate using anti-His tag antibodies (section 2.3.4, Figure 2.17A). ISG65*Pp* purified with bands at approximately 82 kDa and 40 kDa (Figure 2.20B), the same as in the western blot using anti-His tag antibodies (section 2.3.4, Figure 2.18). The purification of *Tbg$_{His}$*ISG65 carried out using Ni-NTA resin was not successful (Figure 2.20C).

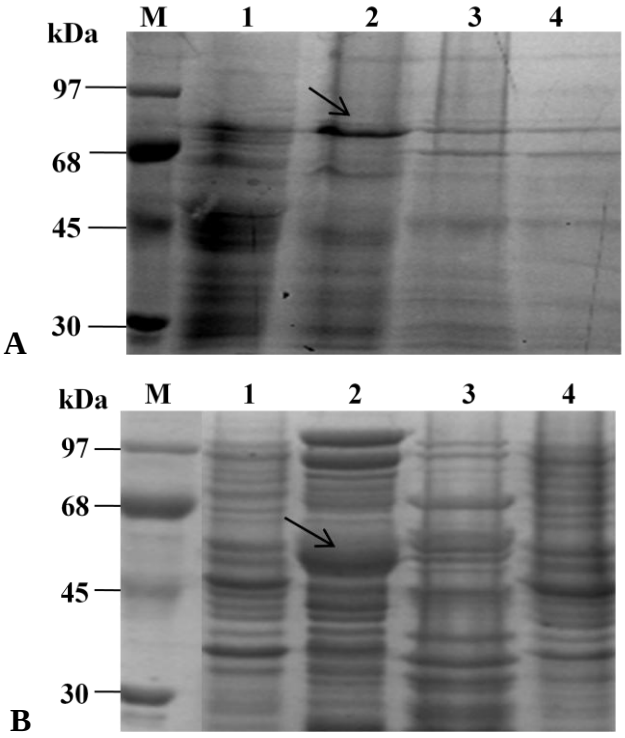


Figure 2.19 Reducing SDS-PAGE analysis of fractions obtained from TPP concentration of expressed ISG65*Pp* and *Tbb*$_{His}$ISG65. Lysates containing ISG65*Pp* and *Tbb*$_{His}$ISG65 were concentrated using TPP with increasing percentages of ammonium sulfate (10–40%). ISG65 in the 20% fraction is identified by arrow. **(A)** TPP concentrated ISG65*Pp*. Lane M, molecular weight markers; lanes 1-4, 10, 20, 30, and 40% ammonium sulfate fractions. **(B)** TPP concentrated *Tbb*$_{His}$ISG65. Lane M, molecular weight markers; lanes 1-4, 10, 20, 30, and 40% ammonium sulfate fractions.

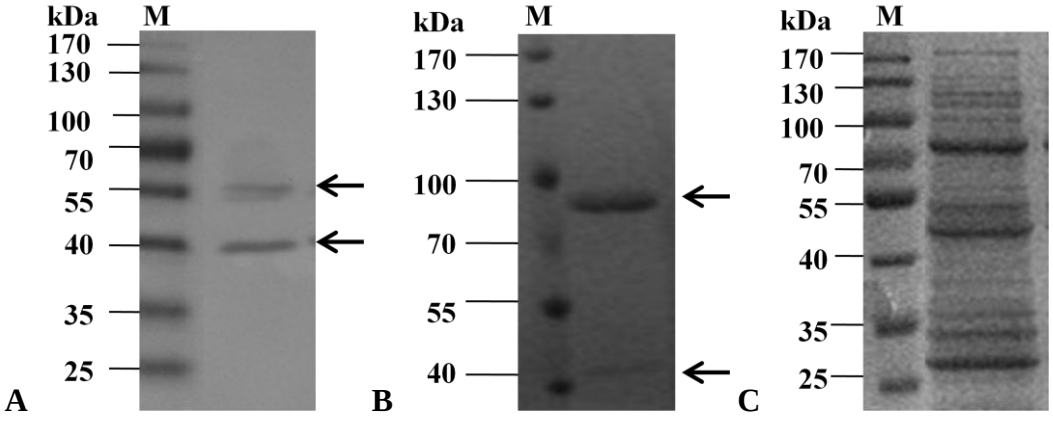


Figure 2.20 Reducing SDS-PAGE analysis of affinity purified ISG65 using nickel chelate chromatography. The eluted fractions from the affinity columns were analysed by 10% reducing SDS-PAGE to assess purification. **(A)** Purified *Tbb*$_{His}$ISG65 (identified by arrow); **(B)** Purified ISG65*Pp* (identified by arrow); **(C)** Purified *Tbg*$_{His}$ISG65. Lane M, pre-stained molecular weight markers.

2.3.5.3 Purification of ISG65 fusion proteins using GST affinity chromatography

The GST fusion proteins, *Tbb*$_{GST}$ISG65 and *Tbg*$_{GST}$ISG65, were purified from contaminating *E. coli* proteins by GST affinity chromatography. Reducing SDS-PAGE analysis of eluates showed that *Tbb*$_{GST}$ISG65 purified as two bands at 79 and 45 kDa (Figure 2.21A). Cleavage of the GST tag left *Tbb*$_{GST}$ISG65 immobilised to the column. Two bands were observed at approximately 66 and 45 kDa for purified *Tbb*$_{GST}$ISG65 without the tag (Figure 2.21B, lanes 1 and 2). The GST tag was also eluted (Figure 2.21B, circled). Reducing SDS-PAGE analysis of the fractions obtained from the purification of *Tbg*$_{GST}$ISG65 showed the expected sizes of 79 kDa and 45 kDa for the purified fusion protein (Figure 2.21C). Additional bands were observed as well: a 66 kDa band representative of the *Tbg*$_{GST}$ISG65 protein cleaved from its GST tag and a 25 kDa band corresponding to the GST tag (circled, Figure 2.21C). A contaminating protein of 82 kDa size was however not separated from the purified *Tbg*$_{GST}$ISG65 (Figure 2.21C).

2.3.5.4 Molecular Exclusion Chromatography purification of *Tbb*$_{His}$ISG65

Following nickel affinity purification and concentration of *Tbb*$_{His}$ISG65 using PEG M_r 20 000, the protein was further purified using MEC to separate the two bands observed for ISG65 in Figure 2.20A. The elution profile from a Sephacryl S-200 HR MEC column is shown in Figure 2.22A. Two bands of 55 and 40 kDa sizes for *Tbb*$_{His}$ISG65 were seen in fractions 23-28 corresponding to elution volumes of 46-56 ml (Figure 2.22B, lanes 2-6). Sephacryl S-200 HR MEC was not successful in the separation of the two bands of ISG65. Some degradation products at approximately 28 kDa and 18 kDa were, however, separated from ISG65 and eluted in fractions 29-32 corresponding to 58-64 ml (Figure 2.22B, lanes 7-10). Interestingly, analysis of a fraction eluted at a volume of 46 ml (Figure 2.22A, see arrow), which is equivalent to 106 kDa (calculated using Fischer's plot, Figure 2.3, Section 2.2.7.4) contained *Tbb*$_{His}$ISG65 migrating at a size of 55 kDa when observed on reducing SDS-PAGE (Figure 2.22B, lane 2).

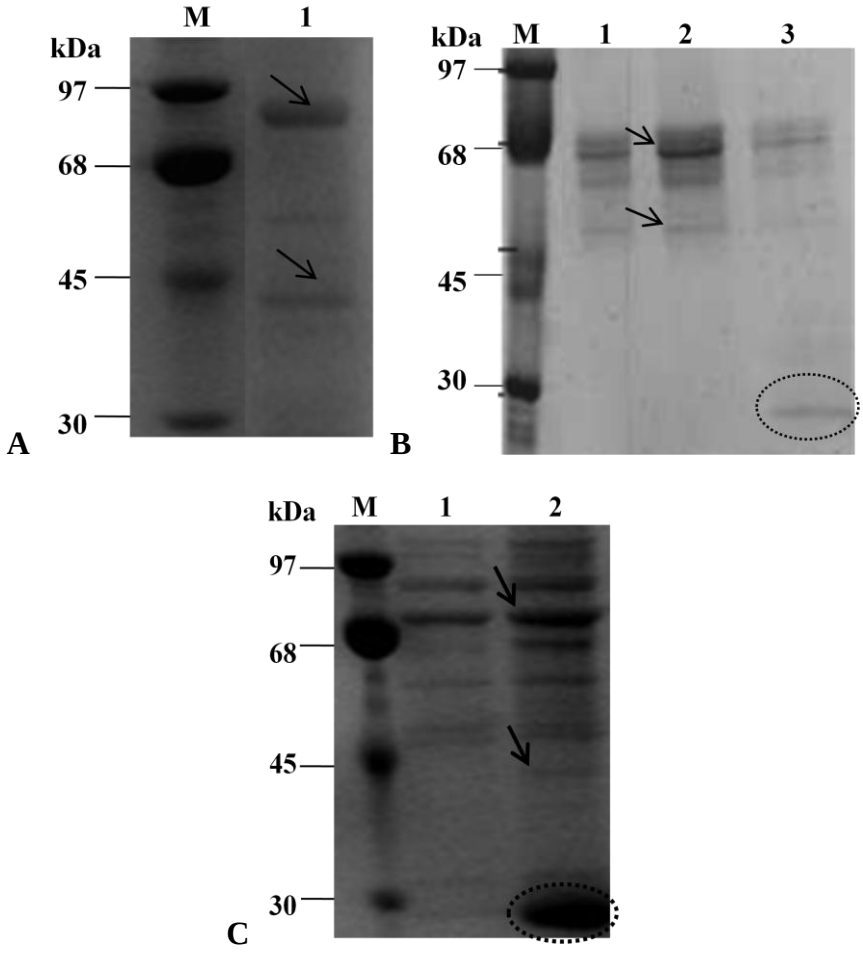


Figure 2.21 Reducing SDS-PAGE analysis of affinity purified ISG65 using glutathione-agarose. The fusion proteins affinity purified using GST resins were analysed by 10% reducing SDS-PAGE to assess purification. **(A)** Purified Tbb_{GST}ISG65. Lane M, molecular weight markers; lane 1, fraction from purification; **(B)** Purified Tbb_{GST}ISG65. Lane M, molecular weight markers; lanes 1-2, fractions 1-2 from purification; lane 3, elution of GST tag (circled); **(C)** Tbg_{GST}ISG65. Lane M, molecular weight markers; lanes 1-2, fractions 1-2 from purification. Target proteins identified by arrows and GST by circles.

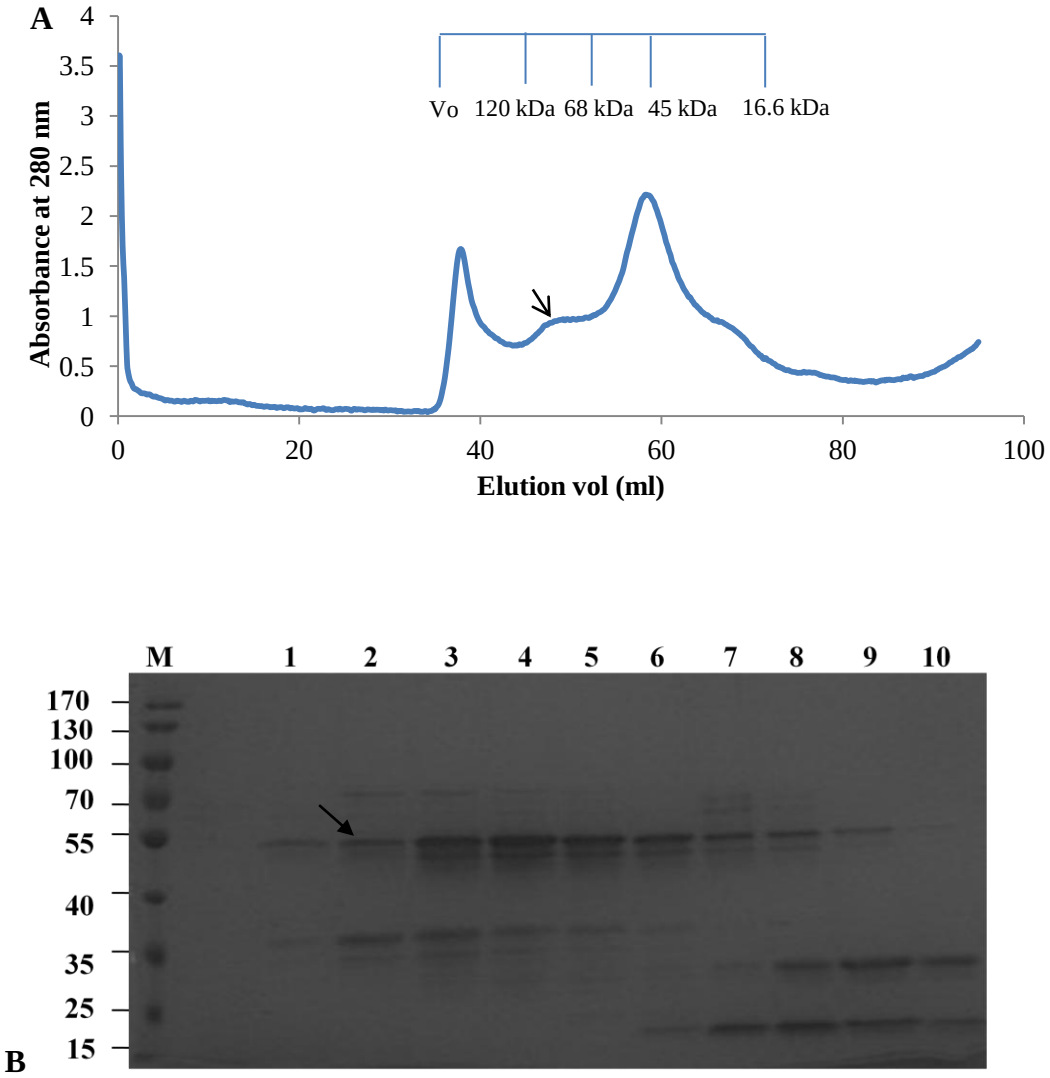


Figure 2.22 Purification of *Tbb*$_{His}$ISG65 on a Sephacryl S-200 molecular exclusion column. **(A)** Elution profile of ISG65 from a Sephacryl® S-200 HR column (16 x 600 mm, flow rate of 0.5 ml/min, 120 ml) in MEC buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8). The A_{280} readings were plotted against the elution volumes. Start of ISG65 elution identified by arrow. **(B)** Reducing SDS-PAGE analysis of 2 ml fractions collected from purification. Lane M, molecular weight markers; lanes 1-10, fractions 23-32 (46-64 ml).

2.3.6 Glycosylation assessment

ISG65 is a glycoprotein with possible N-glycosylation sites in its sequence (Ziegelbauer *et al.*, 1992). Due to the differences in its apparent molecular weight in SDS-PAGE (82 kDa) and the calculated molecular weight from the amino acid sequence (66 kDa), the possibility of glycosylation was considered. Reducing SDS-PAGE analysis of Endoglycosidase H treated and untreated samples of ISG65*Pp* showed differences in the electrophoretic mobilities of both samples (Figure 2.23). The Endoglycosidase H treated sample migrated at the expected size of 66 kDa, which was smaller than the size of the untreated sample (82 kDa). The 16 kDa size difference is most likely due to the removal of the sugar moieties from the ISG65*Pp* fusion protein by Endoglycosidase H.

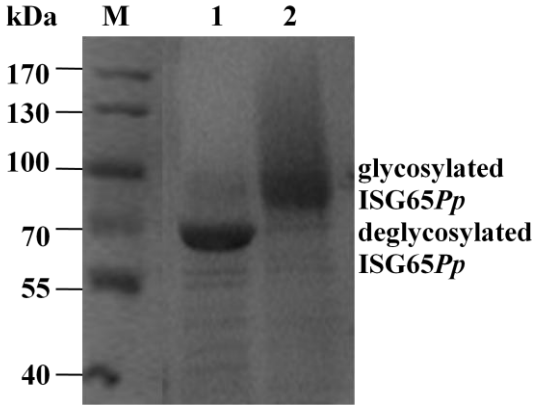


Figure 2.23 Reducing SDS-PAGE of ISG65 glycosylation assessment. Glycosylation of ISG65 was assessed by comparing the electrophoretic mobility of Endoglycosidase H treated and untreated ISG65*Pp* samples. Lane M, molecular weight markers; lane 1, Endoglycosidase H treated ISG65*Pp* sample; lane 2, Endoglycosidase H untreated ISG65*Pp* sample.

2.4 Discussion

Annual economic losses in Africa due to animal trypanosomiasis have been estimated to be in the region of billions of US dollars (Kristjanson *et al.*, 1999; FAO, 1994). Early diagnosis of the disease has been identified as one of the key focus areas for the control of the disease (Welburn *et al.*, 2009). The numerous challenges faced by currently available diagnostic tools have highlighted the demand for more efficient diagnostic tools (Simarro *et al.*, 2008). Invariant surface glycoproteins have been identified as potential diagnostic antigens for the serological diagnosis of trypanosomiasis (Hutchinson *et al.*, 2004). This study focused on the production of soluble ISG65, which could be used for diagnosis and immunological studies. Recombinant production was selected over extraction of the native proteins because recombinant technology provides the opportunity to produce large quantities of pure, soluble and functional proteins at lower cost and with greater ease of isolation (Du *et al.*, 2005). Only the extracellular domain of the *ISG65* gene was expressed in this study. This was motivated by the fact that only this region of the polypeptide would be exposed to the host's immune system in a trypanosomal infection, hence the antigenic epitopes of the ISG65 would be located in this region of the protein.

In this study, the same primer sets were used for the amplification the *ISG65* gene of *T. b. brucei* from *T. b. brucei* genomic DNA as well as for *ISG65* of *T. b. gambiense* from LiTat 1.3 genomic DNA. The nucleotide sequences of EcoR1 and Not1 restriction endonucleases were incorporated into the forward and reverse primers, respectively. This was necessary to facilitate the removal of the *ISG65* gene from the T-vector DNA to facilitate the sub-cloning of the gene into expression vectors. These restriction endonucleases were chosen because of their availability in the laboratory, equivalent optimum performance in buffer 'O' and most importantly because of the absence of their restriction sequence in the nucleotide sequence of the *ISG65* gene. This was in order to prevent the digestive action of the restriction endonucleases occurring at inappropriate sites in the cloned gene, leading to an *ISG65* insert with an incorrect size. Also, the presence of an EcoR1 restriction site in the pTZ57R/T vector map was exploited for the confirmation of the right orientation of the cloned *ISG65* gene in this vector.

In the present study, ISG65 of *T. b. brucei* and *T. b. gambiense* LiTat 1.3 were recombinantly expressed as fusion proteins in *E. coli*. *Escherichia coli* is frequently the host of choice as a "recombinant cell factory" due to its relative simplicity, inexpensiveness, well-known genetics, fast high density cultivation as well as for the availability of compatible molecular tools. Strategies are also in place to overcome obstacles faced during expression. For instance, cases whereby the recombinant proteins expressed are insoluble and/or non-functional. Fusion partners and chaperones, amongst others, can be used to overcome such challenges (Sorensen and Mortensen, 2005). The *E. coli* JM109 strain, a good strain for cloning, was used for the initial transformation of the ligated products of *ISG65* in expression vectors. However, subsequent transformation into the protease deficient *E. coli* BL21 (DE3) strain was necessary before expression to avoid proteolytic degradation of expressed proteins by the bacterial proteases. Also the BL21 (DE3) strain is ideal for high level protein expression for constructs containing T7 RNA polymerase promoter driven vectors such as pET28a used in the present study (Robinson, 2011).

In this study, sub-cloning of *T. b. brucei* ISG65 and *T. b. gambiense* ISG65 into pGEX-4T1 and pET28a expression vectors was conducted to exploit the advantages of these different expression systems. This was in order to identify the best system to meet the aim of producing large quantities of soluble antigens for diagnostic assays. Also, *Tbb*ISG65 and *Tbg*ISG65 were expressed as fusion proteins to facilitate purification (Baneyx, 1999). The pGEX-4T1 and pET28a expression vectors supplied the glutathione *S*-transferase (GST) and poly-histidine (His) tags, respectively. The His-tag consists of 6 histidine residues giving it a small size. This small size and high specific binding capacity to immobilised metal ions such as nickel, in affinity resins, makes it very useful for the purification of recombinant proteins (Du *et al.*, 2005). If the downstream applications of the recombinant ISG65 require the removal of the His tag, this could easily be achieved by proteolytic cleavage using specific enzymes like thrombin, the recognition sequence of which is included in the fusion construct. The glutathione *S*-transferase (GST) tag, however, is quite large ~ 25 kDa so it needs to be proteolytically removed by thrombin hydrolysis of the bond between Arg-Gly residues in the sequence Leu-Val-Pro-Arg-Gly-

Ser (Smith and Johnson, 1988). In this study, this cleavage was carried out when the fusion protein was immobilised on the glutathione column, thus further purification steps to separate the tag from the recombinant protein were obviated.

Expression of Tbb_{His}ISG65 in pET28a gave a yield of 403.7 µg per 40 ml of culture while Tbb_{GST}ISG65 in pGEX-4T1 had a yield of 33.3 µg. In this study, a higher yield of soluble ISG65 was observed for the pET28a expression system than the pGEX-4T1 expression system. Major losses during the concentration steps caused a great reduction in the final yield obtained. TPP was an effective crude purification method for ISG65 expressed in both *E. coli* and *P. pastoris*. It not only served for the concentration of the expression lysate but the technique was also able to separate extraneous bacterial or yeast proteins from the target protein, ISG65. As has been earlier described by Pike and Dennison (1989), this method is relatively cheap, effective and easy to perform.

Expression of ISG65 was also undertaken in the methylotropic yeast *P. pastoris* in order to compare the expression yields with that of *E. coli*. Yeasts are eukaryotes like trypanosomes; hence, it was thought that the expression of ISG65 in this system could offer the added advantage of the expressed proteins being able to undergo post-translational modifications similar to that experienced by the native ISGs in trypanosomes. One such modification is glycosylation. Analysis of the amino acid sequence of ISG65 predicts four potential glycosylation sites at Asn residues 155, 334, 354 and 415 (Ziegelbauer *et al.*, 1992). In the present study, treatment of ISG65*Pp* with Endoglycosidase H which cleaves oligomannose type N-glycans (Mehlert *et al.*, 2012b) resulted in a shift in the electrophoretic mobility of the protein in SDS-PAGE (Figure 2.23). The reduced size of the deglycosylated protein confirmed the removal of the N-linked glycans from the fusion protein ISG65*Pp*.

Purification of ISG65 expressed in *P. pastoris* was significantly easier than that of ISG65 expressed in *E. coli*. This is expected because the expressed proteins in this system are targeted to the secretory pathway. Since *P. pastoris* secretes a very low level of its native proteins, the vast majority of the proteins in the growth medium will be the heterologous protein (Balamurugan *et al.*, 2007). Ordinarily this would have been the expression system

of choice. However, the low expression levels of ISG65 in this system led to a preference for the *E. coli* expression system.

The apparent molecular weight on SDS-PAGE gel for *Tbb*$_{His}$ISG65 fusion protein was 55 kDa (Figure 2.20A). This was larger than the size of 46 kDa predicted from the gene sequence. Previous studies have stated that ISG65 has a slow migration on SDS-PAGE compared to its predicted molecular mass (Leung *et al.*, 2011). Also it has been shown that alanine-rich proteins of *T. brucei* have a heterogeneous mobility and a larger apparent molecular weight in SDS-PAGE (Nolan *et al.*, 2000). So it is possible that the high alanine content of the ISGs could also be a contributing factor to their slow migration rate on SDS-PAGE.

Not much data is available for the recombinant expression of ISG65. Native ISG65 purified from BSF *T. brucei* was reported to elute from a molecular exclusion chromatography column at a size of 150 kDa which was almost double its apparent molecular weight of 70 kDa on SDS-PAGE (Jackson *et al.*, 1993). This led to speculations about the existence of ISG65 as a dimer in solution. In the present study, recombinant *Tbb*$_{His}$ISG65 purified by MEC (Figure 2.22) was eluted at a volume consistent with a larger protein (106 kDa). Reducing SDS-PAGE analysis of these fractions, however, still showed an apparent molecular weight of 55 kDa. The fact that the molecular size of ISG65 from the MEC was 106 kDa which is almost double its apparent size of 55 kDa on SDS-PAGE seems to point to the possibility of dimerisation by this glycoprotein. The question, however, still remains about the explanation of the second band at 40 kDa consistently observed for ISG65 expressed in the present study.

In order to explore the antigenic potential of the recombinant ISG65 produced, there is a need to demonstrate a comparable antigenic character of the recombinant ISG65 with native ISG65. This can be confirmed by demonstrating that anti-ISG antibodies from infection sera are able to recognise the recombinant ISGs. Also the anti-recombinant ISG65 antibodies need to be able to recognise native ISGs. Immunological assays described in subsequent chapters in the present study include: ELISAs, immunofluorescence assays as well as western blots.

This study has been able to provide a necessary step for the production of sufficient quantities of ISG65 for other downstream applications. ISG65 is one of the two major transmembrane domain invariant surface glycoproteins of trypanosomes. Due to the paucity of information about these trypanosomal invariant glycoproteins, further characterisation and possibly tertiary structure determination might be able to provide a clearer picture of the functions of these glycoproteins in trypanosomes and in disease pathogenesis. Hence, it would be worthwhile to embark on the production of its counterpart: ISG75 before the characterisation of both proteins. The results of ISG75 cloning and expression is described in chapter three.

CHAPTER 3

A comparison of the recombinant expression profiles of invariant surface glycoprotein-75 (ISG75) of two sub-species of *Trypanosoma brucei*

3.1 Introduction

With an estimated number of 50,000 molecules per cell, ISG75 is the next most abundant trypanosome surface glycoprotein after the VSGs and ISG65 (Ziegelbauer *et al.*, 1992). The ISG75 gene family is present in multiple copies in the genome of all the pathogenic species and sub-species of the *Trypanozoon* sub-genus (Tran *et al.*, 2009). This includes *T. b. brucei, T. b. gambiense, T. b. rhodesiense, T. congolense, T. evansi and T. equiperdum* (Tran *et al.*, 2006).

In the *Trypanozoon* sub-genus, the ISG75 gene family is divided into two main groups that share at least 75% identity among their cDNA and genomic DNA sequences. Apart from one exception, ISG75 group I sequences consist of 523 residues in contrast to group II sequences that have 522 residues. Invariant surface glycoprotein-75, a trypanosome surface glycoprotein, undergoes post-translational modifications before being targeted to the trypanosome's surface, an example of which is glycosylation. Predictions of N-glycosylation sites within the ISG75 sequences show different trends amongst the group I and group II sequences. All group I sequences have only one glycosylation site at amino acid 134 (tripeptide NAS) while most group II sequences have two glycosylation sites which are at position 134 (tripeptide NSS) and position 115 (tripeptide NRT) (Tran *et al.*, 2006).

The ISG75 polypeptide is characterised by a small signal peptide, a large extracellular domain and small trans-membrane and cytoplasmic domains (Rudramurthy *et al.*, 2013; Tran *et al.*, 2008). The mature ISG75 polypeptide starts at amino acid residue 29 after being cleaved off its signal peptide. The signal peptide is a region of the extracellular domain that is cleaved off from the mature polypeptide when targeted to the surface of the trypanosome. The extracellular domain is the largest region of ISG75 ranging from

residues 29 to 467. Residues 29 to 300 are variable while the remaining portion from residues 301 to 467 is conserved across the *Trypanozoons*. Like other type 1 trans-membrane proteins, ISG75 has a membrane spanning helix from amino acid residues 468 to 490. C-terminal to this region is a short hydrophilic domain exposed on the cytoplasmic surface of the plasma membrane (residues 491-522) termed the intracellular domain. This region and the trans-membrane domain are conserved across the *Trypanozoon* taxa (Figure 3.1).

The functions of ISG75 in trypanosomes are not fully known. Invariant surface glycoprotein-75 which is only present in the bloodstream stage undergoes multiple rounds of endocytosis, trafficking through the parasite's endosomal pathways and recycling back to the parasite's surface (Koumandou *et al.*, 2011; Leung *et al.*, 2011). Invariant surface glycoprotein-75 is turned over through an ubiquitin and ESCRT-dependent mechanism (Chung *et al.*, 2008; Leung *et al.*, 2008). Ubiquitylation is an important process for the down regulation of many trans-membrane domain proteins (Leung *et al.*, 2011). Given the importance of endocytosis and recycling for the survival of trypanosomes in the host's system, ISG75 is thought to play a role in the pathogenesis and immune evasion processes of BSF trypanosomes (Koumandou *et al.*, 2013). Using RNAi target-sequencing (RIT-seq) screen, it was shown that ISG75 is involved in the binding and uptake of suramin, a drug that is used for the treatment of first stage HAT (Alsford *et al.*, 2012). These results have highlighted the possibility of these glycoproteins performing a physiological role as a receptor, but the natural ligand(s), however, is/are yet to be identified (Koumandou *et al.*, 2013).

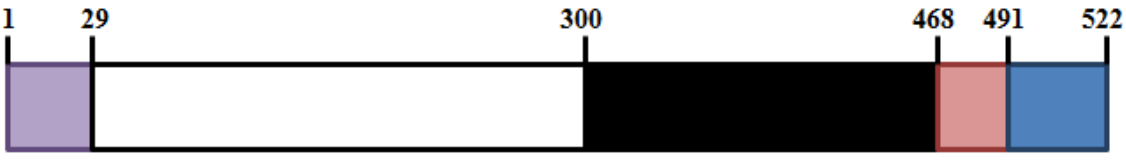


Figure 3.1 Schematic diagram of mature ISG75 polypeptide with predicted domains. From the N-terminus, purple box indicates signal peptide, white and black boxes refer to the variable and conserved regions of the extracellular domain, pink box depicts the trans-membrane region and blue box indicates the intracellular domain [Adapted from Tran *et al.* (2008)].

In this study, the extracellular domains of *T. b. brucei* ISG75 and *T. b. gambiense* ISG75 were cloned and recombinantly expressed in *E. coli* and *P. pastoris* expression systems. The growth profiles and production levels of the fusion proteins in *E. coli* BL21 (DE3) expression system over a four hour period were recorded. The expression of recombinant ISG75 fusion proteins in *E. coli* and *P. pastoris* systems, was confirmed by western blotting analysis using anti-His tag and anti-ISG75 antibodies as primary antibodies. Also nickel affinity chromatography and GST affinity chromatography was employed for the purification of the fusion proteins.

3.2 Materials and Methods

3.2.1 Materials

Common laboratory chemicals and reagents were obtained from Fermentas (Vilnus, Lithuania), Roche Diagnostics (Germany), Sigma-Aldrich (Munich, Germany), and Thermo Scientific (EU, Lithuania).

Cloning and sub-cloning: EcoR1 and Not1 restriction endonucleases, *Taq* polymerase, FastRuler® middle range DNA ladder, Instaclone PCR cloning kit, Zymo DNA clean and concentrator kit, pEq Gold gel extraction kit, TransformAid® kit and GeneJET® miniprep kit were purchased from Fermentas (Vilnus, Lithuania). BL21 (DE3) and JM109 *E. coli* competent cells were from New England Biolabs (Ipswich, MA, USA). Shrimp alkaline phosphatase (SAP) was from Roche Diagnostics (Germany), the pET28a expression vector was obtained from Novagen (San Diego, CA, USA) and O'GeneRuler 1kb DNA ladder was from Thermo Scientific (EU, Lithuania). Constructs of *T. b. gambiense ISG75* in PCR® 4Blunt-TOPO® plasmid and *TbgISG75* in pP-αhSUMO3 plasmid in M5-strain of *P. pastoris* were gifts from Prof. Philippe Büscher, Unit of Parasite Diagnostics, Institute of Tropical Medicine, Antwerp, Belgium.

Protein purification and quantification: Analytical grade thrombin protease and Ni-NTA affinity resin were purchased from Novagen (San Diego, CA, USA). Gluthatione-agarose

was purchased from Sigma-Aldrich (Munich, Germany), HisTrap® FF Crude and GSTrap® FF HiPrep® pre packed columns were from GE Healthcare (Sweden) and BCA Protein Assay Kit and 10 kDa cut off dialysis tubing from Pierce (Rockford, USA).

Antibodies: Mouse anti-His tag antibody, rabbit anti-chicken IgY horseradish peroxidase (HRPO) conjugate and goat anti-mouse IgG HRPO-conjugate were purchased from Jackson ImmunoResearch (USA). HRPO conjugated Nickel chelate was from Separation Scientific (South Africa) and chicken anti-GST tag antibody was an in house preparation.

3.2.2 Cloning of *T. b. brucei ISG75*

The nucleotide sequence for *T. b. brucei ISG75* AnTat 2.2 clone G06 (Accession DQ200189) was obtained from GenBank (http://www.ncbi.nlm.nih.gov accessed on 20-07-11) and used in the design of primers for the amplification of the extracellular domain of ISG75. The forward (5´-CCGAATTCAGCAACTTACCTGTCGCATA-3´) and reverse (3´-TTGCGGCCGCTAACACTTTCGTTGTCCCAAT-5´) primers containing EcoR1 and Not1 restriction sites (underlined) were used in a PCR reaction in the GeneAmp 2400 thermocycler (Applied Biosystems, Singapore). The PCR mix consisted of 0.25 µM of each primer, 1 U of *Taq* polymerase, 0.25 mM dNTPs, 2.5 mM $MgCl_2$, 10 x *Taq* buffer, sterile dH_2O and the genomic DNA of *T. b. brucei* (isolated as described, section 2.2.2) as template. The PCR with a reaction volume of 20 µl was carried out using the following program: initial denaturation at 94^oC for 5 min, denaturation at 94^oC for 1 min, annealing at 50^oC for 1 min, extension at 72^oC for 2 min, 30 cycles and final extension at 72^oC for 7 min.

The *TbbISG75* PCR product was cloned into the pTZ57R/T vector as described previously (section 2.2.3.1) and subsequently sub-cloned into EcoR1 and Not1 linearised pGEX-4T1 vector (Figure 3.2) as described in section 2.2.3.2. The resulting recombinant plasmids were transformed into *E. coli* JM109 and subsequently into chemically competent BL21 (DE3) cells for the maintenance of the recombinant plasmids and protein expression. Transformed colonies were confirmed by PCR using vector and insert specific primers. This construct was denoted as $Tbb_{GST}ISG75$.

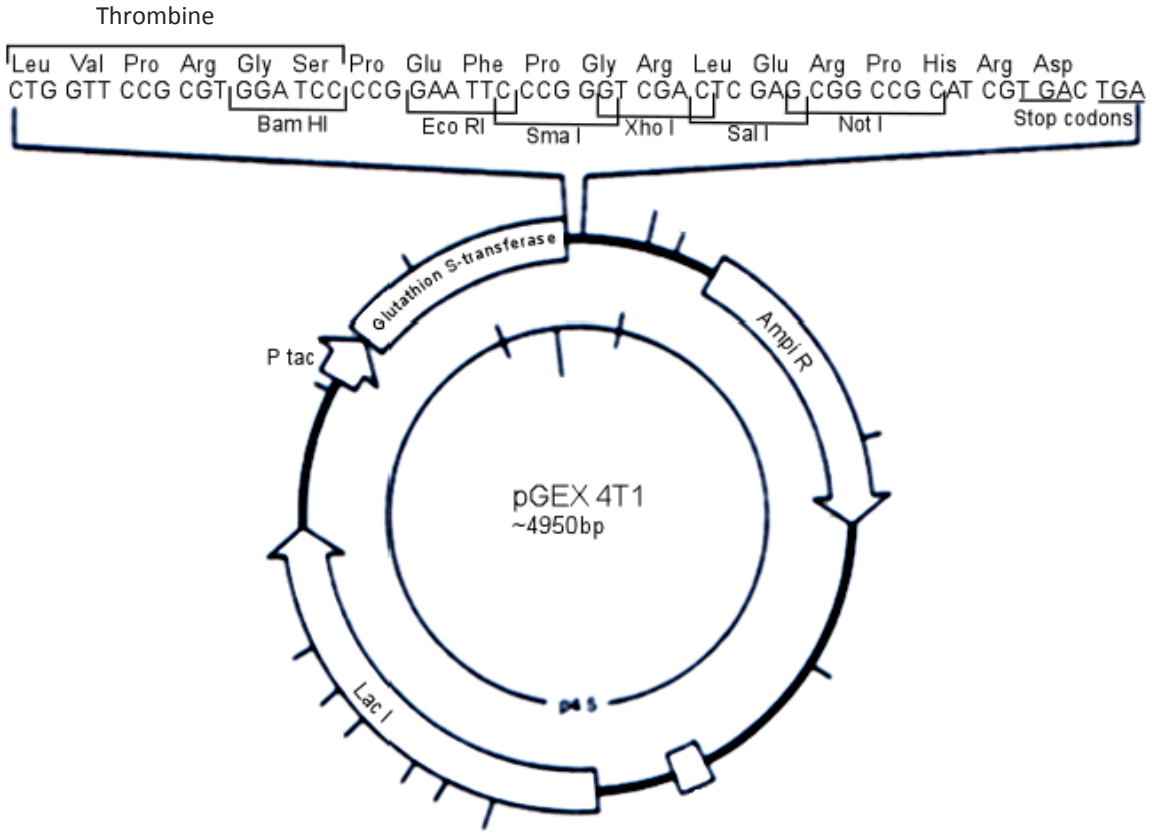


Figure 3.2 Schematic map of pGEX-4T1 vector used for ISG75 expression. It includes a Ptac promoter origin of replication, the glutathione *S*-transferase coding sequence, an ampicillin resistance coding sequence, a *Lac*I repressor coding sequence and a thrombin cleavage site.

3.2.3 Sub-cloning of *T. b. brucei ISG75* and *T. b. gambiense ISG75* into pET28a vector

The *TbbISG75* gene was excised from $Tbb_{GST}ISG75$ plasmid (prepared as per section 3.2.2) using EcoR1 and Not1 restriction endonucleases (Fermentas, Lithuania) in a 50 µl reaction volume containing 11 µg of plasmid DNA, 5 µl of buffer O, 10 U of each enzyme and sterile distilled water. A similar digestion was carried out for *Tbg ISG75* coding region from constructs containing *TbgISG75* in PCR® 4Blunt-TOPO® plasmid (a gift from Prof. Philippe Büscher, Unit of Parasite Diagnostics, Institute of Tropical Medicine, Antwerp, Belgium). The restriction products were analysed on a 1% agarose gel and the bands corresponding to each *ISG75* insert: *TbbISG75* and *TbgISG75* were excised under UV

light. The insert DNA was extracted from agarose using the pEq Gold gel extraction kit (Fermentas, Lithuania) as per the manufacturer's instructions. Briefly, an equivalent volume of 'buffer XP2' was added to the agarose gel slice and incubated at 55^{o}C for 7 min to dissolve it. The sample was loaded onto the spin column provided and centrifuged (10 000 x *g*, 1 min, RT). The column was washed twice with 'CG wash buffer' using centrifugation (10 000 x *g*, 1 min, RT) and dried by further centrifugation (10 000 x *g*, 1 min, RT). The insert DNA was eluted using 50 µl of elution buffer and centrifugation (5 000 x *g*, 1 min, RT).

The expression vector, pET28a (Figure 3.3), was similarly restricted with EcoR1 and Not1 endonucleases and dephosphorylated using shrimp alkaline phosphatase in a 40 µl reaction volume containing 35 µl of vector DNA, 4 µl of 10 x reaction buffer and 1 µl of shrimp alkaline phosphatase. The reaction mixture was incubated at 37^{o}C for 2 h and heated at 65^{o}C (15 min) to inactivate the enzyme (Sorensen and Mortensen, 2005). The vector was cleaned using the Zymo DNA clean and concentrator kit (Fermentas, Lithuania) as described (section 2.2.3.1). The inserts, *TbbISG75* and *TbgISG75*, were ligated as described into the cleaned pET28a expression vector. The ligation products were transformed into *E. coli* JM109 cells and subsequently into *E. coli* BL21 (DE3) competent cells by chemical transformation using the TransformAid® kit (Fermentas, Lithuania) as previously described (section 2.2.3.1). Transformants that contained a gene of the correct size as determined by colony PCR were also confirmed using restriction enzyme digestion. Glycerol stocks were prepared from overnight cultures of recombinant colonies and stored at -80^{o}C. The *T. b. brucei ISG75* and *T. b. gambiense ISG75* genes, cloned into pET28a expression vector, were denoted as $Tbb_{His}ISG75$ and $Tbg_{His}ISG75$ respectively.

3.2.4 Expression and blotting analysis of ISG75 fusion proteins

3.2.4.1 Expression of recombinant ISG75 proteins in *E. coli*

Three way streaks were made from glycerol stocks of transformed *E. coli* BL21 (DE3) cells containing $Tbb_{GST}ISG75$, onto 2x YT agar [1.6% (w/v) peptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) agar, 50 µg/ml ampicillin] plates. Colonies were

picked from the overnight agar plates, inoculated into 2x YT broth and incubated with agitation (280 rpm, 16 h, 37^{o}C). This culture was diluted 1 in 10 with fresh medium and incubated at 37^{o}C until the A_{600} reached 0.7-0.9. Expression was induced with 1 mM IPTG and incubated at 37^{o}C for 4 h.

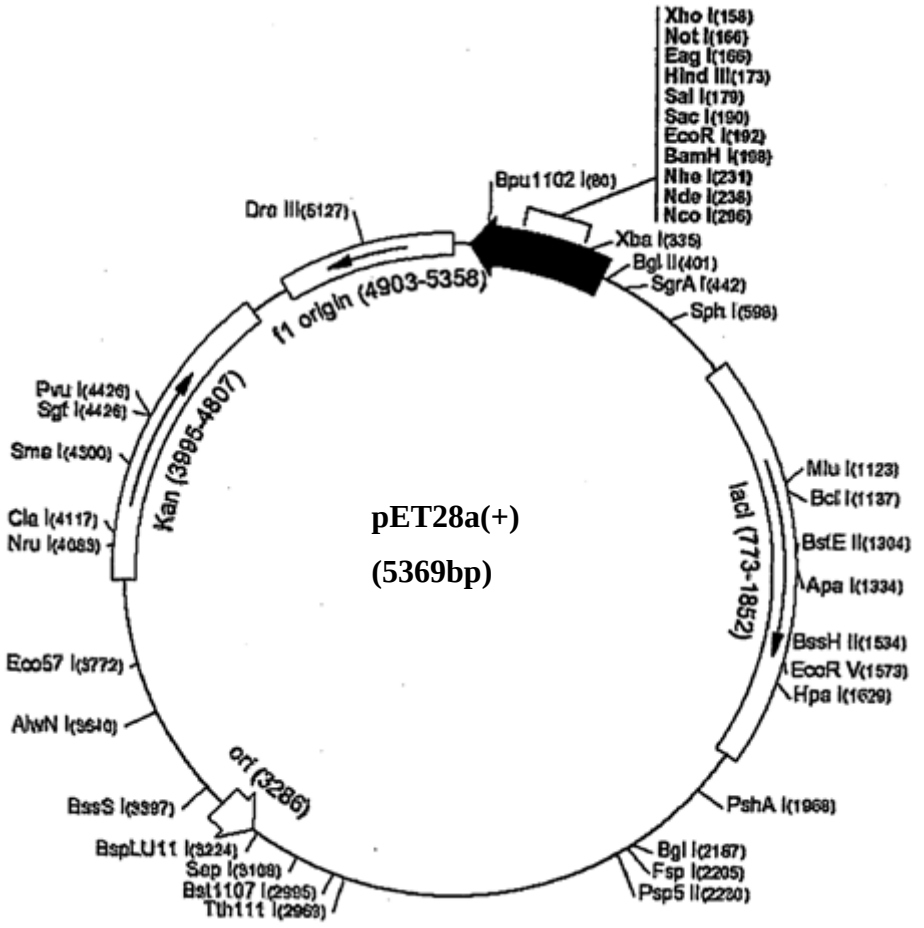


Figure 3.3 Schematic map of pET28a vector used for ISG75 expression. It includes a T7*lac* promoter, a T7 transcription start region, 2 His-tag coding sequences, a multiple cloning site, T7 terminator, *lac*I coding sequence, a pBR322 origin of replication, a kanamycin resistance coding sequence and an fl origin.

Expression of $Tbb_{His}ISG75$ and $Tbg_{His}ISG75$ was similarly carried out using 2x YT broth [1.6% peptone, 1% yeast extract, and 0.5% NaCl] containing kanamycin sulfate (34 µg/ml). One hundred ml aliquots were withdrawn hourly to monitor and quantify protein expression.

3.2.4.2 Preparation of cell lysates and solubility test for expressed proteins

The bacteria cells were harvested from culture by centrifugation (6 500 x *g*, 10 min, 4^{o}C). The pellet was resuspended in lysis buffer [1% (v/v) PBS-Triton X-100, pH 7.4, containing 1 mg/ml lysozyme], incubated at 37^{o}C for 15 min and put through several freeze-thaw and sonication (on ice, 4 x 30 s, with 10 s intervals) cycles to facilitate cell lysis. The lysed cells were centrifuged (6 500 x *g*, 10 min, 4^{o}C), to separate the soluble fraction from insoluble cellular organelles. The supernatant (soluble fraction) and pellets (insoluble fraction) were analysed by 10% reducing SDS-PAGE (Laemmli, 1970) for the presence of the expressed fusion protein.

3.2.4.3 Recombinant expression of *Tbg$_{Pp}$*ISG75 in *P. pastoris*

In order to compare the heterologous expression of ISG75 in prokaryotes with a eukaryotic expression system, constructs containing *T. b. gambiense ISG75* in the pP-αhSUMO3 plasmid in the M5-strain of *P. pastoris* were obtained from Prof. Philippe Büscher (Unit of Parasite Diagnostics, Institute of Tropical Medicine, Antwerp, Belgium). A single colony of transformed *P. pastoris* cells from a yeast extract peptone dextrose (YPD) [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose, 2% (w/v) agar] plate containing 100 µg/ml Zeocin® was inoculated in buffered glycerol-complex medium (BMGY) [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, 0.00004% (w/v) biotin, 1% (v/v) glycerol, 100 µg/ml Zeocin®], to create a biomass by incubating at 29^{o}C with agitation (usually for 48-72 h). Expression was induced by resuspending the cells harvested by centrifugation (2 000 x *g*, 5 min, 4^{o}C) in buffered methanol-complex medium (BMMY) [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, 0.00004 % (w/v) biotin, 0.5% (v/v) methanol, 100 µg/ml Zeocin®] and incubating at 29^{o}C with agitation. Induction was maintained by daily addition of methanol to a final concentration of 0.5% (v/v) for the duration of the 7 days expression. Intracellular and secreted expression was monitored by reducing SDS-PAGE analysis as described (section 2.2.5).

3.2.4.4 Western blotting analysis of ISG75 fusion proteins

Lysates containing ISG75 fusion proteins were separated on a 10% reducing SDS-PAGE gel at 20 mA per gel and blotted onto nitrocellulose by placing the gel on the membrane, in a sandwich of four sheets of blotting paper. Protein transfer was achieved using a western blotting apparatus (Mighty small transphor, Hoefer, USA) and the incubation steps were carried out as previously described (section 2.2.6). The primary and secondary antibodies used for each fusion protein are detailed in Table 3.1. The blot was developed using either 0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2 in TBS (20 mM Tris, 200 mM NaCl, pH 7.4) or an enhanced chemiluminescence detection kit (Pierce, Rockford, USA) and imaging using the Sys Gene® imaging system (Syngene®, USA).

Table 3.1 Details of antibodies used for western blot detection of each ISG75 fusion protein.

Fusion protein	Expression host	Primary antibody	Secondary antibody
Tbb$_{GST}$ISG75	*E. coli*	Chicken anti-GST tag (0.5 µg/ml)	HRPO-conjugated rabbit anti-IgY (1:5 000)
Tbb$_{His}$ISG75	*E. coli*	HRPO-conjugated nickel chelate (1:12 000)	None
Tbg$_{His}$ISG75	*E. coli*	HRPO-conjugated nickel chelate (1:12 000)	None
Tbg$_{Pp}$ISG75	*P. pastoris*	Chicken anti-ISG75 IgY (7.5 µg/ml)	HRPO-conjugated rabbit anti-IgY (1:5 000)

3.2.5 Purification and concentration of expressed ISG75 fusion proteins

3.2.5.1 Fractionation of *Tbg*$_{Pp}$ISG75 by three phase partitioning (TPP)

The *Tbg*$_{Pp}$ISG75 fusion proteins expressed in the *P. pastoris* system were concentrated using three phase partitioning (TPP) as described previously (Pike and Dennison, 1989). The fractions from the different percentages of ammonium sulfate (10-40%) used with 30% (v/v) t-butanol were analysed using 10% reducing SDS-PAGE to determine the optimal percentage of ammonium sulfate for purification.

3.2.5.2 Purification of recombinant ISG75 using nickel affinity chromatography

Purification of *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75 was achieved by affinity chromatography using pre-packed nickel chelate resins: HisTrap® FF crude on an ÄKTA purifier (GE Healthcare, Sweden). The soluble cell fraction from expression was filtered and injected into a HisTrap® FF affinity column (GE Healthcare, Sweden) containing 1 ml of nickel chelate resin at a flow rate of 0.5 ml/min. The packed column was washed with a 10-fold excess volume of binding buffer (0.5 M NaCl, 20 mM imidazole, 20 mM Na_2HPO_4, pH 7.4) and the bound proteins were eluted with elution buffer (0.5 M NaCl, 0.5 M imidazole, 20 mM Na_2HPO_4, pH 7.4) at 1 ml/min. Each fraction consisted of 1 ml of eluate. The A_{280} of fractions (1 ml) was monitored (ÄKTA prime plus, GE Healthcare, Sweden). The purity of the protein samples was assessed by analysing aliquots of the fractions using 10% reducing SDS-PAGE and western blotting with appropriate primary antibodies (anti-His tag or anti GST-tag antibodies).

Lysates of the aliquots (100 ml) collected hourly during the expression of *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75 were purified separately and total protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, USA) as described previously (section 2.2.8).

The fractions of *Tbg*$_{Pp}$ISG75 from TPP were pooled and filtered prior to purification by affinity chromatography using HisTrap® FF crude on an ÄKTA purifier (GE Healthcare, Sweden) as described above.

3.2.5.3 Purification of *Tbb*$_{GST}$ISG75 using GST affinity chromatography

The GST-tagged fusion protein *Tbb*$_{GST}$ISG75, was purified from bacteria lysates using GSTrap® FF affinity columns (GE Healthcare, Sweden) with a liquid chromatography system (ÄKTA prime plus, GE Healthcare, Sweden) and also glutathione-agarose affinity resins (Sigma-Aldrich, Germany) as per the manufacturer's instructions. *Tbb*$_{GST}$ISG75 was cleaved from the GST tag while it was adsorbed to glutathione-agarose column. On-column cleavage was done using 2 U of analytical grade thrombin (1 U/µl, Novagen,

USA) in 200 mM Tris-HCl buffer, pH 8.4 as previously described (section 2.2.7.3). Purified ISG75 was analysed by 10% reducing SDS-PAGE (Laemmli, 1970).

3.2.5.4 Concentration and quantification of recombinant ISG75

The total protein concentration was determined using a BCA Protein Assay kit (Pierce, Rockford, USA) as per the manufacturer's instructions. Bovine serum albumin was used as the reference protein. Concentration of purified ISG75 was achieved by dialysis against polyethylene glycol M_r 20 000, using a dialysis membrane with M_r 10 000 cut-off. PBS (10 x) containing 500 mM each of L-arginine and L-glutamic acid was added to the protein sample to a final concentration of 50 mM of each amino acid (Golovanov *et al.*, 2004). This helped to diminish protein aggregation and improve protein stability during the concentration step.

3.2.5.5 Glycosylation assessment of *Tbg$_{Pp}$*ISG75

The presence of an N-glycosylation site has been reported in the *ISG75* gene sequence (Ziegelbauer *et al.*, 1992) and hence it was worthwhile to examine if the recombinant *Tbg$_{Pp}$*ISG75 was N-glycosylated. This was achieved by treating the fusion protein with Endoglycosidase H (Sigma, USA) as previously described (Caffrey *et al.*, 2001). Endoglycosidase H cleaves between the two N-acetyl glucosamine residues in the diacetyl chitobiose core of the oligosaccharide, generating a truncated sugar molecule with one N-acetyl glucosamine residue remaining on the asparagine. In the assay, the sample was denatured and reduced by treatment with SDS [5% (w/v)] and 2-mercaptoethanol [10% (w/v)] with boiling at 100^{o}C for 5 min. The optimum pH for Endoglycosidase H is pH 5-5.6; thus sodium citrate buffer (0.5 M sodium citrate, pH 5.5) was added to the reaction mixture before the addition of the enzyme. The mixture was incubated at 37^{o}C for 3 h. The resultant samples were analysed by 10% reducing SDS-PAGE (section 2.2.5) alongside untreated samples to compare electrophoretic mobilities in order to deduce if there was a reduction in size due to the Endoglycosidase H treatment. Two other trypanosome proteins ISG65 (section 2.3.6) and TvCATL (Vather, 2010) which were previously shown to be glycosylated were used as positive controls for the experiment.

3.3 Results

3.3.1 Cloning of *TbbISG75*

Successful amplification of *TbbISG75* from genomic DNA was confirmed since a band of the expected size of 1342 bp was observed on a 1% agarose gel (Figure 3.4A). Agarose gel (1%) analysis of the recombinant pTZ57R/T cloning vector shows a band at the expected size of 4228 bp (Figure 3.4B), corresponding to the T-vector DNA (2886 bp) and *TbbISG75* fragment (1342 bp) sizes. Sub-cloning of *TbbISG75* into pGEX-4T1 expression vector was also confirmed by observation of a band of low electrophoretic mobility on a 1% agarose gel (Figure 3.4C).

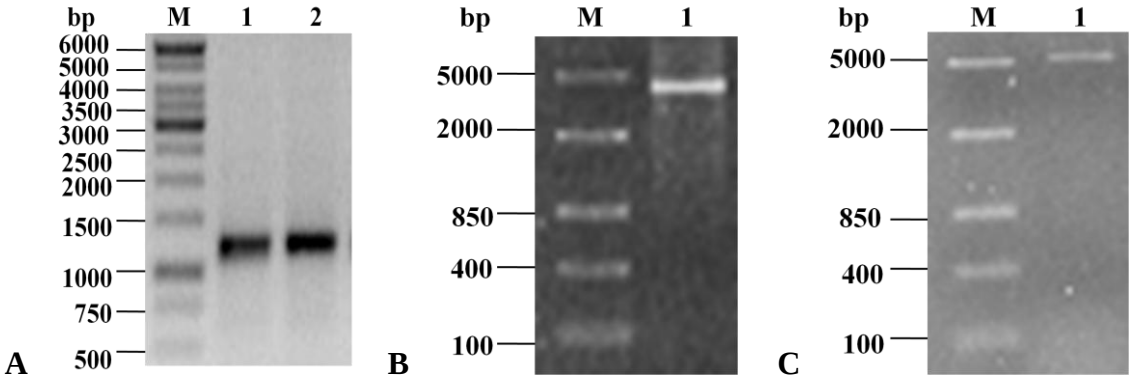


Figure 3.4 Agarose gel (1%) analysis of *TbbISG75* PCR product and recombinant pTZ57R/T and pGEX-4T1 plasmids. (A) Amplified *TbbISG75* fragments (lanes 1-2); lane M, O'GeneRuler 1kb DNA ladder. **(B)** Recombinant pTZ57R/T vector DNA containing *TbbISG75* (lane 1); lane M, FastRuler® middle range DNA ladder. **(C)** Recombinant pGEX-4T1 vector DNA containing *TbbISG75* (lane 1); lane M, FastRuler® middle range DNA.

3.3.2 Sub-cloning of *TbbISG75* and *TbgISG75*

To explore the possibility of obtaining higher yields of *TbbISG75* from a different expression vector, it was necessary to sub-clone the *ISG75* gene into the pET28a expression vector. In order to do this, the *TbbISG75* gene was excised from the recombinant pGEX-4T1 vector DNA shown in Figure 3.4C, by double digestion using EcoR1 and Not1 restriction enzymes. Agarose gel analysis of the double digestion product

showed two bands at 4950 bp and 1342 bp (Figure 3.5A). *Tbb*ISG75, identified as the lower band of 1342 bp (Figure 3.5A), was excised and purified. The successful purification was confirmed by agarose gel analysis which showed only one band of the expected size of 1342 bp (Figure 3.5B).

In order to compare the expression levels of *Tbb*ISG75 with ISG75 from another species of the same sub-genus, recombinant PCR® 4Blunt-TOPO® containing the *Tbg*ISG75 gene (Figure 3.5C, lane 1) was excised with EcoR1 and Not1 restriction endonucleases in a double digestion reaction. *Tbg*ISG75 was identified as the lower band of approximately 1680 bp on a 1% agarose gel of the double digestion product (Figure 3.5C, lane 2). The upper band of approximately 3500 bp corresponds to the linearised TOPO vector DNA (Figure 3.5C, lane 2). Agarose gel analysis of the purified *Tbg*ISG75 fragment showed a band of 1680 bp (Figure 3.5D).

3.3.3 Expression and purification of Tbb_{GST}ISG75

Tbb_{GST}ISG75 was expressed as a fusion protein in BL21 (DE3) *E. coli* expression system. The fusion protein was identified as a discrete band of 88 kDa on a 10% reducing SDS-PAGE gel of the expression lysate (Figure 3.6A, lane 1). This band was not identified in the un-induced control sample (Figure 3.6A, lane 2). Tbb_{GST}ISG75 was expressed both as a soluble and insoluble protein in the *E. coli* cells (Figure 3.6B, arrow). This was confirmed by western blotting analysis using anti-GST tag antibody (Figure 3.6C). In addition to Tbb_{GST}ISG75 at the expected size of 88 kDa, the anti-GST tag antibody also recognised two other bands of 72 and 55 kDa in the insoluble cell components (Figure 3.6C, lane 2).

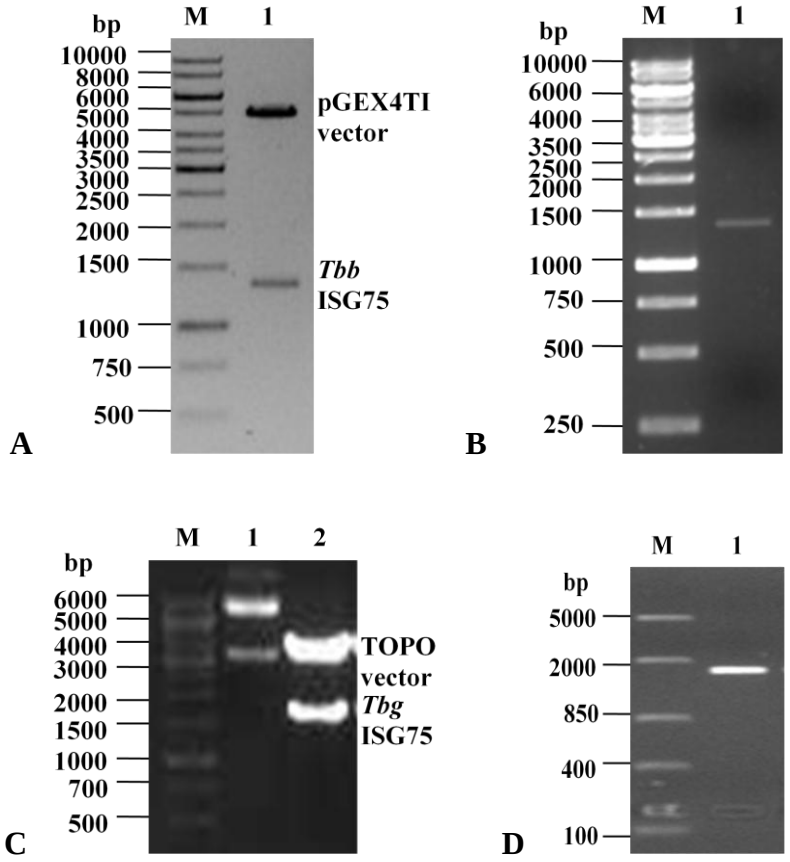


Figure 3.5 Agarose gel (1%) analysis of double digestion products of ISG75 from pGEX-4T1 and PCR®4Blunt-TOPO® vectors. (A) Double digestion product of recombinant pGEX-4T1 vector DNA. Lane M, O'GeneRuler 1kb DNA ladder; lane 1, vector DNA (upper band) and *TbbISG75* (lower band). **(B)** Gel purified *TbbISG75* fragment. Lane M, O'GeneRuler 1kb DNA ladder; lane 1, *Tbb*ISG75 fragment. **(C)** Double digestion product of recombinant PCR® 4Blunt-TOPO®. Lane M, O'GeneRuler 1kb DNA ladder; lane 1, recombinant TOPO plasmid DNA; lane 2, vector DNA (upper band) and *Tbb*ISG75 (lower band). **(D)** Gel purified *Tbg*ISG75 fragment. Lane M, FastRuler® middle range DNA ladder; lane 1, *Tbg*ISG75 insert.

The fusion protein with a GST tag, Tbb_{GST}ISG75 purified by GST-affinity chromatography from cell lysates, was identified on a reducing SDS-PAGE gel at a size of approximately 88 kDa (Figure 3.7A). Upon enzymatic cleavage of the GST tag using thrombin, the size of Tbb_{GST}ISG75 as seen on reducing SDS-PAGE was approximately 66 kDa (Figure 3.7B).

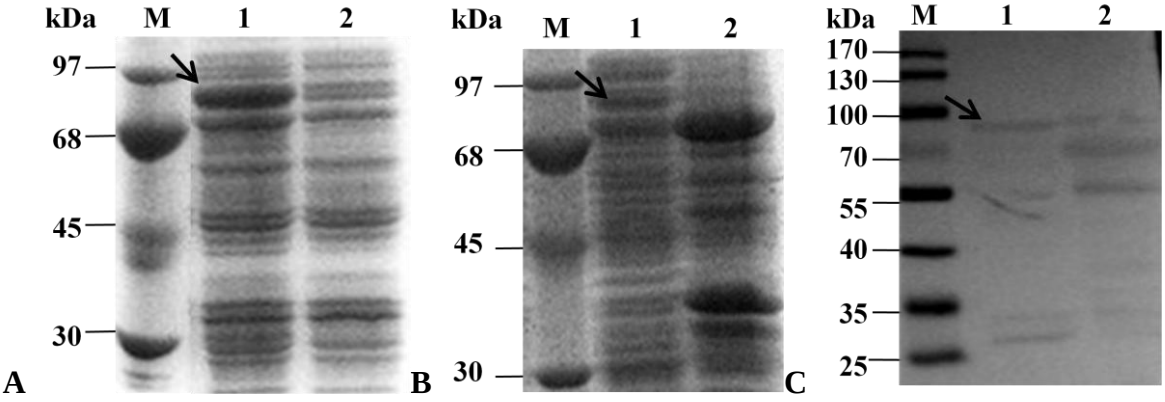


Figure 3.6 Analysis of expression of *Tbb$_{GST}$*ISG75 in BL21 (DE3) *E. coli* by SDS-PAGE (A and B) and western blotting (C). Lane M, molecular weight markers. **(A)** Expression lysate. Lanes 1 and 2, induced and non-induced samples. **(B)** Soluble (lane 1) and insoluble (lane 2) cell fractions. **(C)** Recognition of *Tbb$_{GST}$*ISG75 in soluble (lane 1) and insoluble (lane 2) cell fractions by anti-GST tag antibody. The arrow shows *Tbb$_{GST}$*ISG75.

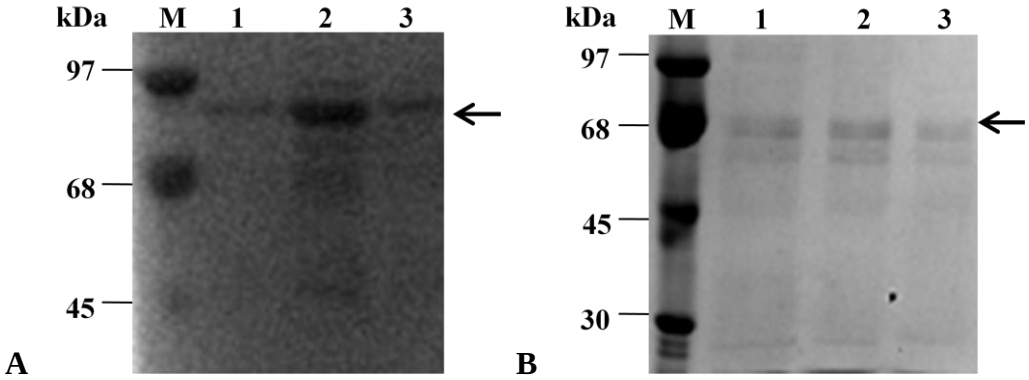


Figure 3.7 Reducing SDS-PAGE analysis of purified *Tbb$_{GST}$*ISG75. (A) Purified *Tbb$_{GST}$*ISG75 fusion protein. Lane M, molecular weight markers; lanes 1-3, fractions 1-3 from purification. **(B)** *Tbg$_{GST}$*ISG75 without a GST tag. Lane M, molecular weight markers; lanes 1-3, fractions 1-3 from purification. Target protein identified by arrow.

3.3.4 Expression of *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75 fusion proteins in *E. coli*

3.3.4.1 Growth profile of *E. coli* cells during the expression of *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75

A plot of the OD_{600} values of the samples collected hourly during the expression of *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75 is shown in Figure 3.8. The growth profile showed a short lag phase (1 h) at an OD_{600} of approximately 0.8 during the expression of both fusion proteins. By the second hour, the cells had entered an exponential phase of growth with the OD_{600} values rising to approximately 1.25. The growth of the cells continued to increase steadily until expression was terminated at the fourth hour, at an OD_{600nm} of approximately 1.7.

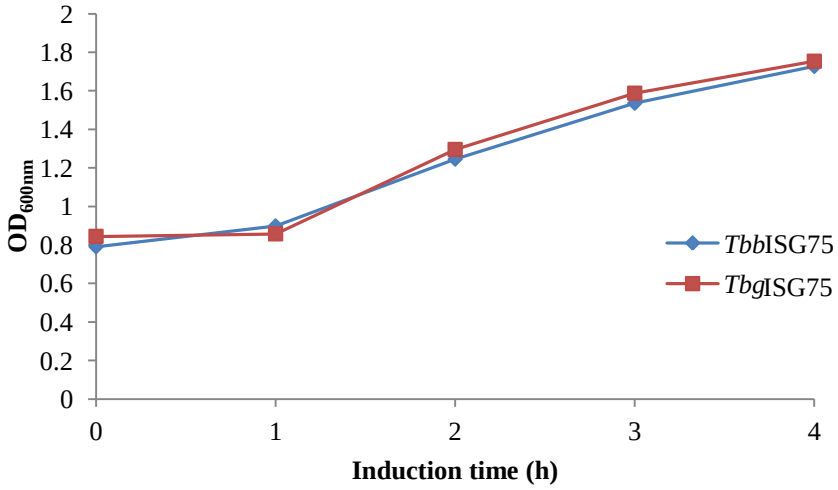


Figure 3.8 Growth profile of BL21 (DE3) *E. coli* cells expressing *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75. The OD_{600} hourly plot during the expression of *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75.

Reducing SDS-PAGE analysis showed that both ISG75 fusion proteins were successfully expressed from the first to the fourth hour post IPTG induction (Figure 3.9). The *Tbb*$_{His}$ISG75 fusion protein was identified as a 72 kDa protein in lanes 2-5 of Figure 3.9 (circled). Expressed *Tbg*$_{His}$ISG75 was identified at approximately 74 kDa in the same 10% reducing SDS-PAGE gel (Figure 3.9, circled in lanes 7-10).

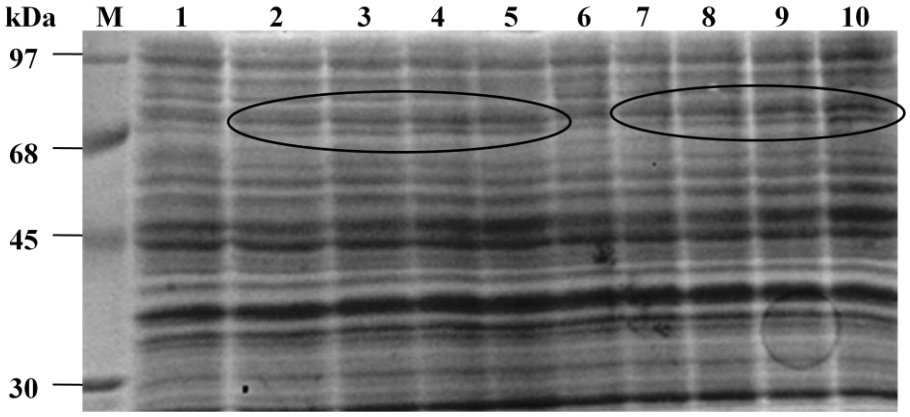


Figure 3.9 Expression profiles of *Tbb$_{His}$*ISG75 and *Tbg$_{His}$*ISG75 fusion proteins in *E. coli* BL21 (DE3) cells. Reducing SDS-PAGE analysis of expression lysates. Lane M, molecular weight markers; lanes 1-5, lysates from 0, 1, 2, 3 and 4 h expression of *Tbb$_{His}$*ISG75; lanes 6-10, lysates from 0, 1, 2, 3 and 4 h expression of *Tbg$_{His}$*ISG75. Target protein was circled.

3.3.4.2 Assessment of the solubility of *Tbb$_{His}$*ISG75 and *Tbg$_{His}$*ISG75 fusion proteins

Reducing SDS-PAGE analysis of the soluble and insoluble cell fractions from expression revealed that *Tbb$_{His}$*ISG75 (Figure 3.10A) and *Tbg$_{His}$*ISG75 (Figure 3.10B) were expressed as soluble proteins. A band of very similar size to the expressed fusion protein was observed in the insoluble cell fractions (Figure 3.10A and B, lanes 2, 4, 6, and 8). However, the presence of *Tbb$_{His}$*ISG75 in the soluble cell fraction only was confirmed using a blot probed with nickel chelate-HRPO (Figure 3.10C, lanes 2 and 3). No band was recognised by nickel chelate-HRPO in the uninduced expression lysate and insoluble cell fraction (Figure 3.10C, lanes 1 and 4).

3.3.4.3 Purification and production levels of *Tbb$_{His}$*ISG75 and *Tbg$_{His}$*ISG75 in *E. coli*

To compare the expression yields in BL21 (DE3) *E. coli* cells of both ISG75 His-tagged fusion proteins, the lysates of the hourly aliquots from ISG75 expression, were purified using nickel affinity chromatography and quantified using the BCA kit. A plot of the purified *Tbb$_{His}$*ISG75 and *Tbg$_{His}$*ISG75 fusion proteins produced hourly are shown (Figure 3.11). A sharp increase in recombinant protein production was observed from 0 mg/ml at the onset of expression, to 1.0 mg/ml for *Tbb$_{His}$*ISG75 and 0.64 mg/ml for *Tbg$_{His}$*ISG75, at the end of the first hour (Figure 3.11).

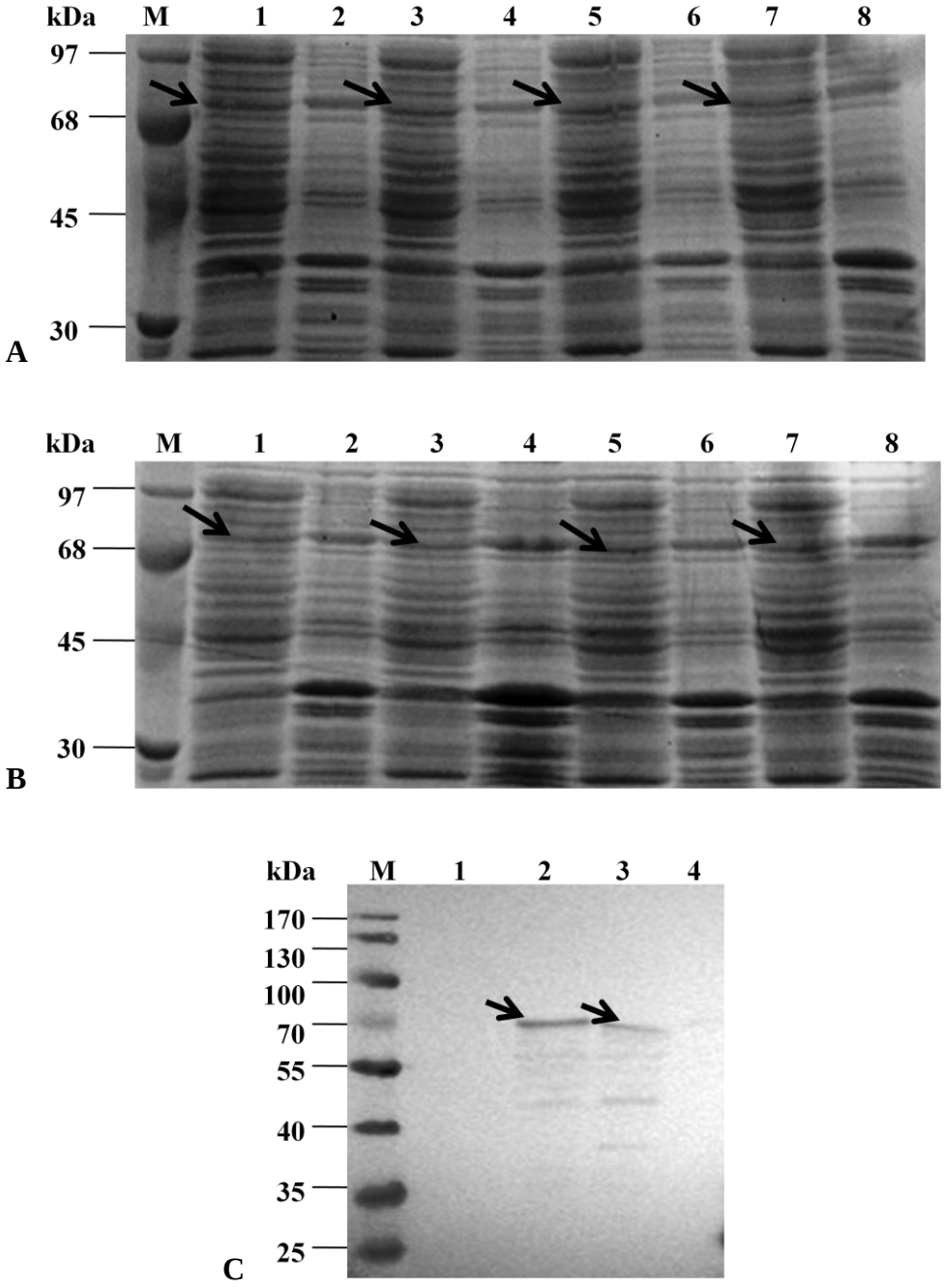


Figure 3.10 Distribution of *E. coli*-expressed Tbb_{His}ISG75 (A) and Tbg_{His}ISG75 (B) in the soluble and insoluble cell fractions. SDS-PAGE **(A and B)** and a blot **(C)** were performed to examine the solubility of the *E. coli* expressed fusion proteins. **(A and B)** Lane M, molecular weight markers; lanes 1, 3, 5 and 7, soluble fractions from 1, 2, 3 and 4 h expression lysates; lanes 2, 4, 6 and 8, insoluble fraction from 1, 2, 3 and 4 h expression lysates. **(C)** A blot probed with nickel chelate-HRPO. Lane M, pre-stained MWM; lane 1, uninduced lysate; lane 2, Tbb_{His}ISG75 lysate 4 h; lane 3, soluble cell fraction; lane 4, insoluble cell fraction. The arrow indicates the target protein.

Recombinant production of both fusion proteins continued increasing until the end of the second hour of expression. A slight drop in protein production by the expressing *E. coli* cells was observed within the second and third hours of expression. This decline was followed by a slight increase in protein production at the last hour of expression (Figure 3.11). A comparison of the production of both fusion proteins showed a higher concentration of accumulated *Tbb*$_{His}$ISG75 at 1.24 mg/ml compared to *Tbg*$_{His}$ISG75 which had a peak concentration of 0.81 mg/ml (Figure 3.11).

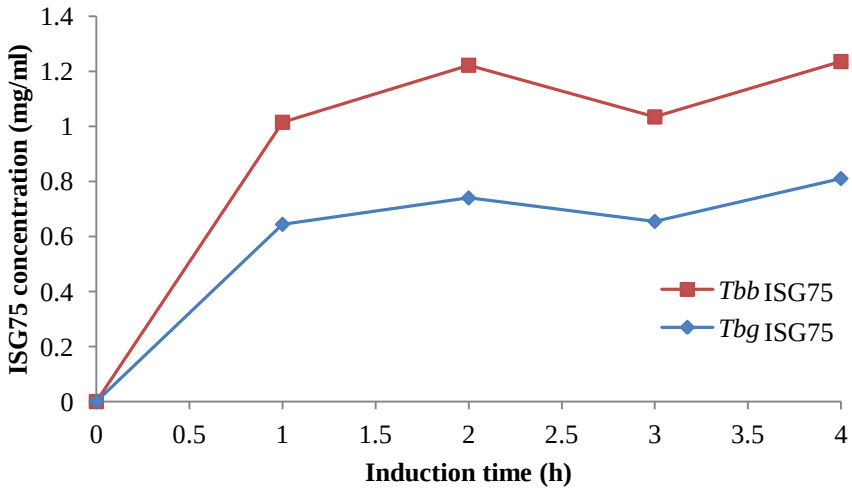


Figure 3.11 Production profiles of *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75 fusion proteins in *E. coli* BL21 (DE3) cells. The production profile for *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75 in BL21 (DE3) *E. coli* cells was evaluated over a time course of 4 h after induction of expression using IPTG.

Purification of *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75 was assessed using reducing SDS-PAGE analysis (Figure 3.12). The fusion protein *Tbb*$_{His}$ISG75 was identified as a 72 kDa band (Figure 3.12A, arrow) while its homologue, *Tbg*$_{His}$ISG75 had an apparent molecular weight of 74 kDa (Figure 3.12B, arrow). A lower band of 45 kDa was also identified in the *Tbb*$_{His}$ISG75 purified fusion protein (Figure 3.12).

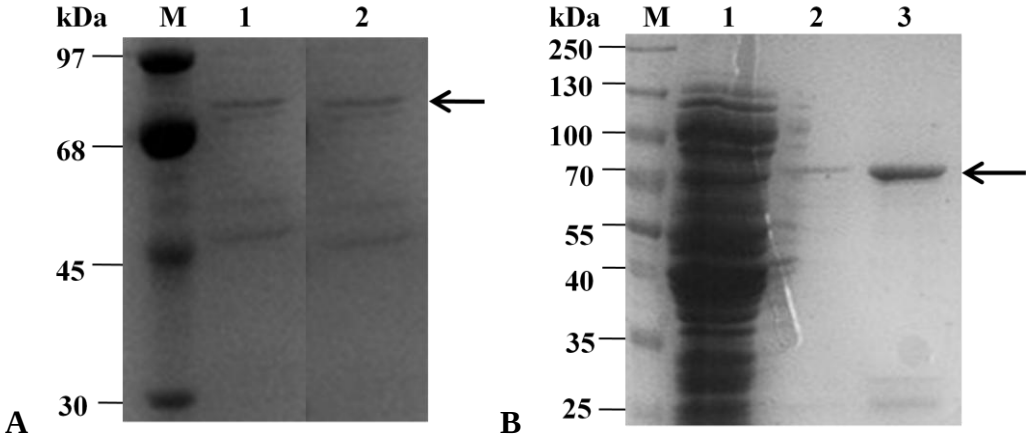


Figure 3.12 Purification of *Tbb*$_{His}$ISG75 and *Tbg*$_{His}$ISG75 using nickel affinity chromatography. Reducing SDS-PAGE analysis of Purified ISG75 samples. **(A)** purified *Tbb*$_{His}$ISG75. Lane M, molecular weight markers; lanes 1-2, Purified *Tbb*$_{His}$ISG75. **(B)** purified *Tbg*$_{His}$ISG75. Lane M, molecular weight markers, lane 1, non-purified lysate; lanes 2-3, purified *Tbg*$_{His}$ISG75. Arrow indicates target protein.

3.3.5 Expression profile and purification of *Tbg*$_{Pp}$ISG75 in *P. pastoris* expression system

3.3.5.1 Expression profile of *Tbg*$_{Pp}$ISG75 in *P. pastoris* expression system

Considering the fact that trypanosomes are eukaryotes as is the methylotropic yeast *P. pastoris*, there was a need to undertake recombinant expression in this similar organism to ensure equivalent glycosylation of proteins. Reducing SDS-PAGE analysis of samples from *Tbg*$_{Pp}$ISG75 expression in the *P. pastoris* expression system showed a progressive increase in protein expression from the first day until the termination of expression on the seventh day (Figure 3.13A). Both secreted (lanes 1-4) and intracellular (lanes 5-8) expression of the protein in pP-αhSUMO3 plasmid were observed (Figure 3.13A). The expression was confirmed by recognition of *Tbg*$_{Pp}$ISG75 by anti-ISG75 IgY (Figure 3.13B) and anti-His tag (Figure 3.13C) antibody. The fusion protein, *Tbg*$_{Pp}$ISG75 was identified as two bands of 90 and 40 kDa sizes (Figure 3.13, arrow).

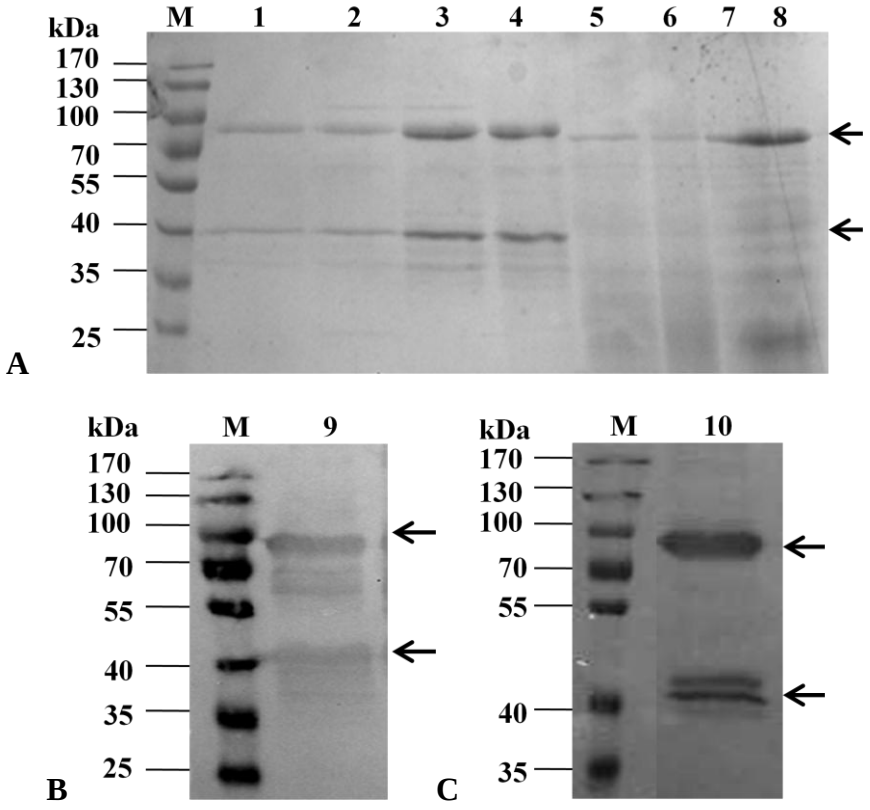


Figure 3.13 ISG75 expression in *P. pastoris* using the pP-αhSUMO3 expression vector. Secreted (in supernatant; SN) and intracellular (in pellet) expression of *Tbg*ISG75 in *P. pastoris* were analysed by **(A)** SDS-PAGE and western blotting using **(B)** anti-ISG75 IgY and **(C)** anti-His tag antibodies. Lane M, molecular weight markers; lanes 1-4, SN for days 1, 3, 5 and 7; lanes 5-8, pellets for days 1, 3, 5 and 7; lanes 9-10, SN for day 7. The arrow indicates Tbg_{Pp}ISG75.

3.3.5.2 Fractionation of Tbg_{Pp}ISG75 using TPP

Reducing SDS-PAGE analysis of the 10-40% ammonium sulfate fractions from the concentration of Tbg_{Pp}ISG75 by TPP showed the presence of the fusion protein in all the fractions. Two bands of 90 and 40 kDa corresponding to Tbg_{Pp}ISG75 were identified in all the fractions (Figure 3.14).

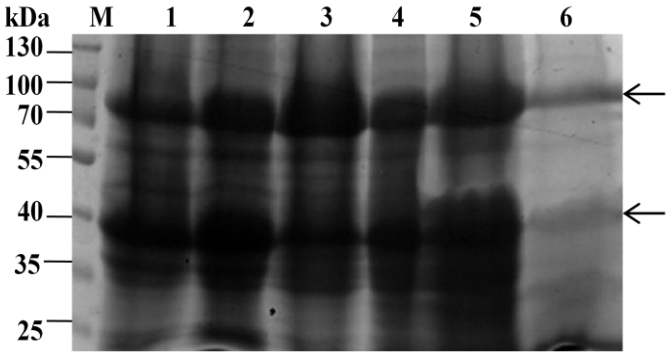


Figure 3.14 Reducing SDS-PAGE analysis of fractions obtained from TPP concentration of secreted *Tbg_{Pp}*ISG75. Fractions containing *Tbg_{Pp}*ISG75 obtained from different percentages (10-40%) of ammonium sulfate incrementally added to a 30% t-butanol mixture of the expression supernatant. Lane M, molecular weight markers; lane 1, 10% $(NH_4)_2SO_4$; lanes 2-3, 20% $(NH_4)_2SO_4$; lanes 4-5, 30% $(NH_4)_2SO_4$; and lane 6, 40% $(NH_4)_2SO_4$ fraction. Target protein identified by arrow.

3.3.5.3 Purification of *Tbg_{Pp}*ISG75 using nickel affinity chromatography

The samples from intracellular and secreted expression of *Tbg_{Pp}*ISG75 in the *P. pastoris* system were purified using nickel affinity chromatography. Purification of *Tbg_{Pp}*ISG75 from the supernatant (secreted expression) was more efficient (Figure 3.15A) and gave a better yield than purification from the cell lysate (intracellular expression; Figure 3.15B). Reducing SDS-PAGE analysis of the purified sample confirmed the expression of *Tbg_{Pp}*ISG75 as two bands of approximately 90 and 40 kDa (Figure 3.15, arrows).

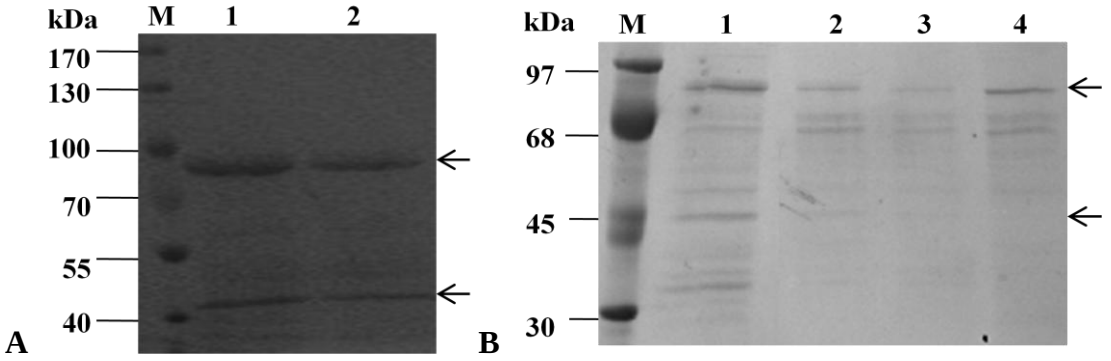


Figure 3.15 Purification of *Tbg_{Pp}*ISG75 using nickel affinity chromatography. Samples purified from **(A)** secreted and **(B)** intracellular expression of *Tbg_{Pp}*ISG75 in *P. pastoris* were analysed by SDS-PAGE. **(A)** Lane M, molecular weight markers; lanes 1-2, eluates from purification of secreted *Tbg_{Pp}*ISG75. **(B)** Lane M, molecular weight markers; lanes 1-4, eluates from purification of intracellular *Tbg_{Pp}*ISG75. The arrow indicates purified *Tbg_{Pp}*ISG75.

3.3.6 Glycosylation assessment

Due to the differences in the apparent molecular weight of *Tbg*$_{Pp}$ISG75 on SDS-PAGE (90 kDa) and the calculated molecular weight calculated from the amino acid sequence (70 kDa), the possibility of the protein being glycosylated was considered. Reducing SDS-PAGE analysis showed no differences in the electrophoretic mobility of the Endoglycosidase H treated and untreated samples *Tbg*$_{Pp}$ISG75 (Figure 3.16), indicating that the protein was not N-glycosylated. Differences were however observed in the electrophoretic mobility of a control protein TvCATL, known to be glycosylated (lanes 1 and 2) and ISG65 (lanes 3 and 4), which were analysed alongside ISG75 as positive controls for the experiment.

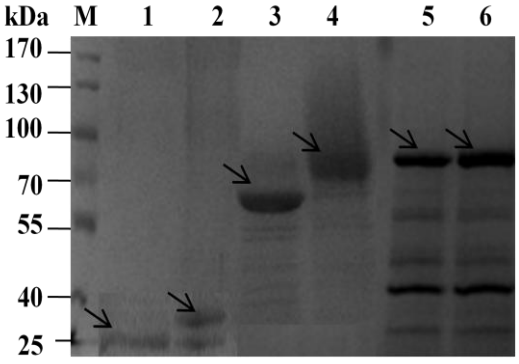


Figure 3.16 Reducing SDS-PAGE of ISG75 glycosylation assessment. Comparison of Endoglycosidase H treated and untreated samples. Lane M, molecular weight markers; lane 1, Endoglycosidase H treated control TvCATL; lane 2, untreated control TvCATL; lane 3, Endoglycosidase H treated ISG65*Pp*; lane 4, untreated ISG65*Pp*; lane 5, Endoglycosidase H treated *TbbPp*ISG75; lane 6, untreated *Tbg*$_{Pp}$ISG75.

3.4 Discussion

Hundreds of thousands of Africans are estimated by the World Health Organisation to be infected with *T. brucei* in endemic areas (Simarro *et al.*, 2008). Treatment of HAT is dependent on the stage of the disease. All drugs currently available for the treatment of the neurological stage (second stage) of the disease are highly toxic therefore administration of such drugs only occurs subsequent to confirmation of the presence of trypanosomes in the CSF of the patient (Giroud *et al.*, 2009). This underscores the need for efficient tools for the diagnosis of the disease. The glycoprotein ISG75, has been earmarked as a potential candidate for the development of novel sero-diagnostic tests for trypanosomiasis (Hutchinson *et al.*, 2004), thus creating a need for the production of this antigen in sufficient quantities to facilitate the exploration of its diagnostic potential.

A relatively inexpensive method for the production of recombinant ISG75 was described in this chapter. Production of native ISG75 would require the *in vivo* cultivation of trypanosomes in laboratory animals, thus exposing laboratory workers to the danger of accidental infections, which could result from needle stick and other injuries (Herwaldt, 2001; Van Nieuwenhove *et al.*, 2011). With a total yield of 90 mg and 56 mg per litre of bacteria culture, production of *T. b. brucei* ISG75 and *T. b. gambiense* ISG75, as described in this study, can be considered as a high yield compared to 0.5 mg per litre obtained from previous studies (Tran *et al.*, 2008).

The *ISG75* gene was cloned into pGEX-4T1 and pET28a expression vectors in the present study. The provision of the GST and His fusion tags by these expression vectors was required for target protein purification by affinity chromatography (Fakruddin *et al.*, 2013; Graslund *et al.*, 2008). *Escherichia coli* produces its own proteins during the heterologous expression process; therefore there is always a need to purify the target protein from these other proteins (Graslund *et al.*, 2008). The presence of the respective fusion tags facilitated purification using GST and nickel chelate affinity chromatography (Nilsson *et al.*, 1997; Smith, 1988). Cleavage of the GST tag from ISG75 in the present study was carried out when the ISG75 fusion protein was immobilised on the glutathione column, same as with

ISG65 in section 2.2.7.3, thus further purification steps to separate the GST tag from the recombinant protein were not necessary.

The commonly used BL21 (DE3) *E. coli* strain was used in the present study. Previous investigators reported very low expression levels for *T. b. gambiense* ISG75 using BL21 (DE3) *E. coli* cells and thus opted for the *trxB* and *gor* mutant strain, Origami B (DE3) to achieve successful expression (Tran *et al.*, 2008). The results obtained in this study nevertheless, seem to be different. Successful expression of *Tbg*ISG75 was recorded in the present study using BL21 (DE3) *E. coli* cells. A number of differences were observed in their approach and those adopted in the present study. Lysogeny broth (LB) was previously used for ISG75 expression (Tran *et al.*, 2008), however 2x YT medium was used in the present study. The 2x YT is a richer medium, it has double the amount of yeast extract and 60% more peptone than LB medium. In a study showing practical protocols for high expression yields in *E. coli*, the recommended OD_{600} value for 2x YT medium was higher at 5-7 than the 3-5, OD_{600} value recommended for expression using LB medium (Sivashanmugam *et al.*, 2009). This capacity for 2x YT to maintain higher *E. coli* densities during expression for a longer growth period, could have contributed to the higher recombinant ISG75 yield obtained in this study. Another difference noted was that the vector-host combination used in the present study was different from that used by Tran *et al.* (2008) previously. The expression plasmid used in the present study was pET28a but pET15b was co-transformed with the pRIL plasmid (to supply additional tRNAs) in the previous study (Tran *et al.*, 2008). Both pET28a and pET15b vectors are similar in that both plasmids have a T7-*lac* promoter that is useful for regulating expression (Robinson, 2011).

A comparison of protein production and the *E. coli* growth kinetics obtained in the present study revealed that despite the steadily increasing growth rate observed for the expressing *E. coli* cells (Figure 3.8), there was a decline in the production of both target proteins, Tbb_{His}ISG75 and Tbg_{His}ISG75, between the second and third hours of expression (Figure 3.11). This suggests that recombinant protein production in BL21 (DE3) *E. coli* cells is not necessarily directly proportional to increasing bacteria biomass. A number of factors could

have contributed to the decrease in production observed. Accumulation of metabolic by-products like acetate could result in a decrease in the rate of heterologous protein synthesis in *E. coli* (Sivashanmugam *et al.*, 2009). Other contributing factors to reduced protein expression are the release of bacterial endotoxins into the expression medium and also proteolysis of recombinant proteins by proteases (Rozkov *et al.*, 2000).

In the present study, the expression of ISG75 was terminated before the bacterial cells reached stationary phase. This was in order to avoid the problems associated with cells going into stationary phase (Voulgaridou *et al.*, 2013). Growth of bacteria cells beyond the stationary phase or even to the death phase could lead to a decrease in the expression of the target protein or even degradation of the expressed protein. As was observed by Tran *et al.* (2008) during the expression of ISG75, no expression was observed for samples incubated for up to 22 hours at 37^{o}C. Nevertheless, at lower temperatures of 25^{o}C and below, the metabolic rate of *E. coli* is slower so expression could be maintained for longer periods at such low temperatures without target protein degradation (Fakruddin *et al.*, 2013; Graslund *et al.*, 2008). The culture media and expression conditions used in the present study were successful for small and large bench scale expressions of ISG75. For large scale production in industrial sized fermentors, or feed-batch fermentation productions, 2x YT broth might not be sufficient and a high density bacterial growth medium containing a buffering system might be required (Lee, 1996).

Previous studies have shown the possibilities of recombinant production of *T. b. gambiense* ISG75 (Roge *et al.*, 2013; Tran *et al.*, 2008) and *T. evansi* ISG75 (Tran *et al.*, 2009) for use in the diagnosis of trypanosomiasis. The present study also provided information on the recombinant expression profile of ISG75 from a different strain, *T. b. brucei*. The successful cloning and heterologous expression of ISG75 from two sub-species of *T. brucei*, further underscores the relative ease of production of ISG75 as an antigen for trypanosomiasis diagnostic assays. There is a need to compare the properties of the recombinant ISG75 produced in this study with that of native ISG75 of trypanosomes. Also characterisation and crystal structure determination of recombinant ISG75 could fill the paucity of information about these invariant trypanosomal surface proteins.

Recombinant ISG75 was expressed as two bands in a reducing SDS-PAGE gel. These two bands were observed following recombinant ISG75 expression, regardless of the expression vector or the expression host used. This trend was previously observed with ISG65 described in chapter 2. An explanation is required for the two bands observed for the recombinant ISGs. Degradation sites were identified by Edman degradation between amino acid residues 145 and 146 of the ISG75 sequence (Roge *et al.*, 2013). There is a possibility that the degradation of ISG75 could be occurring at these sites.

Trypanosomes are eukaryotes and hence expression of ISG75 was also undertaken in another eukaryote, *P. pastoris*. The expectation that post-translational modifications such as glycosylation could occur in the production of ISG75 in this system was a motivating factor. Deglycosylation of Tbg_{Pp}ISG75 using Endoglycosidase H, nonetheless, showed that the recombinant ISG75 was not N-glycosylated (Figure 3.16). Endoglycosidase H only deglycosylates oligomannose type N-linked glycans and it may be necessary to carry out other deglycosylation tests using more robust enzymes like peptide N-glycosidase F that cleaves essentially all types of N-linked glycans (Mehlert *et al.*, 2012b).

Investigations into the use of DNA vaccines for the prevention of trypanosomiasis (Silva *et al.*, 2009) have identified ISGs as one of the potential antigen candidates (Lanca *et al.*, 2011). The cloned ISG75 genes from this study could also be sub-cloned into more appropriate vectors that can be used in DNA vaccine experiments for trypanosomiasis.

A standardised protocol has been outlined for the production from heterologous systems of ISG75 of *T. b. brucei* and *T. b. gambiense* for diagnostic assays. In order to demonstrate the diagnostic potential of these recombinant proteins, the production of anti-ISG antibodies was undertaken and reported in chapter 4. Also immunological assays like ELISAs and immunofluorescence assays undertaken with the recombinant antigens and their respective antibodies will also be reported in chapter 4.

CHAPTER 4

Production and affinity purification of anti-recombinant ISG65 and ISG75 antibodies and use in immunodetection of native ISG65 and ISG75 of *Trypanosoma brucei brucei*

4.1 Introduction

The treatment of trypanosomiasis is dependent on the stage of the infection as well as the causative species (Kuepfer *et al.,* 2011). This is due to differences in the progression of infection and severity of the symptoms of the disease caused by different species of trypanosomes (Maclean *et al.,* 2012). Infections in humans caused by *T. b. gambiense* and *T. b. rhodesiense* are characterised by chronic and acute manifestations respectively of the disease (Maclean *et al.,* 2012). Other trypanosome species like *T. b. brucei, T. congolense, T. vivax,* amongst others, specifically infect only animals (Njiokou *et al.,* 2004).

Diagnosis plays an important role in the control of trypanosomiasis on the African continent. One of the approaches for the diagnosis of trypanosomiasis is the use of serological assays/techniques (Solano *et al.,* 2002). Serological tests are designed to demonstrate the presence of specific parasite antigens or antibodies, as indirect evidence for trypanosome infection. The basis for serology is the detection of either a circulating antigen derived from the parasite or circulating antibodies that recognise an antigen produced by the pathogen (Hutchinson *et al.,* 2004). Current serological tests for trypanosomiasis include ELISA, immunofluorescence assays (IFAs), procyclic agglutination test for trypanosomiasis (PATT), card agglutination test for trypanosomiasis (CATT), latex agglutination test (LATEX), card indirect agglutination test for trypanosomiasis (CIATT), amongst others (Chappuis *et al.,* 2005; Lejon and Büscher, 2005).

The rapid agglutination tests: CATT, PATT, CIATT and LATEX, are widely applicable for field diagnosis of trypanosomiasis. Other tests like the ELISA and IFA are only applicable to laboratory diagnosis of the disease (Chappuis *et al.,* 2005; Lejon and

Büscher, 2005). Large scale epidemiological studies and the follow-up control programs require assays that combine low cost and high specificity. Also highly specific and sensitive point-of-care tests are necessary to improve disease management (Kuepfer *et al.*, 2011; Steverding, 2008). Current research toward meeting these requirements focuses on the identification of suitable trypanosome antigens and the use of recombinant DNA technology (Boulangé *et al.*, 2002; Simarro *et al.*, 2008).

Immunoglobulin Y (IgY) technology which involves the production of antibodies in chickens and the extraction of specific antibodies from egg yolk has been gaining increasing interest in the scientific community (Schade *et al.*, 2005). Immunoglobulin Y is the avian equivalent of mammalian immunoglobulin G (IgG), the most abundant mammalian antibody class (Ayyar *et al.*, 2012; Mine and Kovacs-Nolan, 2002). An outstanding advantage of IgY antibody is the phylogenetic distance between *Aves* and *Mammalia* (Pauly *et al.*, 2011). Hence the IgY from chicken egg yolk will react with more epitopes on a mammalian antigen, leading to the production of more specific antibodies (Carlander *et al.*, 1999; Pauly *et al.*, 2011). Also interference caused by the mammalian complement system, human and bacterial Fc-receptors or binding to Rheumatoid factor in human serum is avoided (Larsson and Mellstedt, 1992). The chicken lays on average one egg per day and so larger quantities of antibodies can be purified from this system at a lower cost compared to other systems like mice and rabbits (Romito *et al.*, 2001; Schade *et al.*, 2005). From an animal ethics point of view it is also a more humane way of harvesting antibodies since bleeding is not required.

Two major surface trans-membrane domain proteins of trypanosomes, ISG65 and ISG75 have been identified as potential diagnostic antigens for trypanosomiasis (Büscher and Lejon, 2004; Hutchinson *et al.*, 2004; Tran *et al.*, 2008). Standardised protocols were outlined for the recombinant expression of ISG65 and ISG75 of *T. b. brucei* and *T. b. gambiense* from heterologous systems (Chapters 2 and 3). There was a need to explore the antigenic potential of the recombinant antigens and their suitability for use in the production of a new diagnostic test for trypanosomiasis. In the current study, recombinant ISG65 and ISG75 of *T. b. brucei* were used to immunise chickens in order to produce anti-

ISG65 and anti-ISG75 antibodies. The antibodies isolated from egg yolk by polyethylene glycol (PEG) precipitation were purified by affinity chromatography using columns prepared by coupling ISG65 and ISG75 to agarose beads and analysed by ELISA. The antibodies were tested in western blots to determine if they would be able to detect the recombinant ISG65 and ISG75 used to produce the antibodies as well as native ISG65 and ISG75 from lysates of *in vitro* cultured *T. b. brucei* Lister 427 parasites. Immunofluorescence microscopy was also used to locate native ISG65 and ISG75 on the surface of *T. b. brucei* Lister 427 strain parasites.

4.2 Materials and Methods

4.2.1 Materials

Bathocuproinedisulfonic acid disodium salt, L-cysteine, hypoxanthine, sodium bi-carbonate, sodium pyruvate, thymidine, sodium cyanoborohydride, poly L-lysine, 2-mercaptoethanol and FITC conjugated rabbit anti-chicken IgY were obtained from Sigma-Aldrich (Munich, Germany). Alexa fluor® 488 goat anti-chicken IgG was purchased from Life Technologies®. Six well tissue culture plates and 96-well ELISA plates were purchased from Nunc Intermed (Denmark). Foetal calf serum and Iscove's Modified Dulbecco's Medium (IMDM) powder were obtained from Gibco (Paisley, UK). AminoLink® coupling resin, enhanced chemiluminescence detection kit and Hoechst 33342 were obtained from Thermo Scientific (Rockford, USA). Rabbit anti-chicken IgY horseradish peroxidase (HRPO) conjugate was purchased from Jackson ImmunoResearch (USA).

4.2.2 Methods

4.2.2.1 Raising of antibodies against ISG65 and ISG75 in chickens and isolation of antibodies

Recombinant proteins *Tbb*ISG65 and *Tbb*ISG75 previously produced as described in chapters two and three respectively of this study, were used to immunise chickens in order to produce anti-ISG65 and anti-ISG75 antibodies. Two chickens were each immunised

intramuscularly with 50 µg of *Tbb*ISG65 and *Tbb*ISG75 emulsified with Freund's complete adjuvant. Booster injections, using Freund's incomplete adjuvant were performed for subsequent weeks. Eggs were collected daily for 12 weeks for IgY isolation. IgY was isolated from the egg yolks as per the method of Polson *et al.* (1964) modified by Goldring and Coetzer (2003). Briefly, egg yolks were separated from the yolk sacs and egg whites, mixed with two volumes of IgY isolation buffer [100 mM Na-phosphate buffer, pH 7.6, 0.02% (w/v) NaN_3] before adding 3.5% (w/v) polyethylene glycol M_r 6 000 (PEG 6 000). The resultant homogenous mixture was centrifuged (4420 x *g*, 30 min, RT) and the supernatant separated by filtration through cotton wool. Polyethylene glycol M_r 6 000 [8.5% (w/v)] was mixed with the supernatant and centrifuged (12 000 x *g*, 10 min, RT). The pellet was resuspended with a volume of IgY isolation buffer equivalent to the filtrate volume and PEG 6 000 [12% (w/v)] mixed in and a pellet obtained by centrifugation (12 000 x *g*, 10 min, RT). This pellet was resuspended in 1/6 of the original yolk volume. The concentration of IgY was determined using the extinction coefficient of IgY of $E_{280\ nm}^{1\ mg/ml} = 1.25$ (Goldring and Coetzer, 2003). The production of antibodies was tested using indirect ELISA.

4.2.2.2 ELISA to monitor the production of anti-ISG65 and anti-ISG75 antibodies

An ELISA was used to monitor the production of anti-ISG65 and anti-ISG75 antibodies over a 12 week period after the immunisation of the chickens. Nunc 96 well ELISA plates were coated overnight at 4°C with 150 µl of 1 µg/ml antigen (either ISG65 or ISG75) in PBS. The plates were washed three times with PBS supplemented with 0.02% (v/v) Tween 20 and unoccupied sites in the wells were blocked with 200 µl of blocking buffer [0.5% (w/v) BSA-PBS] for 1 h at 37°C. After three washes (5 min each), dilutions of the primary antibody samples in blocking buffer were added in duplicate to the plates and incubated for 2 h at 37°C. The primary antibody samples used were IgY isolated from the egg yolks from weeks zero to 12, serially diluted from 0.1 µg/ml to 100 µg/ml. For the ELISA to show the antibody production profiles of the chickens over time, 100 µg/ml primary antibody concentrations were used. The washed plates were incubated for another 1 h at 37°C with 120 µl of secondary antibody, horseradish peroxidase-conjugated rabbit anti-IgY (1:10 000

in BSA-PBS). The plates were washed (3 x 5 min) and incubated with the substrate solution [0.05% (w/v) ABTS, 0.0015 % (v/v) H_2O_2 in 150 mM citrate phosphate buffer, pH 5.0]. Colorimetric development was allowed to occur in the dark for 15-30 min and the optical density was subsequently measured at 405 nm using a FLUOstar OPTIMA (BMG Labtech, Germany) ELISA plate reader.

4.2.2.3 Preparation of ISG65 and ISG75 affinity chromatography columns

ISG65 and ISG75 affinity chromatography columns were prepared for the purification of isolated chicken anti-ISG65 and anti-ISG75 antibodies. *Tbb*$_{His}$ISG65 (see chapter 2) and *Tbb*$_{His}$ISG75 (see chapter 3) were each coupled to a beaded agarose matrix, AminoLink® coupling resin (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. Briefly, 2 ml of the gel slurry was placed in a column, allowed to settle and storage solution drained. The column was equilibrated using 3 volumes of coupling buffer (150 mM sodium citrate, 50 mM sodium carbonate, pH 10). Four milligrams of recombinant protein (either ISG65 or ISG75) in coupling buffer was applied to the column and mixed end-over-end for 4 h. The unbound fraction was collected as flow through and the column was washed with PBS, pH 7.2. Formation of stable secondary amine linkages was achieved by incubation with a mild reducing agent (50 mM sodium cyanoborohydride solution in PBS) for 4 h. The remaining active sites in the resin were blocked by washes using quenching buffer (1 M Tris-HCl, pH 7.4) followed by 30 min incubation with 50 mM sodium cyanoborohydride solution in quenching buffer. After several washes using a wash solution (1 M sodium chloride) the column was stored at 4^{o}C in PBS containing 0.05% (w/v) sodium azide until use. The coupling efficiency of the ISG65 and ISG75 to the resin matrix was calculated by comparing the concentrations of the protein samples prior to and post coupling of the protein to the column. The coupling efficiency obtained was approximately 60% for the ISG65 affinity resin and 65% for ISG75 affinity resin.

4.2.2.4 Purification of anti-ISG65 and anti-ISG75 antibodies using affinity chromatography

The isolated anti-ISG65 and anti-ISG75 antibodies were pooled based on the ELISA results and purified using the prepared ISG65 or ISG75 affinity columns. The affinity column was equilibrated by adding 6 ml of PBS and drained. The pooled antibody sample was applied to the column and allowed to cycle through the resin overnight at RT to achieve maximum binding of specific antibodies. The column was washed with 20 ml of PBS and bound antibodies were eluted using IgY elution buffer (0.1 M glycine-HCl, pH 2.8). One ml eluates were collected in tubes containing 100 µl of neutralisation buffer [1 M NaH_2PO_4, 0.02% (w/v) NaN_3, pH 8.5] and mixed gently to neutralise the pH. Antibody concentrations were calculated by dividing the absorbances at 280 nm with 1.25 (extinction coefficient of IgY). Fractions with the highest $A_{280\ nm}$ values were pooled and the antibody titres were evaluated by ELISA using the protocol described in section 4.2.2.2. The ELISA plates were coated with 1-5 µg/ml of antigen (ISG65 or ISG75). Serial dilutions of the primary antibodies were prepared in the ELISA plates at a range of concentrations from 0.012-25 µg/ml or 0.098-100 µg/ml. The primary antibodies tested included: affinity purified anti-ISG65 and anti-ISG75 antibodies, pre-affinity purification anti-ISG65 and anti-ISG75 antibodies, unbound (anti-ISG65 and anti-ISG75 antibodies, not bound to the affinity columns) and non-immune (pre-immunisation) antibodies.

4.2.2.5 Growth of trypanosomes in culture medium

Bloodstream forms of *T. b. brucei* Lister 427 strain were cultured in HMI-9 based Iscove's Modified Dulbecco's Medium (IMDM) at 37^{o}C with 5% CO_2 in a humid atmosphere in non-adherent culture flasks as described previously (Hirumi and Hirumi, 1989). Briefly, 16.66 g of IMDM was dissolved in 1 L of HMI-9 (3.6 mM $NaHCO_3$, 1 mM hypoxanthine, 1 mM sodium pyruvate, 0.16 mM thymidine, 0.05 mM bathocuproine-disulfonic acid disodium salt, and 1.5 mM L-cysteine and 0.2 mM 2-mercaptoethanol). Heat inactivated foetal calf serum [10% (v/v)] was added and medium filtered using a 0.2 µm filter. A glycerol stock of *T. b. brucei* Lister 427 trypanosomes was thawed and 500 µl was added to 1.5 ml of medium in the culture flask. The trypanosomes were sub-cultured daily by

taking out 1 ml of culture into a fresh well and replacing it with an equivalent amount of medium. The parasites were grown in culture until they reached the stationary phase, after which the suspension was centrifuged (2000 x *g*, 5 min, RT) to isolate the parasites from the medium.

4.2.2.6 Western blot analysis using the affinity purified anti-ISG antibodies

Trypanosoma brucei brucei parasites were harvested from *in vitro* culture by centrifugation (2000 x *g*, 5 min, RT). The pelIet was washed twice with PBS (pH 7.2) and resuspended in 50 µl of lysis buffer [10 mM EDTA, 1% (v/v) Triton X-100, 20 mM Tris-HCl buffer, pH 7.2, containing 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and 10 µM L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E64)], an equal volume of 2 x reducing gel loading buffer was added and samples boiled for 10 min. The mixture was separated on a 10% reducing SDS-PAGE gel at 20 mA per gel, blotted onto nitrocellulose and probed with appropriate antibodies. The primary antibodies used for the individual blots, were affinity purified chicken anti-ISG65 IgY and affinity purified chicken anti-ISG75 IgY at a concentration of 7.5 µg/ml in 0.5% (w/v) BSA-TBS (20 mM Tris, 200 mM NaCl, pH 7.4). Horseradish peroxidase-conjugated rabbit anti-IgY (1:5 000 dilution) was used as secondary antibody. The blot was developed using enhanced chemiluminescence detection kit (Pierce, Rockford, USA) and imaging using the GeneSys™ software with the G.:box (Syngene®, USA).

4.2.2.7 Immunofluorescence microscopy

Trypanosomes (4 x 10^7 cells/ml) were harvested from culture by centrifugation (2000 x *g*, 5 min). Parasites were washed twice with PBS and spread onto 0.01% (w/v) poly-L-lysine (Sigma Aldrich) coated slides, air dried and fixed with 50% (v/v) methanol. The cells were permealised by incubating with 0.5% (v/v) PBS-Triton X-100 for 2 min and washed 3 x 5 min using 0.2% (v/v) PBS-Triton X-100. Non-specific protein binding sites on slides were blocked with 2% (w/v) BSA-PBS and treated with anti-ISG65 or anti-ISG75 antibodies at 5 µg/ml and 7.5 µg/ml concentrations respectively, in 0.2% (w/v) BSA-PBS for 2 h at RT. The slides were washed 4 x 5 min with 0.2% (v/v) PBS-Triton X-100, prior

to and post incubation with FITC conjugated rabbit anti-chicken IgY or Alexa fluor® goat anti-chicken IgG at 1:200 dilution in 0.2% (w/v) BSA-PBS for 1 h at RT. The slides were incubated with Hoechst stain [1 µg/ml in 0.2% (v/v) PBS-Triton X-100], washed 5 x 5 min with PBS, mounted with moviol and the coverslips sealed with nail varnish. Slides were viewed using an LSM 710 confocal microscope (Zeiss, Göttingen, Germany).

4.3 Results

4.3.1 Anti-ISG65 and anti-ISG75 antibodies production

Antibodies against native ISGs have been shown previously to exist in the natural course of trypanosomiasis infection (Chung *et al.*, 2004) hence antibodies against the recombinant ISGs were raised in chickens by immunisation with recombinant ISG65 and ISG75. A rapid immunogenic response to ISG65 was observed as early as the second week post first immunisation (Figure 4.1). For chicken 1, this was followed by a decrease and a peak in IgY production at the seventh week after the first immunisation (Figure 4.1). There was a decline in antibody production for subsequent weeks (Figure 4.1). Immunoglobulin-Y production for the second chicken peaked at the ninth week post first immunisation, was maintained for the tenth week and subsequently decreased (Figure 4.1). Both chickens showed an increase in antibody production after the week 6 booster injection.

Invariant surface glycoprotein-75 also elicited a significant IgY response by the second week of immunisation (Figure 4.2). The antibody production for the first chicken was almost constant for the entire 12 week period, with slight decreases in production for weeks three, seven and ten (Figure 4.2). The antibody levels for the second chicken were higher than that of the first and peaked at the 12th week post first immunisation (Figure 4.2). Step wise increments were observed from the second to the sixth and the 12th week after the first immunisation (Figure 4.2).

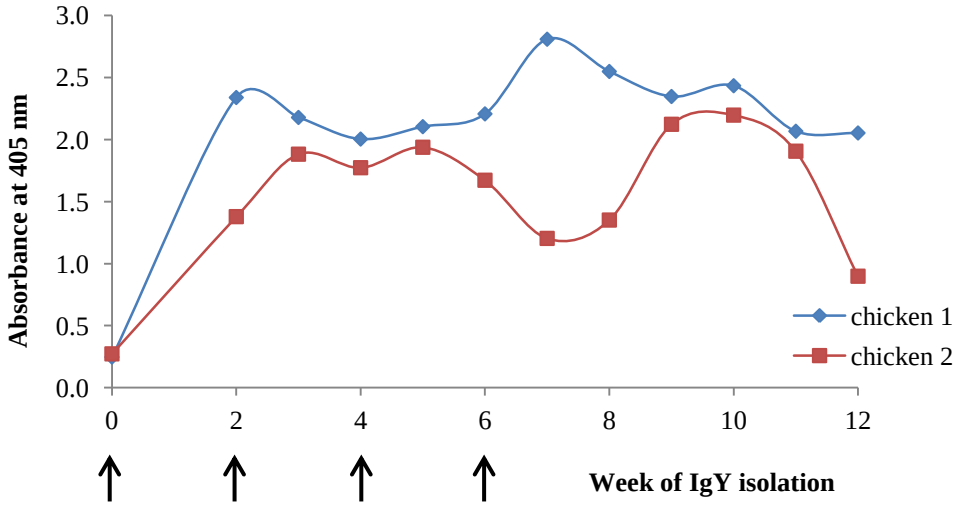


Figure 4.1 Plots of ELISA results showing anti-ISG65 IgY production over a 12-week period. Chicken 1 (◆) and chicken 2 (■) were immunised with ISG65 emulsified in Freund's complete adjuvant at week 0, and in Freund's incomplete adjuvant at weeks 2, 4, and 6 [arrows]. ELISA plates were coated with ISG65 (1 µg/ml, 4°C, 16 h), anti-ISG65 IgY was used as primary antibody (100 µg/ml, 37°C, 2 h) and HRPO-conjugated rabbit anti-IgY (1:10 000 in BSA-PBS) as secondary antibody. Plates were developed with substrate solution [0.05% (w/v) ABTS, 0.0015 % (v/v) H_2O_2 in 150 mM citrate phosphate buffer, pH 5.0]. The absorbance readings at 405 nm represent the average of duplicate experiments.

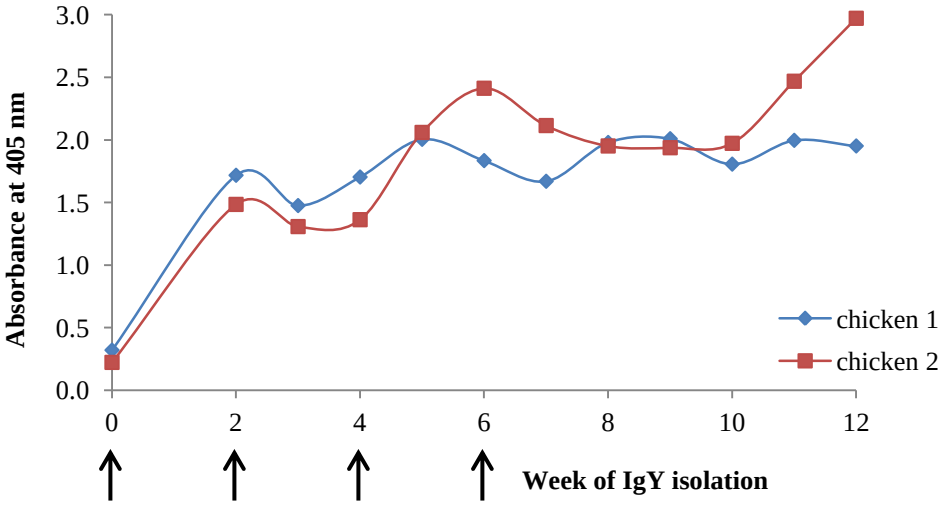


Figure 4.2 Plots of ELISA results showing anti-ISG75 IgY production over a 12-week period. Chicken 1 (◆) and chicken 2 (■) were immunised with ISG75 emulsified in Freund's complete adjuvant at week 0, and in Freund's incomplete adjuvant at weeks 2, 4, and 6 [arrows]. ELISA plates were coated with ISG75 (1 µg/ml, 4°C, 16 h), anti-ISG75 IgY was used as primary antibody (100 µg/ml, 37°C, 2 h) and HRPO-conjugated rabbit anti-IgY (1:10 000 in BSA-PBS) as secondary antibody. Plates were developed with substrate solution [0.05% (w/v) ABTS, 0.0015 % (v/v) H_2O_2 in 150 mM citrate phosphate buffer, pH 5.0]. The absorbance readings at 405 nm represent the average of duplicate experiments.

An evaluation of the titres of the anti-ISG65 antibodies showed that higher titres were obtained for antibodies produced by chicken 1 (Figure 4.3A) compared to chicken 2 (Figure 4.3B). The first chicken produced antibodies with the highest titres at weeks 7 and 8 post first immunisation, while the antibodies produced at weeks 11 and 12 had the lowest titres (Figure 4.3A). A similar trend of low antibody titre was observed for the antibodies produced by the second chicken at the 12th week post first immunisation (Figure 4.3B). However, for the second chicken, the best antibody titres were obtained at weeks 9 and 10 after first immunisation (Figure 4.3B).

For the anti-ISG75 antibodies produced by chicken one, equivalent titres were observed from weeks 2 to 12 (Figure 4.4A). The antibodies produced by the first chicken had the highest titres in the ninth week after first immunisation and the lowest titres were observed in its third week (see Figure 4.4A). The second chicken however, produced antibodies with overall higher titres than the first (Figure 4.4B). The highest titres were observed at the 11th and 12th weeks after first immunisation and the lowest titre was observed in the third week, same as for chicken 1 (Figure 4.4B).

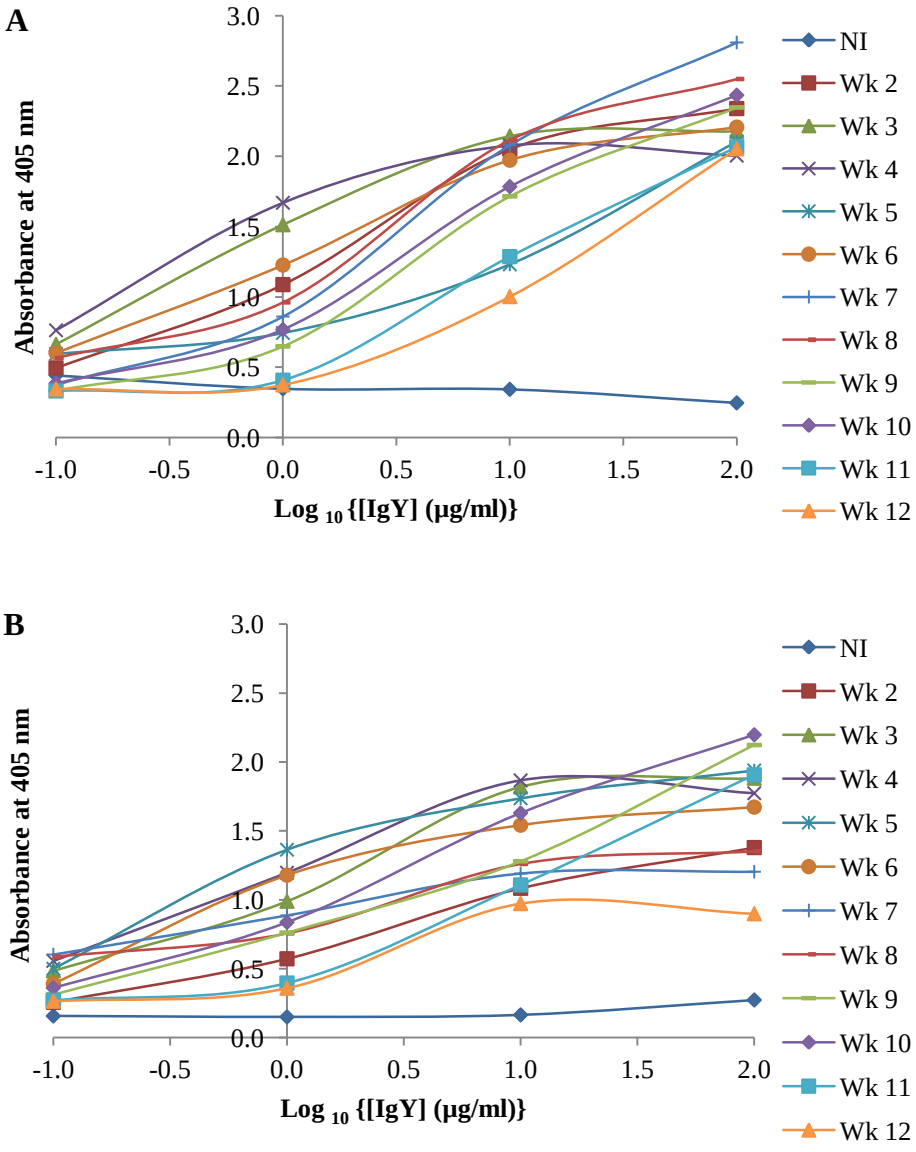


Figure 4.3 ELISA showing IgY response to immunisation with recombinant ISG65. Anti-ISG65 IgY produced by chicken 1 **(A)** and chicken 2 **(B)** for weeks 0 to 12 after first immunisation. ELISA plates were coated with ISG65 (1 µg/ml, 4°C, 16 h), anti-ISG65 IgY was used as primary antibody (0.1-100 µg/ml, 37°C, 2 h) and HRPO conjugated rabbit anti-IgY (1:10 000 in BSA-PBS) as secondary antibody. Plates were developed with substrate solution [0.05% (w/v) ABTS, 0.0015 % (v/v) H_2O_2 in 150 mM citrate phosphate buffer, pH 5.0]. The absorbance readings at 405 nm represent the average of duplicate experiments.

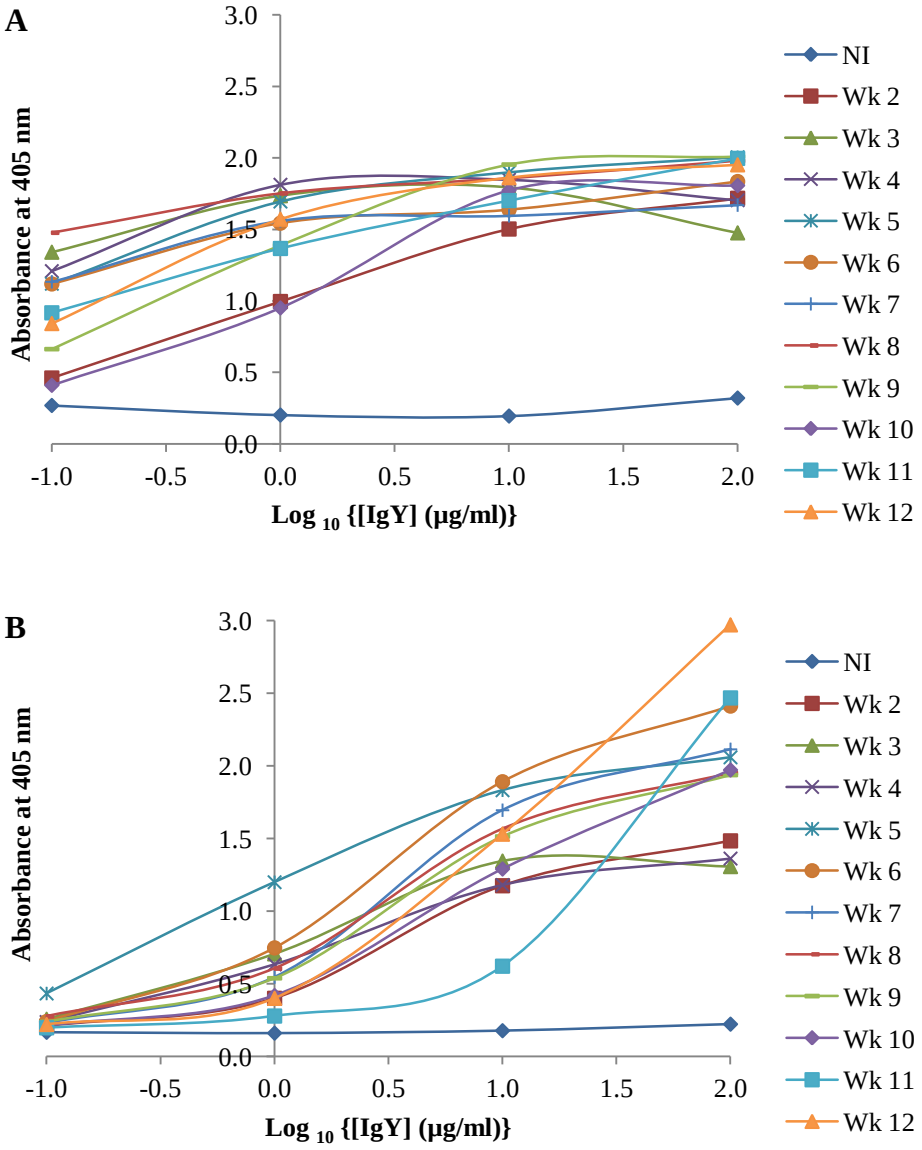


Figure 4.4 ELISA showing IgY response to immunisation with ISG75. Anti-ISG75 IgY produced by chicken 1 **(A)** and chicken 2 **(B)** for weeks 0 to 12 after first immunisation. ELISA plates were coated with ISG75 (1 µg/ml, 4°C, 16 h), anti-ISG75 IgY was used as primary antibody (0.1-100 µg/ml, 37°C, 2 h) and HRPO conjugated rabbit anti-IgY (1:10 000 in BSA-PBS) as secondary antibody. Plates were developed with substrate solution [0.05% (w/v) ABTS, 0.0015 % (v/v) H_2O_2 in 150 mM citrate phosphate buffer, pH 5.0]. The absorbance readings at 405 nm represent the average of duplicate experiments.

4.3.2 Purification of anti-ISG65 and anti-ISG75 antibodies

Specific anti-ISG65 and anti-ISG75 antibodies were affinity purified from the pool of isolated chicken antibodies and the elution profiles plotted using the A_{280nm} readings of the

eluates (Figure 4.5). The anti-ISG65 antibodies for chicken 1 had higher absorbance readings (A_{280nm}) and consequently higher concentrations than those from chicken 2 (Figure 4.5A). For anti-ISG75 antibody, the highest concentration of specific antibodies was purified from the pool of antibodies isolated from eggs of chicken 1 as well (Figure 4.5B).

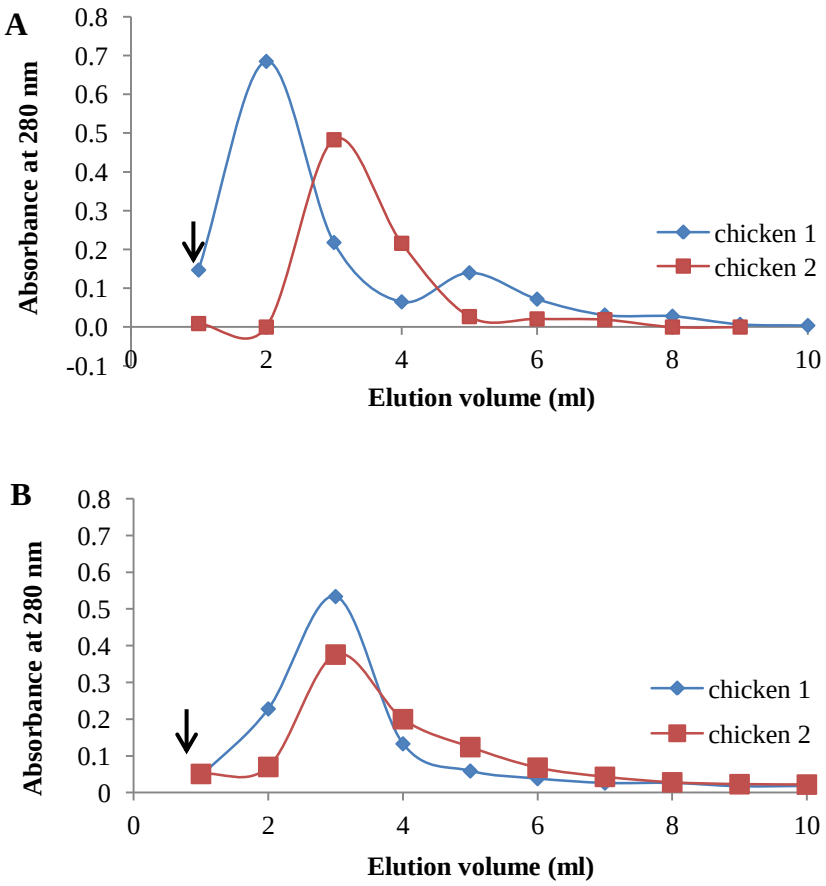


Figure 4.5 Elution profiles of affinity purified chicken anti-ISG65 IgY and anti-ISG75 IgY. **(A)** Anti-ISG65 IgY [chicken 1 (◆) and chicken 2 (■)] was purified on Aminolink® plus coupled with ISG65**. (B)** Anti-ISG75 IgY [chicken 1 (◆) and chicken 2 (■)] was purified on Aminolink® plus coupled with ISG75. The absorbances at 280 nm for the fractions collected were compared. The antibodies were eluted at low pH with 0.1 M glycine-HCl, pH 2.8 (arrows).

4.3.3 Recognition of ISG65 and ISG75 by affinity purified anti-ISG65 and anti-ISG75 antibodies in ELISA

The ability of the affinity purified anti-ISG65 and anti-ISG75 antibodies to recognise the corresponding antigens was assessed using ELISA. The affinity purified anti-ISG65 antibodies had higher titres than all the other antibodies: non-immune, unbound and pre-affinity purification antibodies (Figures 4.6). The affinity purified anti-ISG65 antibodies for chicken 1 (Figure 4.6A and C) had higher titres than chicken 2 (Figure 4.6B and D).

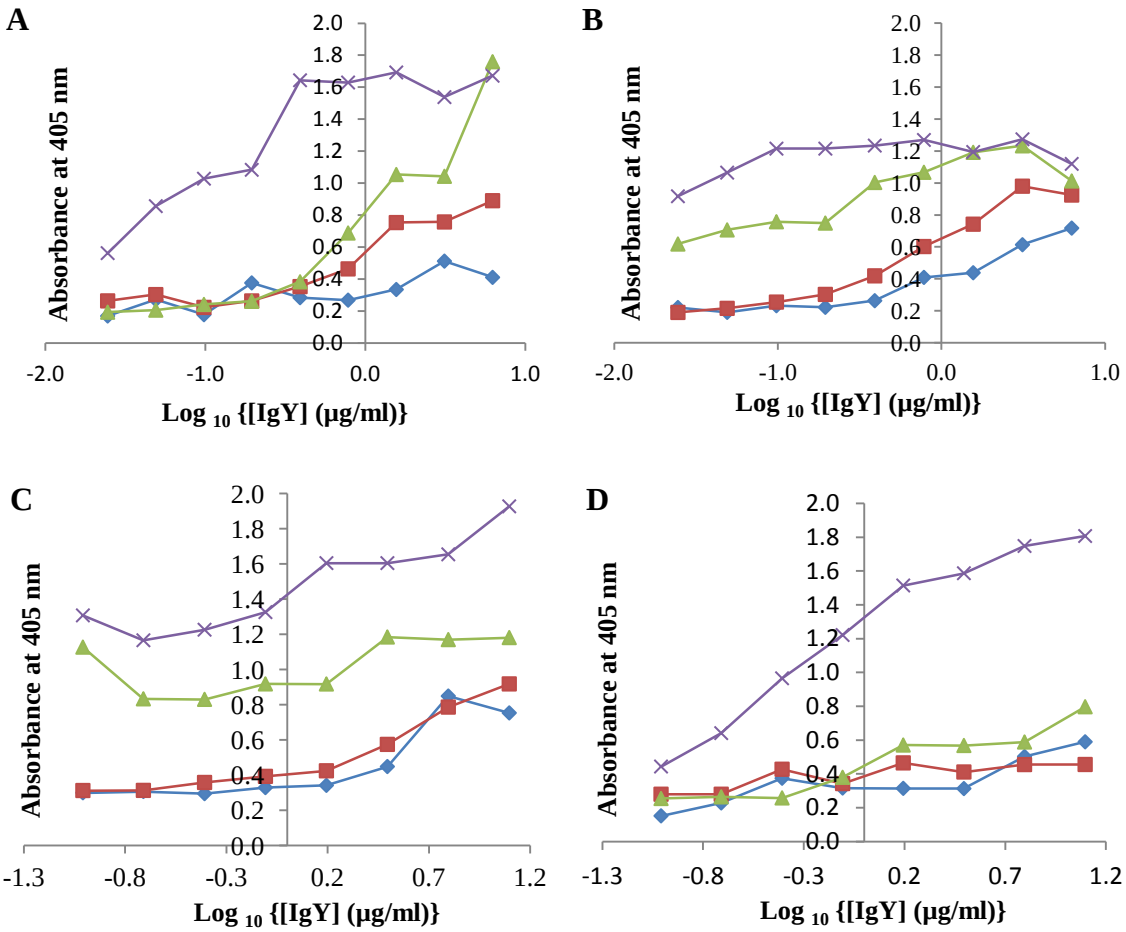


Figure 4.6 Analysis of anti-ISG65 antibodies before and after affinity purification. ELISA plates were coated with ISG65 (1 µg/ml, 4°C, 16 h). Affinity purified (×), pre-affinity purification (▲), unbound (■) and non-immune (◆) antibodies (see section 4.2.2.4 for description of antibodies) were diluted from 0.012-25 µg/ml or 0.098-100 µg/ml. **(A)** Anti-ISG65 antibody pool for chicken 1 weeks 2-7. **(B)** Anti-ISG65 antibody pool for chicken 2 weeks 2-7. **(C)** Anti-ISG65 antibody pool for chicken 1 weeks 8-10. **(D)** Anti-ISG65 antibody pool for chicken 2 weeks 8-10. The absorbance readings at 405 nm represent the average of duplicate experiments.

The affinity purified anti-ISG75 antibodies displayed a higher affinity for the antigen (ISG75) compared to the non-immune, unbound and pre-affinity purification antibodies (Figure 4.7). This trend was consistent for the anti-IGS75 antibodies produced by both chicken 1 (Figure 4.7A) and chicken 2 (Figure 4.7B). Both affinity purified antibody samples had a similar range of absorbance values. The titres were nevertheless, higher for the antibody pool of chicken 1 weeks 6-8 and chicken 2 weeks 2-4 (Figure 4.7C).

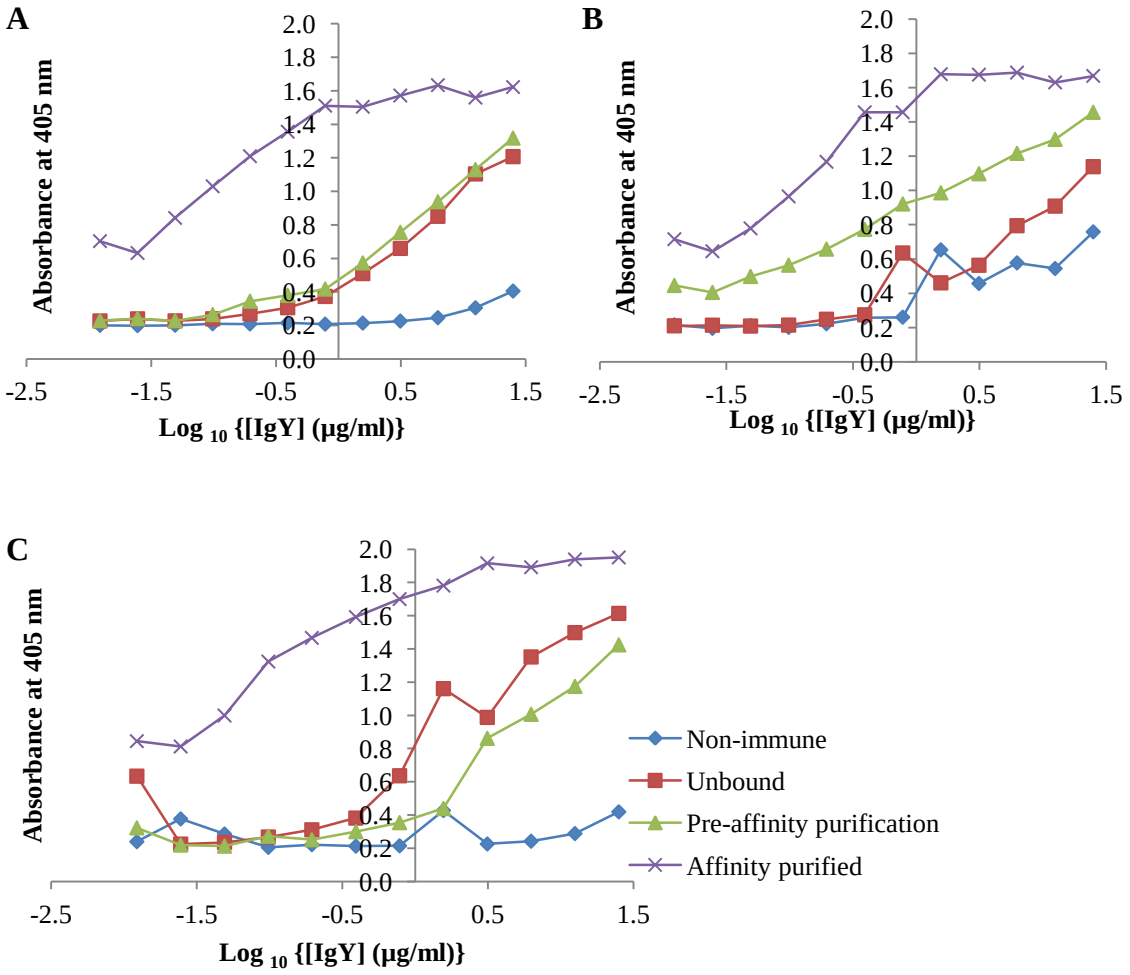


Figure 4.7 Analysis of anti-ISG75 antibodies before and after affinity purification. ELISA plates were coated with ISG75 (1 µg/ml, 4°C, 16 h). Affinity purified (×), pre-affinity purification (▲), unbound (■) and non-immune (◆) antibodies (see section 4.2.2.4 for description of antibodies) were diluted from 0.012-25 µg/ml or 0.098-100 µg/ml. **(A)** Anti-ISG75 antibody pool for chicken 1 weeks 9-12. (**B)** Anti-ISG75 antibody pool for chicken 2 weeks 5,10 and 12. **(C)** Anti-ISG75 antibody pool for chicken 1 weeks 6-8 and chicken 2 weeks 2-4. The absorbance readings at 405 nm represent the average of duplicate experiments.

The affinity purified anti-ISG65 and anti-ISG75antibodies were used in subsequent experiments to show immunological recognition of native ISGs in parasite lysates and using immunofluorescence of fixed parasites.

4.3.4 Western blotting analysis of parasite lysates

The recognition of native ISG65 and ISG75 of *T. b. brucei* by the anti-ISG65 and anti-ISG75 antibodies respectively was examined using western blotting analysis of parasite lysates. Reducing SDS-PAGE analysis of a lysate of the parasites showed an array of proteins subsequent to Coomassie blue staining (Figure 4.8A). Proteins transferred from duplicate gels to nitrocellulose strips and probed with anti-ISG65 (Figure 4.8B) and anti-ISG75 (Figure 4.8C) antibodies showed the recognition of proteins of 65 kDa and 75 kDa (Figure 4.8B and C respectively) by the antibodies. Native ISG65 and recombinant Tbb_{His}ISG65 were recognised by the anti-ISG65 antibodies (Figure 4.8B, lanes 1 and 2). However, an unrelated His tagged fusion protein, used as a negative control, was not recognised by the anti-ISG65 antibodies (Figure 4.8B, lane 3). The size of recombinant ISG65 at 55 kDa was lower than the 65 kDa of the native ISG65 due to the fact that only the extracellular domain of the ISG65 was recombinantly expressed and not the full ISG65 polypeptide as observed for the native protein (Figure 4.8B). A faint band was also identified at a size equivalent to 75 kDa (Figure 4.8B, lane 1, dotted arrow). This could be due to some cross reactivity for ISG75 by the anti-ISG65 antibodies. The anti-ISG75 antibodies recognised native ISG75 of *T. b. brucei* (Figure 4.8C, see arrow). ISG65 was also faintly recognised and another protein of 40 kDa size (two lower bands in Figure 4.8C) by the anti-ISG75 antibodies.

4.3.5 Immunofluorecence microscopy analysis of fixed *T. b. brucei* parasites

Since the anti-ISG65 and anti-ISG75 antibodies were able to recognise native ISG65 and ISG75 in a western blot of trypanosome lysates, it was also worthwhile to examine if the same results would be obtained using fixed parasites. Confocal microscope images obtained showed fluorescing trypanosomes (Figure 4.9). The entire outline of the trypanosome could be seen as green coloured fluorescence in Panels C, G, K and O of

Figure 4.9 same as the differential interference contrast (DIC) images A, E, I and M (Figure 4.9). This fluorescence is attributed to the presence of ISG65 (Figure 4.9, C and K) and ISG75 (Figure 4.9, G and O) on the surface of the trypanosomes.

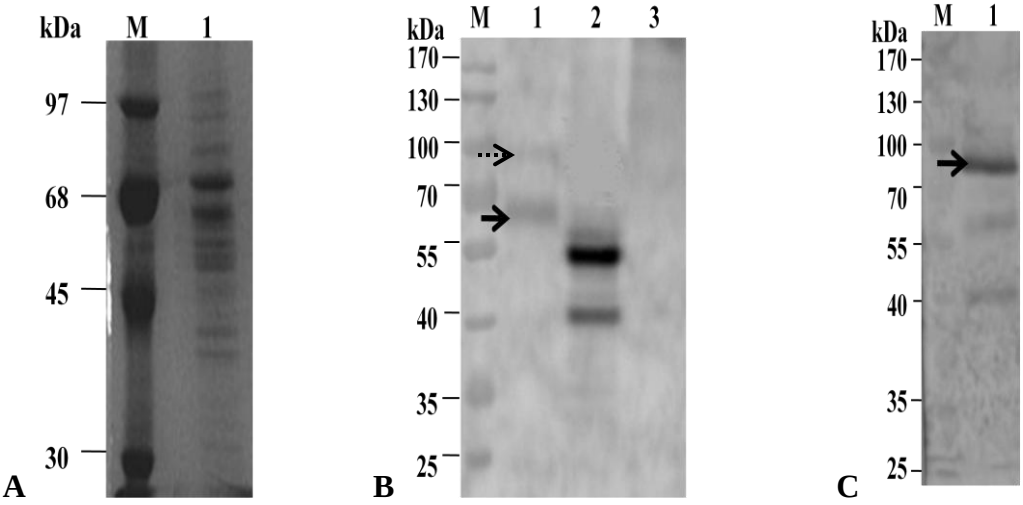


Figure 4.8 SDS-PAGE analysis and immuno-detection of native ISG65 and ISG75 in *T. b. brucei* parasite lysates. **(A)** SDS-PAGE analysis of parasite lysates. Lane M, MWM; lane 1, parasite lysates. **(B)** Western blot of parasite lysates with chicken anti-ISG65 antibodies showing recognition of native and recombinant ISG65. Lane M, pre-stained MWM; lane 1, parasite lysates; lane 2, Tbb_{His}ISG65; lane 3, His tagged fusion protein used as negative control. **(C)** Western blot of parasite lysates with chicken anti-ISG75 antibodies showing recognition of native ISG75. Lane M, pre-stained MWM; lane 1, parasite lysates. Target proteins in lysates identified by arrows.

For slides incubated with pre-immunisation chicken antibodies, no green fluorescence was observed (Figure 4.9, S). Only the DIC (Figure 4.9, Q) and Hoechst stained organelles (Figure 4.9, R) were visible, thus confirming that the fluorescence observed in Panels C, G, K and O was due to the anti-ISG65 and ISG75 antibodies binding to the respective ISG65 and ISG75 on the parasite's surface. The DNA of trypanosomes is localised in two organelles: the nucleus contains the nuclear DNA and the mitochondrial DNA is contained in the kinetoplast. Both the nuclei and kinetoplasts were identified as blue coloured fluorescence in images B, F, J, N and R of Figure 4.9 due to staining by Hoechst 33342.

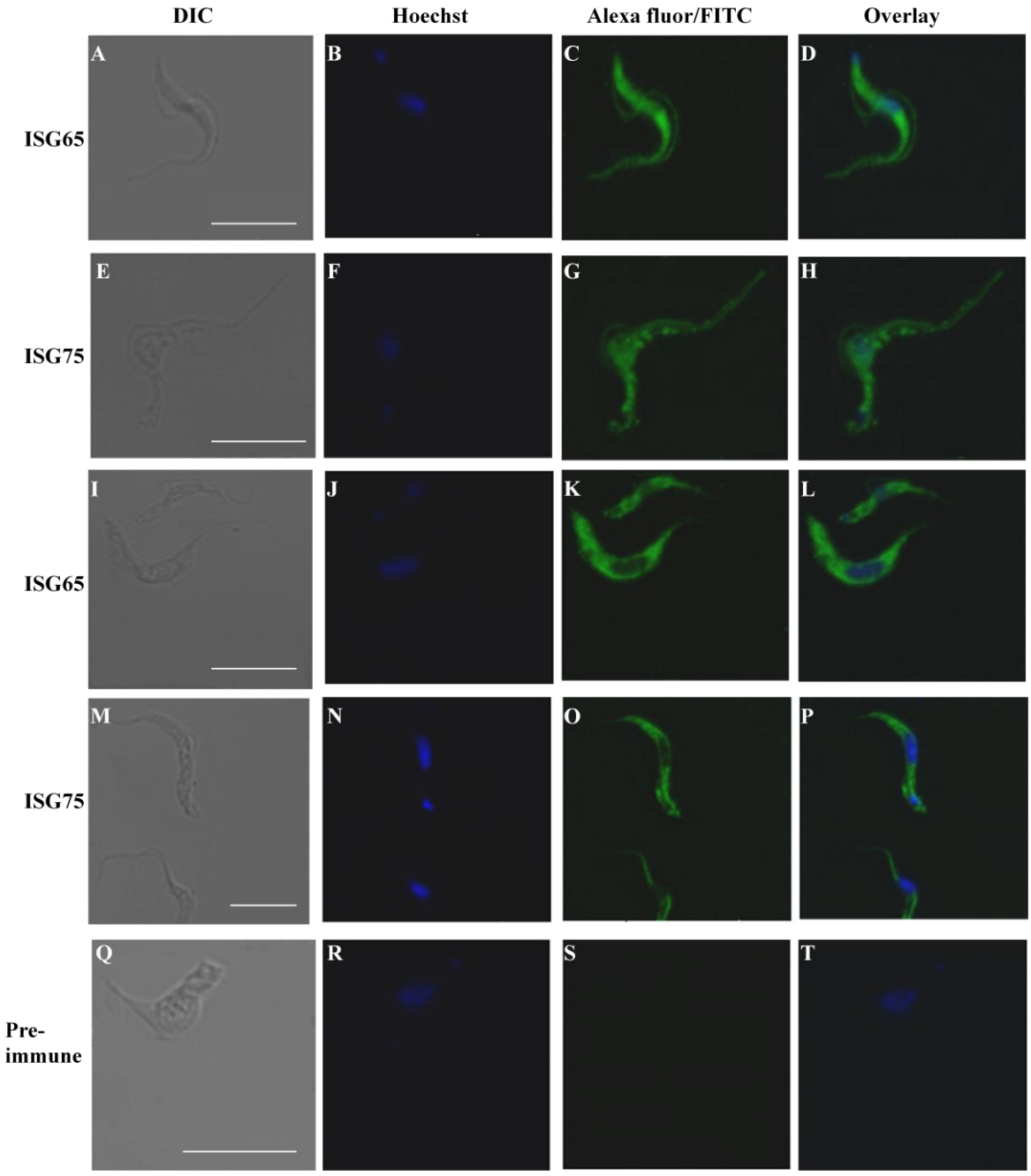


Figure 4.9: Immunofluorescent localisation of native ISG65 and ISG75 in bloodstream forms *T. b. brucei* parasites. Fixed trypanosomes were sequentially probed with anti-ISG65 or anti-ISG75 antibodies and Alexa-fluor or FITC conjugated secondary antibody before viewing under a confocal microscope. Panels A, E, I, M and Q, DIC images; Panels B, F, J, N and R, Hoechst fluorescence images; Panels C and G, Alexa-fluor fluorescence images; Panels K, O and S, FITC fluorescence images; Panels D and H, blue (Hoechst) and green (Alexa-fluor) overlay; Panels L, P and T, blue (Hoechst) and green (FITC) overlay; for ISG65 (A, B, C, D, I, J, K, L); ISG75 (E, F, G, H, M, N, O and P) and pre-immune antibody (Q, R, S and T). Scale bar is 10 µm.

4.4 Discussion

Cross-reactivity between immunoglobulin G (IgG) and immunoglobulin M (IgM) is a source of concern in serum based tests for trypanosomiasis, necessitating the strict use of IgG-specific fluorescent conjugates for serological detection (Büscher and Lejon, 2004). In this study, recombinant ISGs were used to raise antibodies in chickens. Chicken immunoglobulins do not react with mammalian immunoglobulins, thus the use of chicken-raised antibodies in this study has the potential of improved antibody specificity in serological tests. Generally, the use of antibodies from chickens offers numerous advantages when used for the detection of mammalian antigens (Ayyar *et al.*, 2012). Also the cost saving implications of raising antibodies in chickens are another important consideration that motivated the use of chicken antibodies in this study.

Due to the lack of binding to specific affinity ligands, chicken antibodies cannot be purified using conventional antibody purification methods like A or G Sepharose affinity chromatography (Ayyar *et al.*, 2012); hence another method was used in this study. The recombinant ISG65 and ISG75 proteins were separately covalently attached as ligands to the Thermo Scientific AminoLink® beaded agarose support that contains aldehyde groups for linking to free amine groups on proteins. Apart from the fact that the ISG65 and ISG75 affinity columns produced were reusable, this method also offered other advantages to the antibody production process. The use of the same antigen for immunising the chicken as the immobilised ligand on the chromatographic matrix facilitated the purification of antibodies with the greatest affinity and specificity from the pool of PEG isolated chicken antibodies. Strong antigen-antibody interactions require a high degree of complementarity between antigen and antibody. As seen by the indirect ELISA results, higher levels of antibody binding were obtained by the affinity purified anti-ISG65 and anti-ISG75 antibodies in comparison to the binding by the pre-affinity purification antibodies (compare the purple and green plots in the charts in Figures 4.6 and 4.7). The higher absorbance values obtained for the affinity purified antibodies compared to the pre-affinity purification antibodies seem to suggest that when the specific antibodies were in a pool with other non-specific antibodies, their affinity for the antigen (ISG65 or ISG75) was

reduced. The reduction in specific antigen-antibody interaction could be due to steric hindrance by the other non-specific antibodies in the antibody pool. Their presence in the antibody pool may prevent the close interactions required for proper antigen-antibody interactions.

Challenges were encountered during the preparation of the affinity column. The AminoLink® coupling resin was initially used as per the instructions in the user manual. This, however, gave a low coupling efficiency of the antigen to the resin and the antibodies purified had very low concentration and low titres. A new protocol was then used which allowed for the formation of Schiff base intermediates between the primary amines of ISG65 or ISG75 and the aldehyde functional groups of the agarose beads at a high pH of 10 and not the pH of 7 in the former protocol. Subsequent reduction of these Schiff base intermediates to stable secondary amine linkages with $NaCNBH_4$ was achieved at a pH of 7. This new protocol adapted from the AminoLink® plus coupling resin manual (Thermo Scientific, USA) improved the coupling efficiency of the ISG65 and ISG75 to the resin. Also the new columns prepared were more efficient in the purification of the anti-ISG65 and anti-ISG75 antibodies. Higher titres were obtained for the affinity purified antibodies compared to the unbound and pre-affinity purification antibodies (see Figures 4.6 and 4.7). The only drawback experienced was the decline of antibody concentrations by a hundred fold from 20-30 mg/ml before purification to 0.2-0.3 mg/ml after purification. Nevertheless, this was more than compensated for by the high specificity of the affinity purified antibodies as seen from the ELISA charts in Figures 4.6 and 4.7 in the results section.

Histidine tags rarely affect the characteristics of the recombinant protein (Graslund *et al.*, 2008) therefore it was not removed from the recombinant ISG65 and ISG75 fusion proteins prior to immunisation of chickens for antibody production. Previous investigators also used the ISG75 fusion protein with an intact GST tag to raise antibodies (Ziegelbauer *et al.*, 1992). To address the speculation/concern that anti-ISG65 and anti-ISG75 antibodies could also recognise the His-tag, negative controls were used when conducting western blots. ISG65 was analysed alongside another His-tagged protein (Figure 4.8B).

The affinity purified anti-ISG65 antibodies were unable to recognise other His-tagged recombinant proteins but were able to selectively recognise recombinant ISG65.

Native ISG65 and ISG75 of trypanosomes were so named based on their apparent molecular weight determined from migration on SDS-PAGE (Ziegelbauer and Overath, 1992). In the present study, the anti-ISG65 and anti-ISG75 antibodies were able to recognise the native ISG65 and ISG75 of trypanosomes in a western blot of trypanosome lysates at the expected apparent molecular weight of 65 and 75 kDa. Considering the existence of an intracellular pool of ISGs in addition to surface localisation in trypanosomes, both the surface and endosomal populations of ISG65 and ISG75 were present in the trypanosomal lysate and recognised by the antibodies used for the western blot. The ability of anti-ISG65 and anti-ISG75 antibodies to recognise native ISG65 and ISG75 was also confirmed by immunofluorescence microscopy analysis. This underscores the potential of the recombinant ISG65 and ISG75 to replace the native antigens currently used in sero-diagnostic tests for trypanosomiasis.

Parasitological detection of the presence of trypanosomes in a test sample is a pre-requisite to conclusive diagnosis of trypanosomiasis (Chappuis *et al.*, 2005). Most of the methods currently in use have low sensitivity and are laborious (Büscher and Lejon, 2004) due to the difficulty in identifying the trypanosomes which are very small in size (20-30 µm by 1.5-3.5 µm) (Chappuis *et al.*, 2005). Immunofluorescence assays are currently available for the diagnosis of *T. b. gambiense* HAT nevertheless there is still the necessity for better antigens especially for the diagnosis of *T. b. rhodesiense* HAT (Büscher and Lejon, 2004). Here we propose ISG65 and ISG75, which are distributed over the surface of the trypanosomes, as potential antigens to be used together with their respective anti-recombinant ISG antibodies in immunofluorescence assays. As shown in this study, indirect fluorescence by fluorochromic conjugated secondary antibodies (Alexa fluor® and FITC were used in the present study) led to the visualisation of the entire profile of fixed BSF *T. b. brucei* parasites. This technique has the potential to reduce the limitations currently experienced in the parasitological confirmation stage of trypanosomiasis diagnosis, because fluorescing trypanosomes can be easily and quickly identified under the

microscope. The major limitation of this method would be the requirement for expensive fluorescence microscopes. This cost can nonetheless, be reduced by resorting to cheaper alternatives that use external light sources like fluorescent light emitting diodes (Büscher and Lejon, 2004). The Primo Star iLED, a LED-based fluorescence microscope jointly developed by Foundation for Innovative New Diagnostics (FIND) and Carl Zeiss is one of such simpler and less expensive alternatives. It uses LED bulbs which have a long life span of more than 10,000 hours and use very little energy; hence the microscope can be powered using solar energy or generators in remote/rural areas where HAT/AAT is endemic (FIND, 2012a; FIND, 2012c; Ndungu *et al.*, 2010).

The Lister 427 strain of *T. b. brucei* has been cultivated with great success over the years in the laboratory (Brems *et al.*, 2005) and was thus the strain of choice for the immunofluorescence assays in the present study. Also, being a monomorphic strain, it fulfilled the requirement of the present study for only BSF trypanosomes since ISGs are specific for the bloodstream stage of the trypanosomes' life-cycle. It is interesting to note that the flagellum of the trypanosomes was also visualised along with the entire profile of the trypanosomes (see Figure 4.9). This seems to suggest the presence of ISG65 and ISG75 on the outer surface of the flagellum. The fixed parasites were permealised using 0.5% (v/v) PBS-Triton X-100, enabling the Hoechst stain to pass through the membrane of the trypanosomes into the cytoplasm. This facilitated the staining of the nuclei and kinetoplast of the parasites as seen in Figure 4.9B, F, J, N and R.

Previous studies have been undertaken on the diagnostic potential of ISG75 (Tran *et al.*, 2008; Roge *et al.*, 2013) but nothing has been published about ISG65 to the best of the author's knowledge. Here the antigenic potential of both ISGs as diagnostic antigens is demonstrated. The study previously done on ISG75 did not include immunofluorescence microscopy analysis hence this study introduces additional information not previously available. Some studies have highlighted the greater sensitivity of the classical immunofluorescent antibody test over CATT for the diagnosis of HAT (Noireau *et al.*, 1988). The present study offers improvements to the classical immunofluorescent antibody test by introducing a potentially better antigen for the test: ISG65 and/or ISG75.

The detection of the parasites under the confocal microscope was, however, through indirect means requiring a fluorescing secondary antibody to recognise the ISG-primary antibody complex. Indirect immunofluorescense was chosen over a direct immunofluorescence assay due to the ease of acquisition of the secondary Alexa-fluor and FITC conjugated antibodies. Future work would include attempting to do a direct immunofluorescence assay which would involve labelling the anti-ISG65 and anti-ISG75 antibodies with fluorochromes. This could potentially shorten the time of the diagnostic assay by eliminating one of the incubation steps.

Some measure of cross-reactivity was obtained for ISG75 by the anti-ISG65 antibodies and for ISG65 by the anti-ISG75 antibodies as seen by the western blots in Figure 4.8. Given the fact that the two proteins, ISG65 and ISG75, are similar proteins with somewhat similar sequences, it could be possible that both proteins have similar epitopes. EMBOSS Needle Pairwise sequence alignment of *T. b. brucei* ISG65 (Accession XM_946493) and *T. b. brucei* ISG75 (Accession DQ200189) nucleotide sequences used in this study show 42.3% identity between the proteins (Appendix 7). Future studies will include the production of anti-peptide antibodies against both proteins using peptides unique to the respective proteins, as these will be less likely to show cross-reactivity between ISG65 and ISG75. Notwithstanding, considering the fact that the anti-ISG65 and anti-ISG75 antibodies produced in the present study are to be used for the diagnosis of trypanosomiasis, the cross-reactivity between the antibodies is not a disadvantage but actually improves chances of accurate diagnosis of the disease.

In the CATT and LATEX which are commonly used as field tests for *T. b. gambiense* HAT, whole BSF parasites and VSGs are used as antigens (Chappuis *et al.*, 2005). The successful expression of ISG75 and ISG65 in this study provides an alternative antigen that could replace the currently used antigens in a new rapid diagnostic test. Recombinant ISGs do not only offer the advantage of ease of production, but also have the potential to improve specificity and sensitivity of the tests due to their invariant nature. Future studies to be undertaken concerning the development of rapid diagnostics tests for trypanosomiasis would include developing a lateral flow or agglutination assay for point of treatment

diagnosis and also laboratory based tests such as inhibition ELISA and direct immunofluorescence assays would be explored using the recombinant ISG65 and ISG75 as well as the antibodies produced in the present study.

CHAPTER 5

General Discussion

African trypanosomiasis is prevalent in poor and underdeveloped areas of Africa; hence there is a constant need for simple to use, cheap and reliable point-of-care diagnostic tests (Hutchinson *et al.*, 2004). The prevalence of different strains of trypanosomes infecting humans and animals in different parts of the continent complicates the ability to design a pan-species or generic rapid diagnostic test for field diagnosis of the disease. Currently available serological diagnostic tests are specific for different strains of the parasite for example CATT/*T. b. gambiense* or CATT/*T. evansi*, which use lyophilised BSF parasites as antigens (Chappuis *et al.*, 2005). This requires the labour-intensive process of *in vivo* culturing of the parasites in rats and their extraction from the rats' blood (Chappuis *et al.*, 2005). Other tests like the LATEX/*T. b. gambiense* use three VSGs coupled to latex particles. However, due to antigenic variation, the specificity of this test is decreased in infections where different VSGs are expressed by the proliferating trypanosomes. These limitations have fuelled research aimed at discovering new diagnostic markers (Simarro *et al.*, 2008).

Apart from *T. congolense*, the most infective trypanosomes in Africa belong to the sub-genus *Trypanozoon*, hence it will be useful to have a generic diagnostic test for all members of this sub-genus (Deborggraeve *et al.*, 2006). A pan-*trypanozoon* diagnostic kit would be very useful for field diagnosis especially for mass population screenings for the disease (Tran *et al.*, 2009). This would also offer the advantage of reduced cost in the production of diagnostic kits as well as improved logistics for the deployment of the diagnostic tests to trypanosomiasis prevalent areas.

Invariant surface glycoproteins of trypanosomes constitute an abundant group of trans-membrane proteins evenly distributed over the surface of trypanosomes. They are bloodstream stage specific and unlike the VSGs, they are invariant and do not undergo antigenic variation (Nolan *et al.*, 1997). These ISGs have been identified as potential diagnostic antigens (Hutchinson *et al.*, 2004) and also potential DNA vaccine candidates

for trypanosomiasis (Lanca *et al.*, 2011). The two most common ISGs, ISG65 and ISG75, are present in the genome of all the members of the *Trypanozoon* sub-genus: *T. b. brucei, T. b. gambiense, T. b. rhodesiense, T. equiperdum* and *T. evansi* (Tran *et al.*, 2008).

The present study was aimed at designing a high yield heterologous production system for ISG65 and ISG75 of *T. b. brucei* and *T. b. gambiense*, in order to explore their antigenic potential and their possible use for the production of a pan-*trypanozoon* diagnostic test for African trypanosomiasis.

There are up to 70 000 and 50 000 molecules of ISG65 and ISG75 respectively on the surface of *T. b. brucei* (Jackson *et al.*, 1993; Leung *et al.*, 2011; Ziegelbauer and Overath, 1992) therefore the native proteins could have been isolated from trypanosomes. However, this requires labour intensive propagation of the parasites *in vivo* or *in vitro*. Therefore, the focus of the present study was on the production of recombinant ISG65 and ISG75. This was to exploit the numerous advantages offered by recombinant DNA technology to design a more efficient antigen production process. Also needle stick injuries, over exploitation of laboratory animals and all the other risks involved with *in vivo* culturing of pathogenic trypanosomes would be avoided (Herwaldt, 2001; Roge *et al.*, 2013).

Although previous studies have proposed ISG75 as a diagnostic target for surra caused by *T. evansi* (Tran *et al.*, 2009) and trypanosomiasis caused by *T. brucei* (Tran *et al.*, 2008; Roge *et al.*, 2013), no studies have been reported using ISG65 for diagnosis of trypanosomiasis. A blast search (http://blast.ncbi.nlm.nih.gov; accessed on 12-06-11) using the *T. b. brucei* ISG65 sequence (Accession XM_946493) cloned in the present study showed a 99% identity to *T. b. gambiense* ISG65 (Appendix 8). The ISG75 sequence (Accession DQ200189) showed a 99% identity to several other ISG75 sequences from the *Trypanozoon* sub genus, including *T. b. gambiense*, *T. b. rhodesiense* and *T. equiperdum* (Appendix 9). It also showed 96% identity with ISG75 sequences for *T. evansi.* More so, a blast search (http://blast.ncbi.nlm.nih.gov; accessed on 12-06-11) using the ISG65 and ISG75 nucleotide sequences used in this study showed that only 4% or less of the ISG65 and ISG75 sequences were found in other organisms apart from trypanosomes. This further

accentuates the possibility of using ISG65 and ISG75 in the design of a generic diagnostic test for the *Trypanozoons*.

Invariant surface glycoproteins are attached to the trypanosome membrane by means of a trans-membrane alpha-helix formed by hydrophobic amino acid residues close to the C-terminus of the ISG polypeptide. This region as well as a short hydrophilic intracellular domain is conserved across the *Trypanozoons* (Ziegelbauer *et al.*, 1992). Notwithstanding, these regions were not the focus of the present study since they are embedded in the membrane of the trypanosome and most likely would not be exposed to the host's immune system. Another region of the *ISG* gene that was not cloned in the present study is the region coding for the signal peptide which comprises the first 21 to 28 amino acid residues of the protein sequence. This region is cleaved from the ISG polypeptide upon maturation. In the present study, the genes coding for the extracellular domains of ISG65 and ISG75 were cloned. This region is exposed to the host's immune system and would most likely contain the antigenic epitopes of the glycoproteins (Roge *et al.*, 2013; Tran *et al.*, 2008).

Recombinant expression of both ISG65 and ISG75 was carried out in both prokaryotic [strain BL21 (DE3) *E. coli*] and eukaryotic (*P. pastoris*) systems. The BL21 (DE3) strain is a commonly used protease deficient *E. coli* strain appropriate for high-level protein production (Graslund *et al.*, 2008). The methylotrophic *P. pastoris* yeast expression system was chosen due to its ability to express high levels of foreign proteins, but most of all for its ability to produce multiple eukaryotic post-translational modifications. These include disulfide bond formation, glycosylation, protein folding and proteolytic processing (Aloulou *et al.*, 2006; Balamurugan *et al.*, 2007; Macauley-Patrick *et al.*, 2005). The M5 strain of this yeast was used in the present study. It has an engineered N-glycosylation pathway which results in homogenous Man5GlcNAc2 N-glycosylation which resembles the predominant Man9-5GlcNAc2 oligomannose structures in *T. brucei* (Roge *et al.*, 2013). Also a number of trypanosomal proteins including congopain have been successfully expressed in *P. pastoris* (Pillay et al., 2010).

Very low expression levels were previously reported for *T. b. gambiense* ISG75 using BL21 (DE3) *E. coli* cells (Tran *et al.*, 2008), however, successful expression of ISG75

using this strain was obtained in the present study. The success may be attributed to several differences in the approach adopted. Firstly, the pET28a expression vector was used in the present study as opposed to pET15b co-transformed with pRIL plasmid (to supply additional tRNAs) in the previous study (Tran *et al.*, 2008). Secondly, 2x YT, a richer medium was the medium of choice for heterologous expression in the present study as against lysogeny broth preferred in the previous study. The 2x YT medium has the capacity to maintain higher *E. coli* densities during expression for a longer growth period (Sivashanmugam *et al.*, 2009). Thirdly, the selection of high expressing colonies for expression and proper preparation of the starting culture could have also contributed to the successful expression of ISG75 in the present study (Sivashanmugam *et al.*, 2009).

An interesting observation was made during the expression of *T. b. brucei* and *T. b. gambiense* ISG75. The growth curve obtained for the four hour expression period showed a steady increase in bacterial biomass as estimated from the OD_{600} of the expression medium. However, a slight drop in protein production was observed during the third hour of expression and then a subsequent rise in production in the fourth hour (section 3.3.4). This suggests that recombinant protein production was not necessarily directly proportional to increasing bacterial biomass. As explained in chapter 3, accumulation of metabolic by-products like acetate could have resulted in the decreased rate of heterologous protein synthesis in the *E. coli* cells (Sivashanmugam *et al.*, 2009). Moreover, the expression was taking place in the uncontrolled environment of the shaker flasks hence there could have been localised variations in the availability of oxygen and nutrients for the bacterial cells. The subsequent increase in ISG75 production during the fourth hour of expression is noteworthy. Consequently, the expression of ISG75 was routinely terminated at the fourth hour of expression.

A high level of protein aggregation and instability was observed during the purification and concentration processes of recombinant ISG65 and ISG75 in the present study. Addition of equimolar amounts of L-arginine and L-glutamic acid (50 mM) to the lysis buffer and also to the purified protein sample before concentration reduced protein aggregation. The amino acid with the most basic pKa value, L-arginine, has been found to

increase the solubility of aggregate-prone, unfolded protein molecules and folding intermediates (Tischer *et al.*, 2010). Simultaneous addition of both L-arginine and L-glutamic acid has been previously shown to increase the maximum achievable concentration of a soluble protein (Golovanov *et al.*, 2004). This enhanced protein solubility is thought to be due to enhanced crowding around the protein molecules by these amino acids; hence protein-protein association is suppressed. Also a synergistic effect caused by the additive-additive interactions in solution, led to increasing additive-protein interaction (Shukla and Trout, 2011). In previous studies, this same solution was utilised for the stability and solubility challenges encountered with recombinant ISG75 production. In that study, lyophilisation of the purified protein was undertaken as a solution to the high level of protein aggregation observed during the concentration of ISG75 (Tran *et al.*, 2008).

Two bands were observed on reducing SDS-PAGE for all the different ISG65 (chapter 2) and ISG75 (chapter 3) constructs expressed in the present study. A previous study identified degradation sites in the ISG75 amino acid sequence and the two bands observed for ISG75 in that study was explained as being due to degradation at those sites (Roge *et al.*, 2013). Degradation at specific sites could be responsible for the two bands observed for ISG65 and ISG75 in the present study. Another possible explanation could be that ISG65 and ISG75 exist as dimers in their native forms in the trypanosomes' membrane. Other studies have made reference to the possibility of oligomerisation of the ISGs (Tran *et al.*, 2008; Leung *et al.*, 2011). Although the size of the first larger size band is not double that of the smaller band as would be expected in a monomer-dimer relationship, the possibility of ISG65 and ISG75 existing as dimers cannot be completely discounted.

Studies involving recombinant expression of another trypanosome protein, *T. cruzi* oligopeptidase B, showed anomalous migration on SDS-PAGE similar to that observed for the ISGs. The protein was observed as two bands of 80 and 120 kDa in size which are inconsistent with a monomer:dimer size estimation. Nonetheless, through MEC and analytical ultracentrifugation it was shown that the protein, *T. cruzi* oligopeptidase B, was dimeric (Motta *et al.*, 2012). More so, in the present study, recombinant Tbb_{His}ISG65

purified by MEC (section 2.3.5.4) eluted at a volume expected for a larger protein of approximately 106 kDa. Reducing SDS-PAGE analysis of these fractions showed an apparent molecular weight of 55 kDa. The elution of ISG65 from the MEC column at a size of 106 kDa, which is almost double its apparent size of 55 kDa on SDS-PAGE, seems to point to the possibility of dimerisation by this glycoprotein. Native ISG65 was also reported by Jackson *et al.* (1993) to elute from an MEC column at a size consistent with a dimer.

The possibility of ISGs undergoing some form of polypeptide/protein processing before their arrangement on the cell surface cannot be discounted. The *ISG75* gene is present in multiple foci in the trypanosome genome (Tran *et al.*, 2006). Also, the existence of two major groups of *ISG75* sequences, denoted as group I and group II sequences, have been established. There is speculation that *ISG75* from both group I and group II genes are simultaneously expressed and that both proteins associate in the membrane to form dimers/multimers (Tran *et al.*, 2006).

Another aberration observed during SDS-PAGE analysis of both ISG65 and ISG75 in the present study is its anomalous migration in these gels. The proteins migrated at sizes larger than expected calculated from the amino acid sequences of the proteins. This trend was also observed for ISG65 and ISG75 by previous investigators (Jackson *et al.*, 1993; Tran *et al.*, 2009; Ziegelbauer *et al.*, 1992). Both ISG65 and ISG75 possess a high negative charge which reduces their binding affinity to SDS as well as their migratory ability on the SDS-PAGE gels compared to the standard molecular weight markers (Leung *et al.*, 2011; Ziegelbauer *et al.*, 1992). This characteristic (high negative charge) has previously been identified as a contributing factor to uncommon charge to friction ratios of certain proteins which result in abnormal migration on SDS-PAGE (Westerhuis *et al.*, 2000). Some other trypanosome proteins like oligopeptidase B have been shown to exhibit the same irregular migration patterns on SDS-PAGE (De Matos Guedes *et al.*, 2007; Motta *et al.*, 2012). The amino acid composition of ISG65 and ISG75 is another important consideration. It was previously shown that alanine rich proteins of *T. brucei* BARP (brucei alanine rich protein)

and GARP (glutamate and alanine rich protein) have heterogeneous mobility and a larger apparent molecular weight in SDS-PAGE than predicted (Nolan *et al.*, 2000).

Protein glycosylation is a common post-translational modification that can occur in heterologous proteins expressed in the *P. pastoris* expression system (Balamurugan *et al.*, 2007). Considering the higher than expected apparent molecular sizes of 82 kDa and 90 kDa observed for ISG65*Pp* and *Tbg*$_{Pp}$ISG75 on reducing SDS-PAGE, the possibility of glycosylation of these fusion proteins seemed likely. Enzymatic deglycosylation has been previously successfully applied to the assessment of the glycosylation status of some trypanosome proteins. The enzyme N-glycosidase F was successfully used for the deglycosylation of native ISG65 in a membrane fraction of lysed trypanosomes (Ziegelbauer *et al.*, 1992). Another enzyme, peptide N-glycosidase F, has also been successfully used to remove glycan moieties from VSGs (Mehlert *et al.*, 2002) and *T. cruzi* trans-sialidase (Ratier *et al.*, 2008). In the present study, Endoglycosidase H was the glycosidase of choice and it successfully removed the glycan molecules attached to the fusion protein ISG65*Pp* (Figure 2.23). It, however, was not successful in the deglycosylation of *Tbg*$_{Pp}$ISG75 in the present study (Figure 3.16). Other more robust enzymes like peptide N-glycosidase F which cleaves essentially all types of N-linked glycans may prove to be more successful (Mehlert *et al.*, 2012b)

There is potential for the recombinant ISG65 and ISG75 expressed in the present study to be used as diagnostic antigens for trypanosomiasis. One of such proposed formats is in an inhibition ELISA. The hassles of using species-specific enzyme conjugated secondary antibodies will be avoided in the inhibition ELISA format unlike with the indirect ELISA format. In the design of an inhibition ELISA, the plates are coated with the recombinant antigens prior to incubation with the test serum sample (Figure 5.1A and B). This is followed by incubation with the chicken anti-ISG IgY antibodies that will only bind to the immobilised ISG if there are no anti-ISG antibodies present in the test serum to inhibit their binding. The binding of chicken anti-ISG IgY is visualised using enzyme labelled anti-chicken antibody and the same detection antibody can therefore be used for testing sera from different species. This is in contrast to the indirect ELISA design which will

require an enzyme conjugated antibody that is specific for the species from which the test serum was obtained, for example anti-bovine HRPO conjugate specified in the schematic (Figure 5.1C).

Antibodies have been produced against several trypanosomal proteins for example anti-congopain antibodies were produced in rabbits (Huson *et al.,* 2009), anti-peptide antibodies were produced against trypanopain in rabbits and chickens (Troeberg *et al.,* 1997) and anti-VSG antisera were produced in rabbits (Schwede *et al.,* 2011). Recombinant ISG65 and ISG75 were immunogenic as shown by their ability to elicit antibodies in chickens. High antibody titres were obtained as early as two weeks post the first immunisation (section 4.3.1). The high level of immunogenicity observed for ISGs in chickens can be attributed to the evolutionary distance between the immune system of the trypanosomes and the birds. In the inhibition ELISA format proposed for the diagnosis of trypanosomiasis (Figure 5.1), competing antibodies, produced in experimental animals, will be incubated with mammalian serum containing anti-ISG antibodies. The possibility of non-specific reactions between the competing and mammalian patient serum antibodies would be avoided with the use of the chicken antibodies as competing antibodies. Moreover, the high antibody production capacity of chickens as well as the ease of isolation of the antibodies from egg yolks makes this system more cost effective (Mine and Kovacs-Nolan, 2002; Schade *et al.*, 2005).

The anti-ISG65 and anti-ISG75 antibodies produced in the present study can also be useful for research purposes. Since it has been shown in the present study that the antibodies are able to recognise native ISG65 and ISG75, the antibodies may be used for the purification of these native proteins from trypanosomal lysates via immunoprecipitation or through the use of immuno-affinity columns prepared using the antibodies. All these possibilities will be explored in future studies.

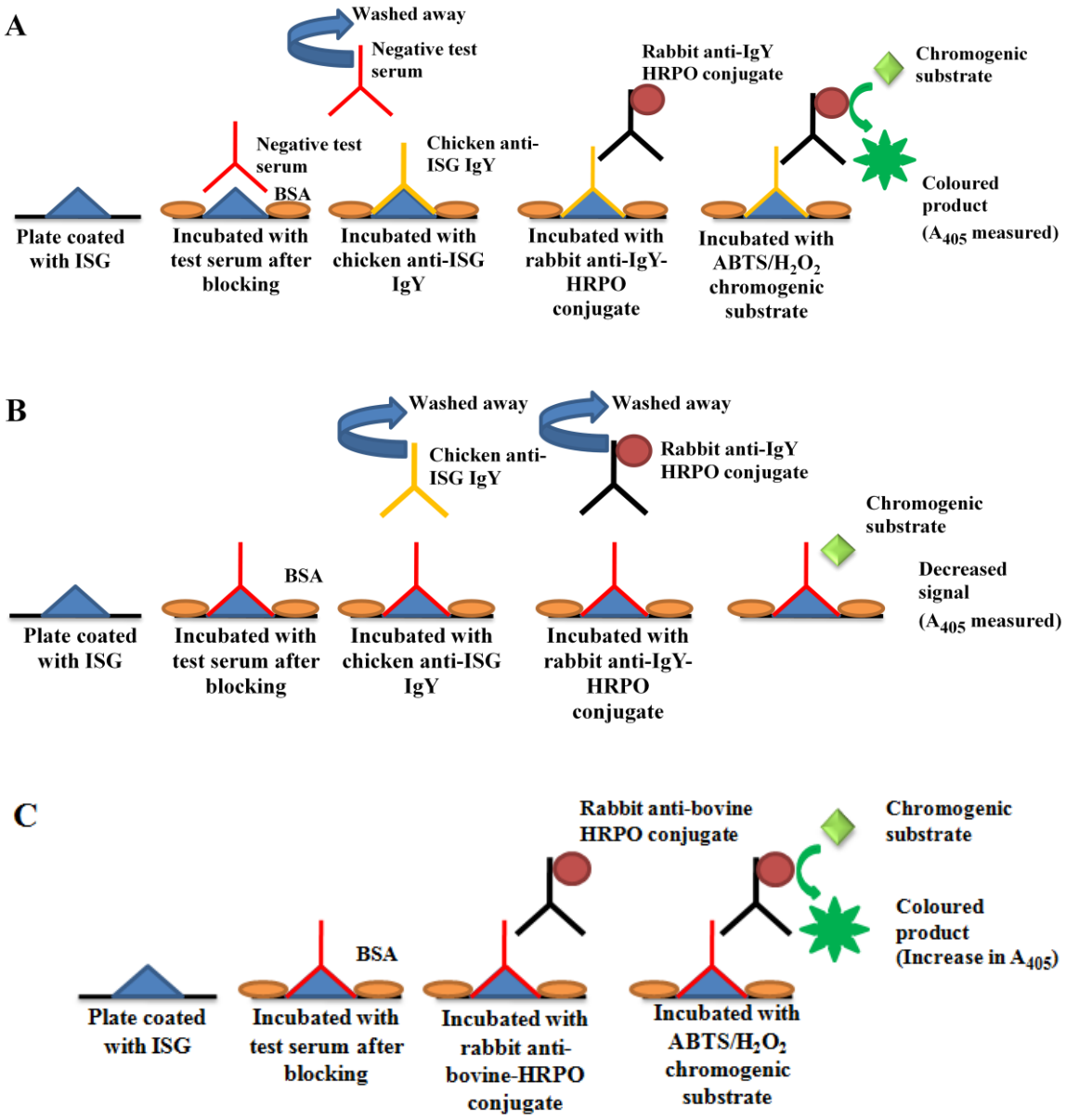


Figure 5.1 Schematic showing the principle of inhibition and indirect ELISAs for the diagnosis of trypanosomiasis. Test samples for each ELISA are serum samples from uninfected **(A)** and infected **(B and C)** mammalian subjects. The ELISA plates will be coated with ISG and incubated with test serum after blocking active sites with an appropriate blocking agent. **(A)** Negative serum. **(B)** Positive serum. **(C)** Indirect ELISA with positive serum.

Polyclonal antibodies were produced in the present study as opposed to monoclonal antibodies. This was in order to reduce the extra costs involved in the production of

monoclonal antibodies, as well as to take advantage of certain characteristics of polyclonal antibodies. These include the ability of polyclonal antibodies to recognise multiple epitopes on a particular antigen and also their stability over a broad pH range and salt concentrations (Lipman *et al*., 2005). The advantages of monoclonal over polyclonal antibodies in certain instances cannot be discounted. However, the polyclonal antibodies were sufficient for the objectives of the present study and their specificity for the antigen, especially after affinity purification, was demonstrated (Chapter 4).

Previous studies have shown that ISGs are distributed over the entire surface of trypanosomes (Ziegelbauer *et al.*, 1992). The immunofluorescence microscopy assay undertaken in the present study using the chicken antibodies produced against the recombinant ISGs gave confirmation of the surface distribution of ISGs on the trypanosomes (Figure 4.9). Diagnosis of second stage HAT is undertaken through the examination of the CNS for the presence of trypanosomes. A need is highlighted for the exploration of the application of the immunofluorescence microscopy diagnostic assay described in the present study for CNS fluids and determining the sensitivity of the method. This would be undertaken in future studies.

Trypanosomes are extracellular parasites and their surface is very important in their strategies for evading the host's immune system. Resolution of the structure of ISGs will contribute to current information about the parasite surface that is in contact with the host's environment. Other *T. b. brucei* proteins expressed in *E. coli*, for example oligopeptidase B, have been successfully characterised using X-ray crystallography (Rea *et al.*, 2006). In order to characterise the ISG65 and ISG75 produced in this study using X-ray crystallography, it will be necessary to purify the recombinant proteins to homogeneity. This would involve the separation of the two bands consistently purified for both ISGs. Future studies would involve attempting to separate the two bands using ion exchange chromatography or molecular exclusion chromatography with a column that will give better resolution than Sephacryl S200 used in the present study (section 2.3.5.4). Site-directed mutagenesis will also be employed to replace the amino acid residues constituting the cleavage site in mature ISG.

Characterisation of ISGs could also open the door for their application in other aspects of trypanosomiasis control including vaccine design as well as in drug design for trypanosomiasis. Although the use of ISG molecules as vaccines did not offer any protection to trypanosomiasis challenged mice (Ziegelbauer and Overath, 1993), pre-immunisation of Balb/c mice using plasmid constructs containing the *ISG* gene offered some measure of protection against trypanosomiasis (Lanca *et al.*, 2011). Also, the identification of ISG75 as a receptor for the uptake of suramin, a trypanocidal drug, has highlighted the possibilities of targeting ISGs in order to gain access to the parasite and deliver trypanocides. The aim is to identify molecules that are similar to suramin and that can also bind to ISGs to be used as trypanocides. Alternatively, designing immuno-toxins with specific affinity to ISGs may be viable (Alsford *et al.*, 2012; Alsford *et al.*, 2013).

In conclusion, an optimised and standardised procedure for the recombinant expression and purification of the respective extracellular domains of ISG65 and ISG75 of *T. b. brucei* and *T. b. gambiense* in prokaryotic [strain BL21 (DE3) *E. coli*] and eukaryotic (*P. pastoris*) systems was described. The proteins were characterised and used for immunological assays and they showed potential for application in the diagnosis of African trypanosomiasis.

REFERENCES

Agbo, E. C., Majiwa, P. A., Büscher, P., Claassen, E. and Te Pas, M. F. (2003). *Trypanosoma brucei* genomics and the challenge of identifying drug and vaccine targets. *Trends in Microbiology,* 11**,** 322-329.

Agur, Z., Abiri, D. and Van Der Ploeg, L. H. (1989). Ordered appearance of antigenic variants of African trypanosomes explained in a mathematical model based on a stochastic switch process and immune-selection against putative switch intermediates. *Proceedings of the National Academy of Sciences,* 86**,** 9626–9630.

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

Aloulou, A., Grandval, P., De Caro, J., De Caro, A. and Carrière, F. (2006). Constitutive expression of human pancreatic lipase-related protein 1 in *Pichia pastoris*. *Protein Expression and Purification,* 47**,** 415-421.

Alsford, S., Eckert, S., Baker, N., Glover, L., Sanchez-Flores, A., Leung, K. L., Turner, D. J., Field, M. C., Berriman, M. and Horn, D. (2012). High-throughput decoding of anti-trypanosomal drug efficacy and resistance. *Nature,* 482**,** 232-236.

Alsford, S., Field, M. C. and Horn, D. (2013). Receptor-mediated endocytosis for drug delivery in African trypanosomes: fulfilling Paul Ehrlich's vision of chemotherapy. *Trends in Parasitology,* 29**,** 207-212.

Amin, D. N., Ngoyi, D. M., Nhkwachi, G. M., Palomba, M., Rottenberg, M., Büscher, P., Kristensson, K. and Masocha, W. (2010). Identification of stage biomarkers for human African trypanosomiasis. *The American Journal of Tropical Medicine and Hygiene,* 82**,** 983-990.

Anene, B. M., Onah, D. N. and Nawa, Y. (2001). Drug resistance in pathogenic African trypanosomes: what hopes for the future? *Veterinary Parasitology,* 96**,** 83–100.

Ansede, J. H., Voyksner, R. D., Ismail, M. A., Boykin, D. W. and Tidwell, R. R., Et Al. (2005). In vitro metabolism of an orally active O-methyl amidoxime prodrug for the treatment of CNS trypanosomiasis. *Xenobiotica,* 35**,** 211-226.

Aradaib, I. E. and Majid, A. A. (2006). A simple and rapid method for detection of Trypanosoma evansi in the dromedary camel using a nested polymerase chain reaction. *Kinetoplastid Biology and Disease,* 5**,** 2.

Asoganyi, T., Bedifeh, B. A., Ade, S. S. and Ngu, J. L. (1994). An evaluation of the reactivity of the Card agglutination test for trypanosomiasis (CATT) reagent in the Fontem focus, Cameroon. *African Journal of Medicine and Medical Sciences,* 23**,** 39-46.

Ayyar, B. V., Arora, S., Murphy, C. and O'Kennedy, R. (2012). Affinity chromatography as a tool for antibody purification. *Methods,* 56**,** 116-129.

Bacchi, C. J. (1993). Resistance to clinical drugs in African trypanosomes. *Parasitology Today,* 9**,** 190-193.

Balamurugan, V., Reddy, G. R. and Suryanarayana, V. V. S. (2007). *Pichia pastoris:* A notable heterologous expression system for the production of foreign proteins-Vaccines. *Indian Journal of Biochemistry,* 6**,** 175-186.

Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology,* 10**,** 411-421.

Baral, T. N. (2010). Immunobiology of African Trypanosomes: Need of Alternative Interventions. *Journal of Biomedicine and Biotechnology,* 2010**,** 1-24.

Barrett, M. P., Burchmore, R. J., Stich, A., Lazzari, J. O., Frasch, A. C., Cazzulo, J. J. and Krishna, S. (2003). The Trypanosomiases. *Lancet,* 362**,** 1469-1480.

Barry, J. D. and Mcculloch, R. (2001). Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. *Advances in Parasitology,* 49**,** 1-70.

Berriman, M., Ghedin, E., Hertz-Fowler, C., Blandin, G., Renauld, H., Bartholomeu, D. C., Lennard, N. J., Caler, E., Hamlin, N. E., Haas, B., Bohme, U., Hannick, L., Aslett, M. A., Shallom, J., Marcello, L., Hou, L., Wickstead, B., Alsmark, U. C., Arrowsmith, C., Atkin, R. J., Barron, A. J., Bringaud, F., Brooks, K., Carrington, M., Cherevach, I., Chillingworth, T. J., Churcher, C., Clark, L. N., Corton, C. H., Cronin, A., Davies, R. M., Doggett, J., Djikeng, A., Feldblyum, T., Field, M. C., Fraser, A., Goodhead, I., Hance, Z., Harper, D., Harris, B. R., Hauser, H., Hostetler, J., Ivens, A., Jagels, K., Johnson, D., Johnson, J., Jones, K., Kerhornou, A. X., Koo, H., Larke, N. et al. (2005). The genome of the African trypanosome *Trypanosoma brucei*. *Science* 309, 416-422.

Black, S. J., Sicard, E. L., Murphy, N. and Nolan, D. (2001). Innate and acquired control of trypanosome parasitaemia in Cape buffalo. *International Journal of Parasitology,* 31**,** 562-565.

Blum, H., Beier, H., and Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis,* 8**,** 93-99.

Bode, C., Zhao, G., Steinhagen, F., Kinjo, T. and Klinman, D. M. (2011). CpG DNA as a vaccine adjuvant. *Expert Review of Vaccines,* 10**,** 499-511.

Boeger, H., Bushnell, D. A., Davis, R., Griesenbeck, J., Lorch, Y., Strattan, J. S., Westover, K. D. and Kornberg, R. D. (2005). Structural basis of eukaryotic gene transcription. *FEBS Letters,* 579**,** 899-903.

Boulangé, A., Katende, J. and Authié, E. (2002). *Trypanosoma congolense*: Expression of a heat shock protein 70 and initial evaluation as a diagnostic antigen for bovine trypanosomiasis. *Experimental Parasitology,* 100**,** 6-11.

Brems, S., Guilbride, D. L., Gundlesdodjir-Planck, D., Van-Duc Luu, C. B., Schanne, M., Hoheisel, J. and Clayton, C. (2005). The transcriptomes of *Trypanosoma brucei* Lister 427 and TREU927 bloodstream and procyclic trypomastigotes. *Molecular & Biochemical Parasitology,* 139**,** 163-172.

Brown, K. (2008). From Ubombo to Mkhuzi: disease, colonial science, and the control of Nagana (livestock trypanosomosis) in Zululand, South Africa, C. 1894-1953. *Journal of the History of Medicine and Allied Sciences,* 63**,** 285-322.

Brun, R., Hecker, H. and Lun, Z. R. (1998). *Trypanosoma evansi* and *T. equiperdum*: distribution, biology, treatment and phylogenetic relationship. *Veterinary Parasitolgy,* 79**,** 95-107.

Brun, R., Blum, J., Chappuis, F. and Burri, C. (2010). Human African trypanosomiasis. *Lancet,* 375**,** 148-159.

Büscher, P. and Lejon, V. (2004). Diagnosis of human African trypanosomiasis. *In*: Maudlin, I., Holmes, P. and Miles, M. A. (eds.) The Trypanosomiases. Oxfordshire, UK: *CABI Publishing,* pp 203-218.

Bulow, R., Overath, P. and Davoust, J. (1988). Rapid lateral diffusion of the variant surface glycoprotein in the coat of *Trypanosoma brucei*. *Biochemistry,* 27**,** 2384–2388.

Caffrey, C. R., Hansell, E., Lucas, K. D., Brinen, L. S., Alvarez Hernandez, A., Cheng, J., Gwaltney, S. L., 2nd, Roush, W. R., Stierhof, Y. D., Bogyo, M., Steverding, D. and McKerrow, J. H. (2001). Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. *Molecular and Biochemical Parasitology,* 118**,** 61-73.

Carlander, D., Stålberg, J. and Larsson, A. (1999). Chicken antibodies: a clinical chemistry perspective. *Upsala Journal of Medical Sciences,* 104**,** 179-189.

Carrington, M., Miller, N., Blum, M., Roditi, I., Wiley, D. and Turner, M. (1991). Variant specific glycoprotein of *Trypanosoma brucei* consists of two domains each having an independently conserved pattern of cysteine residues. *Journal of Molecular Biology,* 221**,** 823–835.

Castro, A., De Mecca, M. M. and Bartel, L. C. (2006). Toxic side effects of drugs used to treat Chagas' disease (American trypanosomiasis). *Human and Experimental Toxicology,* 25**,** 471-479.

Chappuis, F., Loutan, L., Simarro, P., Lejon, V. and Büscher, P. (2005). Options for Field Diagnosis of Human African Trypanosomiasis. *Clinical Microbiology Reviews,* 18**,** 133-146.

Chretien, J. P., Bonnie, L. and Smoak, B. L. (2005). African Trypanosomiasis: Changing Epidemiology and Consequences. *Current Infectious Disease Reports 2005,* 7**,** 54–60.

Chung, W., Carrington, M. and Field, M. C. (2004). Cytoplasmic Targeting Signals in Trans-membrane Invariant Surface Glycoproteins of Trypanosomes. *The Journal of Biological Chemistry,* 279**,** 54887–54895.

Chung, W. L., Leung, K. F., Carrington, M. and Field, M. C. (2008). Ubiquitylation is required for degradation of *trans*-membrane surface proteins in trypanosomes. *Traffic,* 9**,** 1681-1697.

Claes, F., Büscher, P., Touratier, L. and Goddeeris, B. M. (2005). *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends in Parasitology,* 21**,** 316-321.

Coustou, V., Guegan, F., Plazolles, N. and Baltz, T. (2010). Complete in vitro life cycle of *Trypanosoma congolense*: development of genetic tools. *PLoS Neglected Tropical Diseases,* 4**,** e618.

D'Leteren, G. D. M., Authié, E., Wissocq, N. and Murray, M. (1998). Trypanotolerance, an option for sustainable livestock production in areas at risk from trypanosomosis. *Revue Scientifique et Technique de l'OIE,* 17 pp. 154-175.

Daniels, J. P., Gull, K. and Wickstead, B. (2010). Cell Biology of the Trypanosome Genome. *Microbiology and Molecular Biology Reviews,* 74**,** 552–569.

De Matos Guedes, H. L., Duarte Carneiro, M. P., De Oliveira Gomes, D. C., Rossi-Bergmann, B. and Giovanni De-Simone, S. (2007). Oligopeptidase B from Leishmania amazonensis: molecular cloning, gene expression analysis and molecular model. *Parasitology Research,* 101**,** 865-875.

De Souza, W. (1984). Cell biology of *Trypanosoma cruzi*. *International Review of Cytology,* 86**,** 197-283.

De Souza, W. (2002). From the cell biology to the development of new chemotherapeutic approaches against trypanosomatids: dreams and reality. *Kinetoplastid Biology and Disease*.

Deborggraeve, S., Claes, F., Laurent, T., Mertens, P., Leclipteux, T., Dujardin, J. C., Herdewijn, P. and Büscher, P. (2006). Molecular dipstick test for diagnosis of sleeping sickness. *Journal of Clinical Microbiology,* 44**,** 2884-2889.

Donelson, J. E., Hill, K. L. and El-Sayed, N. M. (1998). Multiple mechanisms of immune evasion by African trypanosomes. *Molecular and Biochemical Parasitology,* 91**,** 51-66.

Du, P., Loulakis, P., Luo, C., Mistry, A., Simons, S. P., Lemotte, P. K., Rajamohan, F., Rafidi, K., Coleman, K. G., Geoghegan, K. F. and Xie, Z. (2005). Phosphorylation of serine residues in histidine-tag sequences attached to recombinant protein kinases: a cause of heterogeneity in mass and complications in function. *Protein Expression and Purification,* 44**,** 121-129.

Dukes, P., Gashumba, J. K., Hudson, K. M., Kaukas, A., Bromidge, T. J. and Asonganyi, T. (1992). Absence of LiTat 1.3 (CATT antigen) gene in *Trypanosoma brucei gambiense* stocks from Cameroon. *Acta Tropica,* 51**,** 123-134.

Duszenko, M., Figarella, K., Macleod, E. T. and Welburn, S. C. (2006). Death of a trypanosome: a selfish altruism. *Trends in Parasitology,* 22**,** 536-542.

El-Sayed, N. M., Hegde, P., Quackenbush, J., Melville, S. E. and Donelson, J. E. (2000). The African trypanosome genome. *International Journal for Parasitology,* 30**,** 329-345.

Engstler, M., Pfohl, T., Herminghaus, S., Boshart, M., Wiegertjes, G., Heddergott, N. and Overath, P. (2007). Hydrodynamic flow-mediated protein sorting on the cell surface of trypanosomes. *Cell,* 131**,** 505-515.

Eyford, B. A., Sakurai, T., Smith, D., Loveless, B., Hertz-Fowler, C., Donelson, J. E., Inoue, N. and Pearson, T. W. (2011). Differential protein expression throughout the life cycle of *Trypanosoma congolense*, a major parasite of cattle in Africa. *Molecular and Biochemical Parasitology,* 177**,** 116-125.

Fakruddin, M., Mazumdar, R. M., Bin Mannan, K. S., Chowdhury, A. and Nur Hossain, M. (2013). Critical Factors Affecting the Success of Cloning, Expression, and Mass Production of Enzymes by Recombinant *E. coli*. *ISRN Biotechnology,* 2013**,** 7 pages.

FAO (1994). Opening address of Y. Cheneau. In A systematic approach to tsetse and trypanosomiasis control, Proceedings of the FAO Panels of Experts, Rome, 1-3 December 1993. *FAO Animal and Production Health Paper*, No. 121, p. 1-3. Rome.

Fèvre, E. M., Coleman, P. G., Odiit, M., Magona, J. W., Welburn, S. C. and Woolhouse, M. E. J. (2001). The origins of a new *Trypanosoma brucei rhodesiense* sleeping sickness outbreak in eastern Uganda. *Lancet,* 358**,** 625–628.

Fèvre, E. M., Coleman, P. G., Welburn, S. C. and Maudlin, I. (2004). Reanalyzing the 1900-1920 sleeping sickness epidemic in Uganda. *Emerging Infectious Diseases*, 10**,** 567-573.

Fèvre, E. M., Wissmann, B. V., Welburn, S. C. and Lutumba, P. (2008). The Burden of Human African Trypanosomiasis. *PLoS Neglected Tropical Diseases,* 2:e333.

Field, M. C. and Carrington, M. (2009). The trypanosome flagellar pocket. *Nature Reviews Microbiology,* 7**,** 775-786.

Field, M. C., Sergeenko, T., Wang, Y.-N., Böhm , S. and Carrington, M. (2010). Chaperone Requirements for Biosynthesis of the Trypanosome Variant Surface Glycoprotein. *PLoS One,* 5**,** e8468.

FIND (2012a). Practical fluorescence microscopy for detection of trypanosomes. *http ://www.finddiagnostics.org/programs/hat-ond/hat/parasite_detection/fluorescence_ microscopy/accessed on 020313*.

FIND (2012b). The first rapid test to screen for sleeping sickness is launched. *http ://www.finddiagnostics.org/media/press/121206.html accessed on 020313*.

FIND (2012c). Accelerating the adoption of new tools in the battle against sleeping sickness. *http ://www.finddiagnostics.org/media/news/121214.html accessed on 020313*.

Freymann, D., Down, J., Carrington, M., Roditi, I., Turner, M. and Wiley, D. (1990). 2.9 A resolution structure of the N-terminal domain of a variant surface glycoprotein from *Trypanosoma brucei*. *Journal of Molecular Biology,* 216**,** 141-160.

Gehrig, S. and Efferth, T. (2008). Development of drug resistance in *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. Treatment of human African trypanosomiasis with natural products (Review). *International Journal of Molecular Medicine,* 22**,** 411-419.

Gibson, W.C. (2005). The *SRA* gene: the key to understanding the nature of *Trypanosoma brucei rhodesiense*. *Parasitology*, 131, 143-150.

Giroud, C., Ottones, F., Coustou, V., Dacheux, D., Biteau, N., Miezan, B., Van Reet, N., Carrington, M., Doua, F. and Baltz, T. (2009). Murine Models for *Trypanosoma brucei gambiense* disease progression--from silent to chronic infections and early brain tropism. *PLoS Neglected Tropical Diseases,* 3**,** e509.

Goldring, J. P. D. and Coetzer, T. H. T. (2003). Isolation of chicken immunoglobulins (IgY) from egg yolk. *Biochemistry and Molecular Biology Education,* 31**,** 185-187.

Golovanov, A. P., Hautbergue, G. M., Wilson, S. A. and Lian, L. Y. (2004). A simple method for improving protein solubility and long-term stability. *Journal of the American Chemical Society,* 126**,** 8933-8939.

Graham, S. V. and Barry, J. D. (1995). Transcriptional Regulation of Metacyclic Variant Surface Glycoprotein Gene Expression during the Life Cycle of *Trypanosoma brucei*. *Molecular and Cellular Biology,* 15**,** 5945–5956.

Graslund, S., Nordlund, P., Weigelt, J., Hallberg, B. M., Bray, J., Gileadi, O., Knapp, S., Oppermann, U., Arrowsmith, C., Hui, R., Ming, J., Dhe-Paganon, S., Park, H. W., Savchenko, A., Yee, A., Edwards, A., Vincentelli, R., Cambillau, C., Kim, R., Kim, S. H., Rao, Z., Shi, Y., Terwilliger, T. C., Kim, C. Y., Hung, L. W., Waldo, G. S., Peleg, Y., Albeck, S., Unger, T., Dym, O., Prilusky, J., Sussman, J. L., Stevens, R. C., Lesley, S. A., Wilson, I. A., Joachimiak, A., Collart, F., Dementieva, I., Donnelly, M. I., Eschenfeldt, W. H., Kim, Y., Stols, L., Wu, R., Zhou, M., Burley, S. K., Emtage, J. S., Sauder, J. M., Thompson, D., Bain, K., Luz, J., Gheyi, T., Zhang, F., Atwell, S., Almo, S. C., Bonanno, J. B., Fiser, A., Swaminathan, S., Studier, F. W., Chance, M. R., Sali, A., Acton, T. B., Xiao, R., Zhao, L., Ma, L. C., Hunt, J. F., Tong, L., Cunningham, K., Inouye, M., Anderson, S., Janjua, H., Shastry, R., Ho, C. K., Wang, D., Wang, H., Jiang, M., Montelione, G. T., Stuart, D. I., Owens, R. J., Daenke, S., Schutz, A., Heinemann, U., Yokoyama, S., Bussow, K. and Gunsalus, K. C. (2008). Protein production and purification. *Nature Methods,* 5**,** 135-146.

Guirnalda, P., Murphy, N. B., Nolan, D. and Black, S. J. (2007). Anti-*Trypanosoma brucei* activity in Cape buffalo serum during the cryptic phase of parasitemia is mediated by antibodies. *International Journal for Parasitology,* 37**,** 1391-1399.

Haag, J., O'Huigin, C. and Overath, P. (1998). The molecular phylogeny of trypanosomes: evidence for an early divergence of the Salivaria. *Molecular and Biochemical Parasitology,* 91**,** 37-49.

Hädge, D. and Ambrosius, H. (1984). Evolution of low molecular weight immunoglobulins--IV. IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of mammalian IgA. *Molecular Immunology,* 21**,** 699-707.

Hall, B., Allen, C. L., Goulding, D. and Field, M. C. (2004). Both of the Rab5 subfamily small GTPases of *Trypanosoma brucei* are essential and required for endocytosis. *Molecular and Biochemical Parasitology,* 138**,** 67-77.

Hall, J. P., Wang, H. and Barry, J. D. (2013). Mosaic VSGs and the Scale of *Trypanosoma brucei* Antigenic Variation. *PLoS Pathogens,* 9**,** e1003502.

Handman, E., Noormohammadi, A. H., Curtis, J. M., Baldwin, T. and Sjolander, A. (2000). Therapy of murine cutaneous leishmaniasis by DNA vaccination. *Vaccine,* 18**,** 3011-3017.

Herwaldt, B. L. (2001). Laboratory-acquired parasitic infections from accidental exposures. *Clinical Microbiology Reviews,* 14**,** 659-688.

Heukeshoven, J. and Dernick, R. (1985). Characterization of a solvent system for separation of water-insoluble poliovirus proteins by reversed-phase high-performance liquid chromatography. *Journal of Chromatography,* 326**,** 91-101.

Hirumi, H. and Hirumi, K. (1989). Continuous cultivation of *Trypanosoma brucei* bloodstream forms in a medium containing a low concentration of serum protein without feeder cell layers. *Journal of Parasitology,* 75**,** 985-999.

Hoare, C. A. (1972). The Trypanosomes of Mammals. *Oxford: Blackwell Scientific Publications***,** 1–749.

Hsieh, P., Rosner, M. R. and Robbins, P. W. (1983). Selective Cleavageb y Endo-/I-N-acetylglucosaminidaseH at Individual Glycosylation Sites of Sindbis Virion Envelope Glycoproteins. *The Journal of Biological Chemistry,* 258**,** 2555-2561.

Huson, L. E., Authié, E., Boulangé, A. F., Goldring, J. P. and Coetzer, T. H. (2009). Modulation of the immunogenicity of the *Trypanosoma congolense* cysteine protease, congopain, through complexation with alpha(2)-macroglobulin. *Veterinary Research,* 40**,** 52.

Hutchinson, O. C., Webb, H., Picozzi, K., Welburn, S. and Carrington, M. (2004). Candidate protein selection for diagnostic markers of African trypanosomiasis. *Trends in Parasitology,* 20**,** 519-523.

Jackson, D. G., Windle, H. J. and Voorheis, H. P. (1993). The identification, purification, and characterization of two invariant surface glycoproteins located beneath the surface coat barrier of bloodstream forms of *Trypanosoma brucei*. *The Journal of Biological Chemistry,* 268**,** 8085-8095.

Jannin, J. and Cattand, P. (2004). Treatment and control of human African trypanosomiasis. *Current opinion in Infectious diseases,* 17**,** 565-571.

Kelley, R. J., Alexander, D. L., Cowan, C., Balber, A. E. and Bangs, J. D. (1999). Molecular cloning of p67, a lysosomal membrane glycoprotein from *Trypanosoma brucei*. *Molecular and Biochemical Parasitology,* 98**,** 17-28.

Kieft, R., Capewell, P., T, Urner, C. M., Veitch, N. J., Macleod, A. and Hajduk, S. (2010). Mechanism of *Trypanosoma brucei gambiense* (group 1) resistance to human trypanosome lytic factor. *Proceedings of the National Academy of Sciences of the United States of America,* 107**,** 16137-16141.

Koumandou, V. L., Klute, M. J., Herman, E. K., Nunez-Miguel, R., Dacks, J. B. and Field, M. C. (2011). Evolutionary reconstruction of the retromer complex and its function in *Trypanosoma brucei*. *Journal Cell Science,* 124**,** 1496-1509.

Koumandou, V. L., Boehm, C., Horder, K. A. and Field, M. C. (2013). Evidence for recycling of invariant surface *trans*-membrane domain proteins in African trypanosomes. *Eukaryotic Cell,* 12**,** 330–342.

Kristensson, K., Nygard, M., Bertini, G. and Bentivoglio, M. (2010). African trypanosome infections of the nervous system: parasite entry and effects on sleep and synaptic functions. *Progress in Neurobiology,* 91**,** 152-171.

Kristjanson, P., Swallow, B., Rowlands, G., Kruska, R. and De Leeuw, P. (1999). Measuring the costs of African animal trypanosomosis, the potential benefits of control and returns to research. *Agricultural Systems,* 59**,** 79-98.

Kuboki, N., Inoue, N., Sakurai, T., Di Cello, F. and Grab, D. J., Et Al. (2003). Loopmediated isothermal amplification for detection of African trypanosomes. *Journal of Clinical Microbiology,* 41**,** 5517–5524.

Kuepfer, I., Hhary, E. P., Allan, M., Edielu, A., Burri, C. and Blum, J. A. (2011). Clinical presentation of *T.b. rhodesiense* sleeping sickness in second stage patients from Tanzania and Uganda. *PLoS Neglected Tropical Diseases,* 5**,** e968.

Kuzoe, F. A. (1993). Current situation of African trypanosomiasis. *Acta Tropica,* 54**,** 153-162.

Laddy, D. J. and Weiner, D. B. (2006). From plasmids to protection: a review of DNA vaccines against infectious diseases. *Internationl Reviews of Immunology,* 25**,** 99-123.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227**,** 680-685.

Lanca, A. S., De Sousa, K. P., Atouguia, J., Prazeres, D. M., Monteiro, G. A. and Silva, M. S. (2011). *Trypanosoma brucei*: immunisation with plasmid DNA encoding invariant surface glycoprotein gene is able to induce partial protection in experimental African trypanosomiasis. *Experimental Parasitology,* 127**,** 18-24.

Larsson, A. and Mellstedt, H. (1992). Chicken antibodies: a tool to avoid interference by human anti-mouse antibodies in ELISA after in vivo treatment with murine monoclonal antibodies. *Hybridoma,* 11**,** 33-39.

Leak, S. G. A. (1999). Tsetse Biology and Ecology: Their Role in the Epidemiology and Control of Trypanosomosis. Wallingford and New York: *CABI Publishing*. 568 pp.

Lee, M. G., Bihain, B. E., Russell, D. G., Deckelbaum, R. J. and Van Der Ploeg, L. H. (1990). Characterization of a cDNA encoding a cysteine-rich cell surface protein located in the flagellar pocket of the protozoan *Trypanosoma brucei*. *Molecular and Cellular Biology,* 10**,** 4506-4517.

Lee, S. Y. (1996). High cell-density culture of *Escherichia coli*. *Trends in Biotechnology,* 14**,** 98-105.

Lejon, V. and Büscher, P. (2005). Review Article: cerebrospinal fluid in human African trypanosomiasis: a key to diagnosis, therapeutic decision and post-treatment follow-up. *Tropical Medicine and International Health,* 10**,** 395-403.

Leung, K. F., Dacks, J. B. and Field, M. C. (2008). Evolution of the multivesicular body ESCRT machinery; retention across the eukaryotic lineage. *Traffic,* 9**,** 1698-1716.

Leung, K. F., Riley, F. S., Carrington, M. and Field, M. C. (2011). Ubiquitylation and developmental regulation of invariant surface protein expression in trypanosomes. *Eukaryotic Cell,* 10**,** 916-931.

Lipman, N. S., Jackson, L. R., Trudel, L. J., and Weis-Garcia, F. (2005). Monoclonal versus polyclonal antibodies: distinguishing characteristics, applications, and information resources, *Institute for Laboratory Animal Research Journal,* 46, 258-268.

Lipsitch, M. and O'Hagan, J. J. (2007). Patterns of antigenic diversity and the mechanisms that maintain them. *Journal of the Royal Society Interface,* 4**,** 787-802

Losos, G. J. and Ikede, B. O. (1972). Review of Pathology of diseases in domestic and laboratory animals caused by *Trypanosoma congolense*, *T. vivax*, *T. brucei*, *T. rhodesiense* and *T. gambiense*. *Veterinary Pathology,* 9**,** 1-79.

Lythgoe, K. A., Morrison, L. J., Read, A. F. and Barry, J. D. (2007). Parasite-intrinsic factors can explain ordered progression of trypanosome antigenic variation. *Proceedings of the National Academy of Sciences U S A,* 104**,** 8095–8100.

Macauley-Patrick, S., Fazenda, M. L., Mcneil, B. and Harvey, L. M. (2005). Heterologous protein production using the *Pichia pastoris* expression system. *Yeast,* 22**,** 249-270.

Maclean, L., Reiber, H., Kennedy, P. G. and Sternberg, J. M. (2012). Stage progression and neurological symptoms in *Trypanosoma brucei rhodesiense* sleeping sickness: role of the CNS inflammatory response. *PLoS Neglected Tropical Diseases,* 6**,** e1857.

Magnus, E., Vervoort, T. and Van Meirvenne, N. (1978). A card-agglutination test with stained trypanosomes (C.A.T.T.) for the serological diagnosis of *T. b. gambiense* trypanosomiasis. *Annals de la Societe Belge de Medecine Tropicale,* 58**,** 169-176.

Malvy, D. and Chappuis, F. (2011). Sleeping sickness. *Clinical microbiology and infection,* 17**,** 986-995.

Manful, T., Mulindwa, J., Frank, F. M., Clayton, C. E. and Matovu, E. (2010). A search for *Trypanosoma brucei rhodesiense* diagnostic antigens by proteomic screening and targeted cloning. *PLoS One,* 5**,** e9630.

Marcello, L. and Barry, J. D. (2007). Analysis of the VSG gene silent archive in *Trypanosoma brucei* reveals that mosaic gene expression is prominent in antigenic variation and is favored by archive substructure. *Genome research,* 17**,** 1344-1352.

Masake, R. A., Majiwa, P. A., Moloo, S. K., Makau, J. M., Njuguna, J. T., Maina, M., Kabata, J., Ole-Moiyoi, O. K. and Nantulya, V. M. (1997). Sensitive and specific detection of *Trypanosoma vivax* using the polymerase chain reaction. *Experimental Parasitology,* 85**,** 193-205.

Matovu, E., Kazibwe, A. J., Mugasa, C. M., Ndungu, J. M. and Njiru, Z. K. (2012). Towards Point-of-Care Diagnostic and Staging Tools for Human African Trypanosomiaisis. *Journal of Tropical Medicine,* 2012.

Matthews, K., And Gull, K. (1997). Commitment to differentiation and cell cycle re-entry are coincident but separable events in the transformation of African trypanosomes from their bloodstream to their insect form. *Journal of Cell Science,* 110**,** 2609-2618.

Matthews, K. R., Ellis, J. R. and Paterou, A. (2004). Molecular regulation of the life cycle of African trypanosomes. *Trends in Parasitology,* 20**,** 40-47.

Mattioli, R. C., Feldmann, G., Hendrickx, W., Wint, J. and Jannin, J., Et Al. (2004). Tsetse and trypanosomiasis intervention policies supporting sustainable animal-agricultural development *Journal of Food Agriculture and Environment,* 2**,** 310-314.

Maudlin, I., Holmes, P. H. and Miles, M. A. (eds.) (2004). The Trypanosomiases, Oxfordshire, UK: *CABI Publishing*, pp 1.

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

Medina-Acosta, E. C., G. A. (1993). Rapid isolation of DNA from trypanosomatid protozoa using a simple 'mini-prep' procedure. *Molecular Biochemistry and Parasitology,* 59**,** 327-329.

Mehlert, A., Zitzmann, N., Richardson, J. M., Treumann, A. and Ferguson, M. A. (1998). The glycosylation of the variant surface glycoproteins and procyclic acidic repetitive proteins of *Trypanosoma brucei*. *Molecular and Biochemical Parasitology,* 91**,** 145-152.

Mehlert, A., Bond, C. S. and Ferguson, M. A. (2002). The glycoforms of a *Trypanosoma brucei* variant surface glycoprotein and molecular modeling of a glycosylated surface coat. *Glycobiology,* 12**,** 607-612.

Mehlert, A., Wormald, M. R. and Ferguson, M. A. (2012a). Modeling of the N-glycosylated transferrin receptor suggests how transferrin binding can occur within the surface coat of Trypanosoma brucei. *PLoS Pathogens,* 8**,** e1002618.

Mehlert, A., Wormald, M. R. and Ferguson, M. A. (2012b). Modeling of the N-Glycosylated Transferrin Receptor Suggests How Transferrin Binding Can Occur within the Surface Coat of *Trypanosoma brucei*. *PLoS Pathogens,* 8.

Mehlhorn, H. (2008). Surface Coat. *In:* Mehlhorn, H. (ed.) Encyclopedia of Parasitology. *Springer Berlin Heidelberg*.

Michels, P. A. (1988). Compartmentation of glycolysis in trypanosomes: a potential target for new trypanocidal drugs. *Biology of the Cell,* 64**,** 157-164.

Mine, Y. and Kovacs-Nolan, J. (2002). Chicken egg yolk antibodies as therapeutics in enteric infectious disease: a review. *Journal of Medicinal Food,* 5**,** 159-169.

Mitashi, P., Hasker, E., Lejon, V., Kande, V., Muyembe, J. J., Lutumba, P. and Boelaert, M. (2012). Human african trypanosomiasis diagnosis in first-line health services of endemic countries, a systematic review. *PLoS Neglected Tropical Diseases,* 6**,** e1919.

Motta, F. N., Bastos, I. M., Faudry, E., Ebel, C., Lima, M. M., Neves, D., Ragno, M., Barbosa, J. A., De Freitas, S. M. and Santana, J. M. (2012). The *Trypanosoma cruzi* virulence factor oligopeptidase B (OPBTc) assembles into an active and stable dimer. *PLoS One,* 7**,** e30431.

Mugasa, C. M., Adams, E. R., Boer, K. R., Dyserinck, H. C., Büscher, P., Schallig, H. D. and Leeflang, M. M. (2012). Diagnostic accuracy of molecular amplification tests for human African trypanosomiasis--systematic review. *PLoS Neglected Tropical Diseases,* 6**,** e1438.

Mwandiringana, E., Gori, E., Nyengerai, T. and Chidzwondo, F. (2012). Polymerase chain reaction (PCR) detection of mixed trypanosome infection and blood meal origin in field-captured tsetse flies from Zambia. *African Journal of Biotechnology,* 11**,** 14490-14497.

Naessens, J. (2006). Bovine trypanotolerance: A natural ability to prevent severe anaemia and haemophagocytic syndrome? *International Journal for Parasitology,* 36**,** 521-528.

Nantulya, V. M. (1990). Trypanosomiasis in domestic animals:the problems of diagnosis. *Revue Scientifique et Technique de l`office International des Epizooties OIE,* 9**,** 357-367.

Natesan, S. K. A., Peacock, L., Matthews, K., Gibson, W. and Field, M. C. (2007). Activation of Endocytosis as an Adaptation to the Mammalian Host by Trypanosomes. *Eukaryotic Cell,* 6**,** 2029–2037.

Natesan, S. K. A., Black, A., Matthews, K. R., Mottram, J. C. and Field, M. C. (2011). *Trypanosoma brucei brucei*: Endocytic recycling is important for mouse infectivity. *Experimental Parasitology,* 127**,** 777-783.

Ndoutamia, G., Moloo, S. K., Murphy, N. B. and Peregrine, A. S. (1993). Derivation and characterisation of a quinapyramine resistant clone of *T. congolense. Antimicrobial Agents and chemotherapeutics,* 37**,** 1163-1166.

Ndungu, J. M., Bieler, S. and Roscigno, G. (2010). ‘‘Piggy-Backing’’ on Diagnostic Platforms Brings Hope to Neglected Diseases: The Case of Sleeping Sickness. *PLoS Neglected Tropical Diseases,* 4**,** e715.

Nilsson, J., Stahl, S., Lundeberg, J., Uhlen, M. and Nygren, P. A. (1997). Affinity fusion strategies for detection, purification, and immobilization of recombinant proteins. *Protein Expression and Purification,* 11**,** 1-16.

Njiokou, F., Simo, G., Nkinin, S. W., Laveissiere, C. and Herder, S. (2004). Infection rate of *Trypanosoma brucei* s.l., *T. vivax, T. congolense* "forest type", and *T. simiae* in small wild vertebrates in south Cameroon. *Acta Tropica,* 92**,** 139-146.

Njiru, Z. K., Mikosza, A. S., Matovu, E., Enyaru, J. C. and Ouma, J. O., Et Al. (2008). African trypanosomiasis: sensitive and rapid detection of the sub-genus Trypanozoon by loop-mediated isothermal amplification (LAMP) of parasite DNA. *International Journal for Parasitology,* 38**,** 589–599.

Noireau, F., Lemesre, J. L., Nzoukoudi, M. Y., Louembet, M. T., Gouteux, J. P. and Frezil, J. L. (1988). Serodiagnosis of sleeping sickness in the Republic of the Congo: comparison of indirect immunofluorescent antibody test and card agglutination test. . *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 82**,** 237-240.

Nolan, D. P., Jackson , D. G., Windle, H. J., Pays, A., Geuskens, M., Michel, A., Voorheis, H. P. and Pays, E. (1997). Characterization of a Novel, Stage-specific Invariant Surface Protein in *Trypanosoma brucei* Containing an Internal Serine-rich Repetitive Motif. *The Journal of biological chemistry,* 272**,** 29212-29221.

Nolan, D. P., Jackson, D. G., Biggs, M. J., Brabazon, E. D., Pays, A., Van Laethem, F., Paturiaux-Hanocq, F., Elliott, J. F., Voorheis, H. P. and Pays, E. (2000). Characterization of a novel alanine-rich protein located in surface microdomains in *Trypanosoma brucei*. *The Journal of Biological Chemistry,* 275**,** 4072-4080.

Odiit, M., Kansiime, F. and Enyaru, J. C. K. (1997). Duration of symptoms and case fatality of sleeping sickness caused by *Trypanosoma brucei rhodesiense* in Tororo, Uganda. *East African Medical Journal,* 74**,** 792–795.

OIE. (2010). *Trypanosoma evansi* (surra). In: Manual of diagnostic tests and vaccines for terrestrial animals. World organization for animal health, Paris,. *cited at: http://www.oie.int* [Online].

OIE. (2012). Trypanosoma evansi infections (including surra) Chapter 2.1.17. In: Manual of Diagnostic Tests and Vaccine for Terrestrial Animals. *http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.01.17_TRYPANOSURRA.pdf* [Online].

Overath, P., Chaudhri, M., Steverding, D. and Ziegelbauer, K. (1994a). Invariant surface proteins in bloodstream forms of *Trypanosoma brucei*. *Parasitology Today,* 10**,** 53-58.

Overath, P., Steverding, D., Chaudhri, M., Stierhof, Y. D. and Ziegelbauer, K. (1994b). Structure and function of GPI-anchored surface proteins of *Trypanosoma brucei*. *Brazilian Journal Of Medical and Biological Research,* 27**,** 343-347.

Paine, M. F., Wang, M. Z., Generaux, C. N., Boykin, D. W. and Wilson, W. D., Et Al. (2010). Diamidines for human African trypanosomiasis. *Current Opinion in Investigational Drugs,* 11**,** 876-883.

Paquet, C., Ancelle, T., Gastellu-Etchegorry, M., Castilla, J. and Harndt, I. (1992). Persistence of antibodies to *Trypanosoma brucei gambiense* after treatment of human trypanosomiasis in Uganda *Lancet,* 340**,** 250.

Pauly, D., Chacana, P. A., Calzado, E. G., Brembs, B. and Schade, R. (2011). IgY Technology: Extraction of Chicken Antibodies from Egg Yolk by Polyethylene Glycol (PEG) Precipitation. *Journal of Visualized Experiments,* 51.

Pays, E. (1991). Genetics of antigenic variation in African trypanosomes. *Research in Microbiology,* 142**,** 731-735.

Pays, E. and Nolan, D. P. (1998). Expression and function of surface proteins in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology,* 91**,** 3-36.

Penchenier, L., Gre´Baut, P., Njokou, F., Eboo Eyenga, V. and Büscher, P. (2003). Evaluation of LATEX/*T. b. gambiense* for mass screening of *Trypanosoma brucei gambiense* sleeping sickness in Central Africa. *Acta Tropica,* 85**,** 31–37.

Pike, R. N. and Dennison, C. (1989). Protein fractionation by three phase partitioning (TPP) in aqueous/t-butanol mixtures. *Biotechnology and Bioengineering,* 33**,** 221-228.

Pillay, D., Boulangé, A. F. and Coetzer, T. H. T. (2010). Expression, purification and characterisation of two variant cysteine peptidases from *Trypanosoma congolense* with active site substitutions. *Protein Expression and Purification,* 74**,** 264-271.

Polson, A., Potgieter, G. M., Largier, J. F., Mears, G. E. F. and Joubert, F. J. (1964). The fractionation of protein mixtures by linear polymers of high molecular weight. *Biochimica et Biophysica Acta,* 82**,** 463-475.

Popa, O. P., Murariu, D. and Popa, L. O. (2007). Comparison of four DNA extraction methods from invasive freshwater bivalve species (Mollusca: Bivalvia) in Romanian fauna. *Travaux du Muséum National d'Histoire Naturelle Grigore Antipa,* 6**,** 527-536.

Priotto, G., Fogg, C., Balasegaram, M., Erphas, O., Louga, A., Checchi, F., Ghabri, S. and Piola, P. (2006). Three drug combinations for late-stage *Trypanosoma brucei gambiense* sleeping sickness: a randomized clinical trial in Uganda. *PLoS Clinical Trials,* 1**,** e39.

Priotto, G., Pinoges, L., Fursa, I. B., Burke, B., Nicolay, N., Grillet, G., Hewison, C. and Balasegaram, M. (2008). Safety and effectiveness of first line eflornithine for Trypanosoma brucei gambiense sleeping sickness in Sudan: cohort study. *British Medical Journal,* 336**,** 705-708.

Priotto, G., Kasparian, S. and Mutombo, W. (2009). Nifurtimox-eflornithine combination therapy for second-stage African *Trypanosoma brucei gambiense* trypanosomiasis: a multicentre, randomised, phase III, non-inferiority trial. *Lancet,* 374**,** 56-64.

Radwanska, M., Magez, S., Dumont, N., Pays, A., Nolan, D. and Pays, E. (2000). Antibodies raised against the flagellar pocket fraction of *Trypanosoma brucei* preferentially recognize HSP60 in cDNA expression library. *Parasite Immunology,* 22**,** 639-650.

Radwanska, M., Claes, F., Magez, S., Magnus, E., Perez-Morga, D., Pays, E. and Büscher, P. (2002). Novel primer sequences for polymerase chain reaction-based detection of *Trypanosoma brucei gambiense*. *The American Journal of Tropical Medicine and Hygiene,* 67**,** 289–295.

Ratier, L., Urrutia, M., Paris, G., Zarebski, L., Frasch, A. C. and Goldbaum, F. A. (2008). Relevance of the diversity among members of the Trypanosoma cruzi trans-sialidase family analyzed with camelids single-domain antibodies. *PLoS One,* 3**,** e3524.

Rea, D., Hazell, C., Andrew, N. W., Morty, R. E. and Fulop, V. (2006). Expression, purification and preliminary crystallographic analysis of oligopeptidase B from *Trypanosoma brucei*. *Acta. Crystallographica Section F: Structural Biology and Crystallization Communications,* 62**,** 808-810.

Reyna-Bello, A., Garcia, F., Rivera, M., Sanso, B. and Aso, P. (1998). Enzyme-linked immunosorbent assay (ELISA) for detection of anti-*Trypanosoma evansi* equine antibodies. *Veterinary Parasitology,* 1483**,** 1-9.

Reynolds, J. A. and Tanford, C. (1970). The gross conformation of protein-sodium dodecyl sulfate complexes. *The Journal of Biological Chemistry,* 245**,** 5161-5165.

Roberts, R. J., Belfort, M., Bestor, T., Bhagwat, A. S., Bickle, T. A., Bitinaite, J., Blumenthal, R. M., Degtyarev, S., Dryden, D. T., Dybvig, K., Firman, K., Gromova, E. S., Gumport, R. I., Halford, S. E., Hattman, S., Heitman, J., Hornby, D. P., Janulaitis, A., Jeltsch, A., Josephsen, J., Kiss, A., Klaenhammer, T. R., Kobayashi, I., Kong, H., Kruger, D. H., Lacks, S., Marinus, M. G., Miyahara, M., Morgan, R. D., Murray, N. E., Nagaraja, V., Piekarowicz, A., Pingoud, A., Raleigh, E., Rao, D. N., Reich, N., Repin, V. E., Selker, E. U., Shaw, P. C., Stein, D. C., Stoddard, B. L., Szybalski, W., Trautner, T. A., Van Etten, J. L., Vitor, J. M., Wilson, G. G., and Xu, S. Y. (2003). A nomenclature for restriction enzymes, DNA methyltransferases, homing endonucleases and their genes, *Nucleic acids research* 31, 1805-1812.

Robinson, A. S. (ed.) (2011). Production of Membrane Proteins: Strategies for Expression and Isolation: *Wiley-VCH Verlag GmbH & Co. KGaA*. 420 pp.

Roge, S., Van Reet, N., Odiwuor, S., Tran, T., Schildermans, K., Vandamme, S., Vandenberghe, I., Vervecken, W., Gillingwater, K., Claes, F., Devreese, B., Guisez, Y. and Büscher, P. (2013). Recombinant expression of trypanosome surface glycoproteins in *Pichia pastoris* for the diagnosis of *Trypanosoma evansi* infection. *Veterinary Parasitology*.

Rogers, D. J., Hay, S. I. and Packer, M. J. (1996). Predicting the distribution of tsetse flies in West Africa using temporal Fourier processed meteorological satellite data. *Annals of Tropical Medicine and Parasitology,* 90**,** 225-241.

Romito, M., Viljoen, G. J. and Du Plessis, D. H. (2001). Eliciting antigen-specific egg-yolk IgY with naked DNA. *Biotechniques,* 31**,** 670, 672, 674-675.

Rozkov, A., Schweder, T., Veide, A. and Enfors, S. (2000). Dynamics of proteolysis and its influence on the accumulation of intracellular recombinant proteins. *Enzyme Microbiology Technology,* 27**,** 743-748.

Rudramurthy, G. R., Sengupta, P. P., Balamurugan, V., Prabhudas, K. and Rahman, H. (2013). PCR based diagnosis of trypanosomiasis exploring invariant surface glycoprotein (ISG) 75 gene. *Veterinary Parasitology,* 193**,** 47-58.

Ryan, K. A., Shapiro, T. A., Rauch, C. A. and Englund, P. T. (1988). Replication of Kinetoplast DNA in Trypanosomes. *Annual Review of Microbiology,* 42**,** 339-358.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: a laboratory manual, *Cold Spring harbour Laboratory Press NY 2nd ed*.

Sambrook, J. and Gething, M. J. (1989). Protein structure. Chaperones, paperones. *Nature,* 342**,** 224-225.

Savage, A. F., Cerqueira, G. C., Regmi, S., Wu, Y., El Sayed, N. M. and Aksoy, S. (2012). Transcript Expression Analysis of Putative *Trypanosoma brucei* GPI-Anchored Surface Proteins during Development in the Tsetse and Mammalian Hosts. *PLoS Neglected Tropical Diseases,* 6**,** e1708.

Schade, R., Calzado, E. G., Sarmiento, R., Chacana, P. A., Porankiewicz-Asplund, J. and Terzolo, H. R. (2005). Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and human and veterinary medicine. *Alternatives To Laboratory Animals,* 33**,** 129-154.

Schwede, A., Jones, N., Engstler, M. and Carrington, M. (2011). The VSG C-terminal domain is inaccessible to antibodies on live trypanosomes. *Molecular and Biochemical Parasitology,* 175**,** 201–204.

Seke Etet, P. F. and Mahomoodally, M. F. (2012). New insights in staging and chemotherapy of African trypanosomiasis and possible contribution of medicinal plants. *Scientific World Journal,* Article ID 343652, 16 pages.

Shukla, D. and Trout, B. L. (2011). Understanding the synergistic effect of arginine and glutamic acid mixtures on protein solubility. *The Journal of Physical Chemistry B,* 115**,** 11831-11839.

Silva, J., Carvalho, A. S., Vitorino, R., Domingues, P., Teixeira, P. and Gibbs, P. (2004). Protein extraction and comparison of stain protocols for analysis of two-dimensional electrophoresis gels. *Protein and Peptide Letters,* 11**,** 1-6.

Silva, M. S., Prazeres, D. M., Lanca, A., Atouguia, J. and Monteiro, G. A. (2009). Trans-sialidase from Trypanosoma brucei as a potential target for DNA vaccine development against African trypanosomiasis. *Parasitology Research,* 105**,** 1223-1229.

Simarro, P. P., Jannin, J. and Cattand, P. (2008). Eliminating human African trypanosomiasis: where do we stand and what comes next? . *PLoS Medicine,* 5**,** 174-180.

Simarro, P. P., Cecchi, G., Paone, M., Franco, J. R., Diarra, A., Ruiz, J. A., Fèvre, E. M., Courtin, F., Mattioli, R. C. and Jannin, J. G. (2010). The Atlas of human African trypanosomiasis: a contribution to global mapping of neglected tropical diseases. *International Journal of Health Geographics,* 9.

Sivashanmugam, A., Murray, V., Cui, C., Zhang, Y., Wang, J. and Li, Q. (2009). Practical protocols for production of very high yields of recombinant proteins using *Escherichia coli*. *Protein Science,* 18**,** 936-948.

Smith, D. B. and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene,* 67**,** 31-40.

Solano, P., Jamonneau, V., N'guessan, P., N'dri, L., Dje, N. N., Miezan, T. W., Lejon V., Büscher, P. and Garcia, A. (2002). Comparison of different DNA preparation protocols for PCR diagnosis of Human African Trypanosomosis in Cote d'Ivoire. *Acta Tropica 82***,** 349-356.

Sorensen, H. P. and Mortensen, K. K. (2005). Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. *Microbial Cell Factories,* 4**,** 1.

Steverding, D. (2008). The history of African trypanosomiasis. *Parasites and Vectors,* 1**,** 3.

Stockdale, C., Swiderski, M. R., Barry, J. D. and Mcculloch, R. (2008). Antigenic Variation in *Trypanosoma brucei*: Joining the DOTs. *PLoS Biology,* 6**,** 1386-1391.

Tanowitz, H. B., Wittner, M., Werner, C., Weiss, L. M., Kirchhoff, L. V. and Bacchi, C. (2000). Trypanosomes. *In: Encyclopedia of Microbiology*. *Lederberg J (ed),* Vol 4 Q-Z**,** pp 725-741.

Tarentino, A. L. and Maley, F. (1974). Purification and properties of an endo-B-acetyl glucosaminidase from *Streptomyces griseus. The Journal of Biological Chemistry,* 249**,** 811-817.

Thuita, J. K., Wang, M. Z., Kagira, J. M., Denton, C. L. and Paine, M. F., Et Al. (2012). Pharmacology of DB844, an Orally Active aza Analogue of Pafuramidine, in a Monkey Model of Second Stage Human African Trypanosomiasis. *PLoS Neglected Tropical Diseases,* 6(7), e1734.

Thuita, J. K., Wolf, K. K., Murilla, G. A., Liu, Q., Mutuku, J. N., Chen, Y., Bridges, A. S., Mdachi, R. E., Ismail, M. A., Ching, S., Boykin, D. W., Hall, J. E., Tidwell, R. R., Paine, M. F., Brun, R. and Wang, M. Z. (2013). Safety, Pharmacokinetic, and Efficacy Studies of Oral DB868 in a First Stage Vervet Monkey Model of Human African Trypanosomiasis. *PLoS Neglected Tropical Diseases,* 7**,** e2230.

Tiberti, N., Lejon, V., Hainard, A., Courtioux, B., Robin, X., Turck, N., Kristensson, K., Matovu, E., Enyaru, J. C., Mumba Ngoyi, D., Krishna, S., Bisser, S., Ndung'u, J. M., Büscher, P. and Sanchez, J. C. (2013). Neopterin is a cerebrospinal fluid marker for treatment outcome evaluation in patients affected by *Trypanosoma brucei gambiense* sleeping sickness. *PLoS Neglected Tropical Diseases,* 7**,** e2088.

Tischer, A., Lilie, H., Rudolph, R. and Lange, C. (2010). L-arginine hydrochloride increases the solubility of folded and unfolded recombinant plasminogen activator rPA. *Protein Science,* 19**,** 1783-1795.

Tran, T., Claes, F., Dujardin, J. C. and Büscher, P. (2006). The invariant surface glycoprotein ISG75 gene family consists of two main groups in the *Trypanozoon* subgenus. *Parasitology,* 133**,** 613-621.

Tran, T., Büscher, P., Vandenbussche, G., Wyns, L., Messens, J. and De Greve, H. (2008). Heterologous expression, purification and characterisation of the extracellular domain of trypanosome invariant surface glycoprotein ISG75. *Journal of Biotechnology,* 135**,** 247-254.

Tran, T., Claes, F., Verloo, D., De Greve, H. and Büscher, P. (2009). Towards a new reference test for surra in camels. *Clinical and Vaccine Immunology,* 16**,** 999-1002.

Troeberg, L., Pike, R. N., Lonsdale-Eccles, J. D. and Coetzer, T. H. (1997). Production of anti-peptide antibodies against trypanopain-Tb from *Trypanosoma brucei brucei*: effects of antibodies on enzyme activity against Z-Phe-Arg-AMC. *Immunopharmacology,* 36**,** 295-303.

Truc, P., Lejon, V., Magnus, E., Jamonneau, V., Nangouma, A., Verloo, D., Penchenier, L. and Büscher, P. (2002). Evaluation of the micro-CATT, CATT/*Trypanosoma brucei gambiense*, and LATEX/*T. b. gambiense* methods for serodiagnosis and surveillance of human African trypanosomiasis in West and Central Africa. *Bulletin of the World Health Organization,* 80**,** 882–886.

Urwyler, S., Studer, E., Renggli, C. K. and Roditi, I. (2007). A family of stage-specific alanine-rich proteins on the surface of epimastigote forms of *Trypanosoma brucei*. *Molecular Microbiology,* 63**,** 218–228.

Uzcanga, G., Mendoza, M., Aso, P. M. and Bubis, J. (2001). Purification of a 64kDa antigen from *Trypanosoma evansi* that exhibits cross-reactivity with *Trypanosoma vivax*. *Parasitology,* 124**,** 287-299.

Van Den Bossche, P., Doran, M. and Connor, R. J. (2000). An analysis of trypanocidal drug use in the Eastern Province of Zambia. *Acta Tropica,* 75**,** 247-258.

Van Den Bossche, P. (2001). Some general aspects of the distribution and epidemiology of bovine trypanosomosis in southern Africa. *International Journal for Parasitology,* 31**,** 592-598.

Van Nieuwenhove, L., Stijn Rogé, S., Fatima Balharbi, F., Tessa Dieltjens, T., Thierry Laurent, T., Guisez, Y., Büscher, P. and Lejon, V. (2011). Identification of Peptide Mimotopes of *Trypanosoma brucei gambiense* Variant Surface Glycoproteins. *PLoS Neglected Tropical Diseases,* 5**,** e1189.

Vather, P. (2010). Vivapain: A cysteine peptidase from *Trypanosoma vivax*. MSc dissertation, University of KwaZulu-Natal (Pietermaritzburg campus).

Vickerman, K. (1969). The fine structure of *Trypanosoma congolense* in its bloodstream phase. *Journal of Protozoology,* 16**,** 54-69.

Vickerman, K. (2000). Order Kinetoplastea. In The Illustrated Guide to the Protozoa. *Edited by J. J. Lee, G. F. Leedale & P. Bradbury. Lawrence, KS: Allen Press***,** pp. 1159–1185.

Voulgaridou, G.-P., Mantso, T., Chlichlia, K., Panayiotidis, M. I. and Pappa, A. (2013). Efficient *E. coli* Expression Strategies for Production of Soluble Human Crystallin ALDH3A1. *PLoS One,* 8**,** e56582.

Vreysen, M. J. B., Saleh, K. M., Ali, M. Y., Abdulla, A. M., Zhu, Z. R., Juma, K. G., Dyck, V. A., Msangi, A. R., Mkonyi, P. A. and Feldmann, H. U. (2000). Glossina austeni (Diptera: Glossinidae) Eradicated on the Island of Unguja, Zanzibar, Using the Sterile Insect Technique. *Journal of Economic Entomology,* 93**,** 123-135.

Walrad, P., Paterou, A., Acosta-Serrano, A. and Matthews, K. R. (2009). Differential Trypanosome Surface Coat Regulation by a CCCH Protein That Co-Associates with procyclin mRNA cis-Elements. *PLoS Pathogens,* 5**,** e1000317.

Weirather, J. L., Wilson, M. E. and Donelson, J. E. (2012). Mapping of VSG similarities in Trypanosoma brucei. *Molecular and Biochemical Parasitology,* 181**,** 141-152.

Welburn, S. C., Fèvre, E. M., Coleman, P. G., Odiit, M. and Maudlin, I. (2001a). Sleeping sickness: a tale of two diseases. *Trends in Parasitology,* 17**,** 19–24.

Welburn, S. C., Picozzi, K., Fèvre, E. M., Coleman, P. G., Odiit, M., Carrington, M. and Maudlin, I. (2001b). Identification of human-infective trypanosomes in animal reservoir of sleeping sickness in Uganda by means of serum-resistance-associated (SRA) gene. *The Lancet,* 358**,** 2017-2019.

Welburn, S. C., Maudlin, I. and Simarro, P. P. (2009). Controlling sleeping sickness – a review. *Parasitology,* 136**,** 1943–1949.

Wertheim, H., And, P. H. and Woodall, J. (eds.) (2012). Atlas of Human Infectious Diseases *Wiley-Blackwell, Oxford* pp182.

Westerhuis, W. H., Sturgis, J. N. and Niederman, R. A. (2000). Reevaluation of the electrophoretic migration behavior of soluble globular proteins in the native and detergent-denatured states in polyacrylamide gels. *Analytical Biochemistry,* 284**,** 143-152.

WHO (1983). Trypanosomiasis Control Manual. *African Medical and Research Foundation Nairobi, Kenya, Geneva***,** pp. 1-142.

WHO (1998). Control and surveillance of African trypanosomiasis: report of a WHO expert committe. *World Health Organisation Technical Report Series 881,* I-VI**,** 1-114.

WHO (2010). African Trypanosomiasis (sleeping sickness). http://www.who.int/mediacentre /factsheets/fs259/en/accessed on 091111.

WHO (2013). African Trypanosomiasis (sleeping sickness). *World Health Organisation fact sheet.*

Widener, J., Nielsen, M. J., Shiflett, A., Moestrup, S. K. and Hajduk, S. (2007). Hemoglobin Is a Co-Factor of Human Trypanosome Lytic Factor. *PLoS Pathogens,* 3**,** e129.

Williams, B. I. (1996). African trypanosomiasis. *In The Wellcome Trust illustrated history of tropical diseases. F.E.A.G. Cox, editor. The Wellcome Trust. London, United Kingdom.***,** 178–191.

Williamson, C. C., Stoltsz, W. H., Mattheus, A. and Schiele, G. J. (1988). An investigation into alternative methods for the serodiagnosis of dourine. *Onderstepoort Journal of Veterinary Research,* 55**,** 117-119.

Yang, H., Russell, D. G., Zheng, B., Eiki, M. and Lee, M. G. (2000). Sequence requirements for trafficking of the CRAM transmembrane protein to the flagellar pocket of African trypanosomes. *Molecular and Cell Biology,* 20**,** 5149-5163.

Yansouni, C. P., Bottieau, E., Lutumba, P., Winkler, A. S., Lynen, L., Büscher, P., Jacobs, J., Gillet, P., Lejon, V., Alirol, E., Polman, K., Utzinger, J., Miles, M. A., Peeling, R. W., Muyembe, J. J., Chappuis, F. and Boelaert, M. (2013). Rapid diagnostic tests for neurological infections in central Africa. *Lancet Infectious Diseases,* 13**,** 546-558.

Zablotskij, V. T., Georgiu, C., De Waal, T., Clausen, P. H., Claes, F. and Touratier, L. (2003). The current challenges of dourine: difficulties in differentiating *Trypanosoma equiperdum* within the subgenus *Trypanozoon*. *Revue scientifique et technique de l`office international des epizooties OIE,* 22**,** 1087-1096.

Zerial, M. and Mcbride, H. (2001). Rab proteins as membrane organizers. *Nature Reviews Molecular Cell Biology,* 2**,** 107-117.

Ziegelbauer, K., Multhaup, G. and Overath, P. (1992). Molecular characterization of two invariant surface glycoproteins specific for the bloodstream stage of *Trypanosoma brucei*. *The Journal of Biological Chemistry,* 267**,** 10797-10803.

Ziegelbauer, K. and Overath, P. (1992). Identification of invariant surface glycoproteins in the bloodstream stage of *Trypanosoma brucei*. *The Journal of Biological Chemistry,* 267**,** 10791-10796.

Ziegelbauer, K. and Overath, P. (1993). Organization of two invariant surface glycoproteins in the surface coat of *Trypanosoma brucei*. *Infection and Immunity,* 61**,** 4540-4545.

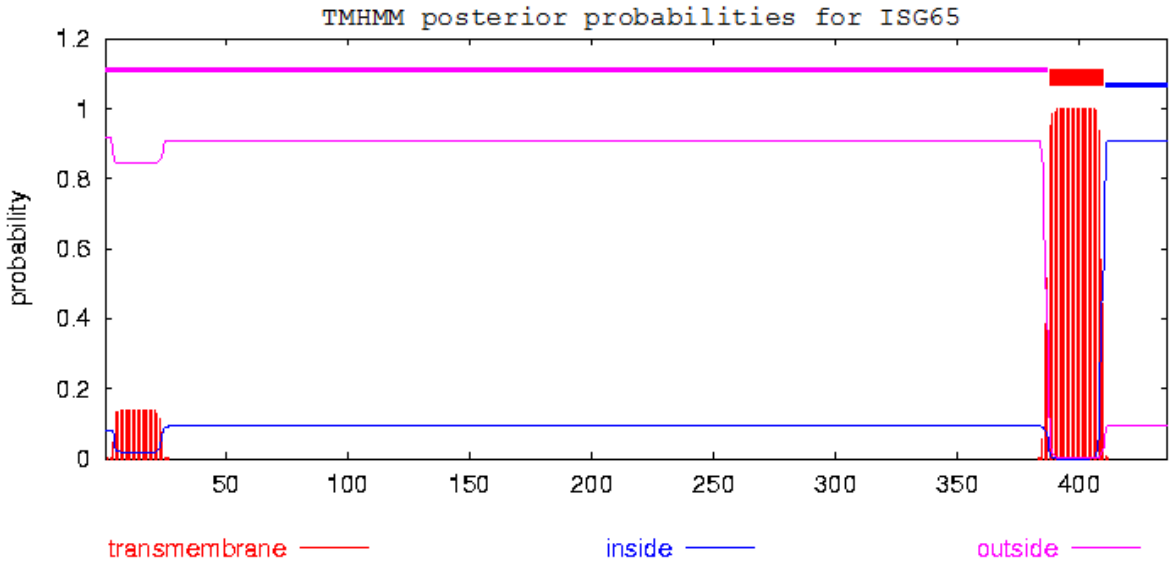
Ziegelbauer, K., Rudenko, G., Kieft, R. and Overath, P. (1995). Genomic organization of an invariant surface glycoprotein gene family of *Trypanosoma brucei*. *Molecular and Biochemical Parasitology,* 69**,** 53-63.

Zucca, M. and Savoia, D. (2011). Current Developments in the Therapy of Protozoan Infections. *The Open Medicinal Chemistry Journal,* 5**,** 4-10.

APPENDICES 1-9

Appendix 1

Domain predictions for *T. b. brucei* 927/4 GUTat10.1 strain ISG65 (Accession XM_946493) using the web-based TMHMM server v. 2.0 (http://www.cbs.dtu.dk/ services/TMHMM-2.0/ accessed on 12-06-11).

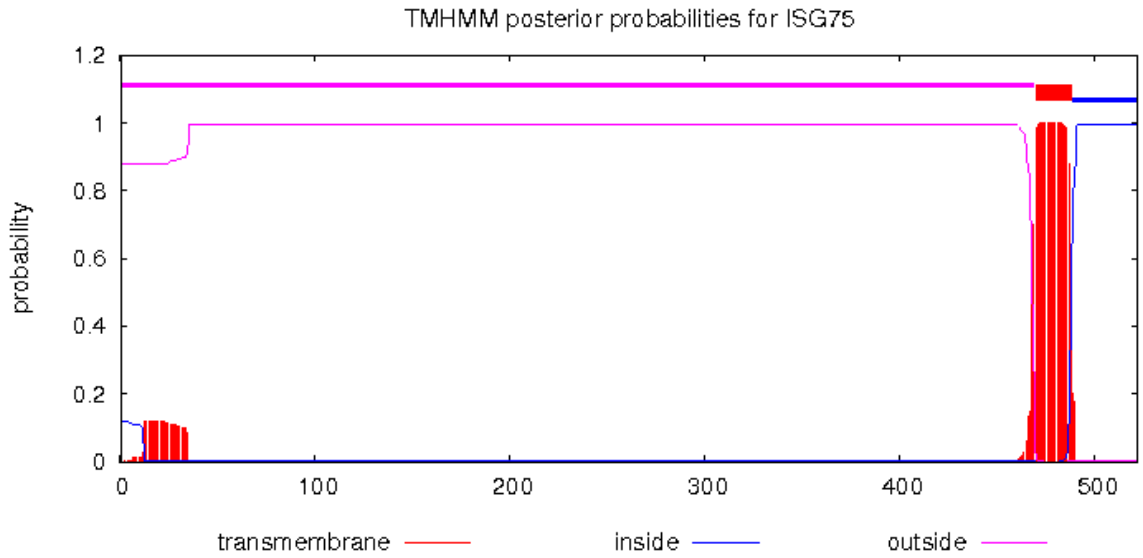



```
# ISG65 Length: 436
# ISG65 Number of predicted TMHs:  1
# ISG65 Exp number of AAs in TMHs: 25.57609
# ISG65 Exp number, first 60 AAs:  2.74091
# ISG65 Total prob of N-in:        0.08244
ISG65   TMHMM2.0       outside      1   387
ISG65   TMHMM2.0       TMhelix    388   410
ISG65   TMHMM2.0       inside     411   436

TMH-transmembrane helix
AA-amino acid
```

Appendix 2

Domain predictions for *T. b. brucei* AnTat 2.2 clone G06 strain ISG75 (Accession DQ200189) using the web-based TMHMM server v. 2.0 (http://www.cbs.dtu.dk/ services/TMHMM-2.0/ accessed on 20-07-11).




```
# ISG75 Length: 522
# ISG75 Number of predicted TMHs:  1
# ISG75 Exp number of AAs in TMHs: 23.04024
# ISG75 Exp number, first 60 AAs:  2.64121
# ISG75 Total prob of N-in:        0.11856
ISG75   TMHMM1.0       outside      1   469
ISG75   TMHMM1.0       TMhelix    470   488
ISG75   TMHMM1.0       inside     489   522
```

Appendix 3

Translation of *T. b. brucei* ISG65 sequence (Accession XM_946493) showing an overlap of the amino acid and nucleotide sequences. Highlighted residues were used to design primers. Extracellular domain of ISG65 was from amino acid residue 21 (red) 387 (blue).

```
1    atgatgaagtatttgctggtatttgcaattattgccacaagaatccctgtgttgttggcg
      M  M  K  Y  L  L  V  F  A  I  I  A  T  R  I  P  V  L  L  A
61   actaatggtggagataaacatgtgaaagctaacaagaagttgacgaaagagggagcagcg
      T  N  G  G  D  K  H  V  K  A  N  K  K  L  T  K  E  G  A  A
121  gcactctgtaaaatgaagcatcttgctgataaagttgcagaggagagatcacaagagttg
      A  L  C  K  M  K  H  L  A  D  K  V  A  E  E  R  S  Q  E  L
181  aaggatagaactcaaatttttgctggttatatagagttcgagttgtatagaatagattat
      K  D  R  T  Q  I  F  A  G  Y  I  E  F  E  L  Y  R  I  D  Y
241  tggttggaaaagctgaacggtccgaaggggcgaaaagacggttatgctaagctgtctgac
      W  L  E  K  L  N  G  P  K  G  R  K  D  G  Y  A  K  L  S  D
301  tctgatatagaaaaagtaaaggagatattcaacaaggcaaaagatggaatagctaagcaa
      S  D  I  E  K  V  K  E  I  F  N  K  A  K  D  G  I  A  K  Q
361  ctccctgaggcaaagaaagccggtgaggaagctgaaaaactacacaccgaagtgaagaag
      L  P  E  A  K  K  A  G  E  E  A  E  K  L  H  T  E  V  K  K
421  gccgcggcgaatgcacgtggagtgaggttaagtgaaggaacgaactctagtggcctttac
      A  A  A  N  A  R  G  V  R  L  S  E  G  T  N  S  S  G  L  Y
481  aggattttggattggtattgttttaaagaaggggagaatgcgggtaagagcgacaactgt
      R  I  L  D  W  Y  C  F  K  E  G  E  N  A  G  K  S  D  N  C
541  gatggcgtcaagttcagcgtgcattacgaaacgcacagaagaaggaatgttattgactgc
      D  G  V  K  F  S  V  H  Y  E  T  H  R  R  R  N  V  I  D  C
601  agcagcaccggctatgaggagaattatgattggtctgcgaacgcgctgcaggtggcccta
      S  S  T  G  Y  E  E  N  Y  D  W  S  A  N  A  L  Q  V  A  L
661  aatagctgggagaatgtgaagccaaaaaaattggagtcagccgggtcggacatgaattgc
      N  S  W  E  N  V  K  P  K  K  L  E  S  A  G  S  D  M  N  C
721  aacatcggccaatcttccgagagccatccatgcaccatgacagaggagtggcagactcca
      N  I  G  Q  S  S  E  S  H  P  C  T  M  T  E  E  W  Q  T  P
781  tacaaggaaactgtcgaaaagctaagggaacttgaggatgcgtaccaaaggggaaagaag
      Y  K  E  T  V  E  K  L  R  E  L  E  D  A  Y  Q  R  G  K  K
841  gctcatgatgctatgttgggttacgctaataccgcatatgctgtgaacacgaaagtggaa
      A  H  D  A  M  L  G  Y  A  N  T  A  Y  A  V  N  T  K  V  E
901  caggaaaagccgctgacagaggtgatagcggcagccaaggaagcagggaaaaagggcgcg
      Q  E  K  P  L  T  E  V  I  A  A  A  K  E  A  G  K  K  G  A
961  aaaattataatacccgcagctgccccagcaacaccgactaacagcacaaaaaatgaagat
      K  I  I  I  P  A  A  A  P  A  T  P  T  N  S  T  K  N  E  D
1021 agtgcttcaaccgagcatgttgatagagggattgcaacaaatgaaacacaggtggaagtt
      S  A  S  T  E  H  V  D  R  G  I  A  T  N  E  T  Q  V  E  V
1081 ggtattgatgctgactttgatagtctcctagatgccacagaggctgcagaggtaacgcgt
      G  I  D  A  D  F  D  S  L  L  D  A  T  E  A  A  E  V  T  R
1141 agacatcagagaacagcaatgattatattggcagtccttgtacctgccattattcttgtg
      R  H  Q  R  T  A  M  I  I  L  A  V  L  V  P  A  I  I  L  V
1201 gttacggccgttgcattcttcataatggtgaaacgaaggaggaatagctcccaggatgtg
      V  T  A  V  A  F  F  I  M  V  K  R  R  R  N  S  S  Q  D  V
1261 gacaccggaaaagcggagggtggggtttctagcgtaaaagtagtaatgtag
      D  T  G  K  A  E  G  G  V  S  S  V  K  V  V  M  -
```

Appendix 4

Translation of *T. b. brucei* ISG75 sequence (Accession DQ200189) showing an overlap of the amino acid and nucleotide sequences. Highlighted residues were used to design primers. Extracellular domain of ISG75 was from amino acid residue 28 (red) to 469 (blue).

```
1    ttttctacggccaaggtaacgtgaggttgagactgacaagctgaggaagtggtgatcaga
      F  S  T  A  K  V  T  -  G  -  D  -  Q  A  E  E  V  V  I  R
61   ggacggatgtcaacgatgcctgttacattacggactactgcaacagtatttctcctctgc
      -  -  M  S  T  M  P  V  T  L  R  T  T  A  T  V  F  L  L  C
121  ggcatatgtgcgctcgatgtgtccaaggcaagcaacttacctgtcgcatataagcagtat
      G  I  C  A  L  D  V  S  K  A  S  N  L  P  V  A  Y  K  Q  Y
181  gtaacaatgaatgggcggaaacttgatggtgaagctgtgttgacactgtgtaccatgaaa
      V  T  M  N  G  R  K  L  D  G  E  A  V  L  T  L  C  T  M  K
241  aagttgcttgacggtgttgctgatagagcagactttctagagaggcaatcctggaaatat
      K  L  L  D  G  V  A  D  R  A  D  F  L  E  R  Q  S  W  K  Y
301  ttgaaggtcgcgagagaaatttttcagaaagtgtcagagcatggtgatatgaaggcggcg
      L  K  V  A  R  E  I  F  Q  K  V  S  E  H  G  D  M  K  A  A
361  ctgggacaaaagatgttggatcagatgcgtggtgtccaacgtgtagcgaaccgaacgaaa
      L  G  Q  K  M  L  D  Q  M  R  G  V  Q  R  V  A  N  R  T  K
421  aggagcgttgaggagacatgggagaaggcgaagagggctgcagcgaactcgagcaaagtt
      R  S  V  E  E  T  W  E  K  A  K  R  A  A  A  N  S  S  K  V
481  ctcaaggagttgctaaaatggcactgtataaacaaggaagcaatccgtgattcgtttgac
      L  K  E  L  L  K  W  H  C  I  N  K  E  A  I  R  D  S  F  D
541  catatggctaacgcaaactgcgatcccagtgcgtataaacatgactatcatcgaaatttt
      H  M  A  N  A  N  C  D  P  S  A  Y  K  H  D  Y  H  R  N  F
601  ggacatgatgacgctcgcgcatacgctatatattgcgagtacaagtctattccggctgca
      G  H  D  D  A  R  A  Y  A  I  Y  C  E  Y  K  S  I  P  A  A
661  aaaactgatgtgaccttctccaacatggaggggggcagtcgaggcatggaatcgagcaaag
      K  T  D  V  T  F  S  N  M  E  G  A  V  E  A  W  N  R  A  K
721  cccaaggctgatgcaagggatgctgttgagtgcagcagcggccattcgtcaagaagtgcg
      P  K  A  D  A  R  D  A  V  E  C  S  S  G  H  S  S  R  S  A
781  tcctcctccgataaaccatgcacgctgcttgaaagttggcggtgggactacgatgcagca
      S  S  S  D  K  P  C  T  L  L  E  S  W  R  W  D  Y  D  A  A
841  aggtatgcgatactcaaacttgaaactctagtccgcaattccttgggggtgactcactat
      R  Y  A  I  L  K  L  E  T  L  V  R  N  S  L  G  V  T  H  Y
901  gctcagagatttcaacagattggaagggagtctcttgcacagtatctggaatggaaaaaa
      A  Q  R  F  Q  Q  I  G  R  E  S  L  A  Q  Y  L  E  W  K  K
961  gcagcagaggcgcgggcggcggaggaggaggcgaaacgccaggctgctgagaaggctgct
      A  A  E  A  R  A  A  E  E  E  A  K  R  Q  A  A  E  K  A  A
1021 gaggaagcgagaaaggctttggaggaggctgaggcgagacgagtggctgctgaggaacag
      E  E  A  R  K  A  L  E  E  A  E  A  R  R  V  A  A  E  E  Q
1081 gcggaggccaggcgcttggaagctgagaaggctgagaaggcaaaggaggcgggtcagccg
      A  E  A  R  R  L  E  A  E  K  A  E  K  A  K  E  A  G  Q  P
1141 gtgagtgaagaaaagaaaaagatgttgctggaggctgttgagaaagctgaggcaactgaa
      V  S  E  E  K  K  K  M  L  L  E  A  V  E  K  A  E  A  T  E
1201 aaggctgcagaaaagcaggcaaaagattcgagaaaggcatttgaggaagcggaggaggag
      K  A  A  E  K  Q  A  K  D  S  R  K  A  F  E  E  A  E  E  E
1261 cgcatcaaagccaccgaagatgcagaagttgcgcggtgggacaaggagggcgccgaggaa
      R  I  K  A  T  E  D  A  E  V  A  R  W  D  K  E  G  A  E  E
1321 tctgaagagaaactgaagaaggatgtagaaaaattggcggaggaattgaaagaggagtcg
      S  E  E  K  L  K  K  D  V  E  K  L  A  E  E  L  K  E  E  S
1381 aaagaaagtggtgaagaggacgacgtaaatgctgaccatgatgatgagggcagcgaggcc
      K  E  S  G  E  E  D  D  V  N  A  D  H  D  D  E  G  S  E  A
```

```
1441 aagagtggctggattgggacaacgaaagtgttaatatttttaattcctttgcttttgctg
      K  S  G  W  I  G  T  T  K  V  L  I  F  L  I  P  L  L  L  L
1501 ttgcttgggttgcttgtgttctttgttattaggggccgtaggaaggctgaggtgaaggat
      L  L  G  L  L  V  F  F  V  I  R  G  R  R  K  A  E  V  K  D
1561 gatataagtataggggaagctaatgctaaaagcaaaaatacgaagaccgcagcaggtttc
      D  I  S  I  G  E  A  N  A  K  S  K  N  T  K  T  A  A  G  F
1621 gacagtgatatttaaacactatcacaggtgcccgaac
      D  S  D  I  -  T  L  S  Q  V  P  E
```

Appendix 5

EMBOSS Needle Pairwise sequence alignment of *Tbb ISG65* (obtained from sequencing of pET 28a-*Tbb ISG65*) [ISG65_001] with *T. b. brucei ISG65* (Accession XM_946493) [ISG65_002].

Key: (| = identical), (. = different)

```
ISG65_001         1 ACTAATGGTGGAGATAAACATGTAAAAGCTAACAAGAAACTTGATCAAGA     50
                    |||||||||||||||||||||||.|||||||||||||..|.....||||
ISG65_002         1 actaatggtggagataaacatgtgaaagctaacaagaagttgacgaaaga     50

ISG65_001        51 AGGTGC--CAACTTGCTCTGTAAAATGAAGCATCTTGCTGATAAAGTTGC     98
                    .||.||  |..|  .||||||||||||||||||||||||||||||||||
ISG65_002        51 gggagcagcggc--actctgtaaaatgaagcatcttgctgataaagttgc     98

ISG65_001        99 GAATAAAGGAGGAGAAGACCTAAAGAAGAAAACTAAAGGTTTTGAAGATG    148
                    ..|..|..||..|.||||..|.|||.|.|.||||.||..|||||..|.|.
ISG65_002        99 agaggagagatcacaagagttgaaggatagaactcaaatttttgctggtt    148

ISG65_001       149 ACGTACTTTTGGAGGTGGAAAGAGTGAATAATTGGTTGGAAAAGCTGAAC    198
                    |..||...||.|||.||.|.|||.|..||.||||||||||||||||||||
ISG65_002       149 atatagagttcgagttgtatagaatagattattggttggaaaagctgaac    198

ISG65_001       199 GGTCCGAAGGGGCGAAAAGACGGTTATGCTAAGCTGTCTGACTCTGATAT    248
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
ISG65_002       199 ggtccgaaggggcgaaaagacggttatgctaagctgtctgactctgatat    248

ISG65_001       249 AGAAAAAGTGAAGGGAATATTTACTAAGGCAAAAGATGGAATAACTAAGC    298
                    |||||||||.|||||.|||||.|..|||||||||||||||.|||||||
ISG65_002       249 agaaaaagtaaaggagatattcaacaaggcaaaagatggaatagctaagc    298

ISG65_001       299 AACTCCCTGAGGCAAAGAAGGCTGCGGACGACGCTAAGAAACTGTACGAT    348
                    |||||||||||||||||.||.|..||.||.|||.|.||||||.||...
ISG65_002       299 aactccctgaggcaaagaaagccggtgaggaagctgaaaaactacacacc    348

ISG65_001       349 GAAGTGAAGAAAGCGGCGGAGCATGCACGCGGAGTGAAAGTGAGTGACGA    398
                    ||||||||||.||.||||.|.|||||||.|||||||...|.||||||.|.
ISG65_002       349 gaagtgaagaaggccgcggcgaatgcacgtggagtgaggttaagtgaagg    398

ISG65_001       399 AACGAACTCTAGTGGCCTTTACAGGATTTTGGATTGGTATTGTTTTAAAG    448
                    ||||||||||||||||||||||||||||||||||||||||||||||||||
ISG65_002       399 aacgaactctagtggcctttacaggattttggattggtattgttttaaag    448

ISG65_001       449 AAGGGGAGAACGCGGGTAAGAGCGACAACTGTGATGGCGTCAAGTTCAGC    498
                    ||||||||||.||||||||||||||||||||||||||||||||||||||
ISG65_002       449 aaggggagaatgcgggtaagagcgacaactgtgatggcgtcaagttcagc    498

ISG65_001       499 GAGCATCATGGAACGCACAGAAGAAGGAATGTTATTGACTGCGGTGACGA    548
                    |.||||.|.|.|||||||||||||||||||||||||||||||.|
ISG65_002       499 gtgcattacgaaacgcacagaagaaggaatgttattgactgcag------    542

ISG65_001       549 GAAAGCGAACAAATATGGGG------ATGCT---TCATCAAAAACATTGG    589
                      .||| |.|...||||.||      |||.|   ||..|.||..|..||.
ISG65_002       543 --cagc-accggctatgaggagaattatgattggtctgcgaacgcgctgc    589

ISG65_001       590 AAGACACCTTGAAACAATGGGAAAGCAAGAAACCGCAAGCCGCAGGCGCA    639
                    |.|...||.|.||....|||||.|....|||.|| .||......||.|..
ISG65_002       590 aggtggccctaaatagctgggagaatgtgaagcc-aaaaaaattggagtc    638
```

```
ISG65_001       640 AGCGGTGGGAATGACGTG---TGCAAAGCCACCGCATCTTCCGAGAGCCA    686
                    |||  .|||...|||.||   |||||...|..|..|||||||||||||||
ISG65_002       639 agc--cgggtcggacatgaattgcaacatcggccaatcttccgagagcca    686

ISG65_001       687 TCCATGCACCATGACAGAGGAGTGGCAGACTCCATACAAGGAAACTGTCA    736
                    ||||||||||||||||||||||||||||||||||||||||||||||||.
ISG65_002       687 tccatgcaccatgacagaggagtggcagactccatacaaggaaactgtcg    736

ISG65_001       737 AAAAGCTAAAAGAACTCGAGGGTGCGCACGAGAGGGGCAAGAAGGCTCAT    786
                    |||||||||..|||||.||||.||||.||.|.|||||.||||||||||||
ISG65_002       737 aaaagctaagggaacttgaggatgcgtaccaaaggggaaagaaggctcat    786

ISG65_001       787 GATGCTATGTTGGGTTACGCTAATACTGCACATGC-GACAACAGGAAAGT    835
                    |||||||||||||||||||||||||.|||.|||| |..||||.||||||
ISG65_002       787 gatgctatgttgggttacgctaataccgcatatgctgtgaacacgaaagt    836

ISG65_001       836 GGAACAGGCAAA-CCACTGACAGAGGTGATAGCGGCAGCTAAGGAAGCA-    883
                    ||||||||.||| ||.|||||||||||||||||||||.|||||||||
ISG65_002       837 ggaacaggaaaagccgctgacagaggtgatagcggcagccaaggaagcag    886

ISG65_001       884 GGAAAAAGGGCGCG-AAATTAT-ATACCCGCAGCTGCCYCATCCACACCG    931
                    |||||||||||||| ||||||| |||||||||||||.||.|.||||||
ISG65_002       887 ggaaaaagggcgcgaaaattataatacccgcagctgccccagcaacaccg    936

ISG65_001       932 ACT-ACAGCAC-GAAAATGGAAATAGTGC-TCAA-CGAGCCATGATGGAT    977
                    ||| ||||||| .|||||||.|.||||||| |||| |||| ||||.||...
ISG65_002       937 actaacagcacaaaaaatgaagatagtgcttcaaccgag-catgttgata    985

ISG65_001       978 GAGGGATT    985
                    ||||||||
ISG65_002       986 gagggatt    993
```

```
#=======================================
#
# Program: needle
# Rundate: 30 Jul 2012 14:27:12
# Aligned_sequences: 2
# 1: ISG65_001
# 2: ISG65_002
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 1008
# Identity:     805/1008 (79.9%)
# Similarity:   805/1008 (79.9%)
# Gaps:          38/1008 ( 3.8%)
# Score: 4218.5
#
#=======================================
```

Appendix 6

EMBOSS Needle Pairwise sequence alignment of *Tbb ISG75* (obtained from sequencing of *pGEX-4T1-Tbg ISG75*) [ISG75_001] with *T. b. brucei ISG75* (Accession DQ200189) [ISG75_002].

Key: (| = identical), (. = different)

```
ISG75_001          1 GTTGAATCGAAAAGGGGCGGT-CTGGTGTCACATAACGATGGTTGGACTC     49
                     |.|||...|||    |||||. |||| |.||   ||.||| |||||| ||
ISG75_002          1 ggtgatatgaa----ggcggcgctgg-gaca---aaagat-gttgga-tc     40

ISG75_001         50 CAAGTTGCG----GATCCACGTGATAGTCGAACCTGACGAAACAGAGGCG     95
                       ||.||||    |..|.||||| ||| |||||||..|||||| ||..|||
ISG75_002         41 --agatgcgtggtgtccaacgtg-tag-cgaaccgaacgaaa-aggagcg     85

ISG75_001         96 TAG-GGAGACATAGGGAGAAGGCCGAAGACGACTGCA--TTATTTGAGCT    142
                     |.| |||||||| ||||||||| ||||||.|.|||||  ..|.|.|||||.
ISG75_002         86 ttgaggagacat-gggagaagg-cgaagagggctgcagcgaactcgagca    133

ISG75_001        143 AAGT--TCAATGGGCTGCTAAAAT-GCACTGTATAACCAAGGAAGCAATC    189
                     ||||  ||||.|.|.||||||||| ||||||||||.|||||||||||||
ISG75_002        134 aagttctcaaggagttgctaaaatggcactgtataaacaaggaagcaatc    183

ISG75_001        190 CG-GA-TCGTTTG-CCATATGGCTAACGCAAACTGCG-TCC---AGCGTA    232
                     || || ||||||| ||||||||||||||||||||||| |||   .|||||
ISG75_002        184 cgtgattcgtttgaccatatggctaacgcaaactgcgatcccagtgcgta    233

ISG75_001        233 TAAACATGACTATCATCGAAATTTTGGACATGATGACGCTCGCGCAT-CG    281
                     |||||||||||||||||||||||||||||||||||||||||||||||| ||
ISG75_002        234 taaacatgactatcatcgaaattttggacatgatgacgctcgcgcatacg    283

ISG75_001        282 -TATATATTGCGAGT-CAAGTCTATTCCGGCTGCAAAAACTGATGTGACC    329
                      ||||||||||||||| |||||||||||||||||||||||||||||||||
ISG75_002        284 ctatatattgcgagtacaagtctattccggctgcaaaaactgatgtgacc    333

ISG75_001        330 TTCTCCAACATGGAGGGGGCAGTCGAGGGCATGGAATCGAGCAAAGCCCA    379
                     |||||||||||||||||||||||| |||||||||||||||||||||||||
ISG75_002        334 ttctccaacatggaggggcagtcga-ggcatggaatcgagcaaagccca    382

ISG75_001        380 AGGCTGATGCAAGGGATGCTGTTGAGTGCAGCAGCGGCCATTCGTCAAGA    429
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        383 aggctgatgcaagggatgctgttgagtgcagcagcggccattcgtcaaga    432

ISG75_001        430 AGTGCGTCCTCCTCCGATAAACCATGCACGCTGCTTGAAAGTTGGCGGTG    479
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        433 agtgcgtcctcctccgataaaccatgcacgctgcttgaaagttggcggtg    482

ISG75_001        480 GGACTACGATGCAGCAAGGTATGCGATACTCAAACTTGAAACTCTAGTCC    529
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        483 ggactacgatgcagcaaggtatgcgatactcaaacttgaaactctagtcc    532

ISG75_001        530 GCAATTCCTTGGGGGTGACTCACTATGCTCAGAGATTTCAACAGATTGGA    579
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        533 gcaattccttgggggtgactcactatgctcagagatttcaacagattgga    582

ISG75_001        580 AGGGAGTCTCTTGCACAGTATCTGGAATGGAAAAAAGCAGCAGAGGCGCG    629
                     |||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        583 agggagtctcttgcacagtatctggaatggaaaaaagcagcagaggcgcg    632
```

```
ISG75_001        630 GGCGGCGGAGGAGGAGGCGAAACGCCAGGCTGCTGAGAAGGCTGCTGAGG    679
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        633 ggcggcggaggaggaggcgaaacgccaggctgctgagaaggctgctgagg    682

ISG75_001        680 AAGCGAGAAAGGCTTTGGAGGAGGCTGAGGCGAGACGAGTGGCTGCTGAG    729
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        683 aagcgagaaaggctttggaggaggctgaggcgagacgagtggctgctgag    732

ISG75_001        730 GAACAGGCGGAGGCCAGGCGCTTGGAAGCTGAGAAGGCTGAGAAGGCAAA    779
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        733 gaacaggcggaggccaggcgcttggaagctgagaaggctgagaaggcaaa    782

ISG75_001        780 GGAGGCGGGTCAGCCGGTGAGTGAAGAAAAGAAAAAGATGTTGCTGGAGG    829
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        783 ggaggcgggtcagccggtgagtgaagaaaagaaaaagatgttgctggagg    832

ISG75_001        830 CTGTTGAGAAAGCTGAGGCAACTGAAAAGGCTGCAGAAAAGCAGGTGAAA    879
                     ||||||||||||||||||||||||||||||||||||||||||||||..|||
ISG75_002        833 ctgttgagaaagctgaggcaactgaaaaggctgcagaaaagcaggcaaaa    882

ISG75_001        880 GATTCGAGAAAGGCATTTGAGGAAGCGGAGGAGGAGCGCATCAAAGCCAC    929
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        883 gattcgagaaaggcatttgaggaagcggaggaggagcgcatcaaagccac    932

ISG75_001        930 CGAAGATGCAGAAGTTGCGCGGTGGGACAAGGAGGGCGCCGAGGAATCTG    979
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        933 cgaagatgcagaagttgcgcggtgggacaaggagggcgccgaggaatctg    982

ISG75_001        980 AAGAGAAACTGAAGAAGGATGTAGAAAAATTGGCGGAGGAATTGAAAGAG   1029
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||
ISG75_002        983 aagagaaactgaagaaggatgtagaaaaattggcggaggaattgaaagag   1032

ISG75_001       1030 GAGTCGAAAGAAAGTGGTGAAGAGGACGACGTAAATGCTGACCACGATGA   1079
                     ||||||||||||||||||||||||||||||||||||||||||||.||||||
ISG75_002       1033 gagtcgaaagaaagtggtgaagaggacgacgtaaatgctgaccatgatga   1082

ISG75_001       1080 TGAGGGCAGCGAGGCCAAGAG-GGCTGGATTGGGACAACGAAAGTGTTA    1127
                     |||||||||||||||||||| |||||||||||||||||||||||||||
ISG75_002       1083 tgagggcagcgaggccaagagtggctggattgggacaacgaaagtgtta    1131

#=======================================
#
#Program: needle
#Rundate: 30 Jul 2012 10:52:50
#Aligned_sequences: 2
# 1: ISG75_001
# 2: ISG75_002
# Matrix: EBLOSUM62
# Gap_penalty: 10.0
# Extend_penalty: 0.5
#
# Length: 1149
# Identity:    1078/1149 (93.8%)
# Similarity:  1078/1149 (93.8%)
# Gaps:          40/1149 ( 3.5%)
# Score: 5915.5
#
#=======================================
```

Appendix 7

EMBOSS Needle Pairwise sequence alignment of *ISG65* (Accession XM_946493) and *ISG75* (Accession DQ200189) nucleotide sequences.

Key: (| = identical), (. = different)

```
ISG75             1 ----------------------------------------atgatgaagt     10
                                                            .|||.|||||
ISG65             1 ttttctacggccaaggtaacgtgaggttgagactgacaagctgaggaagt     50

ISG75            11 -------------------------------------att------tgctg     18
                                                       |||      |.|||
ISG65            51 ggtgatcagaggacggatgtcaacgatgcctgttacattacggactactg    100

ISG75            19 -----gtattt----------gcaattat-tgc-cacaagaatccctgtg     51
                         ||||||          |||  ||| ||| |       ||..||||
ISG65           101 caacagtatttctcctctgcggca--tatgtgcgc-------tcgatgtg    141

ISG75            52 ttgttggcgactaa------tggtggagataaaca-tgtgaaagctaaca     94
                    |....|||.|..||      ||..|.|.||||.|| |.||     |||||
ISG65           142 tccaaggcaagcaacttacctgtcgcatataagcagtatg-----taaca    186

ISG75            95 a-gaagttgacgaaa----gagggagcagc------ggcactctgtaaaa    133
                    | ||| |.|.||.||    ||.||.|.|||      |.||||.||||..|
ISG65           187 atgaa-tgggcggaaacttgatggtgaagctgtgttgacactgtgtacca    235

ISG75           134 tgaagca------------tcttgctgataaagttgcaga------ggag    165
                    ||||..|            |.|||||||||.|   |||||      .|||
ISG65           236 tgaaaaagttgcttgacggtgttgctgataga---gcagactttctagag    282

ISG75           166 ag---atcac----aagagttgaag-----gatagaactcaaatttttt--    201
                    ||   ||| |    ||.|.||||||     ||.||     ||||||||
ISG65           283 aggcaatc-ctggaaatatttgaaggtcgcgagag-----aaatttttca    326

ISG75           202 ------------gc-tggttatatagagttcgagttgtatagaatagatt    238
                             || ||||.||||..||...|.|.|| ..|.||.||||
ISG65           327 gaaagtgtcagagcatggtgatatgaaggcggcgctg-ggacaaaagat-    374

ISG75           239 attggttggaaaagctg----------aacg-gt------ccgaaggggc    271
                        ||||||..||.||          |||| ||      |||||    |
ISG65           375 ----gttggatcagatgcgtggtgtccaacgtgtagcgaaccgaa----c    416

ISG75           272 gaaaa-gacggtt----------------atgctaagctgtctg------    298
                    ||||| ||..|||                |.||.|||..|.|||
ISG65           417 gaaaaggagcgttgaggagacatgggagaaggcgaagagggctgcagcga    466

ISG75           299 actctgatataga-aaaag---taaaggagatattcaacaaggca-----    339
                    ||||       || .||||   |.||||||.|..| ||.|.||||
ISG65           467 actc-------gagcaaagttctcaaggagttgct-aaaatggcactgta    508

ISG75           340 -aaagatggaatagctaagcaactccctga---------------ggc-a    372
                     |||.|.||       |||||| |||.|||               ||| |
ISG65           509 taaacaagg-------aagcaa-tccgtgattcgtttgaccatatggcta    550

ISG75           373 aagaaa--------gccggtgaggaagctgaaaa--actacacaccgaa-    411
                    |.|.||        .||.|||.|.|   |.||.|  |||| .||.||||
ISG65           551 acgcaaactgcgatcccagtgcgta---taaacatgacta-tcatcgaaa    596
```

```
ISG75          412 --------gtgaagaaggccgcg---------------gcgaatgcacgt    438
                           .|||.||.|..||||               ||||.|.||.||
ISG65          597 ttttggacatgatgacgctcgcgcatacgctatatattgcgagtacaagt    646

ISG75          439 ggagtgaggttaagtgaaggaacgaactctagtggcctt-tacaggattt    487
                   ..|.|..||.|        |.|..||||...|||.|||| |.||..||
ISG65          647 ctattccggct--------gcaaaaactgatgtgaccttctccaacat--    686

ISG75          488 tggattggtattgttttaaagaaggggagaatgcgggtaagagcgacaac    537
                                 |.||||
ISG65          687 ---------------------ggaggg------------------------    692

ISG75          538 tgtgatggc-gtcaagttcagcgtgcattacgaaacgcacag---aag--    581
                         ||| |||.||    ||.||.|.| |||   |||.||   |||
ISG65          693 ------ggcagtcgag----gcatggaat-cga---gcaaagcccaaggc    728

ISG75          582 ------aaggaatgttattgactgcagcagcaccggctat--gaggagaa    623
                         ||||.|||.|.||||.||||||||   ||||.||  |...||||
ISG65          729 tgatgcaagggatgctgttgagtgcagcag---cggccattcgtcaagaa    775

ISG75          624 ttatgattggt--------ctgcga--------acgcgctgc-----agg    652
                           ||        ||.|||        .|.||||||     ||.
ISG65          776 ---------gtgcgtcctcctccgataaaccatgcacgctgcttgaaagt    816

ISG75          653 tggccctaaatagctggga---------------gaatgtgaagccaaaa    687
                   ||||        |.|||||               |.|||.||..|..|||
ISG65          817 tggc--------ggtgggactacgatgcagcaaggtatgcgatactcaaa    858

ISG75          688 ----aaattggagtcagc---------cgggtcggac-----atg-----    714
                       |||.|..||||.||         .||||  |||     |||
ISG65          859 cttgaaactctagtccgcaattccttgggggt--gactcactatgctcag    906

ISG75          715 -aattgcaac--atcggccaa-------tcttccgagagccatc------    748
                    .|||.||||  ||.||  ||       ||||.| |.|| .|||
ISG65          907 agatttcaacagattgg--aagggagtctcttgc-acag-tatctggaat    952

ISG75          749 -----catgcaccatga-----------cagagga-gtggc-agactcca    780
                        .|.|||.|| ||           |.||||| |.||| |.||.||
ISG65          953 ggaaaaaagcagca-gaggcgcgggcggcggaggaggaggcgaaacgcc-   1000

ISG75          781 tacaaggaaactgtcgaaaa----gctaagg------gaa---ctt--ga    815
                       |||   |||..||.||    |||.|||      |||   |||  ||
ISG65         1001 ----agg---ctgctgagaaggctgctgaggaagcgagaaaggctttgga   1043

ISG75          816 ggatgc-gtaccaaaggggaaagaag-----gctca---------tgatg    850
                   |||.|| |      |||.||.|..||     |||.|         .||.|
ISG65         1044 ggaggctg------aggcgagacgagtggctgctgaggaacaggcggagg   1087

ISG75          851 ctatg---ttgggttacgctaataccgcatatgctgtgaacacgaa----    893
                   |.|.|   ||||   |.|||.|.|      |.||||.|||..|.||
ISG65         1088 ccaggcgcttgg---aagctgaga------aggctgagaaggcaaaggag   1128

ISG75          894 ---------------agtg--gaacaggaaaa-------------gccgc    913
                               ||||  |||.||.||||             ||.|.
ISG65         1129 gcgggtcagccggtgagtgaagaaaagaaaaagatgttgctggaggctgt   1178

ISG75          914 tgacagaggtgatagcggca---gccaagg--------aagcagggaaaa    952
                   |||.|.||.|||    ||||   |..||||        |||||||.||||
ISG65         1179 tgagaaagctga----ggcaactgaaaaggctgcagaaaagcaggcaaaa   1224
```

```
ISG75           953 agggcgcgaaa---att----------------------ataata---cc    974
                    ....||.||||   |||                      ||.|.|   .|
ISG65          1225 gattcgagaaaggcatttgaggaagcggaggaggagcgcatcaaagccac   1274

ISG75           975 cgcagctgccccag-------------caa------caccgactaa-cag   1004
                    ||.||.|||...||             |||      |.||||..|| |.|
ISG65          1275 cgaagatgcagaagttgcgcggtgggacaaggagggcgccgaggaatctg   1324

ISG75          1005 cacaaaaaatgaagatagtgcttcaaccgagcatgttgata--------g   1046
                    .|.|.|||.||||||              ||.||||.||.|        |
ISG65          1325 aagagaaactgaaga--------------aggatgtagaaaaattggcgg   1360

ISG75          1047 agggattgcaa-------caaatgaaacacaggtg--gaagttg-----g   1082
                    |||.||||.||       |.||.||||     |||  ||||..|     |
ISG65          1361 aggaattgaaagaggagtcgaaagaaa-----gtggtgaagaggacgacg   1405

ISG75          1083 tattgatgctgactttgat---------agtctcctagatgccacaga--   1121
                    ||  .||||||||..||||         ||      .||.|||| |||
ISG65          1406 ta--aatgctgaccatgatgatgagggcag------cgaggcca-agagt   1446

ISG75          1122 ggctgcagaggtaacgcgtagacatcagagaacagcaatgattata-ttg   1170
                    |||||.|..||         ||||.|   |||    |.||.|.||| ||.
ISG65          1447 ggctggattgg---------gacaac---gaa----agtgttaatatttt   1480

ISG75          1171 gcagtccttgtacctgccatt----attcttgtggttacggccgttgcat   1216
                    ..|.|||||     |||..||    .|.|||| ||||.|     |||..|
ISG65          1481 taattcctt-----tgcttttgctgttgcttg-ggttgc-----ttgtgt   1519

ISG75          1217 tcttcataat--ggtgaaacgaaggaggaatagctcccaggatgtggaca   1264
                    ||||..|.||  ||.|  .||.||||.|    |||     ||.|||.|
ISG65          1520 tctttgttattagggg--ccgtaggaag----gct-----gaggtgaa--   1556

ISG75          1265 ccgga----------aaagcggagggtggggtttcta--gcgtaaaagta   1302
                      |||          |.||.|||.|         |||  || ||||||.|
ISG65          1557 --ggatgatataagtatagggggaag---------ctaatgc-taaaagca   1594

ISG75          1303 gtaat--gtag-----------------------------------------   1311
                    ..|||  |.||
ISG65          1595 aaaatacgaagaccgcagcaggtttcgacagtgatatttaaacactatca   1644

ISG75          1312 -------------   1311

ISG65          1645 caggtgcccgaac   1657
```

```
#=======================================
# Program: needle
# Rundate: 12 Jun 2011 11:21:37
# Commandline: needle
# Aligned_sequences: 2
# 1: ISG75
# 2: ISG65
# Matrix: EDNAFULL
# Gap_penalty: 10.0
# Extend_penalty: 0.5
# Length: 1913
# Identity:      809/1913 (42.3%)
# Similarity:   809/1913 (42.3%)
# Gaps:           858/1913 (44.9%)
# Score: 1386.0
#=======================================
```

Appendix 8

Table presenting sequences producing significant alignments to *T. b. brucei ISG65* sequence (Accession XM_946493) using NCBI Blast.

Description	Max score	Total score	Query cover	E value	Identity	Accession
***Trypanosoma brucei* chromosome 2 clone RPCI93-10C8, complete sequence**	2422	12573	100%	0.0	100%	AC007862.7
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 65 kDa invariant surface glycoprotein (Tb927.2.3310) partial mRNA**	2422	2422	100%	0.0	100%	XM_946493.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 65 kDa invariant surface glycoprotein (Tb927.2.330 0) partial mRNA**	2383	2383	100%	0.0	99%	XM_946492.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 65 kDa invariant surface glycoprotein (Tb927.2.329 0) partial mRNA**	2377	2377	100%	0.0	99%	XM_946491.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 65 kDa invariant surface glycoprotein (Tb927.2.328 0) partial mRNA**	2377	2377	100%	0.0	99%	XM_946490.1
***Trypanosoma brucei gambiense* DAL972 chromosome 2, complete sequence**	2211	22752	100%	0.0	97%	FN554965.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 65 kDa invariant surface glycoprotein (Tb927.2.327 0) partial mRNA**	1929	1929	100%	0.0	93%	XM_946489.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 65 kDa invariant surface glycoprotein (Tb927.2.332 0) partial mRNA**	1083	1083	100%	0.0	82%	XM_946494.1
***Trypanosoma brucei* chromosome 2 clone RPCI93-28H13, complete sequence**	861	861	54%	0.0	88%	AC007864.5
***Trypanosoma brucei* 65 kDa invariant surface glycoprotein mRNA, complete cds**	832	1337	91%	0.0	91%	M86709.1
***Trypanosoma brucei* variant surface glycoprotein gene, partial cds**	787	1149	83%	0.0	91%	AF107887.1

Appendix 9

Table presenting sequences producing significant alignments to *T. b. brucei ISG75* sequence (Accession DQ200189) using NCBI Blast.

Description	Max score	Total score	Query cover	E value	Identity	Accession
***Trypanosoma brucei brucei* strain AnTat 2.2 clone G06 ISG75 gene, complete cds**	3061	3061	100%	0.0	100%	DQ200189.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone G14 ISG75 gene, complete cds**	3055	3055	100%	0.0	99%	DQ200186.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone G01 ISG75 gene, complete cds**	3049	3049	100%	0.0	99%	DQ200190.1
***Trypanosoma equiperdum* strain Alfort clone CB37 ISG75 mRNA, complete cds**	3038	3038	100%	0.0	99%	DQ200228.1
***Trypanosoma equiperdum* strain Alfort clone G05 ISG75 gene, complete cds**	3038	3038	100%	0.0	99%	DQ200225.1
***Trypanosoma equiperdum* strain Alfort clone CB36 ISG75 mRNA, complete cds**	3038	3038	100%	0.0	99%	DQ200192.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone CA4 ISG75 mRNA, complete cds**	3038	3038	100%	0.0	99%	DQ200185.1
***Trypanosoma brucei rhodesiense* strain STIB850 clone CA2 ISG75 mRNA, complete cds**	3033	3033	100%	0.0	99%	DQ200188.1
***Trypanosoma equiperdum* strain Alfort clone G06 ISG75 gene, complete cds**	3027	3027	100%	0.0	99%	DQ200227.1
***Trypanosoma brucei rhodesiense* strain AnTat 12.1 clone G18 ISG75 gene, complete cds**	3005	3005	100%	0.0	99%	DQ200191.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone G02 ISG75 gene, complete cds**	3000	3000	100%	0.0	99%	DQ200222.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone C6 ISG75 mRNA, complete cds**	2988	2988	100%	0.0	99%	DQ200193.1
***Trypanosoma equiperdum* strain Alfort clone G20 ISG75 gene, complete cds**	2979	2979	98%	0.0	99%	DQ200226.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone G03 ISG75 gene, complete cds**	2972	2972	100%	0.0	99%	DQ200209.1
***Trypanosoma equiperdum* strain OVI clone G23 ISG75 gene, complete cds**	2972	2972	100%	0.0	99%	DQ200208.1

***Trypanosoma equiperdum* strain BoTat 1.1 clone G02 ISG75 gene, complete cds**	2966	2966	100%	0.0	99%	DQ200210.1
***Trypanosoma equiperdum* strain OVI clone G02 ISG75 gene, complete cds**	2966	2966	100%	0.0	99%	DQ200207.1
***Trypanosoma equiperdum* strain OVI clone G15 ISG75 gene, complete cds**	2966	2966	100%	0.0	99%	DQ200206.1
***Trypanosoma brucei gambiense* DAL972 chromosome 5, complete sequence**	2961	13829	99%	0.0	99%	FN554968.1
***Trypanosoma equiperdum* strain OVI clone G05 ISG75 gene, complete cds**	2955	2955	100%	0.0	99%	DQ200205.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone C14 ISG75 mRNA, complete cds**	2955	2955	100%	0.0	99%	DQ200202.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone G04 ISG75 gene, complete cds**	2944	2944	100%	0.0	99%	DQ200204.1
***Trypanosoma equiperdum* strain Botat 1.1 clone C2 ISG75 mRNA, complete cds**	2939	2939	100%	0.0	99%	DQ200201.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone G06 ISG75 gene, complete cds**	2928	2928	100%	0.0	99%	DQ200203.1
***Trypanosoma evansi* strain RoTat 1.2 clone CB1 ISG75 gene, complete cds**	2911	2911	100%	0.0	98%	DQ200195.1
***Trypanosoma equiperdum* strain Alfort clone CA43 ISG75 mRNA, complete cds**	2904	2904	96%	0.0	99%	DQ200224.1
***Trypanosoma equiperdum* strain OVI clone G08 ISG75 gene, complete cds**	2900	2900	100%	0.0	98%	DQ200194.1
***Trypanosoma evansi* strain RoTat 1.2 clone G02 ISG75 gene, complete cds**	2883	2883	100%	0.0	98%	DQ200197.1
***Trypanosoma brucei* TREU927 BAC26D11 sequence, strain TREU 927/4 GUTat10.1**	2878	9348	99%	0.0	98%	FM160648.1
***Trypanosoma brucei* chromosome 5 clone RPCI93-29K2, complete sequence**	2878	9304	99%	0.0	98%	AC159436.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone G11B ISG75 gene, complete cds**	2870	2870	96%	0.0	99%	DQ200200.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone G10B ISG75 mRNA, complete cds**	2854	2854	96%	0.0	99%	DQ200181.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone G07 ISG75 gene, partial cds**	2843	2843	95%	0.0	99%	DQ200223.1

***Trypanosoma brucei* 75kDa invariant surface glycoprotein (ISG75) mRNA, complete cds**	2839	2839	99%	0.0	97%	L07867.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 75 kDa invariant surface glycoprotein partial mRNA**	2809	2809	94%	0.0	99%	XM_839616.1
***Trypanosoma equiperdum* strain OVI clone G17 ISG75 gene, complete cds**	2772	2772	100%	0.0	97%	DQ200196.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone G01 ISG75 gene, complete cds**	2468	2468	100%	0.0	94%	DQ200199.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone C4 ISG75 mRNA, complete cds**	2423	2423	100%	0.0	93%	DQ200178.1
***Trypanosoma equiperdum* strain BoTat 1.1 clone C7 ISG75 mRNA, complete cds**	2420	2420	100%	0.0	93%	DQ200198.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 75 kDa invariant surface glycoprotein partial mRNA**	2311	2311	94%	0.0	93%	XM_839621.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone G03 ISG75 gene, complete cds**	1299	1597	59%	0.0	99%	DQ200187.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone CB1 ISG75 mRNA, complete cds**	1271	1570	59%	0.0	98%	DQ200237.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone CA6 ISG75 mRNA, complete cds**	1271	1570	59%	0.0	98%	DQ200177.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone CB2 ISG75 mRNA, complete cds**	1249	1547	59%	0.0	98%	DQ200239.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone G23 ISG75 gene, complete cds**	1221	1509	59%	0.0	97%	DQ200243.1
***Trypanosoma brucei rhodesiense* strain AnTat 12.1 clone G23 ISG75 gene, complete cds**	1221	1509	59%	0.0	97%	DQ200244.1
***Trypanosoma brucei rhodesiense* strain AnTat 12.1 clone G04 ISG75 gene, complete cds**	1216	1503	59%	0.0	97%	DQ200247.1
***Trypanosoma brucei rhodesiense* strain AnTat 12.1 clone G20 ISG75 gene, complete cds**	1216	1503	59%	0.0	97%	DQ200246.1
***Trypanosoma brucei rhodesiense* strain STIB850 clone CA1 ISG75 mRNA, complete cds**	1216	1498	59%	0.0	97%	DQ200245.1

***Trypanosoma evansi* strain RoTat 1.2 clone CB7 ISG75 gene, complete cds**	1216	1486	59%	0.0	97%	DQ200240.1
***Trypanosoma brucei* 75 kDa invariant surface glycoprotein mRNA, complete cds**	1216	1216	44%	0.0	97%	M86711.1
***Trypanosoma equiperdum* strain Alfort clone CB3 ISG75 mRNA, complete cds**	1210	1509	59%	0.0	97%	DQ200184.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone CA2 ISG75 mRNA, complete cds**	1205	1400	56%	0.0	96%	DQ200220.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone CB7 ISG75 mRNA, complete cds**	1205	1503	59%	0.0	96%	DQ200211.1
***Trypanosoma evansi* strain RoTat 1.2 clone G18 ISG75 gene, complete cds**	1205	1455	57%	0.0	96%	DQ200242.1
***Trypanosoma evansi* strain RoTat 1.2 clone G19 ISG75 gene, complete cds**	1205	1398	55%	0.0	96%	DQ200216.1
***Trypanosoma equiperdum* strain Alfort clone CA44 ISG75 mRNA, complete cds**	1199	1374	49%	0.0	96%	DQ200213.1
***Trypanosoma brucei rhodesiense* strain STIB850 clone CA4 ISG75 mRNA, complete cds**	1199	1498	59%	0.0	96%	DQ200232.1
***Trypanosoma equiperdum* strain Alfort clone G01 ISG75 gene, complete cds**	1194	1492	59%	0.0	96%	DQ200241.1
***Trypanosoma brucei rhodesiense* strain STIB850 clone CA8 ISG75 mRNA, complete cds**	1194	1481	59%	0.0	96%	DQ200248.1
***Trypanosoma equiperdum* strain Alfort clone G09 ISG75 gene, complete cds**	1190	1446	52%	0.0	96%	DQ200214.1
***Trypanosoma brucei rhodesiense* strain STIB850 clone G03 ISG75 gene, complete cds**	1182	1470	59%	0.0	96%	DQ200182.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone CB3 ISG75 mRNA, complete cds**	1182	1475	59%	0.0	96%	DQ200253.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone G06 ISG75 gene, complete cds**	1177	1475	59%	0.0	96%	DQ200255.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone CB3 ISG75 mRNA, complete cds**	1177	1366	55%	0.0	96%	DQ200234.1
***T. b. gambiense* strain LiTat 1.3 clone G10 ISG75 gene, complete cds**	1177	1475	59%	0.0	96%	DQ200212.1

***Trypanosoma equiperdum* strain Alfort clone CA5 ISG75 mRNA, complete cds**	1177	1475	59%	0.0	96%	DQ200235.1
***Trypanosoma brucei rhodesiense* strain AnTat 12.1 clone G19 ISG75 gene, complete cds**	1177	1464	59%	0.0	96%	DQ200180.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone CA5 ISG75 mRNA, complete cds**	1177	1475	59%	0.0	96%	DQ200251.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone G14 ISG75 gene, complete cds**	1177	1475	59%	0.0	96%	DQ200250.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone G01 ISG75 gene, complete cds**	1177	1483	59%	0.0	96%	DQ200249.1
***Trypanosoma evansi* strain RoTat 1.2 clone CB6 ISG75 mRNA, complete cds**	1177	1475	59%	0.0	96%	DQ200256.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone CB4 ISG75 mRNA, complete cds**	1171	1470	59%	0.0	96%	DQ200254.1
***Trypanosoma brucei rhodesiense* strain AnTat 12.1 clone G01 ISG75 gene, complete cds**	1171	1459	59%	0.0	96%	DQ200179.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone CB2 ISG75 mRNA, complete cds**	1171	1348	55%	0.0	96%	DQ200252.1
***Trypanosoma evansi* strain RoTat 1.2 clone G21 ISG75 gene, complete cds**	1171	1464	59%	0.0	96%	DQ200175.1
***T. brucei* 75kDa invariant surface glycoprotein (ISG75) mRNA, complete cds**	1171	1376	59%	0.0	96%	L07866.1
***Trypanosoma equiperdum* strain Alfort clone CB6 ISG75 mRNA, complete cds**	1166	1420	58%	0.0	95%	DQ200183.1
***Trypanosoma equiperdum* strain Alfort clone G21 ISG75 gene, complete cds**	1166	1464	59%	0.0	95%	DQ200176.1
***Trypanosoma brucei rhodesiense* strain STIB850 clone CB2 ISG75 mRNA, complete cds**	1166	1464	59%	0.0	95%	DQ200215.1
***Trypanosoma evansi* strain RoTat 1.2 clone G17 ISG75 gene, complete cds**	1166	1464	59%	0.0	95%	DQ200236.1
***Trypanosoma evansi* strain RoTat 1.2 clone CB5 ISG75 mRNA, complete cds**	1166	1442	59%	0.0	95%	DQ200221.1
***Trypanosoma evansi* strain RoTat 1.2 clone CB8 ISG75 mRNA, complete cds**	1166	1464	59%	0.0	95%	DQ200219.1

***Trypanosoma evansi* strain RoTat 1.2 clone CB4 ISG75 mRNA, complete cds**	1166	1527	57%	0.0	95%	DQ200217.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone CB4 ISG75 mRNA, complete cds**	1160	1453	59%	0.0	95%	DQ200231.1
***Trypanosoma brucei* 75 kDa invariant surface glycoprotein, complete cds**	1160	1322	52%	0.0	95%	M86710.1
***Trypanosoma brucei gambiense* strain LiTat 1.3 clone G04 ISG75 gene, complete cds**	1157	1455	59%	0.0	95%	DQ200238.1
***Trypanosoma equiperdum* strain Alfort clone G03 ISG75 gene, complete cds**	1155	1398	58%	0.0	95%	DQ200230.1
***Trypanosoma brucei rhodesiense* strain STIB850 clone CB6 ISG75 mRNA, complete cds**	1155	1453	59%	0.0	95%	DQ200229.1
***Trypanosoma brucei brucei* strain AnTat 2.2 clone G26 ISG75 gene, complete cds**	1155	1448	59%	0.0	95%	DQ200233.1
***Trypanosoma evansi* strain RoTat 1.2 clone G16 ISG75 gene, complete cds**	1155	1461	55%	0.0	95%	DQ200218.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 75 kDa invariant surface glycoprotein partial mRNA**	1142	1276	47%	0.0	96%	XM_839617.1
***Trypanosoma evansi* strain RoTat 1.2 invariant surface glycoprotein mRNA, complete cds**	1125	1259	47%	0.0	95%	JN797772.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 75 kDa invariant surface glycoprotein partial mRNA**	1077	1281	54%	0.0	94%	XM_839618.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 75 kDa invariant surface glycoprotein partial mRNA**	1075	1209	47%	0.0	94%	XM_839620.1
***Trypanosoma brucei brucei* strain 927/4 GUTat10.1 75 kDa invariant surface glycoprotein partial mRNA**	113	113	4%	4e-21	97%	XM_839619.1